

# Inhibitory effect of pregnancy on stress-induced immunosuppression through corticotropin releasing hormone (CRH) and dopaminergic systems

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## Abstract

To clarify the involvement of pregnancy in the response of the neuroendocrine-immune system to stress, we examined splenic natural killer cell activity (NKCA) and its relevant central and blood parameters in female virgin and pregnant rats (10 to 11 days gestation) exposed to forced water-immersion stress with durations of 90 min and 180 min. Decreases in splenic NKCA, corticotropin releasing hormone (CRH) in the hypothalamus, and increases in progesterone (P),  $\beta$ -endorphin ( $\beta$  EP), and dopamine (DA) metabolic ratios in the frontal cortex and nucleus accumbens produced by stress were recognized in the virgin rats, but not in the pregnant rats. Pregnancy reduced splenic NKCA in rats without stress, but elevated it in the rats exposed to stress with a duration of 180 min. These findings suggest inhibitory effects of pregnancy on stress-induced immunosuppression and neuroendocrine changes, thereby promoting homeostasis in the neuroendocrine-immune system against stress. Such enhanced homeostasis associated with pregnancy seemed to be mediated by the activation of placental P and placental or pituitary  $\beta$  EP in cooperation with mesocortical and mesolimbic DA systems and hypothalamic CRH.

**Keywords:**  $\beta$ -endorphin; Corticotropin releasing hormone; Natural killer cell activity; Pregnancy; Progesterone; Stress

## 1. Introduction

Physical and psychological stress can influence immunological parameters, especially natural killer (NK) cells and their functions (Shavit et al., 1985). NK cells act early in the immune response before specificity can be generated. They mediate first-line defense by direct cytotoxicity against various types of target cells without apparent prior immunization (Hercend and Schmidt, 1988). Emotional stress was reported to be associated with a decrease in NK cell activity (NKCA) in animal studies (Shavit et al., 1984; Zalzman et al., 1991) and in human (Locke et al., 1984; Irwin et al., 1987). Central corticotropin releasing hormone (CRH) (Audhya et al., 1991; Van Oers et al., 1992; Perez

and Lysle, 1995) and pituitary opiate peptide  $\beta$ -endorphin ( $\beta$  EP) (Mori et al., 1989; Weber and Pert, 1989) play a role in modulating neuroendocrine and immune systems as neurotransmitters. Such functional networks among nervous, endocrine, and immune systems are now interpreted as a neuroendocrine-immune system (Shavit et al., 1985).

Progesterone (P) as well as estradiol ( $E_2$ ) are mostly of ovarian origin in non-pregnant stage (Ryan, 1980). During pregnancy the placenta takes over as the main synthesis site of these hormones from maternal and fetal precursors, subsequently releasing large amounts of P and  $E_2$  (Branch, 1992). Although accumulating evidences suggest a reduction in NKCA during pregnancy to be associated with enhanced activity of sex hormones (Inman, 1978; Giglio et al., 1994), the involvement of pregnancy in the stress-induced immunosuppression, especially in the neuroendocrine-immune system, remains to be elucidated.

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Estrogens have been reported to both antagonize and potentiate the activity of the central dopamine (DA) system (Maggi and Perez, 1985) which is assumed to be closely related to emotional changes induced by stress (Nakamura et al., 1992). Taken together with functional networks among DA, CRH and  $\beta$  EP during stress (Jankovic, 1989), the clarification of the roles of pregnancy in immune and neurotransmitting responses to stress will provide us with systemic information on influence of stress on pregnant organisms. In the present study, therefore, we assayed splenic NKCA and its relevant factors, DA and CRH in discrete regional brains, and blood  $\beta$  EP, in addition to blood corticosterone (CS) as an indicator of the hypothalamic–pituitary–adrenal (HPA) axis, and  $E_2$  and P in female virgin and pregnant rats exposed to stress.

## 2. Materials and methods

### 2.1. Preparation of virgin and pregnant rats for the study

Eighteen female virgin Wistar rats weighing  $268.4 \pm 13.8$  g (mean  $\pm$  SD) and 18 Wistar rats at 10 to 11 days gestation weighing  $270.6 \pm 13.6$  g (mean  $\pm$  SD) were studied. Breeding was performed by introducing 1 male rat into a cage with two females; the environment was controlled in all cases ( $23 \pm 2^\circ\text{C}$ , 50% humidity with alternate cycles of 12 h light (8:00–20:00) and 12 h darkness (20:00–8:00). The onset of pregnancy was determined by means of vaginal smears. All animals had access to commercial food and tap water ad libitum. The rats were fasted, but given water for 24 h prior to the experiment and deprived of food and drink throughout the experiment. This study was approved by the Ethics Committee on Animal Experimentation of Kanazawa University, Takaramachi Campus. In all cases the experimental protocol began at 11:00 am.

### 2.2. Exposure to stress

Rats were subjected to forced water-immersion stress according to the method of Kitagawa et al. (1979); rats were restrained individually in a wire mesh cage in the prone position and were forced to be immersed in water at  $22^\circ\text{C}$  to the depth of the xiphoid for 90 min or 180 min. The method is known as a much severe form of emotional stress to animals (Kitagawa et al., 1979; Mori et al., 1986). Rats which were not exposed to the stress comprised the control group.

### 2.3. Measurements of blood CS, $\beta$ EP, $E_2$ , and P

Blood samples were collected by decapitation of rats immediately after the end of the protocol. Plasma was immediately prepared by transfer of samples to cooled

conical centrifuge tubes containing 0.1 mM EDTA followed by centrifugation. Plasma was frozen at  $-80^\circ\text{C}$  until analyses were performed.

The CS was measured by the fluorometric method of Silber et al. (1958).  $\beta$  EP was measured by the radioimmunoassay (RIA) described by Yoshimi et al. (1978). In the method highly purified human  $\beta$  EP which was labeled with  $\text{Na}^{125}\text{I}$  using chloramine T. The purification of labeled  $\beta$  EP was performed on a carboxymethyl cellulose column. The antiserum against  $\beta$  EP used showed negligible cross-reactivity with other fragments of  $\beta$ -lipotropin,  $\alpha$ -MSH and ACTH.

$E_2$  and P were analyzed by RIA using the tube solid phase method according to Ratcliffe et al. (1988).

### 2.4. Measurement of CRH, DA, and homovanillic acid (HVA) in the 4 discrete regions of the rat brain

After the experiment ended, their brains were quickly removed from the cranium and subsequently dissected into 4 discrete regions, i.e., the frontal cortex, nucleus accumbens, amygdala, and hypothalamus, according to the method described by Marley et al. (1984). The brain regions dissected were sonicated in 1 ml of 0.1 N acetic acid, boiled for 10 min, and then centrifuged twice at 3000 rpm and  $4^\circ\text{C}$  for 20 min. Aliquots of the supernatants were lyophilized and reconstituted in assay buffer for measurement of DA and its main metabolite, HVA as well as CRH. The pellets were dissolved in 1 N NaOH for protein estimation. Protein concentrations were determined as described by Lowry et al. (1951) using bovine serum albumin as the standard.

CRH was measured by a sensitive and specific RIA using the double-antibody method reported by Moldow and Fishman (1982). Briefly, synthetic CRH was conjugated to bovine thyroglobulin and emulsified with complete Freund's adjuvant. The labeled N-Tyr-CRH which was iodinated and used as tracer was purified on Sephadex G50.

DA and HVA were determined using a high-performance liquid chromatography system equipped with a system for electrochemical detection, as described elsewhere (Nakamura et al., 1992). Dihydroxybenzylamine (DHBA) and *p*-hydroxyphenylacetic acid (PHPA) were used as internal standards for DA and HVA, respectively.

### 2.5. Splenic NKCA

To measure splenic NKCA, the spleen was surgically excised and dissociated into a single cell suspension. The splenocytes were suspended in 40 ml PBS and centrifuged in 50 ml tubes at 400 g at room temperature for 30 min over 12 ml Ficoll-Paque (Pharmacia, Piscataway, NJ) to yield mononuclear cells (Reynolds et al., 1981). Splenic lymphocytes were collected at the interface, washed twice

in PBS solution, and suspended in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% v/v fetal bovine serum (FBS, GIBCO), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, all from GIBCO.

NKCA was measured in a standard 4 h chromium (Cr) release assay that was performed in 0.2 ml volumes in U-bottom microplates. The YAC-1 mouse lymphoma cell line was used as the target for detecting NK cell cytotoxicity. The cells, suspended in culture in RPMI 1640 medium, were labeled with  $\text{Na}_2^{51}\text{CrO}_4$  of 1 mCi/ml (New England Nuclear, Boston, MA) for 1 h at 37°C. Cells were washed 4 times in a tissue culture medium consisting of RPMI 1640 and resuspended in fresh medium, counted and aliquoted at  $1 \times 10^4$  target cells/well into 96-well U-bottom microtiter plates containing lymphocytes as effector cells at predetermined concentrations. The effector:target cell ratios (E/T) were 40:1, 20:1, 10:1, to 5:1. After plates were incubated in 5%  $\text{CO}_2$  in air at 37°C for 4 h, the assay was terminated by centrifuging the plate at 400g for 5 min, after which the medium was harvested from each well using a supernatant-harvesting apparatus (Flow, McLean, VA). All determinations were done in triplicate. Radioactivity was counted in a gamma counter. The spontaneous  $^{51}\text{Cr}$  release, determined by incubating labeled target cells in the medium alone, did not exceed 10% of the maximum release that was determined by adding 1% Triton X-100. The NKCA as percentage specific lysis was determined according to the formula  $100 \times (\text{mean experimental cpm} - \text{mean spontaneous cpm}) / (\text{mean maximal cpm} - \text{mean spontaneous release cpm})$ . The percent cytotoxicity was calculated at each E/T, and these values were converted to lytic units at 30% ( $\text{LU}_{30}$ ) according to the method of Pross et al. (1981).

Total lymphocyte counts were made by standard techniques using a microscope, hemocytometer, and typan blue-stained smear of the splenocyte suspension.

## 2.6. Statistical analysis

Statistical analysis of the difference in the mean values of splenic NKCA, and blood and brain parameters in the virgin and pregnant rats with and without stress exposure were performed by one way analysis of variance

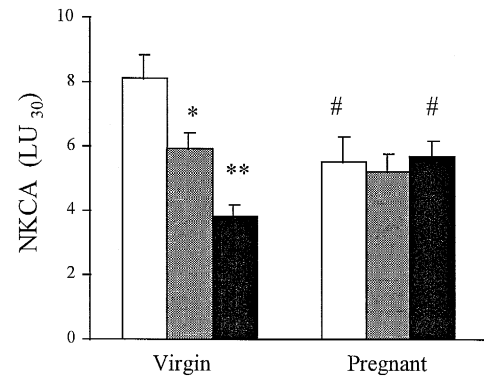


Fig. 1. Effects of stress on splenic NKCA in the virgin and pregnant rats. White: non-stress, grey: stress exposure with a duration of 90 min, black: stress exposure with a duration of 180 min. Values represent mean  $\pm$  standard error. Statistical analysis of differences was performed by one way ANOVA, followed by the Tukey test for multiple comparison. Significant differences from the values of the rat group with the same conditions except for the stress. \*  $p < 0.05$ . \*\*  $p < 0.01$ . Significant differences from the values of the virgin rat group with the same stress conditions. #  $p < 0.05$ .

(ANOVA), followed by the Tukey test for multiple comparison. All statistical tests were two-tailed.  $p$  values less than 0.05 were regarded as indicating statistical significance.

## 3. Results

### 3.1. Splenic NKCA

Splenic NKCA were compared among virgin and pregnant rats without and with stress (Fig. 1). When splenic NKCA were compared between the virgin and pregnant rats without stress, they were recognized to be reduced associated with pregnancy. Conversely, pregnancy elevated splenic NKCA in the rats exposed to stress with a duration of 180 min. NKCA in both virgin rat groups with stresses of 90 min and 180 min were significantly lower than that without stress. The NKCA in the virgin rats was reduced in a stress dependent exposure time. In the pregnant rats, however, decreases in splenic NKCA were recognized not to be significant. Splenic weight and absolute number of splenic lymphocytes are shown in Table 1. One

Table 1  
Effects of stress on splenic weight and the absolute number of splenic lymphocytes in virgin and pregnant rats

Rat group	Number of rats examined	Values (mean $\pm$ SEM)	
		splenic weight (g)	total lymphocytes in spleen ( $\times 10^7$ )
Virgin rat without stress	6	0.74 $\pm$ 0.065	20.8 $\pm$ 1.49
Virgin rat with stress (90 min)	6	0.76 $\pm$ 0.049	22.3 $\pm$ 1.73
Virgin rat with stress (180 min)	6	0.70 $\pm$ 0.055	21.3 $\pm$ 1.88
Pregnant rat without stress	6	0.72 $\pm$ 0.031	21.5 $\pm$ 1.26
Pregnant rat with stress (90 min)	6	0.72 $\pm$ 0.056	20.9 $\pm$ 1.42
Pregnant rat with stress (180 min)	6	0.79 $\pm$ 0.068	22.9 $\pm$ 1.51

Table 2  
Effects of stress on CRH and DA in the regional brain of virgin and pregnant rats

Rat group	Number of rats examined	CRH (mean $\pm$ SEM, ng/mg protein)				DA (mean $\pm$ SEM, ng/mg protein)			
		frontal cortex	nucleus accumbens	amygdala	hypothalamus	frontal cortex	nucleus accumbens	amygdala	hypothalamus
Virgin rat without stress	6	0.027 $\pm$ 0.004	0.393 $\pm$ 0.036	0.138 $\pm$ 0.014	2.73 $\pm$ 0.25	2.43 $\pm$ 0.37	32.2 $\pm$ 2.95	18.0 $\pm$ 1.10	22.5 $\pm$ 1.98
Virgin rat with stress (90 min)	6	0.026 $\pm$ 0.007	0.382 $\pm$ 0.039	0.149 $\pm$ 0.017	1.71 $\pm$ 0.20 **	1.92 $\pm$ 0.16	25.3 $\pm$ 3.62	19.3 $\pm$ 1.41	21.7 $\pm$ 2.77
Virgin rat with stress (180 min)	6	0.033 $\pm$ 0.004	0.353 $\pm$ 0.025	0.148 $\pm$ 0.010	1.23 $\pm$ 0.09 **	2.18 $\pm$ 0.22	27.2 $\pm$ 3.97	21.0 $\pm$ 1.98	29.0 $\pm$ 1.39
Pregnant rat without stress	6	0.024 $\pm$ 0.005	0.357 $\pm$ 0.054	0.149 $\pm$ 0.010	2.56 $\pm$ 0.23	2.62 $\pm$ 0.28	33.0 $\pm$ 3.97	20.2 $\pm$ 3.16	23.7 $\pm$ 2.78
Pregnant rat with stress (90 min)	6	0.023 $\pm$ 0.005	0.342 $\pm$ 0.027	0.150 $\pm$ 0.011	2.88 $\pm$ 0.20	2.87 $\pm$ 0.41	31.7 $\pm$ 4.30	19.2 $\pm$ 1.56	25.7 $\pm$ 3.28
Pregnant rat with stress (180 min)	6	0.028 $\pm$ 0.002	0.412 $\pm$ 0.026	0.160 $\pm$ 0.013	2.67 $\pm$ 0.18	2.45 $\pm$ 0.40	33.3 $\pm$ 3.16	22.2 $\pm$ 2.85	28.2 $\pm$ 4.20

The statistical analysis of differences was performed by one way ANOVA, followed by the Tukey test for multiple comparison. Significant differences from the values of the rat group with the same conditions except for the stress exposure. \*\*  $p < 0.01$ .

Table 3  
Effects of stress on HVA and the HVA/DA ratio in the regional brain of virgin and pregnant rats

Rat group	Number of rats examined	HVA (mean $\pm$ SEM, ng/mg protein)				HVA/DA ratio (mean $\pm$ SEM)			
		frontal cortex	nucleus accumbens	amygdala	hypothalamus	frontal cortex	nucleus accumbens	amygdala	hypothalamus
Virgin rat without stress	6	0.47 $\pm$ 0.097	2.38 $\pm$ 0.18	3.07 $\pm$ 0.41	3.25 $\pm$ 0.29	0.20 $\pm$ 0.034	0.078 $\pm$ 0.012	0.17 $\pm$ 0.020	0.15 $\pm$ 0.013
Virgin rat with stress (90 min)	6	0.72 $\pm$ 0.064 *	3.18 $\pm$ 0.30 *	2.72 $\pm$ 0.24	2.92 $\pm$ 0.15	0.39 $\pm$ 0.056 **	0.143 $\pm$ 0.032 *	0.14 $\pm$ 0.015	0.14 $\pm$ 0.016
Virgin rat with stress (180 min)	6	0.79 $\pm$ 0.070 **	3.55 $\pm$ 0.30 **	2.43 $\pm$ 0.36	3.35 $\pm$ 0.35	0.38 $\pm$ 0.044 **	0.150 $\pm$ 0.033 *	0.12 $\pm$ 0.023	0.12 $\pm$ 0.017
Pregnant rat without stress	6	0.53 $\pm$ 0.049	2.65 $\pm$ 0.33	2.78 $\pm$ 0.62	3.10 $\pm$ 0.35	0.21 $\pm$ 0.022	0.090 $\pm$ 0.021	0.14 $\pm$ 0.017	0.14 $\pm$ 0.018
Pregnant rat with stress (90 min)	6	0.40 $\pm$ 0.067	2.87 $\pm$ 0.26	3.02 $\pm$ 0.47	3.02 $\pm$ 0.33	0.18 $\pm$ 0.029	0.096 $\pm$ 0.011	0.16 $\pm$ 0.026	0.13 $\pm$ 0.030
Pregnant rat with stress (180 min)	6	0.49 $\pm$ 0.056	2.80 $\pm$ 0.23	2.92 $\pm$ 0.13	3.02 $\pm$ 0.25	0.23 $\pm$ 0.049	0.087 $\pm$ 0.01	0.14 $\pm$ 0.016	0.12 $\pm$ 0.026

The statistical analysis of differences was performed by one way ANOVA, followed by the Tukey test for multiple comparison. Significant differences from the values of the rat group with the same conditions except for the stress exposure. \*  $p < 0.05$ . \*\*  $p < 0.01$ .

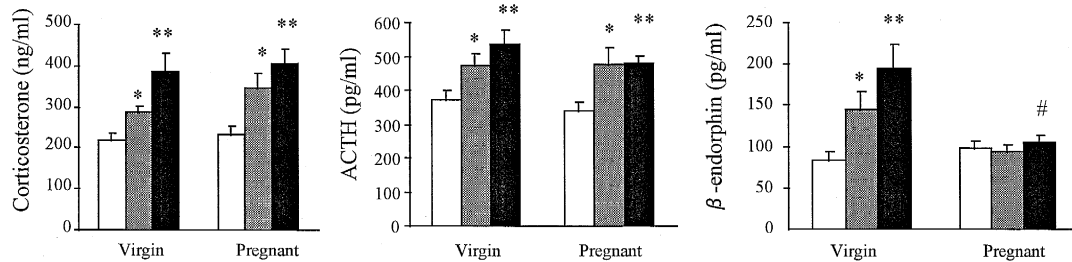


Fig. 2. Effects of stress on blood CS and ACTH, and  $\beta$  EP levels in the virgin and pregnant rats. White: non-stress, grey: stress exposure with a duration of 90 min, black: stress exposure with a duration of 180 min. Values represent mean  $\pm$  standard error. Statistical analysis of differences was performed by one way ANOVA, followed by the Tukey test for multiple comparison. Significant differences from the values of the rat group with the same conditions except for the stress. \*  $p < 0.05$ , \*\*  $p < 0.01$ . Significant differences from the values of the virgin rat group with the same stress conditions. #  $p < 0.05$ .

way ANOVA revealed no statistically significant differences in splenic weight and absolute number of splenic lymphocytes.

### 3.2. Blood CS, ACTH, $\beta$ EP, $E_2$ and P

Changes of CS, ACTH and  $\beta$  EP produced by stress in the virgin and pregnant rats were demonstrated in Fig. 2. CS and ACTH were increased by stress in both virgin and pregnant rats in an exposure time dependent manner, whereas significant increases in  $\beta$  EP by stress were detected only in the virgin rats. Pregnancy was found to reduce  $\beta$  EP in the rats exposed to stress with a duration of 180 min.

$E_2$  was significantly increased by both stresses in the virgin and pregnant rats, while P was increased only in the virgin rats (Fig. 3). When the blood indicators were compared between the virgin and pregnant rats without any stresses, pregnancy was found to increase  $E_2$  and P. Although increases in  $E_2$  associated with pregnancy were recognized in the rats exposed to both stresses of 90 min and 180 min, no significant difference in P between the virgin and pregnant rats with 180 min stress was found following 180 min stress.

### 3.3. CRH, DA, HVA, and HVA/DA ratio in the regional brains

Levels of CRH and DA in the virgin and pregnant rats without and with stress are shown in Table 2. CRH in the hypothalamus in the virgin rats with both stresses of 90 min and 180 min were significantly lower as compared to that in the virgin rats without stress, although the decreases were not found in the pregnant rats. No significant change in DA among the 6 groups was recognized (Table 2). Table 3 showed significant increases in HVA in the frontal cortex and nucleus accumbens in the virgin rats with both stresses, but not in the pregnant rats. Likewise, significant increases in the HVA/DA ratio in the frontal cortex and nucleus accumbens by stress were recognized only in the virgin rats (Table 3).

## 4. Discussion

Persistently low NK cell function has been shown to be associated with a greater risk for health impairments, including infectious diseases and cancer. Stress-induced immunosuppression accounts for individuals who experi-

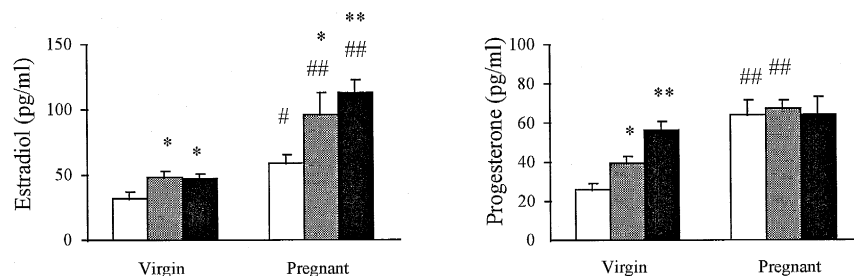


Fig. 3. Effects of stress on blood  $E_2$  and P levels in the virgin and pregnant rats. White: non-stress, grey: stress exposure with a duration of 90 min, black: stress exposure with a duration of 180 min. Values represent mean  $\pm$  standard error. Statistical analysis of differences was performed by one way ANOVA, followed by the Tukey test for multiple comparison. Significant differences from the values of the rat group with the same conditions except for the stress. \*  $p < 0.05$ , \*\*  $p < 0.01$ . Significant differences from the values of the virgin rat group with the same stress conditions. #  $p < 0.05$ , ##  $p < 0.01$ .

ence stressful life events more susceptible to a variety of illness (Bonneau et al., 1990). Although many researchers have demonstrated that stress has been shown to suppress immune activity (Shavit et al., 1984; Locke et al., 1984; Irwin et al., 1987; Zalcman et al., 1991), it is still difficult to specify the conditions under which stress induces immunosuppression; this has led to some controversies (Geiser, 1988). Factors of stress-induced immunosuppression that need clarification include the kind and the exposure time of stress (Sieber et al., 1992). Our results showing that splenic NKCA in the virgin rats decreased in a stress time dependent manner indicate that the forced water-immersion stress employed in this study induced immunosuppression. Caggiula and his associates have demonstrated that immunological responsiveness to stress in non-pregnant women is little different from that for men (Caggiula et al., 1995). The stress-induced immunosuppression seen in the virgin rats are similar to those reported for male animals (Shavit et al., 1984; Zalcman et al., 1991). Taken together, it seems that the stress-induced immunosuppression is not related to the sex difference, excluding the pregnant stage.

Although reduction in NKCA in pregnant women has been demonstrated (Gregory et al., 1985; Salmeron et al., 1991), the involvement of pregnancy in the immunosuppression remains to be elucidated, in part due to the unclear role of decreased NKCA with advanced pregnant terms (Branch, 1992). Reduction in splenic NKCA in the rats without stress associated with pregnancy demonstrated that pregnancy suppressed cellular immunity. On the contrary, NKCA between pregnant rats with and without stress rats did not reveal a significant difference. In addition, we observed that NKCA in the pregnant rats was higher than that in the virgin rats in case of stress exposure of 180 min. This result can be interpreted as less immunosuppression induced by stress in the pregnant rats as compared to that in the virgin rats, suggesting that pregnancy interferes with the inhibitory effects of stress on immune functions. Since stress did not change NKCA which was once reduced by pregnancy, it seems unlikely that stress induces immunosuppression in the pregnant rats. P at concentrations found in the human placenta acts as an immunosuppressive agent on lymphocyte cultures stimulated by allogenic antigen (Grossman, 1985). P increased by stress in the virgin rats was not observed in the pregnant rats as was a similar finding to that for NKCA. Accordingly, P seems to be involved in the inhibitory effect of pregnancy on stress-induced immunosuppression.

Although many central neurotransmitters have been implicated in stress-induced immunosuppression, CRH is the key mediator of the endocrine immune responses to stress (Jankovic, 1989; Jain et al., 1991). Our findings showing stress-reduced hypothalamic CRF in the virgin rats was consistent with a previous report on male rats (Chappel et al., 1986). In addition, CRH administration results in decreases in LH and FSH secretion through the inhibitory

effect on the gonadotropin (Gn)-releasing hormone pulse generator. Decreases in these Gn following administration of CRH characterizes the response to a stressful condition (Ferin, 1993). Coordination of stress response and regulation of the immune/inflammatory reaction as a physiological role of CRH account for sexual dimorphism (Vamvakopoulos and Chrousos, 1993). In the present study, reduction in hypothalamic CRF following stress was not recognized in the pregnant rats. Thus, CRH seems to modulate the inhibitory effect of the pregnancy in the stress-induced immunosuppression which may be related to the sex steroids.

Likewise,  $\beta$  EP was increased by stress only in the virgin rats. The cytotoxicity of splenic NK cells is suppressed in rats exposed to opioid-dependent stress. Since this decreased NKCA is blocked by naloxone, the endogenous opioids are apparently involved (Shavit et al., 1984). In addition to  $\beta$  EP induced immunosuppression (Mori et al., 1989; Weber and Pert, 1989), several studies have shown that circulating  $\beta$  EP increases in pregnant women (Newnham et al., 1983). The increase is related in part to secretion by the placenta as well as the pituitary (Nakai et al., 1978). We did not find the effect of pregnancy on  $\beta$  EP in case of no stress exposure, but conversely found that pregnancy reduced  $\beta$  EP in the rats exposed to stress with a duration of 180 min. Such a consistent relationship between splenic NKCA and  $\beta$  EP seen in the virgin and pregnant rats with and without stress suggests that pregnancy antagonizes stress-induced immunosuppression and neuroendocrine changes, thereby promoting homeostasis in the neuroendocrine-immune system against stress. The HPA axis activated by stress was demonstrated by increases in CS and ACTH by stress both in virgin and pregnant rats. As Branch (1992) points out for enhanced homeostasis with pregnancy, such mechanisms in pregnant organisms exposed to stress seem to result in reinforcing the homeostasis against stress which activates the HPA axis. Since our hypothesis has been drawn from the descriptive study which compared neuroimmune changes produced by stress between non-pregnant and pregnant rats, it should be demonstrated by future studies with experimental designs investigating a direct causal relationship among P,  $\beta$  EP, and the inhibitory effects of pregnancy on stress-induced immunosuppression.

An acute administration of CRH increases DA metabolism in the limbic systems (Dunn and Berridge, 1987; Liposits and Paull, 1989). Stress activates hypothalamic CRH nerves (Chappel et al., 1986) as well as DA systems originating in the mesencephalon innervating the frontal cortex and limbic systems, i.e. mesocortical and mesolimbic DA systems (Roth et al., 1988; Nakamura et al., 1992). Evidence for involvement of striatal and mesolimbic DA pathways in the neuroimmunomodulation has been accumulated by recent researches (Deleplanque et al., 1994; Nistico et al., 1994; Basu et al., 1995). Song et al. (1995) have demonstrated functional networks among

CRH and DA nervous systems and immunosuppression during stress. Increased metabolisms of DA (an increase of HVA and/or HVA/DA ratio) in the frontal cortex and nucleus accumbens in the virgin rats exposed to stress demonstrate activations of mesocortical and mesolimbic DA systems by stress, but the activations were not recognized in the pregnant rats. Based on the functional relationship between CRH and DA, no activation in the mesocortical and mesolimbic DA systems by stress during pregnancy seems to be implicated in the role of pregnancy in the regulation of stress-induced immunosuppression, which may lead to the enhancement of homeostasis, in cooperation with hypothalamic CRH.

## 5. Conclusions

Decreases in splenic NKCA, and CRH in the hypothalamus and increases in P,  $\beta$  EP, and DA metabolic ratios in the frontal cortex and nucleus accumbens produced by stress were recognized in the virgin rats, but not in the pregnant rats. Pregnancy reduced splenic NKCA in rats without stress, but elevated it in the rats exposed to stress with a duration of 180 min. These findings suggest inhibitory effects of pregnancy on stress-induced immunosuppression and neuroendocrine changes, thereby promoting homeostasis in the neuroendocrine-immune system against stress. Such enhanced homeostasis during pregnancy seemed to be mediated by the activation of placental P and placental or pituitary  $\beta$  EP in cooperation with mesocortical and mesolimbic DA systems and hypothalamic CRH.

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## References

- Audhya, T., Jain, R. and Hollander, C.S. (1991) Receptor-mediated immunomodulation by corticotropin-releasing factor. *Cell Immunol.* 134, 77–84.
- Basu, S., Dasgupta, P.S. and Chowdhury, J.R. (1995) Enhanced tumor growth in brain dopamine-depleted mice following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment. *J. Neuroimmunol.* 60, 1–8.
- Bonneau, R.H., Kiecolt-Glaser, J.K. and Glaser, R. (1990) Stress-induced modulation of the immune response. *Ann. NY Acad. Sci.* 594, 253–269.
- Branch, D.W. (1992) Physiologic adaptations of pregnancy. *Am. J. Reprod. Immunol.* 28, 120–122.
- Caggiula, A.R., McAllister, C.G., Matthews, K.A., Berga, S.L., Owens, J.F. and Miller, A.L. (1995) Psychological stress and immunological responsiveness in normally cycling, follicular-stage women. *J. Neuroimmunol.* 59, 103–111.
- Chappel, P.B., Smith, M.A., Kilts, C.D., Bissette, G., Ritchie, J., Anderson, C. and Nemeroff, C.B. (1986) Alterations in corticotropin-releasing factor-like immunoreactivity in discrete rat brain regions after acute and chronic stress. *J. Neurosci.* 6, 2908–2914.
- Deleplanque, B., Vitiello, S., Le-Moal, M. and Neveu, P.J. (1994) Modulation of immune reactivity by unilateral striatal and mesolimbic dopaminergic lesions. *Neurosci. Lett.* 166, 216–220.
- Dunn, A.J. and Berridge, C.W. (1987) Corticotropin-releasing factor administration elicits a stress-like activation of cerebral catecholaminergic systems. *Pharmacol. Biochem. Behav.* 27, 685–91.
- Ferin, M. (1993) Neuropeptides, the stress response, and the hypothalamo-pituitary-gonadal axis in the female rhesus monkey. *Ann. NY Acad. Sci.*, 697, 106–116.
- Geiser, D.S. (1988) Psychosocial influences on human immunity. *Clin. Psychol. Rev.* 9, 689–715.
- Giglio, T., Imro, M.A., Filaci, G., Scudeletti, M., Puppo, F., De Cecco, L., Indiveri, F. and Costantini, S. (1994) Immune cell circulating subsets are affected by gonadal function. *Life Sci.* 54, 1305–1312.
- Gregory, C.D., Shah, L.P., Lee, H., Scott, I.V. and Golding, P.R. (1985) Cytotoxic reactivity of human natural killer (NK) cells during normal pregnancy: a longitudinal study. *J. Clin. Lab. Immunol.* 18, 175–181.
- Grossman, C.J. (1985) Interactions between the gonadal steroids and the immune system. *Science* 227, 257–261.
- Hercend, T. and Schmidt, R.E. (1988) Characteristics and uses of natural killer cells. *Immunol. Today* 9, 291–293.
- Inman, R.D. (1978) Immunologic sex differences and the female predominance in systemic lupus erythematosus. *Arthritis Rheum.* 21, 849–852.
- Irwin, M., Daniels, M., Smith, T.L., Bloom, E. and Weiner, H. (1987) Impaired natural killer activity during bereavement. *Brain Behav. Immun.* 1, 98–104.
- Jain, R., Zwickler, D., Hollander, C.S., Brand, H., Saperstein, A., Hutchinson, B., Brown, C. and Audhya, T. (1991) Corticotropin-releasing factor modulates the immune response to stress in the rat. *Endocrinology* 128, 1329–1336.
- Jankovic, R.D. (1989) Neuroimmunomodulation: facts and dilemmas. *Immunol. Lett.* 21, 101–118.
- Kitagawa, H., Fujiwara, M. and Osumi, Y. (1979) Effects of water-immersion stress and mucosal blood flow in rats. *Gastroenterology* 77, 298–302.
- Liposits, Z. and Paull, W.K. (1989) Association of dopaminergic fibers with corticotropin releasing hormone (CRH)-synthesizing neurons in the paraventricular nucleus of the rat hypothalamus. *Histochemistry* 93, 119–27.
- Locke, S.E., Kraus, L., Leserman, J., Hurst, M.W., Heisel, J.S. and Williams, R.M. (1984) Life change stress, psychiatric symptoms, and natural killer cell activity. *Psychosom. Med.* 46, 441–453.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Maggi, A. and Perez, J. (1985) Role of female gonadal hormones in the CNS: clinical and experimental aspects. *Life Sci.* 37, 893–906.
- Marley, P.D., Rehfeld, J.F. and Emson, P.C. (1984) Distribution and chromatographic characterization of gastrin and cholecystokinin in the rat central nervous system. *J. Neurochem.* 42, 1523–1536.
- Moldow, R.L. and Fishman, A.J. (1982) Radioimmunoassay of CRF-like material in rat hypothalamus. *Peptides* 3, 37–39.
- Mori, A., Hiramatsu, H., Kabuto, H. and Marescau, B. (1986) Effects of emotional stress on E1-mouse convulsions and their biochemical background. *Neurochem. Res.* 11, 37–45.
- Mori, T., Kaizuka, Y., Hori, T. and Nakashima, T. (1989) Effects of intracerebral injection of  $\beta$ -endorphin on the cytotoxic activity of natural killer cells in the spleen. *Jpn. J. Physiol.* 39 (suppl.), s118.
- Nakai, Y., Nakao, K., Oki, S. and Imura, I. (1978). Presence of im-

- munoreactive  $\beta$ -LPH and  $\beta$ -EP in human placenta. *Life Sci.* 23, 2013–2018.
- Nakamura, H., Moroji, T., Nohara, S., Nakamura, H. and Okada, A. (1992) Activation of cerebral dopaminergic systems by noise and whole-body vibration. *Environ. Res.* 57, 10–18.
- Newnham, P.J., Tomlin, S., Ratter, S.J., Bourne, G.L. and Rees, L.H. (1983) Endogenous opioid peptides in pregnancy. *Br. J. Obstet. Gynaecol.* 90, 535–538.
- Nistico, G., Caroleo, M.C., Arbitrio, M. and Pulvirenti, L. (1994) Evidence for an involvement of dopamine D1 receptors in the limbic system in the control of immune mechanisms. *Neuroimmunomodulation* 1, 174–80.
- Perez, L. and Lysle, D.T. (1995) Corticotropin-releasing hormone is involved in conditioned stimulus-induced reduction of natural killer cell activity but not in conditioned alterations in cytokine production or proliferation responses. *J. Neuroimmunol.* 63, 1–8.
- Pross, H.F., Baines, M.G., Rubin, P., Shragge, P. and Patterson, M.S. (1981) Spontaneous human lymphocyte-mediated cytotoxicity against tumor target cells. The quantitation of natural killer cell activity. *J. Clin. Immunol.* 1, 51–63.
- Ratcliffe, W.A., Carter, G.D., Dowsett, M., Hillier, S.G., Middle, J.G. and Reed, M.J. (1988) Estradiol assays: applications and guidelines for the provision of a clinical biochemistry service. *Ann. Clin. Biochem.* 25, 466–483.
- Reynolds, C.W., Timonen, T. and Herberman, R.B. (1981) Natural killer cell activity in the rat: isolation and characterization of the effector cells. *J. Immunol.* 127, 282–287.
- Roth, R.H., Tam, S.Y., Ida, Y., Yang, J.X. and Deutch, A.Y. (1988) Stress and the mesocorticolimbic dopamine system. *Ann. NY Acad. Sci.* 537, 138–147.
- Ryan, K.J. (1980) Placental synthesis of steroid hormones. In: D. Tulchinsky and K.J. Ryan (Eds.), *Maternal–Fetal Endocrinology*. Saunders, Philadelphia, pp. 245–260.
- Salmeron, O.J., Vaquer, S., Salmeron, I., Molto, L., Lapena, P., Manzano, L., de las Heras, J.I. and Alvarez Mon, M. (1991) Pregnancy is associated with a reduction in the pattern of the cytotoxic activity displayed by lymphokine-activated killer cells. *Am. J. Reprod. Immunol.* 26, 150–155.
- Shavit, Y., Lewis, J., Terman, G., Gale, R. and Leibeskind, J. (1984) Opioid peptides mediate the suppressive effect of stress on natural killer cell cytotoxicity. *Science* 223, 188–190.
- Shavit, Y., Terman, G.W., Martin, F.C., Lewis, J.W., Liebeskind, J.C. and Gale, R.P. (1985) Stress, opioid peptides, the immune system, and cancer. *J. Immunol.* 135, 834S–837S.
- Sieber, W.J., Rodin, J., Larson, L., Ortega, S., Cummings, N., Levy, S., Whiteside, T. and Herberman, R. (1992) Modulation of human natural killer cell activity by exposure to uncontrollable stress. *Brain Behav. Immun.* 6, 141–156.
- Silber, R.H., Busch, R.D. and Oslapas, R. (1958) Practical procedure for estimation of corticosterone or hydrocortisone. *Clin. Chem.* 4, 278–285.
- Song, C., Earley, B. and Leonard, B.E. (1995) Behavioral, neurochemical, and immunological responses to CRF administration. Is CRF a mediator of stress? *Ann. NY Acad. Sci.* 771, 55–72.
- Van Oers, J.W., Hinson, J.P., Binnekade, R. and Tilders, F.J. (1992) Physiological role of corticotropin-releasing factor in the control of adrenocorticotropin-mediated corticosterone releasing from the rat adrenal gland. *Endocrinology* 130, 282–288.
- Vamvakopoulos, N.C. and Chrousos, G.P. (1993) I: Evidence of direct estrogenic regulation of human corticotropin-releasing hormone gene expression. Potential implications for the sexual dimorphism of the stress response and immune/inflammatory reaction. *J. Clin. Invest.* 92, 1896–902.
- Weber, R.J. and Pert, A. (1989) The periaqueductal gray matter mediates opiate-induced immunosuppression. *Science* 245, 188–190.
- Yoshimi, H., Matsukawa, S., Sueoka, S., Fukae, M., Yokota, M., Hirata, Y. and Imura, H. (1978) Radioimmunoassay for  $\beta$ -endorphin: presence of immunoreactive ‘big–big’  $\beta$ -endorphin (‘big’-lipotropin) in human and rat pituitaries. *Life Sci.* 22, 2189–2195.
- Zalcman, S., Irwin, J. and Anisman, H. (1991) Stressor-induced alterations of natural killer cell activity and central catecholamines in mice. *Pharmacol. Biochem. Behav.* 39, 361–366.