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Adaptive Response of Stress Hormones and Neuronal Activity of the Paraventricular Nucleus to the Same Repeated Stressor in Rats

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ABSTRACT

To investigate the effects of previous experience on stress response, rats were exposed to a stressor repeatedly and changes in plasma concentrations of stress hormones were measured. Rats were exposed to electric footshocks for 1 day (the first exposure (1S) group) or consecutive 3 days (the repetitive exposure (3S) group). A retardation of the rate of decrease from the peak corticosterone concentration, and an acceleration of the rate of decrease from the peak epinephrine concentration were observed in the repetitive exposure group as compared with the first exposure group. Moreover, a higher peak concentration of norepinephrine was observed in the repetitive exposure group than in the first exposure group. Immunohistochemical studies showed that the ratio of c-Fos and nitric oxide synthase (NOS) double stained cells/ c-Fos-positive cells increased in the paraventricular nucleus of the hypothalamus. The results suggest that previous experience of the same stressor results in the rapid termination of the energy consuming response, and the persistence of the energy- saving response. Therefore, it is speculated that memory of a previous stressful experience contributes to the development of a successful strategy for coping with the facing stressor.

Key words: stress response, stressor, coping strategy, footshock

INTRODUCTION

Stressor is an environmental stimulus which elicits stress response. The environmental stimulus can be a physical or psychic nature (Whang, 1977). Stress response is a force to maintain homeostasis, and it may be considered to have 3 stages, an alarm reaction, a resistance stage and finally an exhaustion stage. This phenomenon is referred to as the "general adaptation syndrome" (Selye, 1978).

Stress response starts with a change in the activity of the noradrenergic system in the locus ceruleus, subsequently many responses occur serially, these include behavioral, neuro-hormonal and immune system responses (Heuther, 1996; Feldman et al., 1997). These responses are under the con-

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trol of the sympathetic adrenomedullary and the hypothalamic-pituitary-adrenocortical systems. The activation of the sympathetic adrenomedullary system results in the release of epinephrine and norepinephrine from the medulla of the adrenal gland, and the activation of the hypothalamic-pituitary-adrenocortical system results in the release of cortisol from the cortex of the adrenal gland. These hormones induce physical responses to allow adaptation to stressful situations, and suppress responses that are not necessary for the efficient management of a stressful situation. Epinephrine sends stored physical energy to muscles to facilitate fight or flight, and improves cardiovascular function, allowing greater access to oxygen and nutrients. On the other hand, cortisol stores physical energy and suppresses digestive, growth, and the inflammatory, immune and reproductive functions (Sapolsky et al., 2000). Cortisol inhibits every steps of the hypothalamic-pituitary-adrenocortical system by utilizing negative feedback mechanisms. In particular, cortisol influences the hippocampus, an important organ for learning and memory, and the hippocampus in turn regulates the activity of the paraventricular nucleus. On the other hand, the sympathetic adrenomedullary system activates the locus ceruleus which has a positive feedback relationship with the paraventricular nucleus (Koob, 1999).

Neuronal activity can be measured by examining expression of c-Fos protein immunohistochemically. Fos was originally a viral transcription factor. Cellular Fos (c-Fos) is the mammalian homolog protein of the viral Fos, and the c-Fos-producing gene c-fos is highly expressed immediately after stimulation in various neuronal cells but is not expressed without such stimulation. Therefore, the expression of c-Fos is regarded as an index of neuronal activity (Purves et al., 2004b). Nitric oxide (NO) is a diffusible gas, with a recently proven neuromodulatory role, which is produced during the metabolic conversion of arginine to citrulline, a reaction catalyzed by nitric oxide synthase (NOS) (Purves et al., 2004a). NO has many roles in several important neuronal functions, especially in learning and memory (Da Cunha et al., 2005; Koylu et al., 2005).

In this study, we examined the effects of a previous stressful experience on stress response by comparing changes in the concentrations corticosterone, epinephrine and norepinephrine after single or repetitive exposure to the same stressor. A role of the central stress response system was studied by immunohistochemical quantifications of c-Fos and NO synthase in the paraventricular nucleus.

MATERIALS AND METHODS

Experimental schedule

Stress responses were measured immediately after exposing Sprague-Dawley rats to electric footshocks for 1 day (the first exposure group, the 1S group) or for consecutive 3 days (the repetitive exposure group, the 3S group). Time-dependent stress response was investigated by determining the blood concentrations of stress hormones (epinephrine, corticosterone and norepinephrine), which were measured immediately after (0 min), and 15 min, 30 min, 60 min and 120 min after exposure to the footshock. Moreover, at the same time, c-Fos-positive and NADPH diaphorase-positive cells were counted in the hypothalamic paraventricular nucleus. The involvement of the serotonin system was also investigated by measuring the expression of serotonin transporter mRNA in the raphe nucleus. Stress due to exposure to the inert footshock equipment was measured in the context group. These animals, which were exposed to the unit for the same times as the other animals, were not administered footshocks.

In the day of experiment, 12 rats of the first exposure group and 12 rats of the repetitive exposure group, which had already been exposed to footshocks for 2 consecutive days, were administered footshocks as described below. Six ml of blood was collected from the heart of the anesthetized rats at set times listed above. Brains were removed after fixation by cardiac perfusion. The experiment was repeated 3 times, then 6 rats were assigned to each group per time point.

Animals

Sprague-Dawley rats were purchased from the Dae-Han Biolink Ltd.(Seoul, Korea), and adapted to the experimental environment at least for 1week. All animal experiments were approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University, and animals were cared for in

accord with The Guide for Animal Experiments edited by the Korean Academy of Medical Sciences (2000), which are consistent with the NIH Guideline Guide for the Care and Use of Laboratory Animals, 1996 revised. In brief, Sprague Dawley rats were cared for in a specific pathogen free (SPF) barrier area under a 12:12 hr light:dark cycle (lights on 07.00 hr) at a temperature (22±1°C) and a humidity of 55%. Rats were randomly assigned to the groups, and identified by ear piercing.

Exposure to footshocks

After 10 min acclimatization, a rat was administered electric shocks to the feet using a footshock unit (Med Associates, USA). Footshocks were delivered 10 times at interval of 30 seconds. Each footshocklasted for 5 seconds at a constant current 0.6 mA. Experimental groups were allocated to the first exposure group (the 1S group, a single day's exposure) and the repetitive exposure group (the 3S group, 3 days of exposure).

Concentration of plasma corticosterone

Blood samples from the left ventricle of the anesthetized rats were placed into EDTA-containing tubes, and the tubes were centrifuged for 10 min at 2,000 rpm. The supernatant was stored in −70°C until required. The concentration of plasma corticosterone was measured by radioimmunoassay. Briefly, the antigen-antibody reaction was performed using the plasma sample and a ^{125}Iodine labeled rat corticosterone Radioimmunoassay Kit (DPC Co., CA, USA), and plasma corticosterone levels were quantified using a gamma counter (Packard Instrument Company Inc., USA).

Concentration of plasma epinephrine and norepinephrine

One ml of stored plasma was mixed with 25 mg of alumina and 50 μl of 3 M Tris EDTA buffer, and then stirred vigorously for 10 min. After the epinephrine and norepinephrine had been completely adsorbed onto the alumina, it was washed 3 times with distilled water, and then the water was removed by spin-dryimg. Epinephrine and norepinephrine were extracted by adding 0.1 M HCl 100 μl, and 30 μl of the extract was injected into a HPLC-ECD system which consisted of a Waters 717 autosampler, 510 pump (Waters Instruments, MA, USA), a column C18 ODS 5 μm; 250 mm×4.5 mm diameter (Bio Analytical System, IN, USA) and an electrochemical detector (Coulochem II #5200A; ESA Inc., MA, USA), operated at a at Guard cell voltage of 320 mV, Electrode cell voltage of 240 mV, and a sensitivity of 200 nA. The mobile phase consisted of 50 mM acetate, 1.25% glacial acetic acid, 1,34 mM sodium octyl sulfonate, 0,27 mM EDTA and 25% Methanol (pH 3.35). The flow rate used was 0.8 ml/min, the peak areas (μV x sec) were equated to concentrations using the external standard method.

NADPH diaphorase staining and c-Fos immuohistochemistry

Rats were anesthetized with 50 mg/kg sodium pentobarbital i.p. and transcardially perfused first with heparinized isotonic saline (0.9% NaCl, 0.5% $NaNO_2$), then with ice-cold paraformaldehyde (PFA) (4% PFA, 0.1 M phosphate buffer, pH 7.2). Brains were immediately excised, postfixed for six hours in the same fixative and cryoprotected with a 30% sucrose solution for 24 hours prior to sectioning. Brains were coronally sectioned at 40 μm thickness using a freezing, sliding microtome (MICROM Laborgerate GmbH, Walldorf, Germany). Free-floating tissue sections, which had been completed NADPH diaphorase staining, were washed twice for 15 min in 0.1 M sodium phosphate buffered saline (PBS), and then permeabilized in 0.2% Triton, 1% bovine serum albumin (BSA) in PBS for 30 min. After washing twice in PBS-BSA, the sections were incubated for 16 hours with monoclonal mouse anti-c-Fos antiserum at a dilution of 1 : 20,000 (Oncogene, USA.). The sections were then washed twice in PBS-BSA and incubated for one hr with biotinylated anti-mouse antibody at a dilution of 1 : 200. Bound secondary antibodies were amplified using the ABC kit (Vectastain ABC Kit, Vector Laboratories, Inc., CA, USA)., and antibody complexes were visualized with 0.05% of diaminobenzidine for 5 min. Immunostained sections were mounted on gelatin-subbed slides in 0.05 M phosphate buffer, air dried, dehydrated through graded ethanol to xylene, and coverslipped. Sections were examined under an optical microscope equipped with an MCID imaging system (MCID, Imaging Research Inc., Ontario, Canada).

RESULTS

Time-dependent concentration changes of plasma corticosterone after exposure to footshocks

Results are illustrated in Fig. 1. The context group showed no differences in plasma concentrations of corticosterone according to the number of sham exposures to the footshock. The 1st and 3rd exposures showed levels of 309.6±5.5 ng/ml and 317.6±25.5 ng/ml, respectively. Corticosterone concentrations increased immediately after footshock in the 1S and 3S groups by 57% or 77%, respectively. Reductions in the peak corticosterone concentrations differentiated the 1S and 3S groups ($p<0.05$, repeated measures ANOVA, 15~120 min after exposure). The 1S group showed a reduced and stable corticosterone concentration at 15 min, while the 3S group showed a similarly reduced concentration at 30 min. Therefore, a significantly higher plasma concentration was present in the 3S group (498.5± 19.4 ng/ml for 3S; 378.8±26.9 ng/ml for 1S) at 15 min after exposure to footshock ($p<0.01$, Student's *t*-test).

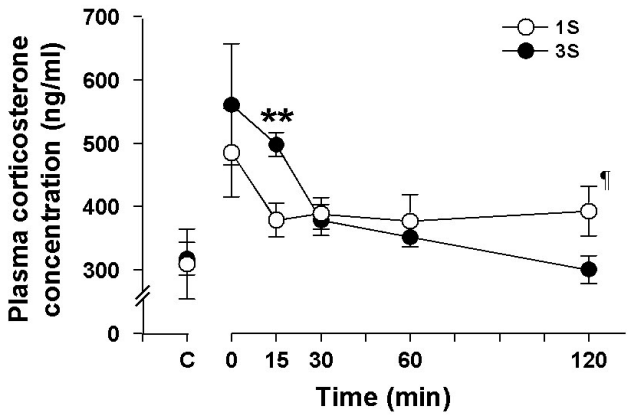

Fig. 1. Time-dependent concentration changes of plasma corticosterone after exposure to footshocks. 1S: the one-day exposure group, 3S: the 3-day exposure group. **: $p<0.01$: compared with the 1S group, Student *t*-test. ¶: $p<0.05$: compared with the 3S group, repeated measures ANOVA (15~120 min).

Time-dependent concentration changes in plasma epinephrine and norepinephrine after exposure to footshocks

Changes in the concentrations of epinephrine are illustrated in Fig. 2. The context group showed no differences in plasma epinephrine concentration according to the number of sham exposures to the footshock. The 1S group showed 55.7±1.0 ng/ml and the 3S group 54.4±0.9 ng/ml. No significant changes were noted immediately after exposure to footshocks in the 1S and 3S groups (1S: 55.6±13.7 ng/ml, 3S: 52.2±1.7 ng/ml). However, the epinephrine concentrations increased by about 20% at 15 min (1S: 63.7±2.3 ng/ml, 3S: 62.8±1.2 ng/ml) in both groups, and then decreased. Interestingly, different patterns of decrease were found in the 1S and 3S groups. At 120 min after exposure, the 3S group showed a marked reduction in concentration (39.4±3.4 ng/ml, i.e., 74% of that of the 1S group) vs. the 1S group (53.1±2,4 ng/ml) ($p<0.05$, Student *t*-test), whereas both groups showed similar concentrations until 60 min after exposure.

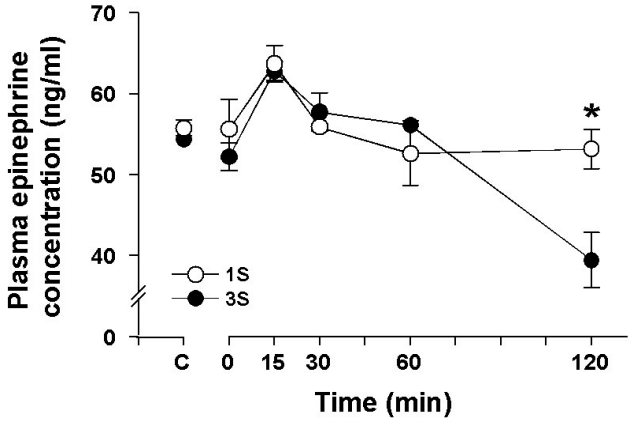

Fig. 2. Time-dependent concentration changes of plasma epinephrine after exposure to footshocks. 1S: the one-day exposure group, 3S: the 3-day exposure group. *: $p<0.05$: compared with the 3S, Student *t*-test.

The patterns of norepinephrine change (Fig. 3) differed from those of epinephrine. In the context group, the number of sham exposure to footshock resulted in different concentrations ($p<0.05$, Student *t*-test) in which the 3S context group (171.1±2.3 ng/ml) showed higher norepinephrine concentrations than the 1S context group (142.4±10.4 ng/ml). Plasma concentrations of norepinephrine were elevated after exposure to footshocks in both of the 1S and 3S groups. However, the peak concentration was reached at 15 min in the 1S group (158.4±12.7 ng/ml), and at 60 min in the 3S group (174.6±3.7 mg/ml). This data indicates that the release of norepinephrine was more protracted in the 3S group.

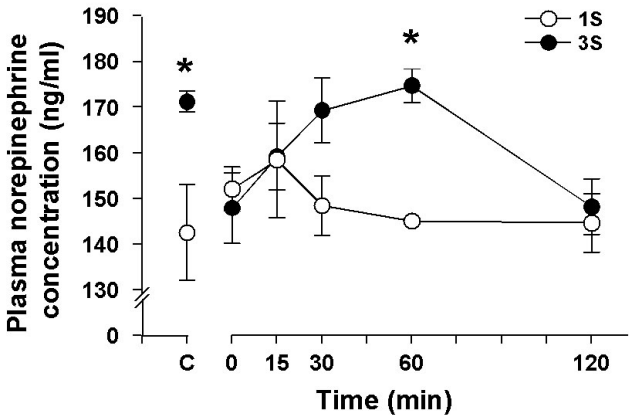

Fig. 3. Time-dependent concentration changes of plasma norepinephrine after exposure to footshocks. 1S: the one-day exposure group, 3S: the 3-day exposure group. *: $p<0.05$: compared with the 1S, Student *t*-test.

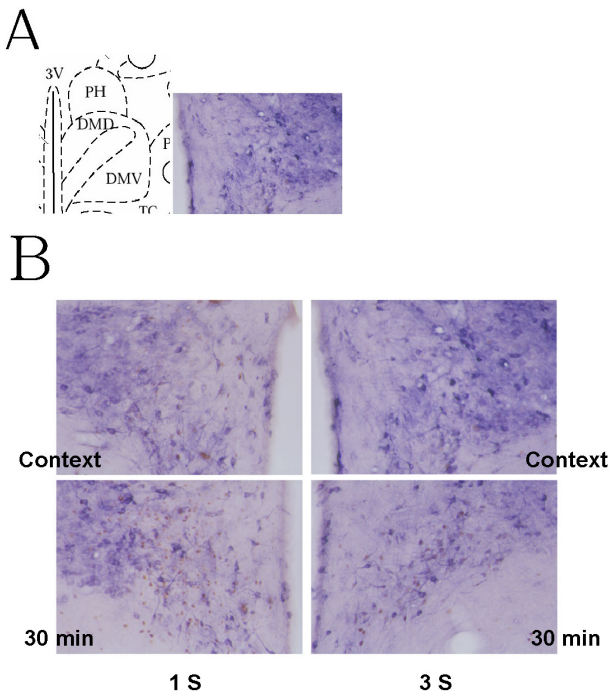

Fig. 4. Paraventriculatr nucleus of the hypothalamus (A), and c-Fos-positive (brown) and NADPH diaphorase-positive (blue) cells in the paraventricular nucleus (B). Context: the context group, 1S: the one-day exposure group, 3S: the 3-day exposure group

Expression of NADPH diaphorase-positive and c-Fos-positive cells in the paraventricular nucleus

The time-dependent expressions of NADPH diaphorase-positive and c-Fos -positive cells were examined in the paraventricular nucleus of the hypothalamus (Fig. 4A).

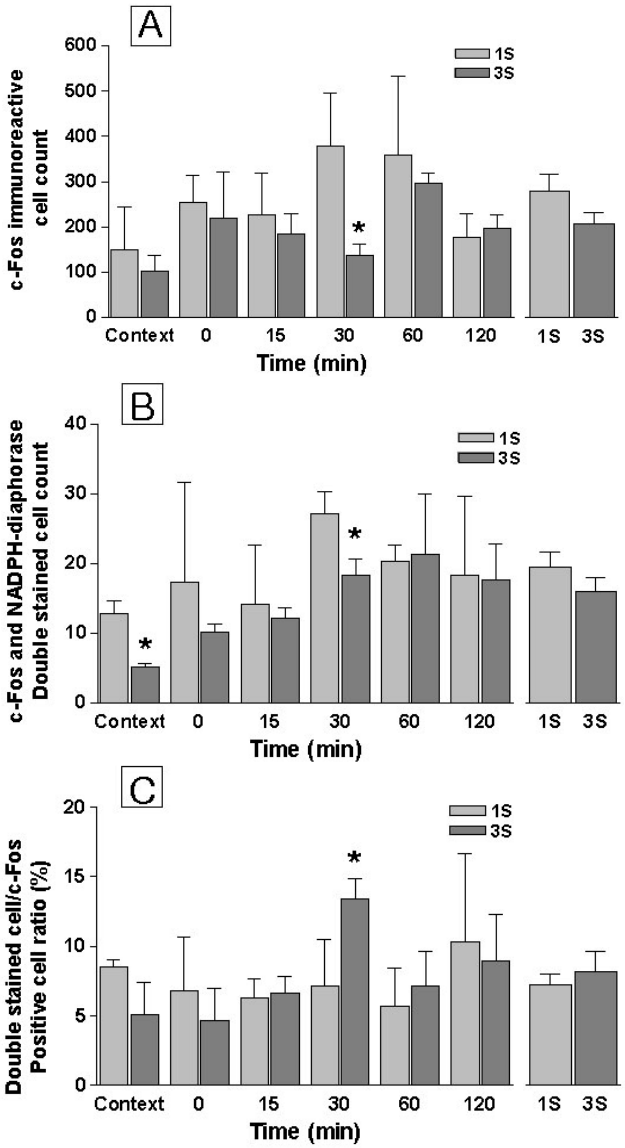

Fig. 5. Time-dependent expression of c-Fos-positive cells (A), c-Fos- and NADPH diaphorase double stained cells (B) and the ratio of double stained cells/c-Fos-positive cells (C) in the paraventricular nucleus. 1S: the one-day exposure group, 3S: the 3-day exposure group. The right panel shows mean values at 0, 15, 30, 60 and 120 min after exposure to footshocks. *: $p<0.05$: compared with the 1S group, Student *t*-test.

Time-dependent expression of c-Fos-positive cells was showed in Fig. 5. The context group showed no differences in the numbers of c-Fos-positive cells according to the number of sham exposure to the footshock. Both the 1S and 3S groups showed increased numbers of c-Fos-positive cells after exposure to footshocks. Marked greater increases in c-Fos-positive cells numbers ($p<0.05$, Student t-test) were observed in the 1S group (379±117 cells)

than in the 3S group (137±26 cells) at 30 min after exposure to footshocks although the summation of c-Fos-positive cells of all 5 measured times did not showed significant differences (Fig. 4B, 5A). c-Fos and NADPH diaphorase double stained cells were measured, and the ratio of double stained cells/ c-Fos-positive cells increased in the 3S group at 30 min after exposure to footshocks (Fig. 5C), whereas time-dependent pattern of double stained cells was generally similar to that of c-Fos-positive cells (Fig. 5B).

DISCUSSION

This study showed that stress responses are dependent upon the number of exposures to a stressor. Exposure to a stressor increases the plasma concentrations of stress hormones. However, a previous experience to the same stressor was found to influence the temporal nature of hormone expression. A retardation of the rate of decrease from the peak corticosterone concentration, and an acceleration of the rate of decrease from the peak epinephrine concentration were observed in the 3S group as compared with the 1S group. Moreover, a higher peak concentration of norepinephrine was observed in the 3S group than in the 1S group. Moreover, the ratio of c-Fos and nitric oxide synthase (NOS) double stained cells/c-Fos-positive cells increased in the paraventricular nucleus of the hypothalamus These results suggest that previous experience is an important determinant of the nature of stress response to the facing stressor.

When an organism is exposed to a stressful situation, it adapts to deal with the situation and may be at risk if the response is inappropriate. Epinephrine induces the energy-consuming response necessary for the fight/flight behavior, whereas corticosterone suppresses excessive physical response and promotes an energy-storing metabolism. Corticosterone also suppresses unnecessary responses, such as immune or inflammation reactions, allowing an animal to prepare for a subsequent stressor (Munck et al., 1984; De Kloet et al., 1998). The findings of this study suggest that previous memory of a stressor promotes an energy-storing reaction on exposure to the same stressor in order to prepare subsequent stressor that may occur. The facilitation of the energy-storing reaction is demonstrated by the retardation of the rate of decrease from the peak corticosterone concentration.

Changes in plasma epinephrine concentrations are highly sensitive, and an increase in epinephrine concentration occurs during the early phase of a stress response. A high epinephrine level after the sham exposure to the footshock in the context group supports this sensitivity. In the present study, a previous memory of the stressor quickly terminated the energy-consuming response reflected by an acceleration of the rate of decrease from the peak epinephrine concentration. It appears that reciprocal inhibition exists between corticosterone and epinephrine. In case of human, dissociation between reactivity of the hypothalamus-pituitary- adrenal axis and the sympathetic-adrenal-medullary system was also observed after repeated exposure to psychological stress (Schommer et al., 2003).

Origins of plasma norepinephrine are the adrenal medulla and the sympathetic nerve endings (Mazzeo et al., 1997). In the present study, the 3S context group showed higher norepinephrine concentration than the 1S context group, suggesting that an unpleasant memory of the previous experience had stimulated sympathetic nervous system. In the 3S group, the norepinephrine concentration is lower than that of the 3S context group immediately after exposure to footshocks. This is an unpredicted phenomenon which may be explained by a transient paralysis of the sympathetic nervous system induced by a strong stressor. And, the activity of the sympathetic nervous system was not as high in the 1S group because they lacked an unpleasant memory of footshock.

The 3S group showed lower neuronal activity after exposure to footshocks in the paraventricular nucleus than the 1S group, which suggests that previous experience had already generated a neuronal circuit that orchestrates an appropriate stress response. Operating an established neuronal circuit may not need as many active neurons as establishing a new neuronal circuit. That is, the previous experience induced a more efficient stress response. Similar to our results, c-FOS immunoreactivity or mRNA expression decreased after repeated restraint stress in the paraventricular nucleus (Carter et al., 2004; Tobe et al., 2005), and it was sug-

gested that amygdale may have some role in this adaptation (Carter et al., 2004). The hippocampus also seems to have an important role in this memory-mediated response, because it is the most important organ in terms of learning and memory (Fuchs and Flugge, 1998). It is possible that both NOS-positive and c-Fos positive cells transfer the previous memory to the hypothalamic-pituitary-adrenocortical system because the ratio of c-Fos and NOS double stained cells/c-Fos-positive cells increased in the 3S group, although this issue requires further clarification.

We observed changed stress response in stress hormones and neuronal activities after exposure to repeated same stressor. We believe that these changes are responsible for the adaptive response of an organism against future stressor because impaired habituation to repeated exposure to the same stressor could reflect a state of increased vulnerability for allostatic load.

In conclusion, the results of this study indicated that prior experience is importantly required to cope efficiently with stressors. The memory of a previous experience seems to alter those brain functions responsible for stress response, thereby allowing an organism to respond efficiently to a stressful situation. In fact, the epinephrine-induced energy-consuming reaction terminated quickly, and the corticosterone-induced energy-storing reaction lasted longer in the 3-day exposure group. It is concluded that an appropriate previous stressful experience may contribute to an organism's ability to successfully cope with stress.

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