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Increased IL-1β and Tyrosine Hydroxylase Immunoreactivity by Acute and Repeated Foot Shock Stress in the Locus Coeruleus

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ABSTRACT

The locus-coeruleus (LC), which is almost composed of noradrenergic neurons, plays an important role in modulating the central stress response. It has been well known that stress causes an increase in the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme for NE synthesis, in the LC and may also influence brain cytokine such as interleukin-1β (IL-1β) expression. However, relevance of IL-1β, the proinflammatory cytokine, and TH in the LC following exposure of stress has not been studied. The aim of the present study was to determine possible involvement of TH and IL-1β in the stress response in the LC. Exposure to acute electric foot shock (ten, 0.8 mA for 5 sec, foot-shocks) resulted in increased IL-1β in the LC, evidenced by increased IL-1β double labeled cells in TH immunoreactive cells, which were similar to those observed after treatment with lipopolysaccharide (LPS), compared with the non-treated group. Repeated daily exposure to the same foot shock stress tended to further increase IL-1β in the TH immunoreactive cells in the LC. The present results demonstrated that acute physical stress stimulated IL-1β production in the NE cells and that repeated stress may potentiate stress-induced IL-1β production in the NE cells in the LC. These findings raise the possibility that NE neurotransmitter system is modulated by IL-1β in the LC under conditions of acute and repeated foot shock stress.

Key words: foot Shock Stress, locus-coeruleus (LC), interleukin-1β (IL-1β), tyrosine hydroxylase (TH), immunoreactivity

INTRODUCTION

The locus coeruleus (LC), one of the most abundant areas containing norepinephrine (NE) neurons in the brainstem, is responsible for mediating sympathetic effects during stress. The LC is activated by stress, and responds by increasing NE secretion. Increased NE in the LC by stress innervates the spinal cord, the brain stem, cerebellum, hypothalamus, amygdala, nucleus accumbens and the cortex. So, NE is thought to play an important role in modulating the central stress response and may be involved in stress-related psychopathological conditions such as depression or anxiety (Moore and Bloom, 1979). Increase in tyrosine hydroxylase (TH), the rate-limiting enzyme for NE synthesis, mRNA expression was observed in the LC following restraint and foot shock stress

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in Sprague-Dawley rats (Smith et al., 1991; Chang et al., 2000; Scott et al., 2000). Also, repeated stress increases the expression of TH mRNA and protein in the LC (Melia and Duman, 1991; Rusnak et al., 2001; Shinya et al., 2002). Thus, activation of NE in the LC following stress may be a signal for stress response. Cathecholamines (such as NE) and cytokines are known to constitute a significant portion of the regulatory neuroimmune networks involved in maintaining homeostasis in the central nervous system (Asai, 1995; Johnson et al., 2005; Kaneko et al., 2005) Interleukin-1, one of pro-inflammatory cytokines, emanating from the immune system and from the brain itself is evoked by either certain psychological or physical stressor. It has been demonstrated that IL-1β, IL-1-converting enzyme, bioactivity and their receptors in the brain are involved in regulating stress responses (Minami et al., 1991; Shintani et al., 1995; Shintani et al., 1997; Nguyen at al., 1998). For instance, it was reported that immobilization stress and inescapable foot shock increased IL-1β mRNA and protein in various brain regions, including the hypothalamus and hippocampus in rats (Minami et al., 1991; Nguyen et al., 1998; Johnson et al., 2004; Deak et al., 2005). Commensurate with the notion that cytokine changes contribute to stressor-provoked neuronal alterations, the central administration of IL-1 receptor antagonist (IL-1Ra) prevented the stressor-elicited *in vivo* release of hypothalamic NE (Nguyen et al., 1998). These data provisionally suggest that IL-1β may play an intermediate role in promoting several cathecholaminergic changes associated with stressors. But no studies have addressed whether physical stressors such as foot shock can alter IL-1β levels and relevance of NE in the LC. Here, we addressed the following questions: (1) Does acute and repeated foot shock stress alter TH and IL-1β levels in the LC? (2) Do animals adapt or become sensitized to repeated foot shock stress as reflected by changes in TH and IL-1β expression? (3) How about the cross reactivity of TH and IL-1β in the LC? These questions performed in four groups of rats: (1) non-treated, (2) subjected acute injection of LPS (lipopolysaccharide; 0.1 mg/kg i.p.) as a positive control, (3) subjected to acute stress (ten, 0.8 mA for 5 sec, foot-shocks), (4) subjected to repeated same stress for 7 days.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighting 220～240 g were obtained from Orient Corp. (Gyeonggi-do, Korea). Animals were housed in groups of three with continuous access to food and water *ad libitum*; they were maintained on a 12 : 12 h light : dark cycle (lights on 08 : 00 h) regulated at 22°C room temperature. The experiments began at least 7 days after they had been acclimatized to their new environment. The experimental procedures were carried out according to the animal care guidelines of the NIH and the Catholic University Medical College Institutional Animal Care.

Experimental design

The experiment consisted of four groups (each groups consist of 10 animals): the nontreated group (nontreated animals), the LPS group (lipopolysaccharide; 0.1 mg/kg., i.p), the acute Foot Shock group (ten, 5-s duration, 0.8-mA foot-shocks), and the repeated Foot shock group (ten, 5-s duration, 0.8-mA 7-days foot-shocks).

Lipopolysaccharide Treatment

LPS group were injected intraperitoneally (i.p.) with 0.1 mg/kg dissolved in sterile, endotoxin-free 0.9% vehicle (LPS: Escherichia coli: 0111:B4 ; from Sigma-Aldrich, Pool, UK). 2 h after the LPS injection, rats were sacrificed.

Application of foot shock stress

To undertake foot shock experiments, we utilized a dark- colored chamber (28×23.5×20 cm), which has a floor grid for foot shock made of stainless steel rods of 3 mm in diameter with 10-mm intervals between the rods. A shock generator with the ability to vary the current intensity was connected to the floor grid through a shock scrambler. The generator and scrambler were switched on and off by means of a time controller set for the predetermined time period used for foot shock. Rats were given an electrical foot shock through the floor grid by delivering electric currents (ten, 0.8-mA, 5-s duration) and sacrificed immediately. Repeated Foot shock group was received same stress for 7 days.

Measurement of core body temperature.

Rats were deeply anesthetized with sodium pentobarbital (80 mg/kg, administered intraperitoneally) and core temperature was measured for three minutes.

Blood sampling and determination of corticosterone

Blood sampling was separated plasma frozen at −70°C until corticosterone assay.

Plasma corticosterone level was determined using of Corticosterone Enzyme Immunoassay Kit (assay designs, USA).

Immunofluorescence histochemistry

After completion of the behavioral testing, all of the animals were deeply anesthetized with sodium pentobarbital (80 mg/kg, administered intraperitoneally) and were then perfused through the ascending aorta with normal saline solution (0.9%), followed by 500 ml of 4% paraformaldehyde in 0.1 mol/L phosphate buffer. The brains were removed, postfixed overnight, and cryoprotected in 20% sucrose in 0.1 mol/L phosphate-buffered saline, at 4°C. The brains were cut, with cryostat sectioning, into 30-um coronal sections, which were histochemically processed as free-floating sections. Each section of the locus coeruleus was used in immunostaining, Primary goat polyclonal antibodies against the following antigens were used: TH (concentration, 1 : 1000; ZYMED, USA) and IL1β polyclonal (concentration, 1 : 200; R & D Systems, Minneapolis, MN). Primary antibodies were diluted with blocking solution (10% fetal bovine serum in phosphate-buffered saline- Triton, pH 7.4), and tissues were incubated for 24 hours at 4°C, with constant agitation. After being rinsed in phosphate-buffered saline, sections were incubated for 1 hours at room temperature with FITC anti-mouse (Vector Laboratories, Burlingame, CA) and Alexa Fluor 546 anti-goat (Invitrogen, Oregon, USA) secondary antibody diluted 100-fold in phosphate-buffered saline-Triton. After additional rinsing with phosphate-buffered saline. Sections were mounted on slides, air-dried, and coverslipped for microscopic observation. All slides were examined with a Confocal microscope (Bio Rad)and the TH cell color was temporarily changed by confocal system to clear double labeled cell color (green to blue). For cell measurements, Cells within the LC area were counted (300×300 μm).

Statistical Analyses

All data were analyzed using the statistical software SPSS (for windows OS). The data were expressed as the mean standard error of the mean (SEM). For the comparison among the groups, one-way ANOVA tests were performed and differences among the groups were considered statistically significant at $p < 0.05$.

RESULTS

Effect of foot shock on fever

The level of body temperature of rats in each group is shown in Fig. 1. Animals exposed to foot shock had significantly higher fever compared with the non-treated group and LPS group ($F(3,45) = 27.749$, $p < 0.001$). Also, rats repeatedly exposed to foot shock had higher fever ($F(3,45) = 27.749$, $p < 0.001$), but it's fever was not significantly different from that of the acute foot shock group (Fig. 1).

Plasma Corticosterone

Plasma corticosterone level was determined in each group of the rats. The acute foot shock stressor manipulation markedly elevated the levels of plasma corticosterone relative to the non-treated group ($F(3,36) = 21.511$, $p < 0.001$). The magnitude of the corticosterone response was similar to that of

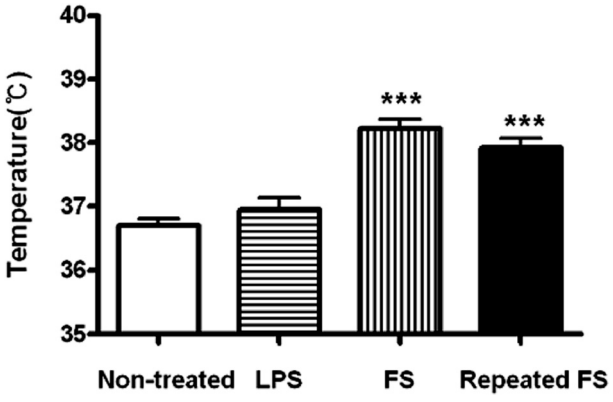

Fig. 1. Core body temperature in each group. Body temperature was measured for three minutes. Significance with Tukey's test following a one-way ANOVA is indicated as ***< 0.001. Vertical lines indicate S.E.M.

the repeated foot shock and LPS groups. With repeated exposure to the foot shock stress, there was a slight attenuation of the corticosterone elevation, but there was no significant difference between groups (Fig. 2).

Effect of foot shock stress on TH immunoreactivity

As shown in Fig. 3～5, TH immunoreactive cells in the LC were increased in the acute foot shock stress group like LPS group compared with the non-treated group ($F(3,21)=7.87$, $p<0.05$). Repeated foot shock group' TH immunoreactive cells in the LC further increased compared with the acute foot shock group but it had not significant difference among the test groups ($F(3,21)=7.87$, $p<0.005$).

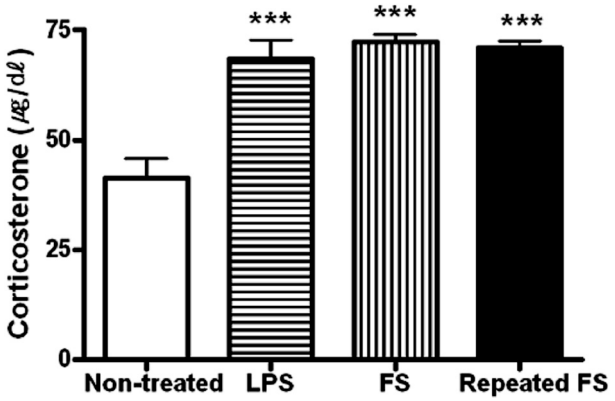

Fig. 2. Plasma corticosterones (㎍/dl) in each group. Significance with Tukey's test following a one-way ANOVA is indicated as ***<0.001 Vertical lines indicate S.E.M.

Effect of foot shock stress on the IL-1β immunoreactivity

As shown in Fig. 3～5, IL-1β immunoreactive cells in the LC were increased in the acute and repeated foot shock groups like as LPS group compared with the non-treated group ($F(3,21)=24.808$, $p<0.005$). IL-1β immunoreactive cells in the LC of the repeated foot shock group tended to be further increased compared with the acute foot shock group but it had not significant difference among the test groups ($F(3,21)=24.808$, $p<0.001$).

Effect of foot shock stress on the double labeled cells of TH and IL-1β

As shown in Fig. 3～5, most of IL- 1β immunoreactive cells were expressed in TH cells in the LC, so number of double labeled cells is same as TH immunoreactive cells.

DISCUSSION

Catecholamines and cytokines are known to constitute a significant portion of the regulatory neuroimmune networks involved in maintaining homeostasis in the central nervous system (Johnson et

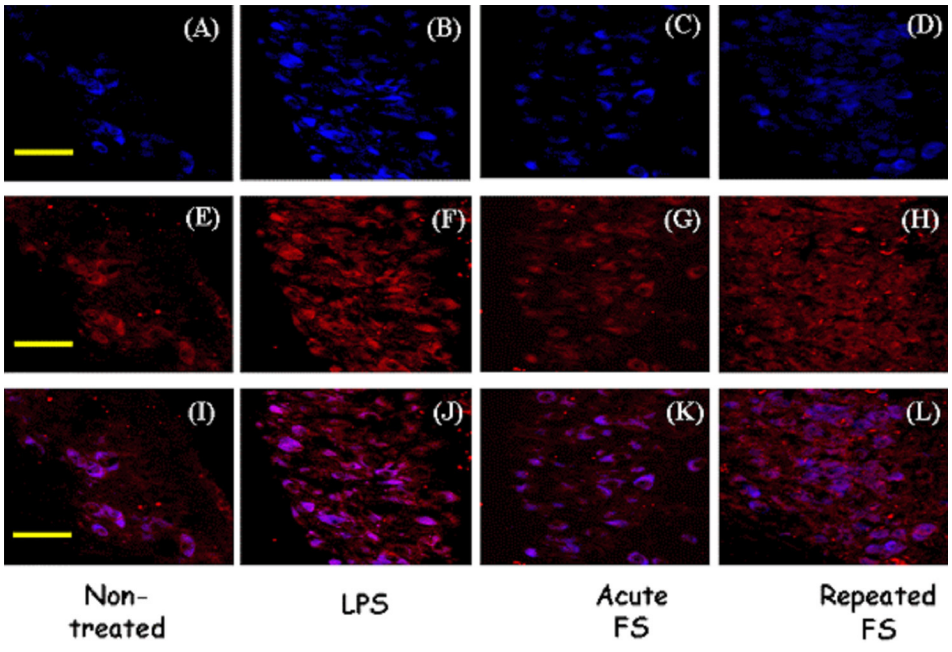

Fig. 3. Representative photographs showing TH and IL1β cells in the locus coeruleus region of rats. Panel (A～D) show TH reactive cells and panel (E～H) show IL1β reactive cells. Panel (I～L) show TH and IL1β double labeled cells. All the scale bar was 50㎛.

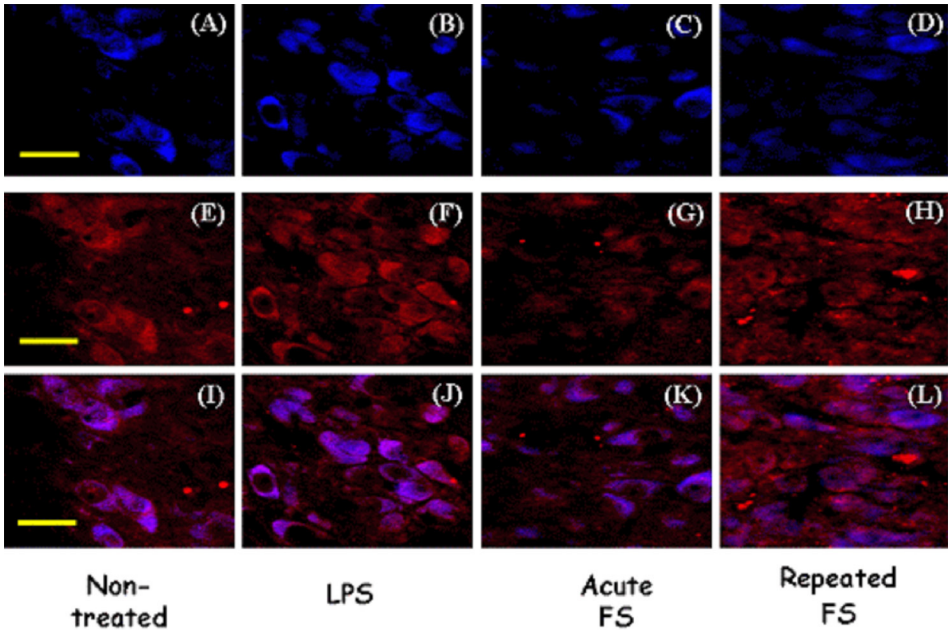

Fig. 4. Representative photographs showing TH and IL1β cells in the locus coeruleus region of rats. Panel (A~D) show TH reactive cells and panel (E~H) show IL1β reactive cells. Panel (I~L) show TH and IL1β double labeled cells. All the scale bar was 100 μm.

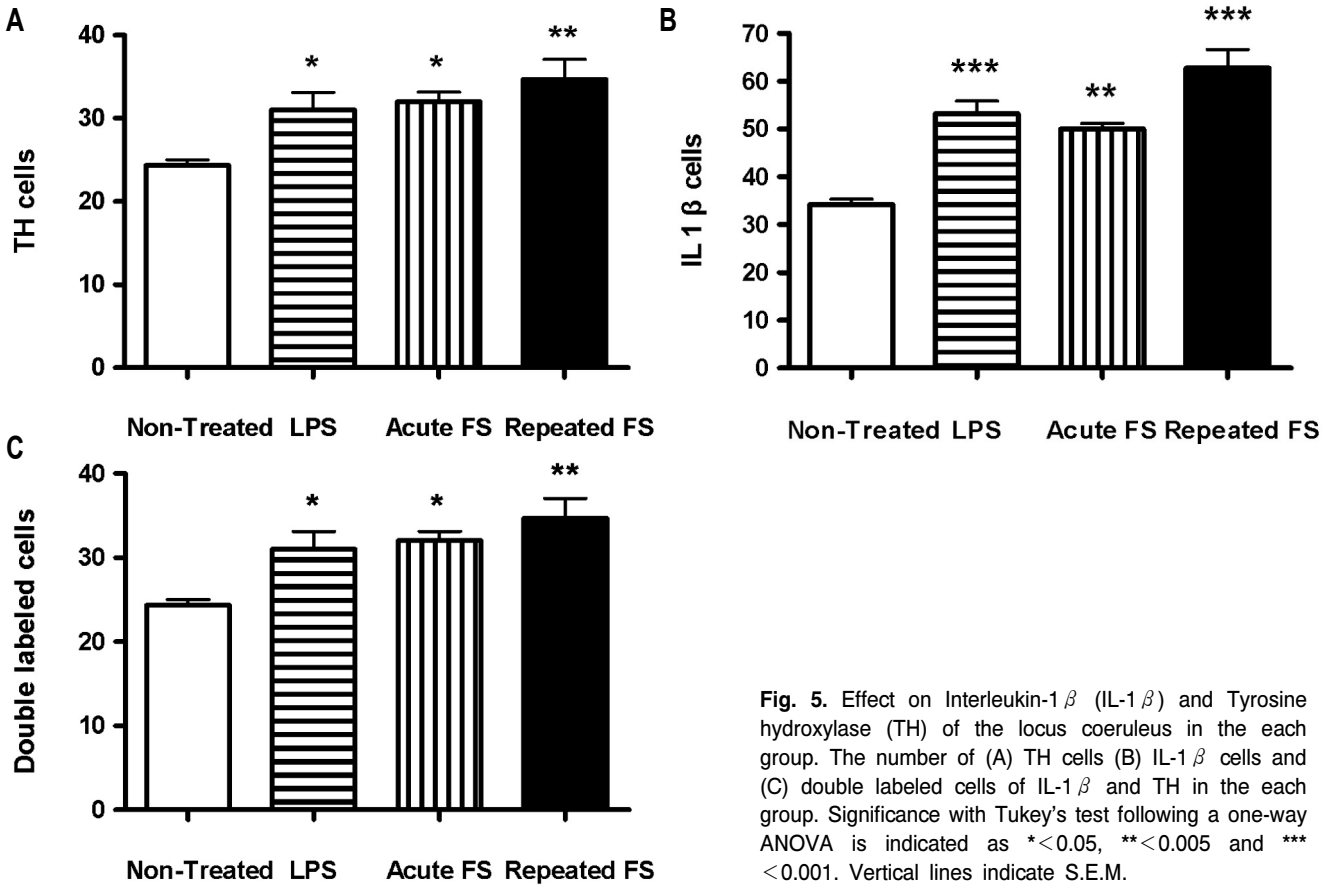

Fig. 5. Effect on Interleukin-1β (IL-1β) and Tyrosine hydroxylase (TH) of the locus coeruleus in the each group. The number of (A) TH cells (B) IL-1β cells and (C) double labeled cells of IL-1β and TH in the each group. Significance with Tukey's test following a one-way ANOVA is indicated as *<0.05, **<0.005 and ***<0.001. Vertical lines indicate S.E.M.

al., 2003; Kaneko et al., 2005, Yoko et al., 2005). Stress is the response to any physical, physiological, emotional and neuroendocrine stimulus that disrupts homeostasis and stress response comprises adaptive physiological processes in repeated threatening situations. The LC, which is almost

composed of noradrenergic neurons, plays an important role in modulating the central stress response and pro-inflammatory cytokines are activated in the LC by stress response. But, relevance of IL-1β, the pro-inflammatory cytokine released from immune cells or brain itself, and TH, the rate-limiting enzyme for NE synthesis, in the LC following exposure of stress has not been studied. So, we examined whether noradrenergic neurons in the LC interact with pro-inflammatory cytokine like IL-1β expression in the acute and repeated foot shock stress. As the first step of the experiment, body temperature was measured since stress can influence physiological processes including hyperthermia (Singer et al., 1986; Briese and Cabanac, 1991; Poole and Stephenson, 1997). Our data showed that a significant elevation of body temperature was caused after the foot shock stress exposure compared with the non-treated group and body temperature was slightly reduced after repeated foot shock stress exposure compared with acute foot shock group but still maintained up-regulation. But unlike the previous studies, body temperature was not increased at 2 h after the intraperitoneal (i.p.) injection of LPS as a positive control (Johnson et al., 2003; Kathryn et al., 2007). LPS group was used as a positive control since peripheral injection of LPS, an endotoxin released from the outer membranes of gram negative bacteria, enhances expression of inflammatory cytokines in the LC and other brain regions (Quan et al., 1994; Kong et al., 1997; Pitossi et al., 1997) and induced alteration of catecholamines in the LC and other brain regions (Lavicky and Dunn, 1995; Lacosta et al., 1999; Yoko et al., 2005). Plasma corticosterone level was also determined since this hormone is a major indicator of stress. After foot shocks plasma corticosterone levels were significantly increased in both acute and repeated foot shock groups like treatment with LPS, consistent with other's results (Yelvingron et al., 1984; Yelvingron et al., 1987; Ratner et al., 1989). Under these stress models, we used measures of TH immunoreactivity levels in the LC as an index of NE activity because TH is the rate limiting enzyme in NE synthesis, and because of data that suggest an association between TH expreassion levels and the LC firing rate (Chang et al., 2000; Scott et al., 2000; Rusnak et al., 2001). Data on immunofluorescence histochemistry showed that the expression levels of TH in the LC were very increased in LPS, acute and repeated foot shock stress groups compared with the non-treated group. To date, in various studies showed acute stress enhances TH levels in the LC (Smith et al., 1991; Chang et al., 2000; Scott et al., 2000) and also repeated stress group does (Rusnak et al., 2001; Shinya et al., 2002). Therefore these data consistent with other's results however repeated exposures to the same daily stress fail to habituate in repeated foot shock stress group. TH expression of repeated foot shock group was slightly increased compared with acute foot shock group but did not achieve statistical significance. Likewise, repeated foot shock group's hypersensitization also showed in IL-1β immunofluorescence histochemistry. Also, acute and repeated foot shock stress as well as LPS increased double labeled IL-1β and TH expression in the LC. Although the source of IL-1β was not addressed in the present study, several experiments have examined that rat microglia express mRNA for IL-1β after peripheral LPS injection (Buttini and Boddeke, 1995; Van Dam et al., 1995). All the expression of TH cells in the LC was effected with IL-1β, so double labeled cells number is same as TH immunoreactive cells. These results suggest that NE synthesis could be increased either by changes in the activity of pre-existing tyrosine hydroxylase or by induction of new TH protein by stimulation of IL-1β.

In summary, acute foot shock produced increase in IL-1β of TH immunoreactive cells in the LC, evidenced by increased IL-1β double labeled cells, which was similar to those observed after treatment with LPS, compared with the non-treated group. Repeated daily exposure to the same foot shock stress group resulted in further increased expression of IL-1β in the TH immunoreactive cells in the LC. The present results demonstrate that acute physical stress stimulates IL-1β production in the NE cells and that repeated stress may potentiate stress-induced IL-1β production in the NE cells in the LC. These findings raise the possibility that NE neurotransmitter system is modulated by IL-1β in the LC under conditions of acute and repeated foot shock stress.

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