Hyperalgesia in an immobilized rat hindlimb: effect of treadmill exercise using non-immobilized limbs

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Abstract

Cast immobilization of limbs causes hyperalgesia, which is a decline of the threshold of mechanical and thermal mechanical stimuli. The immobilization-induced hyperalgesia (IIH) can disturb rehabilitation and activities of daily living in patients with orthopedic disorders. However, it is unclear what therapeutic and preventive approaches can be used to alleviate IIH. Exercise that activates the descending pain modulatory system may be effective for IIH. The purpose of this study was to investigate the effects of treadmill exercise during the immobilization period, using the non-immobilized limbs, on IIH. Thirty-six 8-week-old Wistar rats were randomly divided into 1) control, 2) immobilization (Im), and 3) immobilization and treadmill exercise (Im+Ex) groups. In the Im and Im+Ex groups, the right ankle joints of each rat were immobilized in full plantar flexion with a plaster cast for an 8-week period. In the Im+Ex group, treadmill exercise (15 m/min, 30 min/day, 5 days/week) was administered during the immobilization period while the right hindlimb was kept immobilized. Mechanical hyperalgesia was measured using von Frey filaments every week. To investigate possible activation of the descending

pain modulatory system, beta-endorphin expression levels in hypothalamus and midbrain periaqueductal gray were analyzed. Although IIH clearly occurred in the Im group, the hyperalgesia was partially but significantly reduced in the Im+Ex group. Beta-endorphin, which is one of the endogenous opioids, was selectively increased in the hypothalamus and midbrain periaqueductal gray of the Im+Ex group. Our data suggest that treadmill running using the non-immobilized limbs reduces the amount of hyperalgesia induced in the immobilized limb even if it is not freed. This ameliorating effect might be due to the descending pain modulatory system being activated by upregulation of beta-endorphin in the brain.

Keywords: Immobilization, hyperalgesia, treadmill exercise, beta-endorphin, hypothalamus, midbrain periaqueductal gray

1. Introduction

In experimental studies using animal, cast immobilization of limbs causes hyperalgesia, which is a decline of the threshold of mechanical and thermal mechanical stimuli [1, 2], and limb immobilization for long durations may induce chronic pain associated with central sensitization [3]. The hyperalgesia induced by cast immobilization was also observed in humans [4]. The immobilization-induced hyperalgesia (IIH) can disturb rehabilitation and activities of daily living in patients. Cast immobilization is carried out for restoring damaged tissue and is widely used in the medical treatment of orthopedic disorders such as fracture and sprain. During the immobilization period, exercise is prescribed to prevent muscular disuse atrophy mainly in the non-immobilized limbs, and is not prescribed for prevention of IIH in the immobilized limb. Although exercise may alleviate IIH, the immobilized limb is not moved directly, and the mechanism by which IIH of the affected limb can be treated remains unknown.

Clinical studies suggest that exercise decreases pain symptoms and improves function in patients with chronic pain due to various diseases such as rheumatoid arthritis, fibromyalgia, and complex regional pain syndrome

[5-7]. Recent studies using experimental animals have shown that beta-endorphin is involved in the mechanism by which exercise affects pain. Beta-endorphin is an endogenous opioid with analgesic properties and is distributed throughout the brain by axons emanating from the hypothalamus. Stagg et al. reported that exercise training on a treadmill ameliorated thermal and tactile hyperalgesia in spinal nerve-ligated animals; the effects of exercise were reversed by systemically administered opioid receptor antagonists. Moreover, exercise increased beta-endorphin content in the rostral ventromedial medulla and in the midbrain periaqueductal gray (PAG) [8]. Thus, exercise can activate the descending pain modulatory system via upregulation of beta-endorphin in the central nerve system.

Considering the therapeutic effect of beta-endorphin on pain, one is led to the hypothesis that though the immobilized limb is not moved, the upregulation of endorphins induced by the exercise of the non-immobilized limbs may suppress IIH in the immobilized limb via activation of the descending pain modulatory system. To test this hypothesis, treadmill exercise was administered to rats in which the right hindlimb was

immobilized by casting, and any resulting changes in IIH and content of endorphins in the midbrain relative to cast-treated but unexercised rats were examined.

2. Materials and methods

2.1. Animals

Male Wistar rats (n = 36; 8 weeks old; Kudo Laboratories, Tokyo, Japan) were randomly divided into three groups: immobilization for 8 weeks (Im, n = 10); immobilization for 8 weeks and administered treadmill exercise (Im+Ex, n = 16); and age-matched controls (Con, n = 10). A flow diagram of the experimental design is presented in Figure 1. All rats were housed in twos or threes in plastic cages at 22–24 ºC with a 12-h light/dark cycle. Water and food were available ad libitum. All procedures received approved by the Nagasaki University Animal Care Committee (approval number: 1305201061) and complied with the recommendations of the International Association for the Study of Pain.

2.2. Immobilization

Rats in the Im and Im+Ex groups were anesthetized with sodium pentobarbital (40 mg/kg). Subsequently, their right ankle joints were fixed in full plantar flexion by using plaster casts (ipsilateral hindlimb). Left ankle joints were not immobilized (contralateral hindlimb). The plaster cast was replaced at least every two or three days to prevent loosening, and to prevent edema in the hind paw. The period of cast immobilization was 8 weeks.

2.3. Treadmill exercise

Before the experiment, rats of the Im+Ex group were acclimated to the treadmill lanes without running (30 min/day, 1 week). During the immobilization period, rats of the Im+Ex group were administered forced treadmill running (15 m/min, 30 min/day, 5 days/week). The immobilization of the right hindlimb continued during treadmill running. Thus, these rats ran using their three non-immobilized limbs: forelimbs bilaterally and left hindlimb.

2.4. Behavioral test

All rats were tested for mechanical sensitivity before the immobilization period and once every week following application of the cast. The tests were performed using a home-made restrainer made of cloth, as described previously [1]; this technique was employed because the experimental ankle joint contracture prevented the immobilized rats from placing their right hind paws on the ground. The rats were placed individually in the restrainer after cast removal and allowed to acclimate for 20 min in a quiet room from 10:00 a.m. to 6:00 p.m.; room temperature was maintained at 22–24 ºC. The glabrous skin of the hind paw was probed ten times with 4- and 15-g von Frey filaments (VFF; North Coast Medical, Morgan Hill, CA, USA) every 10 s. Lifting or pulling back of the paw was counted as a paw withdrawal response (PWR) [9]. Where applicable, measurements were performed 23 h after exercise training. Immediately after the behavioral test, a plaster cast was again applied to rats in the immobilization groups to continue the immobilization period.

2.5 Sampling and preparation

At the end of the immobilization period, 23 h after the final period of

treadmill running in the Im+Ex group, all rats were deeply anesthetized with sodium pentobarbital (40 mg/kg). Rats were transcardially perfused with saline for blood removal and the whole brain was extracted. The hypothalamus was immediately dissected from the brain and stored in a deep freezer (-80 ºC) until enzyme-linked immunosorbent assaying for beta-endorphin was performed. Next, the midbrain was dissected out and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 48 h. After fixation, the midbrains were soaked successively in 10% and 20% sucrose in 0.01 M phosphate buffer saline (PBS, pH 7.4) for 24 h. The tissues were then embedded in tragacanth gum and frozen by immersion in liquid nitrogen-cooled isopentane.

2.5.1 Enzyme-linked immunosorbent assay for beta-endorphin in hypothalamus

All rat hypothalami were homogenized in 10 mM Tris-HCl buffer (pH 7.4) at 4 ºC. The homogenate was centrifuged (10,000 rpm, 10 min), and the supernatant stored at -80 ºC. The level of beta-endorphin in a hypothalamus sample was examined using an enzyme immunoassay kit (Phoenix

Pharmaceuticals, Inc., USA, EK-022-33). The range of validity of the kit was 0–100 ng/mL of beta-endorphin. The protein content of each tissue supernatant was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Concentrations of beta-endorphin were normalized to protein content.

2.5.2 Immunohistochemistry

Frozen serial sections (10 μm) were cut using a cryostat (CM1950, Leica, Germany), and coronal sections including the middle levels of the PAG were selected for immunohistochemical analysis. Immunohistochemical staining for beta-endorphin was performed using the polyclonal anti-beta-endorphin antibody. Briefly, sections were blocked with 5% bovine serum albumin in PBS (pH 7.4) for 30 min at room temperature, and then incubated overnight with anti-beta-endorphin antibody (1:3,000; ImmunoStar Inc., Hudson, WI, USA) at 4 ºC. After rinsing in PBS, the sections were incubated with goat anti-rabbit IgG labeled with Alexa Fluor 594® (1:1000, Molecular Probes; Eugene, OR, USA) for 1 h at room temperature.

To analyze the distribution of beta-endorphin in the PAG, two stained

sections were selected from each rat. Using an upright fluorescent microscope (Eclipse H600L; Nikon Corp., Tokyo, Japan) fitted with a high-performance camera (DS-Ri1), micrographs were obtained at 200× magnification. The immunofluorescence intensity in the PAG was measured in five randomly chosen areas (100 × 100 pixels) using image analysis software (NIS-Elements D 3.00; Nikon Corp.). The immunofluorescence intensity was evaluated using two micrographs from each rat. Image analysis data were scaled by the average value of the Con group, which was set to 1.

2.6 Statistical analysis

All data are presented as the mean ± SE. For comparison of groups, a Kruskal–Wallis test (non-parametric one-way ANOVA) followed by Bonferroni's multiple comparison test was used. In the analysis of the behavioral results, a Kruskal-Wallis test was performed at each time point. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Treadmill exercise reversed IIH.

The PWR on the ipsilateral hind paw of the Im group, as measured by a 4-g VFF, increased steadily after immobilization (Fig. 2A). By 3 weeks, this increase reached statistical significance and remained significantly different from the control group up to the end of the immobilization period. Similarly, the increase in the PWR measured by a 15-g VFF in the Im group reached significance only 1 week after immobilization (Fig. 2C). However, in the Im+Ex group, the PWR on the ipsilateral hind paw measured by a 4-g VFF showed values intermediate between the Con and Im groups; a significant increase was not detected between the Con and Im+Ex group or between the Im and Im+Ex group except 4 and 5 weeks post immobilization. Although the PWR of the Im+Ex group measured by a 15-g VFF was significantly increased over that of the Con group starting 2 weeks after immobilization, the extent of the increase was smaller than that of the Im group. Four weeks after immobilization, the Im+Ex group was both significantly increased over the Con group and significantly lower in PWR than the Im group, and remained so for the rest of the experiment. In contrast, meaningful changes

were not observed in the contralateral paw in any group over the entire immobilization period (Fig. 2B,D).

3.2 Increase in beta-endorphin expression induced by treadmill exercise

The concentrations of beta-endorphin in the hypothalamus in the Con, Im, and Im+Ex groups were 0.44 ± 0.04, 0.41 ± 0.04, and 0.60 ± 0.05 ng/mg, respectively (Fig. 3). The Im+Ex group demonstrated a significant increase compared to the Con and Im groups.

Image analysis gave immunofluorescence intensities for beta-endorphin in the PAG as 1.00 ± 0.03, 1.11 ± 0.04, and 1.32 ± 0.04 for the Con, Im, and Im+Ex groups, respectively (Fig. 4A,B). As in the hypothalamus, the Im+Ex group showed a significant increase compared to other two groups.

4. Discussion

Cast immobilization of limbs induces hyperalgesia to mechanical stimulation, a phenomenon known as immobilization-induced hyperalgesia (IIH). This is possibly caused by a histological change in the hindlimb or a central sensitization in the spinal cord in previous studies [1-3, 10]. Here,

IIH was clearly observed in the Im group starting 3 weeks after immobilization. A form of stress in rodents caused by immobilization, restraint stress, might also induce hyperalgesia [11]. In the case of restraint stress, hyperalgesia develops bilaterally in limbs via changes in multiple neural systems in the brain. However, in the Im group, IIH was observed in the ipsilateral hindlimb, but not in the contralateral hindlimb. Therefore, it is likely that restraint stress did not develop in the immobilization model used in this study.

The main finding of this study was that IIH was reduced by treadmill exercise during the immobilization period in the Im+Ex group. The treadmill exercise occurred while the ipsilateral hindlimb was immobilized at the ankle joint. The experimenter confirmed that the ipsilateral hindlimb was not used in running. Thus, IIH of the ipsilateral hindlimb was inhibited although the hindlimb could not move at the immobilized joint. We hypothesize that the effect of treadmill exercise on IIH was not due to peripheral changes in the immobilized hindlimb. In previous studies, exercise training induced an increase in beta-endorphin and μ-μopioid receptors in plasma, midbrain, and hypothalamus [8, 12, 13]. The increase in

beta-endorphin caused by exercise is due to a rise in body temperature, changes in oxygen tension, energy metabolism, activation of the hypothalamo-pituitary-adrenal axis, and changes in plasma ion concentrations [14-16]. The concentration of beta-endorphin in the hypothalamus was increased in the Im+Ex group, but not in the Im group. Although the concentration of beta-endorphin in the blood may have been increased in the Im+Ex group, beta-endorphin does not cross the blood-brain-barrier [15]. In addition, since in this study blood was removed from brain tissue early in processing, changes in the beta-endorphin content of blood could not have affected our hypothalamus results. Therefore, we hypothesize that treadmill exercise leads to upregulation of beta-endorphin in the hypothalamus. Although the factor causing the upregulation was not confirmed, it could have been a rise in body temperature. We found that in the Im+Ex group, the rats' body temperature just before exercise was 36.3 ± 0.2 ºC, and just after exercise was increased to 37.4 ± 0.2 ºC (data not shown).

Previous studies showed that when the PAG was activated by experimental electrical stimulation, an analgesic effect was produced [17] [18]. Therefore, it is thought that the PAG plays an important role in the

descending pain modulatory system of the brain. Additionally, beta-endorphin is secreted from hypothalamic efferents to the PAG [19] and activates the μ-opioid receptor of GABA neurons and the descending pain modulatory system [20]. In the Im+Ex group, the immunofluorescence intensity for beta-endorphin in the PAG was greater than in the Con or Im groups. This result might indicate greater beta-endorphin being synaptically released into the PAG. Therefore, it is possible that IIH was reduced in the Im+Ex group by activation of the descending pain modulatory system via increased beta-endorphin release to the PAG. However, this study analyzed only the PAG, which is only one part of the neural circuits constituting the descending pain modulatory system. Other endogenous opioids and neurotransmitters beside beta-endorphin also participate in the system. Moreover, this study did not prove activation of the descending pain modulatory system. Further research is necessary to establish the mechanism by which treadmill exercise reduces IIH.

5. Conclusions

IIH was partially reduced by treadmill running on the non-immobilized

limbs in rats even if the immobilized joint was not moving. We hypothesize that treadmill running caused the release of beta-endorphin from hypothalamic axons to the descending pain modulatory system in the brain. The most interesting aspect of this study is the finding that IIH could be reduced by a systemic exercise such as treadmill running even if the immobilized limb did no work. When a limb is immobilized after a fracture or sprain in a human, induction of IIH will be an undesirable side effect. The results of this study suggest that systemic exercise or partial exercise using the non-immobilized limbs might reduce IIH.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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and morphine-induced inhibition of the tail-flick response in rats, J Pharmacol Exp Ther 252 (1990) 546-551.

Figure Legends

Figure 1.

Experimental design

Treadmill exercise was performed without release of immobilization of the right hindlimb; rats ran using both the forelimbs and the left hindlimb. After the last behavioral test, the rats were killed for brain tissue sampling.

Figure 2.

Behavioral testing

Results of behavioral testing for mechanical sensitivity in hind paw employing 4- and 15-g von Frey filaments. Con, control group; Im, immobilization group; Im+Ex, immobilization and exercise group. Hyperalgesia is clearly observed in the immobilized hindlimb (ipsilateral) of the Im group 3 weeks after induction of immobilization and thereafter. The

hyperalgesia is reduced in the Im+Ex group by treadmill exercise (A,C). No change is observed in the contralateral paw in any group over the entire immobilization period (B,D). Data are mean ± SE. ∗, significantly different from the Con group; †, significantly different from the Im group; VFF, von Frey filaments.

Figure 3.

Changes in beta-endorphin in hypothalamus

A significant increase in beta-endorphin levels in the hypothalamus is observed in the Im+Ex group. Data are mean ± SE. ∗, significantly different from the Con group.

Figure 4.

Distribution of beta-endorphin in midbrain periaqueductal gray (PAG)

Representative photographs of coronal sections through the PAG stained for beta-endorphin (A). Dorsal region at middle levels of PAG is shown. Note that immunofluorescence intensity is greatest in the Im+Ex group. Semiquantitative analysis of immunofluorescence intensity in the Im+Ex

group shows a significant increase compared to other two groups. Ca, cerebral aqueduct; ∗, significantly different from the Con group.

Fig. 1

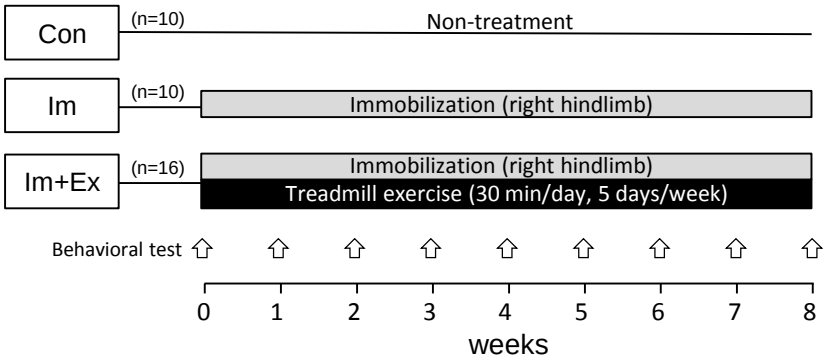

Con
(n=10)
Non-treatment
Im
(n=10)
Immobilization (right hindlimb)
Im+Ex
(n=16)
Immobilization (right hindlimb)
Treadmill exercise (30 min/day, 5 days/week)
Behavioral test
0
1
2
3
4
5
6
7
8
weeks

Fig. 2

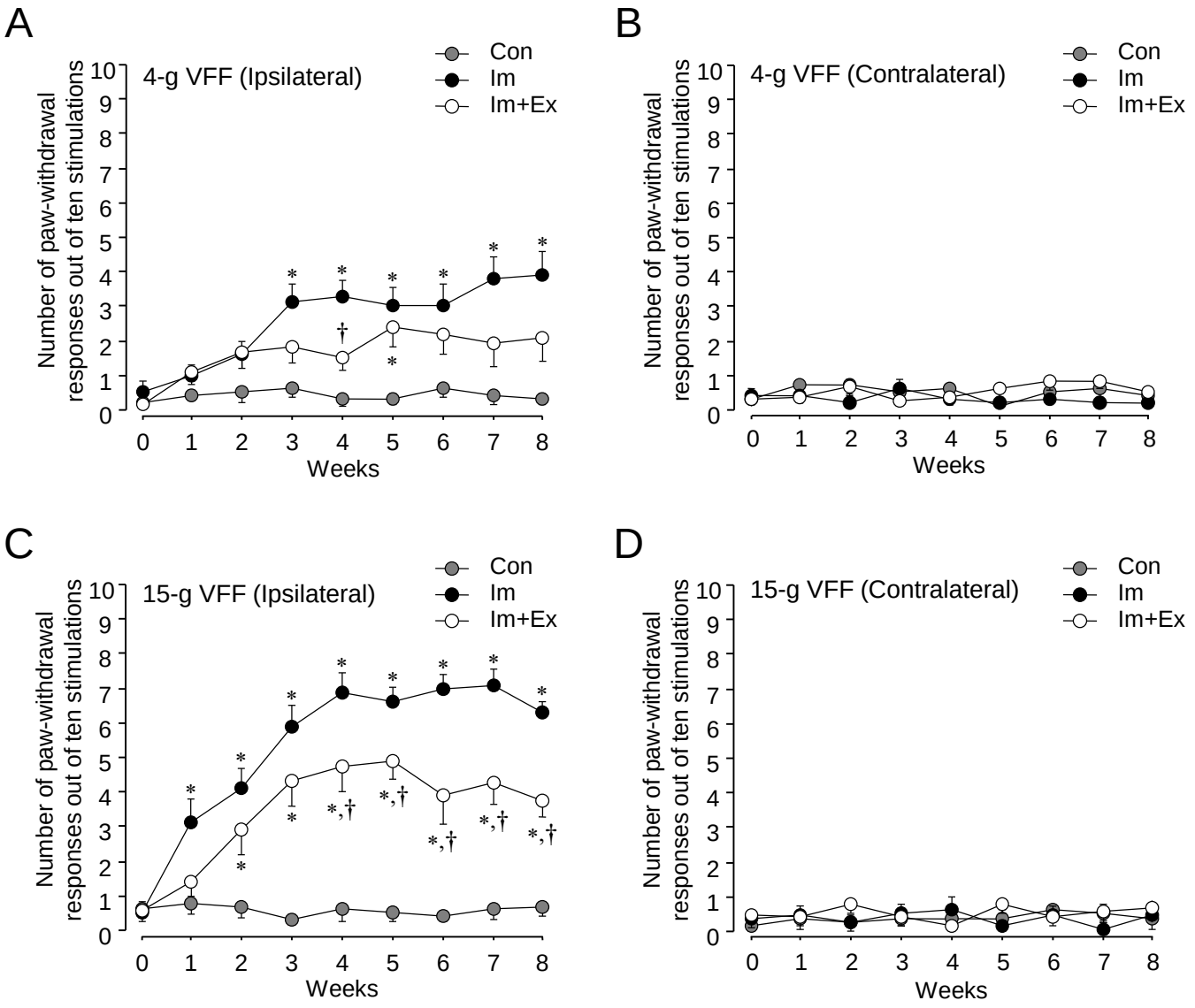

A
4-g VFF (Ipsilateral)
Con
Im
Im+Ex
Number of paw-withdrawal responses out of ten stimulations
Weeks
B
4-g VFF (Contralateral)
C
15-g VFF (Ipsilateral)
D
15-g VFF (Contralateral)

Fig. 3

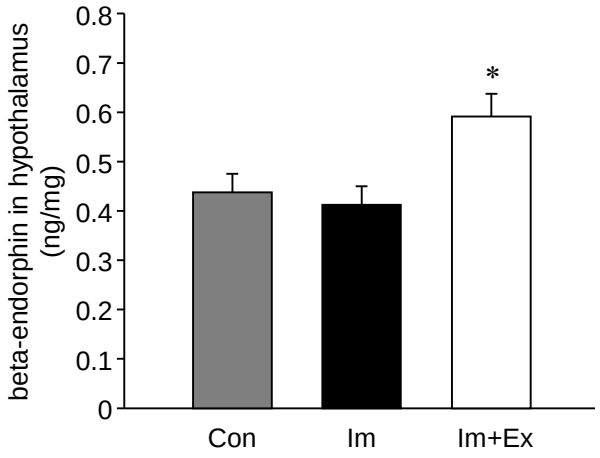
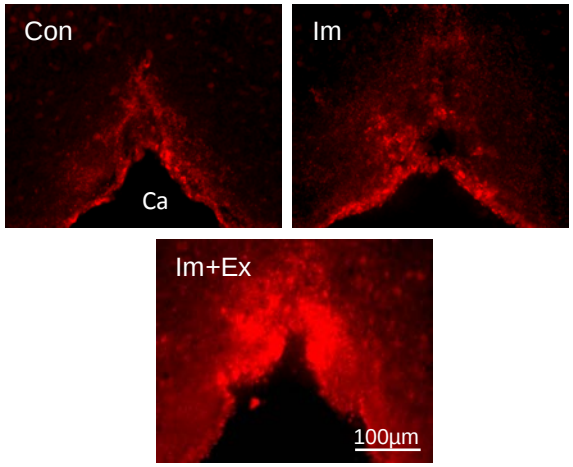
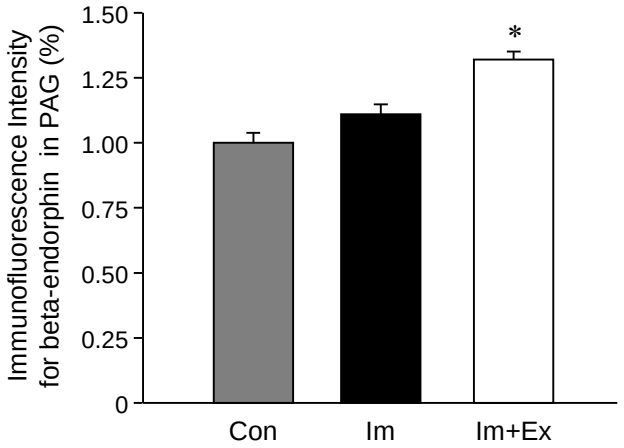

beta-endorphin in hypothalamus
(ng/mg)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Con
Im
Im+Ex

Fig. 4

A


Con
Ca
Im
Im+Ex
100μm

B


Immunofluorescence Intensity for beta-endorphin in PAG (%)
1.50
1.25
1.00
0.75
0.50
0.25
0
*
Con
Im
Im+Ex