Effects of spontaneous and forced running on activation of hypothalamic corticotropin-releasing hormone neurons in rats

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Abstract

Corticotropin-releasing hormone (CRH)-containing neurons in the hypothalamic paraventricular nucleus (PVN) are known to be activated during physical or psychological stress, and play an important role as one of the central activators of integrated stress response. Physical exercise has also been suggested as one of the stressors activating CRH neurons in the PVN. Spontaneous wheel running (SWR) has recently been reported to result in improved mental health or mood, unlike treadmill running that commonly forces the animal to run. Thus, forced running may strongly induce an activation of CRH neurons compared with spontaneous running, and spontaneous running may not represent a strong stressor. However, whether the effects of spontaneous running on activation of CRH neurons in the PVN differ from those of forced running is unknown. The present study examined the activity of CRH neurons in 1-h forced wheel running (FWR) and SWR using c-Fos/CRH immunohistochemistry in male Wistar rats. No significant differences in 1-h running distance were observed between FWR and SWR, indicating that amount of work was almost equal between exercises. Number of double-labeled neurons for c-Fos and CRH in the PVN was markedly higher in FWR than in SWR. In addition, no significant differences in Fos expression in the LC, which is related to various stress responses, were found between FWR and SWR. These results indicate that FWR strongly activates CRH neurons in the PVN compared with SWR, suggesting that spontaneous running is not an intense stressor even though running distance does not differ significantly from forced running.

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Introduction

Corticotropin-releasing hormone (CRH) neurons in the hypothalamic paraventricular nucleus (PVN) play an important role as one of the central activators of the integrated stress response. Previous studies have demonstrated that a variety of stressors, such as immobilization (Covenas et al., 1993; Imaki et al., 1992; Imaki and Vale, 1993), restraint (Hsu et al., 1998; Kalin et al., 1994), foot shock (Imaki et al., 1993; Imaki and Vale, 1993; Pezzone et al., 1992), and psychological stress using a communication box (Shibasaki et al., 1993) activate CRH-containing neurons in the PVN.

Timofeeva et al. (2003) recently reported that acute physical exercise using treadmill running induces strong expression of CRH mRNA in the PVN. In addition, physical exercise is known to intensity- and duration-dependently induce the activation of the hypothalamic-pituitary-adrenal (HPA) axis that is initiated by the activation of CRH neurons (Vale et al., 1981; Pacak, 2000; Kawashima et al., 2004; Chennaoui et al., 2002; Farrell et al., 1983). It is, thus, suggested that physical exercise is one stressor activating CRH neurons in the PVN. Moreover, CRH neurons in the PVN also project to extrahypothalamic brain regions, such as locus coeruleus (LC) neurons, dorsal raphe nucleus (DRN), central nucleus of amygdala (CeA) and bred nucleus of the stria terminalis (BNST), which are regions involved in mood or affect (Asakura et al., 2000; Azmitia and Segal, 1978; Gray, 1993; Lee and Davis, 1997). Therefore, it is possible that physical exercise activating CRH neurons could play an important role on psychological alterations.

Forced physical exercise such as treadmill running and forced swimming often produces negative physiological and psycho-pathological adaptations to stress responses accompanied by activation of CRH neurons (Cullinan et al., 1995; Moraska et al.,...
The results suggest that activation of LC neurons, which are activated by various stressors examined the effects of spontaneous and forced wheel running on and Greenberg, 1990), and CRH in the PVN. In addition, we (Kovacs, 1998; Curran, 1988; Dragunow and Faull, 1989; Sheng and Greenberg, 1990), and CRH in the PVN. In addition, we compared the activation of the hypothalamic CRH neurons during spontaneous physical exercise could be beneficial to both physiological adaptation and psychological health (Burghardt et al., 2004; Dishman, 1997; Greenwood et al., 2003; Moraska and Fleshner, 2001). The effects on activation of CRH neurons in the PVN during spontaneous physical exercise may thus differ from those during forced physical exercise. The purpose of this study was to compare the activation of the hypothalamic CRH neurons during spontaneous and forced acute wheel running in rats with double-staining for c-Fos, which is the protein encoded by the immediately early gene c-fos, and well known to be a transcription factor and a functional marker of neuronal activity (Kovacs, 1998; Curran, 1988; Dragunow and Faull, 1989; Sheng and Greenberg, 1990), and CRH in the PVN. In addition, we examined the effects of spontaneous and forced wheel running on activation of LC neurons, which are activated by various stressors (Chowdhury et al., 2000). The results suggest that activation of CRH neurons in the PVN during spontaneous running is small compared with that during forced running.

Materials and methods

Animals

Twenty adult male Wistar rats (220–250 g) were used for the experiments. All rats were individually housed in cages attached to voluntarily accessible running wheels unlocked in order to habituate to the wheel apparatus and housing environment for 1 week before all experimental procedure as previously described (Greenwood et al., 2003; Timofeeva et al., 2003). All the rats were housed under controlled conditions of temperature (23 °C) and light (12-h light, 12-h dark cycle, light on at 0800 h) with ad libitum access to food and water. All experimental protocols were approved by the Animal Care Committee of Tokyo Metropolitan University, and every effort was made to minimize animal suffering and the number of animals used.

Experimental procedure

Rats were individually performed either 1-h spontaneous wheel running or 1-h forced wheel running in the wheels in the homecages during the dark period of the light/dark cycle (SWR group and FWR group, respectively). Rats in the FWR group received softly prodding stimulation by a stick to force running when the rats stopped running. The number of wheel revolutions in 1 h of wheel running was recorded using a counter attached to the running wheel, and running distance in 1 h of wheel running was calculated by multiplying the number of revolutions by the wheel circumference (1.19 m). Mean running distance for FWR group was manipulated to be basically equivalent to that for SWR group. Control rats were left in locked wheels in the homecages for 1 h without wheel running. In addition, rats received the prodding alone without wheel running in the locked wheels as another control group. The number of prodding stimulation was matched between FWR and prodding group (approximately 25 to 30 times for 1 h). All the rats individually performed the experimental procedure (SWR, FWR, control, and prodding) at the separate days.

Immunohistochemistry

At 90 min after the start of running, all rats were deeply anesthetized using intraperitoneal injection of sodium pentobarbital (50 mg/kg) and perfused transectually with heparin solution (1000 U/I, 0.9% saline), followed by ice-cooled 4% paraformaldehyde, 0.1% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Brains were removed and post-fixed in the same fixative without glutaraldehyde for 24 h at 4 °C, then cryoprotected in phosphate-buffered 30% sucrose solution with 0.1% sodium azide for 24–48 h. Next, brains were frozen and cut in the coronal plane (6 series of 40-μm thick sections) on a microtome and collected in 0.1 M PBS with 0.1% sodium azide.

Immunohistochemical visualization of c-Fos and CRH was performed on free-floating sections using antibodies and avidin–biotin-peroxidase methods as previously described (Kita et al., 2006; Piekut and Phipps, 1998; Serino et al., 1999). After blocking endogenous peroxidase and preincubation in 10% normal horse serum, sections were incubated in primary rabbit polyclonal anti-Fos antiserum (sc-52, Santa Cruz Biotechnology, CA) diluted 1:600 in 0.1M PBS with 0.1% Triton X-100 (PBS-TX) for 16 h at room temperature. After rinsing 3 times for 5 min in PBS-TX, sections were further incubated in secondary biotinylated donkey anti-rabbit IgG (AP182B, Chemicon, Temecula, CA; 1:800) for 90 min at room temperature, rinsed 3 times for 5 min in PBS-TX, and finally treated with an avidin–biotin-peroxidase complex (Vectastain ABC peroxidase kit, Vector Laboratories, CA; 1:400) for 90 min at room temperature, rinsed 3 times for 5 min in PBS-TX, and finally treated with an avidin–biotin-peroxidase complex (Vectastain ABC peroxidase kit, Vector Laboratories, CA; 1:400) for 90 min at room temperature. Sections were then reacted for peroxidase activity in a solution of nickel ammonium sulfate, 0.02% 3,3′-diaminobenzidine (DAB) in 0.1 M Tris–HCl buffer (pH. 7.6) and 0.01% H2O2 for 20 min. Immunoreactivity for c-Fos was localized to cell nuclei, appearing as a dark gray-black stain. For dual immunostaining for CRH, sections were sequentially incubated in 10% normal horse serum, sections were incubated in secondary biotinylated donkey anti-rabbit IgG (AP182B, Chemicon, Temecula, CA; 1:800) for 90 min at room temperature, rinsed 3 times for 5 min in PBS-TX, and finally treated with an avidin–biotin-peroxidase complex (Vectastain ABC peroxidase kit, Vector Laboratories, CA; 1:400) for 90 min. Sections were then reacted for peroxidase activity in a solution of nickel ammonium sulfate, 0.02% 3,3′-diaminobenzidine (DAB) in 0.1 M Tris–HCl buffer (pH. 7.6) and 0.01% H2O2 for 20 min. Immunoreactivity for c-Fos was localized to cell nuclei, appearing as a dark gray-black stain. For dual immunostaining for CRH, sections were sequentially incubated in CRH antibody (T-4047, Peninsula Laboratories, CA; 1:5000). Avidin–biotin horseradish peroxidase complexes

Fig. 1. Mean running distance (±SEM) for 1 h of spontaneous wheel running and forced wheel running. n.s., not significant.
were visualized using DAB in 0.1 M Tris–HCl buffer without nickel sulfate. CRH immunoreactivity was localized to the cell cytoplasm and was visible as light-brown staining. Sections were then washed in 0.1 M PBS, mounted onto gelatine-coated slides, air-dried, dehydrated in graded alcohol, cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific, PA).

The CRH antibody we used was purchased from Peninsula Laboratories. Radioimmunoassay data obtained from Peninsula Laboratories indicated that this anti-CRH (human, rat) cross-reacts 100% with the CRH peptide (human, rat). It does not cross-react (0%) with ovine CRH, bovine CRH, prepro CRH 125–151 (human), Sauvagine (frog), PACAP 38 (ovine), LH-RH, ACTH (human), Arg⁸-Vasopressin, and BNP 45 (rat). The specificity of this antibody has previously been described (Piekut et al., 1996; Piekut and Phipps, 1998).

Cell counts and quantification

Immunoreactive cells on sections were observed using a BX-10 microscope (Olympus, Japan) equipped with a camera (ELMO). Quantitative analysis was performed on all sections.

Fig. 2. Photomicrographs of coronal slices through the PVN. Sections are double-labeled for c-Fos (dark gray-black stain) and CRH (light brown stain). A–B) control; C–D) spontaneous wheel running; E–F) forced wheel running; G–H) prodding. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
containing the PVN. Total number of double-labeled cells for c-Fos and CRH was counted bilaterally in the PVN on sections between \(-0.8\) to \(-2.1\) from the bregma (corresponding to Plates 21–26 in Paxions and Watson rat brain atlas). Similarly, total number of c-Fos-positive cells were counted bilaterally in the LC on sections between \(-9.3\) and \(-10.3\) from the bregma (corresponding to Plates 55–59 in Paxions and Watson rat brain atlas).

**Statistical analysis**

Values for immunoreactive cells are expressed as mean±SEM. Statistical evaluations were performed using one-way analysis of variance (ANOVA) followed by Scheffe’s post-hoc analysis. Values of \(p<0.05\) were considered statistically significant.

**Results**

In this experiment, rats performed 1-h forced or spontaneous wheel running. Mean running distance was 390.5±43.4 m for FWR and 343.8±27.8 m for SWR. Running distance did not differ significantly between FWR and SWR (\(p=0.38\); Fig. 1), indicating that all rats performed broadly equal amount of works.

A marked increase in number of double Fos/CRH-positive cells was observed in the FWR group (Fig. 2). In contrast, small
numbers of double Fos/CRH-positive cells were expressed in the SWR, control, and prodding groups. A significant main effect for groups was observed in one-way ANOVA for the number of double Fos/CRH-positive neurons ($F(3, 13)=22.54, p<0.01$). Post-hoc comparisons revealed that the mean number of double Fos/CRH-positive cells was significantly increased in the FWR group compared with the control and prodding group ($p<0.05$; Fig. 3). On the other hand, no significant differences in number of double Fos/CRH-positive cells were identified between the SWR groups and controls ($p=0.99$). Similarly, the prodding alone by a stick did not significantly increase double Fos/CRH-positive cells in the PVN compared with control group ($p=0.12$). Double Fos/CRH-positive cells in the PVN was significantly larger for FWR group than for SWR group ($p<0.05$).

Fos-positive cells in the LC were strongly induced in both FWR and SWR (Fig. 4). In contrast, small numbers of Fos-positive cells were expressed in the control group and prodding group. One-way ANOVA revealed a significant main effect for groups in the number of Fos-positive cells in the LC ($F(3, 12)=6.33, p<0.05$). Post hoc analysis determined that larger numbers of Fos-positive cells in the LC were induced in the FWR group than in the both control and prodding groups ($p<0.05$; Fig. 4E). Similarly, SWR also significantly enhanced Fos expression in the LC compared with the control and prodding groups ($p<0.05$). No significant differences in Fos-positive cells in the LC were found between FWR and SWR ($p=0.85$). The prodding alone by a stick did not significantly increase Fos-positive cells in the LC compared with the control group ($p=0.72$).

**Discussion**

The present study revealed that forced and spontaneous wheel running produced markedly different effects on activation of CRH neurons in the PVN. We demonstrated that significantly more double Fos/CRH-positive neurons were induced in the PVN by forced wheel running than by spontaneous wheel running, although running distances were almost equal in both groups. These results suggest that forced physical exercise is a strong stressor compared with spontaneous physical exercise, even if the amount of exercise is equivalent.

Several studies have investigated the effects of acute and forced physical exercise on activation of CRH neurons in the PVN. Timofeeva et al. (2003) reported that acute treadmill running induces marked increased expression of mRNA in the PVN. Jiang et al. (2004) and Harbuz and Lightman (1989) demonstrated that forced swimming produces strong induction of CRH mRNA in the PVN. These studies indicate that acute and forced physical exercise could activate CRH neurons in the PVN. Our results also revealed that forced wheel running strongly induces double Fos/CRH-positive neurons in the PVN, consistent with previous studies. Acute and forced physical exercise may thus represent one stressor activating CRH neurons in the PVN.

In the present study, since the rats in the FWR group received soft prodding stimulation by a stick to force running when the rats stopped running, the increase of double Fos/CRH-positive neurons during FWR may be partially due to the prodding stimulation. We also examined the effects of the prodding stimulation in rats that received the prodding alone without wheel running in the locked wheels. The results showed that the number of double Fos/CRH-positive neurons during the prodding was slightly increased compared to that in the control, but not significant. The increase of Fos/CRH-positive neurons in FWR was very large compared to its increase in the prodding. It is, thus, possible that not only the prodding but also the synergistic effect between prodding and wheel running could induce the increase of Fos/CRH-positive neurons in FWR, although we cannot rule out the possibility that the source of CRH activation in FWR at low intensity of exercise partially come from prodding. In addition, since it is possible that all the rats were sufficiently habituated to housing environment and wheel apparatus, the other factors, such as novel environment and wheel apparatus could not much affect the activation of CRH neurons during FWR.

We also demonstrated that spontaneous wheel running of approximately equal running distance to forced wheel running did not induce significant increases in the number of double Fos/CRH-positive neurons in the PVN compared to the controls, suggesting that spontaneous wheel running is a milder stressor than forced wheel running. Previously, many studies have reported that peripheral stress hormones were increased during forced running, and that the increase of stress hormones during acute running is implicated in exercise intensity and duration (Saito and Soya, 2004; Kawashima et al., 2004; Chennauoi et al., 2002; Farrell et al., 1983; Watanabe et al., 1991, 1992; Inder et al., 1998). These results suggest that an activation of HPA axis could be dependent on exercise intensity and duration. Our results showed that a significant difference in the PVN CRH neuronal activation was observed between forced and spontaneous wheel running, suggesting the possibility that the activation of HPA axis might be also affected by modality of exercise (i.e., forced or spontaneous exercise). However, it is still unclear whether the modality of exercise influences the activation of HPA axis, and further investigation needs to demonstrate the interaction between the modality of exercise and the activation of HPA axis.

Some studies have suggested that activation of CRH neurons in forced physical exercise produces negative physiological responses related to stress. Moraska et al. (2000) and Moraska and Fleshner (2001) reported that repeated treadmill running produces negative adaptations of adrenal hypertrophy, thymic involution, decreased serum corticosteroid binding globulin, elevated lymphocyte nitrite concentrations, suppressed lymphocyte proliferation and suppressed antigen-specific IgM in rats, while spontaneous wheel running does not produce these negative adaptations. Negative responses like this are known to occur due to activation of stress-responsive systems such as the HPA axis (Akana et al., 1985; Spencer et al., 1996). Forced physical exercise can therefore strongly activate CRH neurons, as the major regulator of the HPA axis, and chronic stimulation of the HPA axis with repeated forced exercise could produce negative physiological adaptations.

Several studies have indicated that activation of CRH neurons produces not only negative physiological responses, but also psychopathological responses such as depression and anxiety.
(Baker et al., 1999; Bakshi and Kalin, 2000; Bremner et al., 1996; Dunn and Berridge, 1990; Lee et al., 1987). This is also supported by anatomical evidence indicating that CRH neurons in the PVN widely project to extrahypophysial brain regions, such as CeA, LC, DRN and BNST, which are related with regulation of psychological alteration (Asakura et al., 2000; Azmitia and Segal, 1978; Gray, 1993; Lee and Davis, 1997). Taken together with our results showing that forced physical exercise can strongly activate CRH neurons, it is suggested that forced physical exercise may evoke psychopathological responses. However, the suggestion has recently been made that spontaneous physical exercise is beneficial to psychological health, while forced physical exercise is not (Burghardt et al., 2004; Dishman et al., 1996; Dunn et al., 1996; Greenwood et al., 2003; Moraska and Fleshner, 2001). Greenwood et al. (2003) reported that 6 weeks of voluntary running using a running wheel prevents behavioral depression such as shuttle box escape deficit and exaggerated conditioned fear, induced by exposure to uncontrollable stress. Burghardt et al. (2004) have shown that 8 weeks of wheel running produces alterations in anxiety-like behavior and defensive responses. These results indicate that spontaneous exercise training could improve stress tolerance. The present study showed that acute and spontaneous wheel running does not induce strong activation of CRH neurons in the PVN, suggesting that spontaneous physical exercise could represent a mild stressor. Repeated spontaneous physical exercise might be beneficial to psychological health as a mild stressor, although forced physical exercise may evoke psychopathological responses through strong activation of CRH neurons.

We also examined the effects of forced and spontaneous wheel running on activation of the LC neurons. Both forced and spontaneous wheel running significantly enhanced Fos expression in the LC compared with the control and prodding. This indicates that activation of LC neurons induced by physical exercise is not dependent on exercise modality, and conflicts with the effects on activation of CRH neurons in the PVN during forced and spontaneous wheel running.

Adrenergic neurons in the LC play important roles in arousal response and sympathetic drive to various stressful stimuli (Aston-Jones and Cohen, 1999; Bremner et al., 1996; Chowdhury et al., 2000). Physical exercise is known to be accompanied by arousal response and activation of autonomic functions such as blood pressure, heart rate and body temperature, regardless of exercise modality. Physical exercise could thus activate LC neurons. In fact, both forced and spontaneous exercise reportedly increased norepinephrine levels centrally and peripherally (Dunn et al., 1996; Meeusen et al., 2001; Pagliari and Peyrin, 1995). Both forced and spontaneous wheel running represent stressors activating LC neurons, and physical exercise might itself activate LC neurons regardless of exercise modality. Some studies have reported that acute stress, irrespective of stress type, enhances activation of both LC and PVN neurons. Chowdhury et al. (2000) identified marked increases in Fos expression in both the PVN and LC following mild and severe acute restraint stress. This is inconsistent with our result showing that the effects of acute exercise on activation of CRH neurons in the PVN might depend on exercise type (i.e., forced or spontaneous). This discrepancy may involve differences in the modality of stress, such as restraint stress versus physical exercise. However, this remains unclear and the neuronal mechanisms responsible for differences in activation between LC and PVN under various modalities or stressors warrant further investigation.

Although intensity of exercise (i.e., running speed) in spontaneous and forced wheel running in the present study was relatively low, at approximately 8 m/min, forced wheel running enhanced activation of CRH neurons. Saito and Soya (2004) reported that treadmill running at the supra-lactate threshold (25 m/min) activates PVN neurons, while these effects were not observed on treadmill running at low intensity (15 m/min). They examined the effects of acute treadmill running on activation of PVN neurons using trained rats (final speed, 25 m/min) for several weeks, whereas we used rats that did not experience any exercise training in this study. This difference may explain the conflicting results regarding activation of PVN neurons during acute running. Therefore, forced and acute physical exercise may activate CRH neurons in the PVN even if exercise intensity is relatively low.

In conclusion, our results showed that double Fos/CRH-positive neurons in the PVN are strongly induced following forced wheel running. Conversely, spontaneous wheel running did not induce significant increases in double Fos/CRH-positive neurons in the PVN, although running distance was roughly equal to that in forced wheel running. These results suggest that the effects of acute exercise on activation of CRH neurons in the PVN depend on the modality, rather than the intensity, of exercise. Spontaneous physical exercise may thus represent a mild stressor compared with forced physical exercise, even though the amount of exercise is equivalent.

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