Dorsomedial hypothalamic corticotropin-releasing factor mediation of exercise-induced anorexia

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EXERCISE HAS LONG BEEN REPORTED to influence food intake and energy balance. A number of studies have demonstrated that forced exercise such as extensive treadmill running or strenuous swimming results in significant reductions in appetite, food intake, and body weight compared with sedentary animals (1, 7, 20, 22, 26). Despite these demonstrated effects of exercise, the modulation of hypothalamic peptide signaling that may underlie these responses to exercise is unclear.

Rivest and Richard (24) reported that lateral ventricular injection of a CRF antagonist prevents the effects of exercise on food intake and body weight (5, 23). Although Lewis and colleagues (17) demonstrated that exercise increased the concentration of the orexigenic peptide neuropeptide Y (NPY) in multiple hypothalamic sites including the arcuate nucleus (Arc), the dorsomedial nuclei (DMH), the medial preoptic area, and the lateral hypothalamus, these animals were food restricted and it is not clear whether these effects were due to the exercise, the food restriction, or some interaction between exercise and food restriction.

Voluntary exercise deriving from running wheel access also increases physical activity but appears to have different effects on food intake. Although forced exercise produces consistent, long-term inhibitory effects on food intake, voluntary exercise decreases food intake only in the short term (within the initial 1–2 wk), with a subsequent normalization or even increased intake in response to the energy demands of exercise (16, 18, 19, 27). Taking the view that voluntary exercise may be less stressful than forced exercise, we conducted the present experiment to assess whether changes in hypothalamic CRF and NPY may underlie the feeding alterations induced by running wheel activity. We compared the patterns of changes in food intake, body weight, and hypothalamic CRF and NPY mRNA levels between sedentary male rats and rats with short-term access to running wheels. We demonstrate that running wheel access results in decreased food intake and body weight and significant increases in DMH but not PVN CRF mRNA expression. NPY mRNA expression was increased in both the Arc and the DMH. To assess a functional role for the increased DMH CRF mRNA expression in the reduced feeding, we examined the ability of intracerebroventricular (ICV) injection of a CRF antagonist to alter the feeding response to running wheel access. Antagonist administration attenuated the feeding reduction and further increased DMH CRF gene expression in rats with running wheel access. These results provide evidence for a critical role for DMH CRF in the effects of exercise on food intake and energy balance.

METHODS

Sprague-Dawley male rats weighing 250–275 g were purchased from Charles River Laboratories. Rats were individually housed and maintained on a 12:12-h light-dark cycle (lights on at 6:00 AM) in a temperature-controlled environment (22°C) with food and water available ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee at Johns Hopkins University.

Running wheel access, food intake, and body weight. In the first experiment, 12 male rats were transferred into running wheel cages containing an automated pellet dispenser controlled by an infrared

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pellet-sensing photo beam. Pellet dispensers delivered a 45-mg chow pellet (Bio-Serv, Frenchtown, NJ) in response to the removal of the previous pellet. Running wheels were kept in a locked position for the initial 10 days. After 10 days, animals were weight matched and assigned to one of two groups: six rats with access to running wheels (indicated as “unlocked”) and another six rats kept in cages with a locked running wheel for 7 days. All animals had ad libitum access to food and water. Food intake and running wheel activity were computer monitored 24 h/day (MED Associates). Body weights were measured daily. At the end of the experiment, all rats were killed between 9:00 and 11:00 AM by decapitation under ether inhalation anesthesia. Brains were removed and rapidly frozen in isopentane on dry ice for subsequent analyses of hypothalamic gene expression.

Central injection of CRF antagonist. In the second experiment, rats were kept in locked-running wheel cages and unilaterally implanted with chronic indwelling lateral ventricular cannulas. Rats were anesthetized with an mixture of Ketamine (100 mg/kg im) and xylazine (20 mg/kg im) and placed in a stereotaxic device. A 23-gauge stainless steel guide cannula was implanted 5.0 mm below dura, 1.5 mm caudal to bregma, and 1.5 mm lateral to midline (21). A 30-gauge stainless steel obturator was inserted into the cannula to maintain potency. Rats were given penicillin (60,000 U im) after surgery to prevent postoperative infection. After 1 wk of postoperative recovery, cannula placements were assessed by examining water intake in response to ICV angiotensin administration. Rats were deprived of water for 1 h, injected with 50 ng angiotensin II ICV in 5 μl of saline solution and allowed 30-min access to water in graduated drinking tubes. Water intake of 5 ml more with angiotensin than after saline vehicle administration was the criterion for correct cannula placement.

Before unlocking the running wheels, we determined whether CRF antagonist itself affected food intake or body weight. Twelve rats with correct cannula placements were maintained in running wheels with locked positions, weight matched, and assigned to one of two groups: one group received ICV vehicle with two injections (4.5 μl of saline solution each), and other group received two injections of 15 nmol of CRF antagonist α-helical-CRF[9–41] (α-hCRF; Sigma) dissolved in saline in 4.5 μl. The first injection was given at 5:00 PM (1 h before lights off), and the second injection was given at 9:00 AM the next day. This dose of CRF antagonist was previously shown to prevent forced exercise-induced anorexia (24). ICV injections were made with a Gilmore microliter syringe attached to polyethylene tubing and a 30-gauge stainless steel injector. The tip of the injector extended 1.5 mm past the tip of the guide cannula. After injections, food intake was computer monitored 24 h/day and body weight was recorded daily. Meal parameters were analyzed with Tongue Twister version 1.42 software (Dr. T. A. Houp, Florida State University, Tallahassee, FL). A meal was defined as the consumption of at least five pellets preceded and followed by at least 10 min of no feeding. Meal size was defined as the number of pellets delivered during a meal. This monitoring period lasted 7 days. On day 8, rats were given a second treatment. Animals were randomly divided into two groups and received three 4.5-μl injections of either vehicle or CRF antagonist (15 nmol α-hCRF) at 5:00 PM on the first day and at 9:00 AM and 5:00 PM on the following day. After the first injection at 5:00 PM, running wheels were immediately unlocked. Food intake and running wheel activity were monitored for an additional 7-day period. Body weights were recorded daily. Meal patterns were analyzed as described above. Because we noted that the effects of running wheel access on food intake and meal pattern mainly occurred in the first 4 days, we only present data for this period. Data from these animals were compared with data from a third group (n = 6) kept in locked-running wheel cages and receiving vehicle injections.

We cannulated an additional 16 rats for examination of the effect of CRF antagonist treatment on hypothalamic CRF and NPY mRNA expression. Cannulated placements were assessed and rats were habituated as described above. Rats were weight matched and assigned to one of four groups: four vehicle-treated sedentary rats, four vehicle-treated rats with access to running wheels, four α-hCRF-treated sedentary rats, and four α-hCRF-treated rats with access to running wheels. ICV injections were conducted as described above. After the first injection at 5:00 PM, the sedentary group of rats remained sedentary but the running wheel group of rats were immediately allowed access to running wheels. All animals had ad libitum access to pellets and water until the end of experiments. Forty-two hours after the first injection, all rats were killed at 11:00 AM by decapitation under ether inhalation anesthesia. As described above, brains were harvested for subsequent analyses of hypothalamic gene expression.

Cryosections. Coronal sections (14 μm) were cut via a cryostat, mounted on superfrost plus slides (Fisher Scientific) as a series of six series (section 1: slide 1, section 2: slide 2, etc.; section 7: slide 1, etc.), fixed with 4% paraformaldehyde, and stored at −70°C for later in situ hybridization determination. One slide from each series was stained with cresyl violet acetate and used for the selection of sections for the following in situ hybridization. Sections for NPY and DMH CRF mRNA were selected at the region of 3.1–3.5 mm caudal to bregma and for PVN CRF at the region of 1.8–2.1 mm caudal to bregma (21). Three to six sections per brain were selected and anatomically matched among animals for each in situ hybridization determination.

Ribogrobes. As previously described (2), 35S-labeled antisense riboprobes of NPY and CRF were transcribed from rat NPY precursor cDNA (10) and rat CRF precursor cDNA (11), respectively, with in vitro transcription systems (Promega, Madison, WI) and purified with Quick Spin RNA columns (Roche, Indianapolis, IN).

In situ hybridization. As previously described (2), frozen tissue sections were allowed to warm to room temperature, treated with acetic anhydride, and incubated in hybridization buffer containing 50% formamide, 0.3 M NaCl, 10 mM Tris–Cl, pH 8.0, 1 mM EDTA, pH 8.0, 1× Denhardt’s solution (Eppendorf), 10% dextran sulfate, 10 mM DTI, 500 μg/ml yeast tRNA, and 1015cpm/ml of [35S]UTP at 55°C for NPY and PVN CRF and 58°C for DMH CRF overnight. After hybridization, the sections were washed three times with 2× SSC, treated with 20 μg/ml RNase A (Sigma) at 37°C for 30 min, and then rinsed in 2× SSC twice at 55°C and washed twice in 0.1× SSC at 55°C for 15 min (58°C for DMH CRF). Slides were dehydrated in gradient ethanol, air-dried, and exposed with BMR-2 film (Kodak) for 1–3 days (3–7 days for DMH CRF).

Quantitative analysis of the in situ hybridization was done with NIH Scion image software (National Institutes of Health, Bethesda, MD). Autoradiographic images were scanned with an Epson professional scanner (Epson, Long Beach, CA) and stored in a computer for subsequent analyses with the Scion image program using autoradiographic 14C microscales (Amersham) as a standard. In situ radioactivity data were calculated as the product of hybridization area × density (background density was subtracted). Data for each animal were an average of data generated from three to six sections. Data from each group were normalized to vehicle-treated sedentary rats as 100%, and all data are presented as means ± SE.

Data analyses. Food intake and body weight gain in the first experiment were analyzed by two-way mixed-model ANOVA for the factors of running wheel access and time. For the second experiment, we began by assessing the effect of CRF antagonist treatment on baseline food intake and body weight, using a mixed-model ANOVA for the factors of treatment and time. Having shown that this dose of gradient ethanol did not affect baseline intake, we then assessed the effects of CRF antagonist on running wheel-induced alterations in food intake, meal size, meal number, and body weight gain, using two-way mixed-model ANOVA for the factors of treatment (antagonist and/or running wheel access) and time. In situ hybridization data were analyzed by Student t-test in the first experiment and by two-way ANOVA for the factors of antagonist treatment and running wheel access in the third experiment. Running activity data were assessed by one-way ANOVA for the first experiment and by two-way ANOVA for the second experiment. Data were further analyzed by pairwise
RESULTS

Effects of running wheel access. During the 7-day period of access to running wheels, rats had running activities over a range from 1,500 to 2,100 revolutions/day (1.5–2.1 km/day) (Fig. 1A). ANOVA demonstrated that running wheel access and the resulting increased activity significantly decreased food intake and body weight gain (Fig. 1, B and C). There were significant running wheel access by measurement time interactions for food intake \[ F(7,70) = 4.440, P < 0.001 \] and body weight gain \[ F(7,70) = 10.158, P < 0.001 \]. Rats with access to running wheels voluntarily decreased food intake by 48% on day 1 and by 30–35% during days 2–5 compared with sedentary rats, and the food intake of exercising rats increased on days 6 and 7 to the point that it was no longer significantly suppressed (Fig. 1B). Rats with access to running wheels did not gain body weight during the first 3 days and gained body weight more slowly than sedentary controls over the following days \( P < 0.001 \); Fig. 1C). In situ hybridization determination revealed that CRF mRNA levels were significantly elevated in the DMH in exercising rats relative to sedentary controls \( P < 0.05 \) but did not differ in the PVN \( P > 0.05 \) by the end of 7-day running wheel access (Fig. 2). Running wheel access-induced elevation of DMH CRF gene expression was mainly localized in the dorsal part of the DMH (Fig. 3). Both Arc and DMH NPY mRNA levels were significantly increased in response to 7 days of running wheel access (Figs. 2 and 3). Although running wheel access affected food intake, body weight gain, and mRNA levels of DMH CRF and Arc and DMH NPY, there were no significant correlations between levels of running wheel access and food intake, running wheel access and body weight loss, or running wheel access and gene expression \( P > 0.05 \).

Effects of ICV CRF antagonist injection on food intake and meal patterns in exercising rats. Before access to running wheels, ICV injection of the CRF antagonist α-hCRF did not affect food intake \[ F(1,36) = 0.0168, P > 0.05 \], meal size \[ F(1,36) = 0.0305, P > 0.05 \], or meal number \[ F(1,36) = 0.0112, P > 0.05 \]. Running wheel access resulted in a significant decrease in food intake, and this reduction was significantly attenuated by the ICV injection of CRF antagonist α-hCRF (Fig. 4A). ANOVA revealed significant interactions between treatment and measurement time \[ F(8,60) = 8.352, P < 0.001 \]. Fisher LSD comparison revealed that food intake was significantly decreased on days 1, 2, 3, and 4 of running wheel access, and this intake reduction was significantly attenuated by CRF antagonist α-hCRF injection on days 2 and 3 (Fig. 4A). Analyses of meal patterns revealed that there were no significant effects of treatments on meal size \[ F(2,60) = 1.520, P = 0.251 \], but the treatments had significant effects on meal frequency \[ F(2,60) = 8.547, P < 0.05 \]. Fisher LSD comparisons demonstrated that the effects of ICV α-hCRF injection on food intake were mainly due to effects on the size of meals. Although running wheel access decreased meal sizes on days 3 and 4, ICV α-hCRF administration prevented a significant effect of running wheel access (Fig. 4B). However, ICV α-hCRF injection did not prevent exercise-induced decreases in meal frequency (Fig. 4C).
Effects of ICV α-hCRF injection on body weight and running wheel activity in exercising rats. Similarly, ICV injection of a CRF antagonist did not affect body weight of sedentary rats \[F(1,36) = 0.127, P > 0.05\]. Running wheel access resulted in body weight loss, and this weight loss was attenuated by ICV α-hCRF injection (Fig. 5A). ANOVA revealed a significant treatment by measurement time interaction \[F(8,64) = 8.100, P < 0.001\]. Fisher LSD comparisons revealed that body weight was significantly reduced when rats had access to running wheels, and ICV injection of CRF antagonist α-hCRF attenuated this reduction on day 3 of running wheel access (Fig. 5A). Moreover, although running activity was increased by 79% on the initial day of access to running wheels in rats with α-hCRF treatment relative to vehicle-treated exercising rats, there was no main effect of ICV α-hCRF injection \[F(1,36) = 1.848, P = 0.207\]. Thus vehicle- and α-hCRF treated rats had similar activity on the following days (Fig. 5B).

Effects of ICV α-hCRF injection on CRF and NPY mRNA levels in exercising rats. Although CRF mRNA levels in the PVN were not affected by 42 h of running wheel access, CRF mRNA expression was significantly elevated in the DMH in rats with access to running wheels relative to sedentary rats (Fig. 6). Two-way ANOVA revealed a significant main effect of running wheel access on DMH CRF mRNA levels \[F(1,11) = 12.029, P = 0.0053\] but not on PVN CRF mRNA expression \[F(1,11) = 0.09, P = 0.7694\]. Post hoc comparisons demonstrated that DMH CRF mRNA levels were significantly elevated in vehicle-treated exercising rats compared with vehicle-treated sedentary rats, and this elevation was further increased in ICV α-hCRF-treated exercising rats (from 60% to 112% elevation relative to vehicle-treated sedentary controls) (Fig. 6). Forty-two hours of running wheel access did not produce significant changes in ARC NPY mRNA levels, and these levels were not affected by ICV CRF antagonist injection (Fig. 6). Similarly, in response to 42 h of running wheel access, DMH NPY mRNA expression was not altered, and ICV α-hCRF injection did not affect levels of DMH NPY mRNA expression (Fig. 6).

DISCUSSION

The current results demonstrate that running wheel access results in significant decreases in food intake and body weight. This finding is consistent with previous reports showing that food intake is reduced in rats with access to running wheels over a 7- to 10-day period (16, 18, 19). Furthermore, we demonstrate that voluntary exercise reduces both meal size and meal frequency. In response to voluntary exercise, we observed that CRF mRNA expression is significantly elevated in the DMH, but not in the PVN. NPY mRNA expression was increased in both the Arc and DMH after 7 days of voluntary exercise, but not by 42 h of running wheel access. Moreover,
exercise induces endogenous CRF release, leading to decreased meal size and food intake. Because the current data demonstrate that CRF antagonist injection does not affect exercise-induced reductions in meal number, central CRF appears to play an important role, in a specific manner, in the effects of exercise on feeding behavior.

The PVN and CeA have been demonstrated to be important sites in the actions of central CRF. Krahn and colleagues (14) demonstrated that CRF acts in the PVN not only to induce anorexia but also to increase locomotion and grooming. Koob and colleagues (13) reported that direct injections of CRF into the amygdala produce anxiety-like behaviors and microinjection of a CRF antagonist into the CeA produces antistress effects. However, a role for these structures in CRF-mediated exercise-induced anorexia has not been demonstrated. Thus lesions of either the PVN or the CeA do not prevent the anorectic effect of forced exercise in male rats (5, 23). The present data demonstrating no alterations in PVN CRF mRNA expression during the period in which exercise results in decreased food intake provide additional evidence suggesting that PVN CRF may not serve as a signal to mediate the feeding-inhibitory effects of exercise. In contrast, the finding that CRF mRNA expression is significantly elevated in the DMH in response to voluntary exercise and further increased in exercising rats with administration of a CRF antagonist suggests a role for DMH CRF in the mediation of exercise-induced feeding-inhibitory effects.

In the current study, we did not find any alterations in NPY mRNA expression in the Arc and the DMH in rats with 42 h of exercise at a time when exercised rats had significantly decreased food intake. However, we observed that NPY mRNA expression was increased in both the Arc and the DMH in rats having a 7-day period of running wheel access. This elevated NPY mRNA expression and presumed release is consistent with previous results showing that Arc and DMH NPY concentrations are increased in exercised rats (17). At the time of these increases in NPY mRNA expression, we observed that exercised rats had recovered food intake to levels close to what sedentary rats consumed. These data suggested that, although DMH CRF expression is higher in 7 days of running wheel
access than in 42 h of running wheel access, the elevation of Arc and DMH NPY expression by 7 days of running wheel access may counterbalance the effects of increased DMH CRF and result in a recovery of food intake. Central NPY actions in the control of food intake and energy balance have been demonstrated. Central injection of NPY increases food intake (25) and decreases energy expenditure (4, 8). NPY mRNA expression is increased in the Arc in food-deprived and food-restricted rats (3, 6) and only elevated in the DMH in response to chronic food restriction (3). The current finding of increased DMH NPY mRNA expression in rats with 7 days of exercise supports the view that DMH NPY is responsive to chronic negative energy balance. This pattern of results suggests that NPY expression levels are elevated to compensate for exercise-induced negative energy balance. Moreover, central injection of a CRF antagonist did not affect NPY mRNA expression in either the Arc or the DMH, suggesting separate actions for central CRF and NPY signaling pathways.

Despite the findings that voluntary exercise alters food intake, body weight, and DMH CRF gene expression, we did not find any correlations between levels of running activity and food intake, running activity and body weight, or running activity and DMH CRF mRNA levels. Moreover, although ICV CRF antagonist treatment attenuated exercise-induced anorexia and body weight loss and resulted in further increases in DMH CRF gene expression, CRF antagonist did not affect the degree of running activity. The absence of significant correlations implies that the alterations induced by exercise result from the presence of rather than the degree of exercise. This lack of correlation is similar to recent findings of Levin and colleagues (15) examining the effects of exercise on food intake, body weight, and hypothalamic gene expression in obesity-prone rats.

In summary, the present results demonstrate that voluntary exercise results in a temporary period of decreased food intake and body weight. These inhibitory effects of voluntary exercise on food intake and body weight appear to be mediated, at least in part, via increases in DMH CRF gene expression. Elevations of Arc and DMH NPY gene expression in response to 7 days of running wheel access may represent a compensatory response to the increased energy demands resulting from exercise.

GRANTS

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