

Chronic and voluntary exercise enhances learning of conditioned place preference to morphine in rats

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Abstract

Previous research has shown that brief and intermittent activity wheel running attenuates conditioned place preference (CPP) to morphine in rats, which suggests that exercise may produce a cross-tolerance to opiates. On the other hand, a different exercise paradigm, chronic and voluntary wheel running, enhances learning in contextual conditioning tasks. The present experiments tested CPP to 2.5, 5, and 7.5 mg/kg morphine in sedentary rats and rats provided free access to running wheels for three weeks. Sucrose preference was also tested to determine exercise's influence on appetitive processes. Levels of mRNA encoding brain-derived neurotrophic factor and preprogalanin mRNA were quantified using *in situ* hybridization. In rats that exhibited CPP to morphine, exercising rats spent significantly more time per entry in the morphine-paired chamber during the CPP test. CPP to morphine was dose-dependent. The expression of hippocampal brain-derived neurotrophic factor (BDNF) was greater in exercising rats compared to the sedentary group. Preprogalanin (GAL) mRNA expression in the locus coeruleus (LC) was positively correlated with mean distance run. These results suggest that while chronic exercise may produce cross-tolerance to opioids, exercise-induced enhancement of associative learning caused by exercise may override this effect in the conditioned place preference procedure. © 2007 Elsevier Inc. All rights reserved.

Keywords: Exercise; Conditioned place preference; Brain-derived neurotrophic factor; Galanin; Locus coeruleus; Hippocampus

1. Introduction

Brief and intermittent bouts of access to an activity wheel attenuate conditioned place preference (CPP) to a low dose of morphine. This finding has led some investigators to propose that exercise produces cross-tolerance to opioid agonists (Lett et al., 2002). This hypothesis is supported by studies demonstrating that chronic and voluntary wheel running decreases the antinociceptive response to mu-opioid drugs (Kanarek et al., 1998; Mathes and Kanarek, 2001; Smith and Lyle, 2006; Smith and Yancey, 2003). In contrast, chronic and voluntary exercise slightly increases aversive responses to the kappa opioid spiradoline in the CPP task, while simultaneously decreasing the antinociceptive response to spiradoline (Smith et al., 2004).

Despite the finding that some forms of exercise may diminish CPP, there is ample evidence that exercise improves various forms of learning, including contextual conditioning. Three weeks of

chronic and voluntary exercise increases conditioned freezing behavior in the contextual fear conditioning task (Van Hooymissen et al., 2004) and rats with seven weeks of access to running wheels acquire learning in the radial arm maze with fewer trials compared to sedentary rats (Anderson et al., 2000). The question remains as to whether exercise-induced enhancement of contextual conditioning would occur using an opiate as an appetitive stimulus following a more chronic, voluntary form of exercise. While the effect of brief and intermittent exercise on CPP to morphine has been tested (Lett et al., 2002), the effect of chronic and voluntary exercise on CPP to a range of morphine doses has not been investigated. Although there are examples in which acute and/or forced bouts of exercise have benefited learning and memory (Albeck et al., 2006; Fordyce and Farrar, 1991; Fordyce and Wehner, 1993; Navarro et al., 2004; Ogonovszky et al., 2005; Radak et al., 2001; Samorajski et al., 1985; Uysal et al., 2005), these studies do not address the effects of voluntary and chronic activity wheel running on associative or motivational learning. Studies that employ acute, forced or intermittent bouts of access to an activity wheel or other kind of exercise may yield behavior that is confounded by stress or withdrawal from running (Moraska et al., 2000). The behavioral

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effects of chronic and voluntary wheel running, in contrast to acute or forced bouts of wheel running, may more accurately reflect neurobiological changes that are long-lasting and not confounded with stress or exercise withdrawal effects.

The neurobiological effects of exercise support the hypothesis that exercise generally enhances learning through long-term adaptations in specific neurotransmitter systems. For example, exercise reliably increases hippocampal brain-derived neurotrophic factor (BDNF) mRNA expression, which is consistent with the synaptic plasticity required for enhanced learning and memory (Adlard et al., 2004; Farmer et al., 2004; Neeper et al., 1996; Oliff et al., 1998; Tong et al., 2001). In addition to hippocampal BDNF, forebrain noradrenergic systems also play a critical role in learning and memory. The main site of noradrenergic cell bodies in the central nervous system, the locus coeruleus (LC) (Dahlstrom and Fuxe, 1964), contains high concentrations of the peptide galanin (GAL), which coexists with norepinephrine (Holets et al., 1988; Holmes and Crawley, 1995; Skofitsch et al., 1986) and modulates (Ma et al., 2001; Pieribone et al., 1995; Seutin et al., 1989) noradrenergic transmission. Preprogalanin mRNA expression in the LC is increased by chronic exercise (Holmes et al., 2006; O'Neal et al., 2001; Van Hoomissen et al., 2004) and serves as a marker of altered noradrenergic activity. Several studies demonstrate that norepinephrine (NE), the release of which is altered by chronic exercise (Meeusen et al., 1997; Soares et al., 1999), has an important role in appetitively motivated behavior. Blockade of β -adrenoreceptors with the noradrenergic antagonist propranolol reduces the enhancement of the contextual fear conditioning produced by chronic exercise in rats (Van Hoomissen et al., 2004). Reacquisition of morphine self-administration is blocked in rats pretreated systemically with a dopamine β -hydroxylase inhibitor (Davis et al., 1975). Finally, CPP to morphine is reversed in mice with the α -2 adrenoreceptor agonist clonidine (Zarrindast et al., 2002) and blocked in mice missing dopamine β -hydroxylase (Olson et al., 2006). These findings demonstrate that noradrenergic systems are critically involved in CPP to morphine. Modulation of the noradrenergic system, possibly through a GALergic mechanism, may underlie the enhancement in learning caused by exercise.

The aim of this study was to determine the effect of three weeks of voluntary exercise on tasks that involve appetitive stimuli. The CPP to morphine paradigm was employed to determine if chronic and voluntary exercise enhances associative learning in this task or if the cross-tolerance to opiates observed after brief and intermittent bouts of exercise (Lett et al., 2002) generalizes to CPP after chronic and voluntary exercise. A second task, sucrose preference, was employed to further evaluate the effects of chronic and voluntary exercise on responsivity to stimuli with appetitive qualities. Since the hippocampus is known to be involved in the CPP task (Ferbinteanu and McDonald, 2001) and to be affected by exercise (Neeper et al., 1996; Oliff et al., 1998), BDNF mRNA expression levels in the hippocampus were quantified using *in situ* hybridization in sedentary and active rats. To further characterize the possible role of the LC in exercise-induced enhancement of learning, mRNA expression levels for the

peptide GAL, which modulates NE release in the LC (Ma et al., 2001; Pieribone et al., 1995; Seutin et al., 1989), were quantified.

2. Method

2.1. Subjects

Adult male Sprague–Dawley (*Rattus norvegicus*) rats (250–275 g; $N=58$), approximately 60 days old at the time of testing, were purchased from Harlan Inc. (Indianapolis, IN). All procedures were approved by the University of Georgia Animal Care and Use Committee. Animals were individually housed and had access to food and water *ad libitum*. All animals were housed under a reverse 12 h light/dark schedule (lights off at 7:00 a.m. and lights on at 7:00 pm). All testing took place during the dark phase.

2.2. Apparatus and materials

Rats in the activity wheel condition were individually housed in $43 \times 23 \times 20$ cm polycarbonate cages that contained running wheels with a circumference of 107 cm (MiniMitter) ($n=29$). The cages had electromagnetic counters interfaced with a computer to count the number of wheel revolutions, which were recorded daily. Sedentary rats were housed in cages without running wheels but otherwise identical to those described for exercising rats ($n=29$).

A portion of the rats ($n=28$) were tested for sucrose preference while housed in the same conditions used throughout the experiment; rats in the exercise condition remained in the cages fitted with the activity wheels and rats in the sedentary condition remained in the unaltered cages. Each cage was fitted with a bottle containing 250 ml tap water and a bottle containing 250 ml 1% sucrose solution. Food continued to be available *ad libitum*.

CPP was conducted in one of two large polycarbonate boxes set up for this procedure. Each box contained two chambers of equal dimensions (44.5 cm by 16.5 cm by 34 cm) separated by a neutral area (44.5 cm by 11 cm by 34 cm). Dark cutouts of construction paper in various shapes and sizes were affixed to the walls of one chamber, with the wall adjoining the neutral area painted black. This chamber's floor was covered with textured cardboard. Cutouts of white construction paper were affixed to the walls of the other chamber, with the wall adjoining the neutral area painted white. This chamber's floor was not altered and was clear plastic. The neutral area consisted of clear plastic walls and floor and had two openings to each of the chambers through which the rat could travel. These openings were blocked during conditioning sessions.

During conditioning sessions, rats were injected subcutaneously (SC) with 2.5, 5.0, or 7.5 mg/kg morphine sulfate (Sigma, St. Louis, MO) or isotonic saline solution. Morphine sulfate was dissolved in isotonic saline solution. Injection volume was 1 ml/kg.

2.3. Procedure

Approximately one week after arrival at the animal facility, rats were randomly assigned to either the exercise or sedentary

condition. Once assigned to an activity condition, rats were randomly assigned to a drug dosage condition. Rats in the exercise condition had uninterrupted access to activity wheels set up in their cages for three weeks. The number of wheel revolutions for each rat was recorded daily.

After three weeks of access to activity wheels or no wheels, a portion of the rats (AW $n=16$; SED $n=12$) were exposed to two bottles containing 250 ml tap water and 1% sucrose solution. This test was conducted during the dark phase and 2 h after this phase (7:00 a.m. to 9:00 p.m.). At the end of this period, the bottles were collected and replaced with one bottle filled with tap water. Tap water and sucrose solution consumption was measured by weighing the liquid remaining in the bottles. The sucrose preference score was calculated by subtracting the remaining 1% sucrose solution volume from 250 ml and dividing this amount by the total sucrose solution and tap water volume consumed ((sucrose intake/total intake) \times 100).

The day after the sucrose preference test, the CPP to morphine procedure began. After each portion of the CPP procedure was completed, rats were returned to their home cages. The particular CPP paradigm employed was a modification of that described in Will et al. (2004). On the first day of CPP, rats were placed in the neutral area of the CPP chamber and assessed for natural preference for either chamber. Time spent in each chamber was timed with a stopwatch and recorded for 30 min. Over the next two days, rats underwent conditioning. In the morning, all rats were injected SC with saline and enclosed in the non-conditioned chamber for 45 min. The non-conditioned side was determined for each rat by choosing the chamber that the rat spent the majority of time in during the natural preference assessment. In the afternoon, each rat was injected SC with saline (in the 0 mg/kg morphine condition, $n=8$), 2.5 mg/kg ($n=14$), 5.0 mg/kg ($n=24$), or 7.5 mg/kg ($n=12$) morphine sulfate solution and enclosed in the chamber that the rat spent less time in during natural preference assessment. In both morning and afternoon sessions rats were placed in the chambers 5 min after injections. On the final testing day, rats underwent the same procedure used in the natural preference assessment. Time spent in and number of entries into and exits out of each chamber were timed with a stopwatch and recorded. A rat was considered to be inside a chamber when all four paws were inside the chamber. CPP score was determined by subtracting the amount of time spent in the conditioned chamber on the initial assessment day from the amount of time spent in that chamber during the test day. In addition, the CPP test session was divided into three ten-minute intervals and time per entry in each chamber was calculated.

Approximately 24 h after testing, rats were decapitated. Brains were frozen on dry ice and stored at -80°C .

2.4. *In situ* hybridization

Brain tissue was sliced into 12- μm sections at LC and hippocampal levels on a Microm Cryostat (Carl Zeiss, Waldorff, Germany). After thaw mounting the tissue onto glass slides, approximately every 10th section was stained with .1% thionin to determine neuroanatomy. Sections were chosen for hybrid-

ization based on anatomical similarities to hippocampal and LC sections from plates 30 and 58, respectively, in the rat brain atlas of Paxinos and Watson (1986). Tissue sections were fixed in 4% (wt./vol.) formaldehyde in .12 M sodium phosphate-buffered saline (PBS, pH=7.4) for 5 min, rinsed twice in PBS, and placed in .25% (wt./vol.) acetic anhydride in .1 M triethanolamine HCl-.9% (wt./vol.) NaCl (pH=8.0) for 10 min. Ethanol washes (70%, 80%, 95%, 100%; wt./vol.) were employed to dehydrate tissue sections. Delipidation was achieved by placing the tissue in chloroform for 5 min. Tissue was then rinsed in ethanol (100%, 95%) and left to dry.

GAL and BDNF oligonucleotide probe sequences (Oligos Etc., Wilsonville, OR) were complementary to rat GAL cDNA Bases 228–271 (Kaplan et al., 1988) and mouse BDNF mRNA transcript Bases 650–699, respectively. The mouse BDNF mRNA transcript is 98% homologous to that of the rat (Hofer et al., 1990; Maisonpierre et al., 1991). The 3' ends of the probes were labeled with [^{35}S]-dATP (1000–1500 Ci/mmol; New England Nuclear, Boston, MA), terminal deoxynucleotidyl transferase (TdT, 25 U/ml; Roche, Indianapolis, IN), and tailing buffer. Unincorporated nucleotides were separated from the probes by column separation. Sections were incubated for approximately 20 h with a hybridization solution that included 50% (wt./vol.) formamide, 600 mM NaCl, 80 mM Tris-HCl, 4 mM EDTA, .1% (wt./vol.) sodium pyrophosphate, .2% (wt./vol.) sodium dodecyl sulfate, and 10% dextran sulfate in a humid chamber at 37°C . To reduce nonspecific binding, tissue underwent a series of washes (3, $1\times$ SSC; 1, $2\times$ SSC–50% formamide; 3, $2\times$ SSC–50% formamide for 20 min at 40°C ; 2, $1\times$ SSC for 30 min at 22°C). After a rinse in deionized water and 70% ethanol, the slides were left to dry. Slides were exposed to autoradiographic film (BioMax MR, Eastman Kodak, Rochester, NY) and developed with Kodak GBX developer and fixer.

A computerized image analysis system (NIH Image 1.38 software, Rasband, 1995; Power Macintosh 8100 computer, Apple Computer, Cupertino, CA; light box and camera, Imaging Research, St. Catharines, Ontario; video interface, Data Translation, Marlboro, MA) was used to analyze autoradiographic film. A 10×10 pixel oval was placed in the dorsal portion of the LC and randomly placed in the hippocampal formation of each section of each rat and the average optical density (OD) readings were taken. Readings were taken by an investigator blind to subject condition.

2.5. Data analysis

Student's between-subjects *t*-tests were performed to evaluate the effect of exercise on sucrose preference score, amount of water consumed, and amount of sucrose solution consumed. Student's between-subjects *t*-tests were performed to evaluate differences in rats that received positive and negative CPP scores for the dependent measures CPP score, time per entry, and number of entries into the morphine-paired chamber. A paired-subjects *t*-test was performed to compare time spent in the morphine-paired chamber to time spent in the saline-paired chamber on test day by rats treated with morphine. A two

(activity wheel or sedentary) by four (0, 2.5, 5, or 7.5 mg/kg dose of morphine sulfate) analysis of variance (ANOVA) was performed to evaluate the effect of exercise and varying dosages of morphine sulfate on CPP score, total time per entry and number of entries into the morphine-paired chamber on CPP test day. A 2 (activity or sedentary) \times 4 (drug dosage) repeated measures ANOVA was conducted to determine the effect of exercise, varying dosages of morphine, and 10 min time interval during the CPP test on time spent per entry during the CPP test. Tukey least significant difference (LSD) post hoc analyses were performed when appropriately indicated. Student's paired-subjects *t*-tests were conducted to determine differences between time intervals for time spent per entry into the morphine-paired chamber. Two-tailed Student's *t*-tests were conducted to compare optical densities for galanin mRNA and BDNF mRNA in exercisers and sedentary rats. Welch's correction factors were used when heterogeneity of variance existed between two groups. Pearson's correlation analyses were performed to evaluate relationships between mRNA optical densities, wheel revolutions, and various CPP measurements. For all statistical tests, the criterion for significance was set at $\alpha = .05$.

3. Results

3.1. Sucrose preference in exercising and sedentary rats

Exercising rats (AW) tended to prefer sucrose to water to a lesser degree than sedentary (SED) rats, with mean (S.E.M.) preference scores of 68.74% (4.96) and 76.95% (2.18), respectively, although this difference was not significant [$t(26) = -1.589$, $P = .148$]. Further analyses revealed that AW rats consumed significantly more sucrose solution than SED rats. AW and SED rats consumed an average (S.E.M.) of 50.55 ml (8.05 ml) and 30.4 ml (3.8 ml), of sucrose solution, respectively [$t(17) = 2.264$, $P = .037$, Welch's correction]. AW rats consumed significantly more water than SED rats. AW and SED rats consumed an average (S.E.M.) of 22.58 ml (4.87 ml) and 9.21 ml (1.35 ml) of water, respectively [$t(14) = 2.643$, $P = .019$, Welch's correction].

3.2. Conditioned place preference in exercising and sedentary rats

Rats that did not spend more time in the morphine-paired chamber on the CPP test day than during natural preference observation were not included in the initial analyses, eliminating 1 AW rat from the 2.5 mg/kg dose, 2 SED rats from the 5 mg/kg dose, and 3 AW rats from the 7.5 mg/kg dose conditions. These rats received negative CPP scores and between-subjects Student *t*-tests determined that these rats scored significantly lower than rats that received positive CPP scores subsequent to morphine conditioning in the following dependent measurements: CPP scores [$t(20.261) = -9.319$, $P < .001$, Welch's correction], total time spent per entry into the morphine-paired chamber at test day [$t(14.529) = -5.024$, $P < .001$, Welch's correction], and number of entries into the

morphine-paired chamber on the CPP test day [$t(48) = -2.023$, $P = .049$]. These exclusion criteria thus eliminate rats that either failed to condition or showed aversion to the morphine-paired chamber.

Overall, a paired *t*-test that excluded the rats in the saline condition and those that received negative CPP scores revealed that the morphine-paired chamber was preferred over the saline-paired chamber on test day by both AW and SED rats [$t(43) = 9.453$, $P < .001$]. Rats spent an average (S.E.M.) of 16 min 40 s (40 s) in the morphine-paired chamber and an average of 6 min 14 s (29 s) in the saline-paired chamber. With all animals included in the analysis, the paired *t*-test indicated again that both AW and SED rats preferred the morphine-paired chamber over the saline-paired chamber on test day [$t(49) = 5.814$, $P < .001$], with rats spending an average (S.E.M.) of 15 min 25 s (46 s) in the morphine-paired chamber and 7 min 24 s (40 s) in the saline-paired chamber. Overall, as indicated by a 2 (AW vs. SED) \times 4 (saline, 2.5, 5, or 7.5 mg/kg morphine) ANOVA, AW rats did not differ from SED rats in their conditioned place preference score (time in conditioned chamber at test time – time spent in conditioned chamber at habituation time) [$P = .181$]. However, in the analysis in which animals that received negative CPP scores were excluded, there was a main effect of dose on conditioned place preference score [$F(3, 44) = 5.678$, $P = .002$], as depicted in Fig. 1, and Tukey LSD post hoc tests were performed to determine at which dosages CPP scores differed from each other. Rats in the saline control condition received significantly lower CPP scores than those in the 5 mg/kg morphine condition [$P < .001$]. Rats in the 2.5 mg/kg condition received significantly lower CPP score than rats in the 5 mg/kg condition [$P < .001$]. Rats in the 7.5 mg/kg condition did not differ in CPP score from rats in any other dose condition. The activity group \times dose interaction was not significant [$P > .05$]. When all

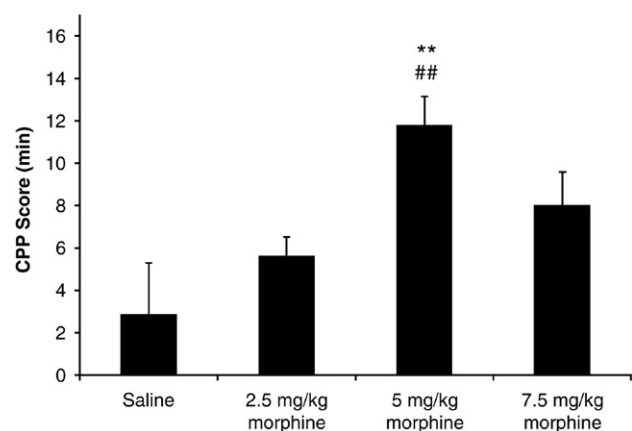


Fig. 1. Dose-dependent effects of morphine on conditioned place preference (CPP) in exercising (AW) and sedentary (SED) rats. CPP scores are expressed as group averages (plus S.E.M.) for time spent in the conditioned chamber before conditioning minus time spent in the conditioned chamber post-conditioning. Across exercise conditions, rats that received 5 mg/kg morphine during conditioning received larger CPP scores than rats that received only saline. Also across exercise conditions, rats that received 5 mg/kg morphine during conditioning received greater CPP scores than rats that received 2.5 mg/kg morphine. ** $P < .01$ indicates difference in comparison to saline-treated rats. ## $P < .01$ indicates difference in comparison to 2.5 mg/kg treated rats.

rats were included in the ANOVA for CPP score, there was again a main effect for dose [$F(3, 50)=4.448, P=.008$] but not for activity group [$P=.605$]. Tukey LSD post hoc tests were performed to determine at which dosages CPP scores differed from each other. Rats in the 5 mg/kg displayed significantly greater magnitude of CPP compared to animals that received saline [$P=.004$], 2.5 mg/kg morphine [$P=.009$], and 7.5 mg/kg morphine [$P=.033$]. The activity group \times dose interaction was not significant [$P>.05$].

On the CPP test day, AW rats spent significantly more time per entry in the morphine-paired chamber than SED rats, as determined by a two (AW vs. SED) \times four (saline, 2.5, 5, or 7.5 mg/kg morphine) ANOVA [$F(1, 44)=4.22, P=.046$] that did not include animals that received negative CPP scores at test day after undergoing conditioning with morphine (Fig. 2). The main effect for dose [$P=.177$] and activity group \times dose interaction [$P>.05$] was not significant. When all animals were included in the ANOVA, the main effect for activity group approached but did not reach statistical significance [$F(1, 50)=2.231, P=.142$]. The main effect for dose and activity group \times dose interaction was not significant [$P>.05$ for both].

The thirty minute CPP test was divided into ten-minute intervals upon further data analysis to characterize the pattern of time spent per entry. A two (AW vs. SED) by four (saline, 2.5, 5, or 7.5 mg/kg morphine dose) repeated measures ANOVA was conducted to determine the effects of morphine dose and activity condition on time spent per entry into the morphine-paired chamber during three ten-minute intervals. Results from the analysis in which animals that received negative CPP scores were excluded are presented first. Time spent per entry increased over time in both groups [$F(2, 44)=2.466, P<.001$] although to a greater extent in AW rats (Fig. 3). Paired-subject Student's *t*-tests were performed to determine at what time points time per entry into the morphine chamber differed. The amount of time spent per entry by AW and SED rats significantly differed at all 3 time intervals [$P<.01$ for all

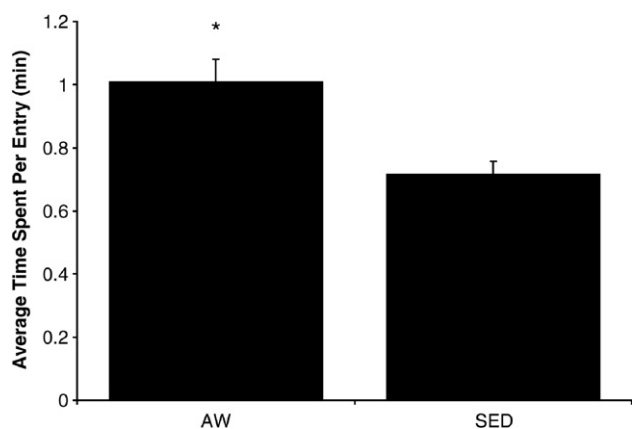


Fig. 2. Effects of exercise on total time spent per entry into the morphine conditioned chamber. Time per entry was obtained by dividing number of seconds spent in the conditioned chamber by the number of entries made into that chamber during the posttest. AW rats spent significantly more time per entry on average (plus S.E.M.) in the morphine-paired chamber than SED rats on test day, as determined by an ANOVA. * $P<.05$ indicates difference in comparison to SED rats.

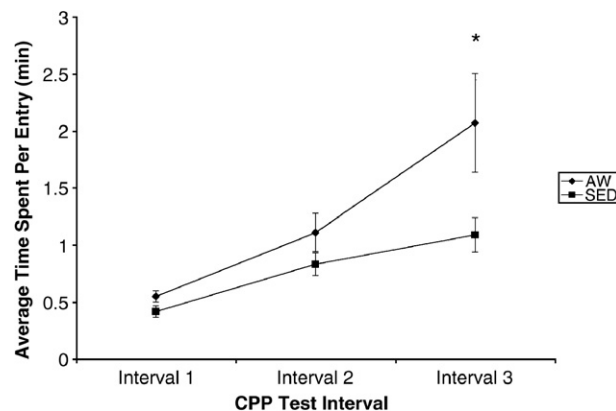


Fig. 3. Mean (plus S.E.M.) time spent per entry increased over time in both AW and SED rats that received saline, 2.5, 5 or 7.5 mg/kg morphine during conditioning, although this increase was evident to a greater extent in AW rats. AW rats spent significantly more time per entry during the last 10 min of the CPP test than SED rats. * $P<.05$ indicates difference in comparison to SED rats at that time point.

comparisons]. A main effect for group [$F(1, 44)=7.619, P=.008$] occurred with AW rats spending an average (S.E.M.) of 32.85 s (3.29 s), 1 min 11 s (9.64 s), and 2 min 7 s (25.98 s) in the morphine-paired chamber during the first, second, and third 10 min time intervals, respectively. Sedentary animals spent an average (S.E.M.) of 24.85 s (2.72 s), 49.45 s (5.65 s), and 1 min 9 s (8.86 s) in the morphine-paired chamber during the first, second, and third 10 min time intervals, respectively. The main effect of dose on time spent per entry was marginally significant [$F(3, 44)=2.673, P=.059$]. Subsequent Tukey post hoc analyses revealed that animals in the saline condition spent significantly less time per entry than animals in the 5 mg/kg morphine condition and animals in the 7.5 mg/kg morphine condition during the 30 min CPP test [$P<.05$ for both comparisons]. Animals in the 2.5 mg/kg morphine condition [$M=41.04$ s, S.E.M.=9.65 s] did not differ in time spent per entry from animals in other dosage conditions [$P>.05$ for all comparisons]. There was a significant time per entry \times activity group interaction [$F(2, 43)=4.693, P=.012$]. Subsequent between-subjects Student's *t*-tests revealed that AW and SED animals significantly differed in time per entry into the morphine-paired chamber during the last 10 min of the CPP test [$t(30)=2.113, P=.043$], spending an average (S.E.M.) of 2 min 7 s (25.98 s) and 1 min 9 s (46 s) per entry into the morphine-paired chamber, respectively. There were no significant interactions between time per entry and dose or between time per entry, dose, and group.

When all animals were included in the two (AW vs. SED) \times four (saline, 2.5, 5, or 7.5 mg/kg morphine dose) repeated measures analysis, rats significantly differed in time spent per entry at the three different time intervals regardless of dose or activity group [$F(2, 50)=12.644, P<.001$]. Paired-subject Student's *t*-tests were performed to determine at what time points time per entry into the morphine chamber differed. Time spent per entry in the morphine-paired chamber significantly differed between each ten-minute interval: the first and second ten-minute intervals [$t(57)=-6.396, P<.001$];

the first and third ten-minute intervals [$t(57)=-5.011, P<.001$]; and the second and third ten-minute intervals [$t(57)=-2.761, P=.008$]. The main effect of activity group approached statistical significance [$F(1, 50)=2.99, P=.09$], with AW rats spending more time per entry in the morphine-paired chamber on average than SED rats. There was no main effect of dose on time spent per entry at any of the three ten-minute intervals. There were no significant interactions between time per entry and dose or time per entry and activity group. Likewise, there was no significant interaction between time per entry, dose, and activity group.

A two (AW vs. SED) \times four (saline, 2.5, 5, or 7.5 mg/kg morphine dose) ANOVA was performed to determine the effects of activity group and dose on number of entries into the morphine-paired chamber at test day. When the analysis excluded animals that received negative CPP scores, the main effect of group approached significance [$F(1, 44)=3.486, P=.069$] with AW and SED rats making an average (S.E.M.) of 19.57 (1.62) and 23.58 (1.41) entries into the morphine-paired chamber on test day, respectively. No significant main effect of dose or activity group \times dose interaction occurred [$P>.05$ for both]. When the analysis included all animals, there was a main effect of activity group [$F(1, 50)=5.95, P=.018$] with AW and SED rats making an average (S.E.M.) of 18.82 (1.38) and 23.56 (1.36) entries into the morphine-paired chamber, respectively. The main effect for dose and the activity group \times dose interaction was not significant [$P>.05$ for both].

3.3. Preprogalanin mRNA in the LC of exercising and sedentary rats

Levels of optical density for preprogalanin mRNA in the LC did not significantly differ in AW and SED rats [$P>.05$]. However, a Pearson's correlation analysis revealed a significant and positive relationship between distance run and preprogalanin mRNA optical density in AW rats [$r(16)=.593, P=.015$] (Fig. 4).

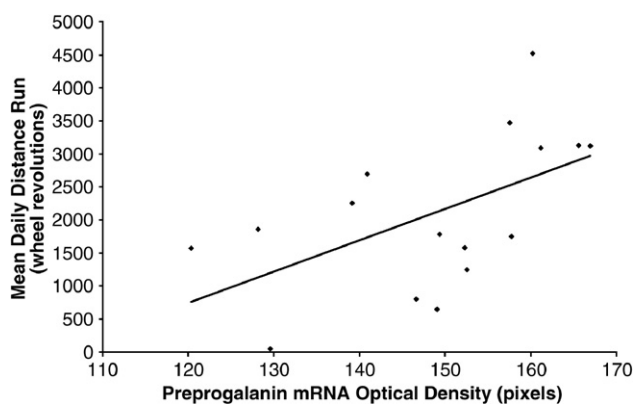


Fig. 4. Running distance is related to galanin mRNA expression in the LC. A significant, positive relationship between GAL mRNA levels and average daily revolutions of AW rats was determined by Pearson's correlation analysis [$r(16)=.593, P=.015$].

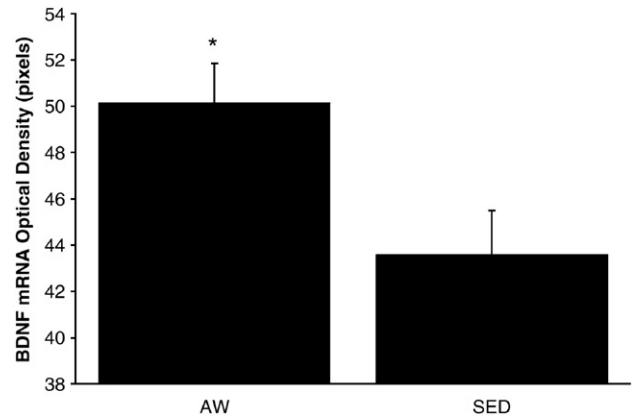


Fig. 5. Effect of exercise on BDNF mRNA. Mean (plus S.E.M.) optical densities for hippocampal BDNF mRNA significantly differed between AW and SED rats in the 5 mg/kg morphine condition. * $P<.05$ indicates significant difference from AW rats as determined by Student's t -test.

3.4. BDNF mRNA in the hippocampi of exercising and sedentary rats

Since rats receiving the 5 mg/kg dose of morphine during conditioning for CPP made up the largest dosage condition, a subset of these hippocampal sections was selected for in situ hybridization (AW $n=9$, SED $n=7$). A Student's between-subjects t -test revealed that optical density for BDNF mRNA was higher in AW [$M=50.14, S.E.M.=1.69$] than SED rats [$M=43.58, S.E.M.=1.93$] [$t(14)=2.56, P<.05$] (Fig. 5). Pearson's correlational analyses indicated a significant and negative relationship between BDNF optical density and number of entries into the morphine-paired chamber for animals in the 5 mg/kg morphine condition [$r(16)=-.510, P<.05$].

3.5. Running distance

AW rats ran an average (S.E.M.) of 1269.1 (86.93), 2278.42 (137.72), and 2824.49 (156.63) m during weeks one, two, and three of the experiment, respectively.

4. Discussion

The results reported in this experiment indicate that exercise exerts complex effects on CPP to morphine. The main results for animals that received positive CPP scores, which include increased time spent per entry and decreased number of entries into the morphine-paired chamber at test day in AW rats, suggest that chronic and voluntary wheel running enhances associative learning in the CPP to morphine task despite exercise's possible inducement of cross-tolerance to opioid agonists. Exercising and sedentary rats did not display significantly different degrees of CPP to morphine when calculated as the difference between time spent in the morphine-paired chamber and time spent in that chamber prior to conditioning. CPP to morphine occurred in a dose-dependent manner in both exercising and sedentary rats. In addition, active and sedentary rats displayed a pattern in which increased time was spent in the preferred chamber during later phases of the 30 min CPP test. Though the effects observed in the

CPP test may be subtle, the present results extend those of other studies showing an enhancement of performance in contextual learning tasks after chronic and voluntary exercise (Adlard et al., 2004; Anderson et al., 2000; Van Hooymissen et al., 2004).

An exclusion criterion was applied to all subjects in order to focus only on rats exhibiting CPP. Based on statistical comparisons, it was apparent that the excluded rats either failed to condition to the morphine-paired chamber or found the morphine-paired chamber aversive and thus were not comparable to the majority of the animals tested. However, one could argue that the negative CPP scores are valid reflections of the degree of place conditioning experienced by the rats. Thus, results of analyses including these animals were also included in our results' summaries. Focusing only on rats meeting the learning criterion, exercising animals displayed reliable CPP to morphine at all doses administered. Notably, the present results conflict with previous results that indicate that exercise attenuates CPP to morphine (Lett et al., 2002). The latter study employed brief and intermittent bouts of exercise in which rats were not housed with exercise wheels. In addition, the Lett et al. study administered morphine at a low dose of 1 mg/kg during conditioning and assessed CPP for 10 min only. As discussed above, the results of the current study indicate that CPP to morphine is most evident with a higher conditioning dose of morphine and after 10 min of the CPP test. The results of this study do not necessarily conflict with those that report cross-tolerance to morphine as indicated by decreased antinociception after administration of opioid agonists (Kanarek et al., 1998; Mathes and Kanarek, 2001; Smith and Lyle, 2006; Smith and Yancey, 2003). The exercise paradigm employed in the current study may have facilitated conditioning and/or associative learning in AW rats that overcame the effects of cross-tolerance. In addition, as suggested by Smith et al. (2004), exercise may have separate effects on nociceptive and reward systems in response to opioid agonists, which may be mediated by different neurotransmitter systems. Future studies should investigate whether animals that display enhanced associative learning in the CPP to morphine task also display cross-tolerance to morphine.

The mechanism involved in improved associative learning in the CPP to morphine task in chronically exercising rats deserves further study. One likely candidate is noradrenergic transmission, which is known to be modulated by exercise (Dishman et al., 2000; Dunn et al., 1996; Pagliani and Peyrin, 1995a,b) and which may in turn regulate BDNF concentrations in the hippocampus (Garcia et al., 2003; Hutter et al., 1996). β -adrenergic blockade attenuates BDNF mRNA expression increases observed after three days of voluntary wheel running in rats (Ivy et al., 2003). Notably, antidepressants that affect noradrenergic and serotonergic receptor levels are posited to increase expression of BDNF through increased phosphorylation of cyclic AMP response element binding protein (CREB) via enhanced activation of signaling cascades (Duman et al., 1997), supporting the hypothesis that NE plays a regulatory role in BDNF expression. In the current study, exercising rats may have experienced enhanced noradrenergic transmission and thus enhanced BDNF expression. This hypothesis is supported by the increased BDNF mRNA expression observed in the AW rats with *in situ*

hybridization. Considering the putative role of noradrenergic transmission in attention and learning, it is not surprising that enhanced noradrenergic transmission arising from the LC would benefit performance in tests of contextual conditioning (Aston-Jones et al., 1999; Olson et al., 2006; Van Hooymissen et al., 2004; Zarrindast et al., 2002).

When considering noradrenergic mechanisms underlying CPP, it is important to also examine the potential role of the peptide galanin in this process, since galanin is the major coexisting neurotransmitter in LC neurons (Holets et al., 1988; Holmes and Crawley, 1995; Skofitsch et al., 1986) and modulates NE release (Ma et al., 2001; Pieribone et al., 1995; Seutin et al., 1989). Although in the present study, activity wheel and sedentary rats did not differ in optical densities for galanin mRNA in the LC, there was a significant and positive relationship between average daily wheel revolutions and galanin mRNA levels, indicating that exercise increases galanin expression in a dose-dependent manner. This finding supports those of previous studies performed by our laboratory in which preprogalanin mRNA expression levels in the LC were higher in active rats than in sedentary rats (Holmes et al., 2006; O'Neal et al., 2001; Van Hooymissen et al., 2004). In the present study, a significant difference was not found for galanin mRNA levels. A possible explanation for this discrepancy is that the average daily running distance for the rats in the present study was much lower than that of the rats in previous studies (Holmes et al., 2006; Van Hooymissen et al., 2004). It is also possible that the morphine treatment may have influenced preprogalanin mRNA levels, although not in a "dose-dependent" manner as with activity. A Pearson's correlational analysis did not indicate the existence of a relationship between preprogalanin mRNA levels and CPP to morphine, suggesting no simple, linear relationship between LC galanin levels and learning.

The observed increase in hippocampal BDNF mRNA expression in AW rats is in line with the results of previous studies (Adlard et al., 2004; Farmer et al., 2004; Molteni et al., 2002; Neeper et al., 1996; Oliff et al., 1998; Tong et al., 2001). Furthermore, BDNF mRNA expression was negatively correlated with the number of entries into the morphine-paired chamber during the CPP test. It has previously been established that the hippocampus is necessary for CPP (Ferbinteanu and McDonald, 2001). The mechanism by which exercise benefits learning through hippocampal BDNF is currently under investigation. Exercise may not only exert short-term effects such as increasing BDNF expression but it may also affect downstream signaling proteins that are involved in long-term synaptic alterations (Chen and Russo-Neustadt, 2005; Farmer et al., 2004; Molteni et al., 2002; Tong et al., 2001). After one week of voluntary exercise, rats pretreated with a noncompetitive antagonist for BDNF do not show the improved performance in the Morris swim maze task that non-pretreated exercising rats do. In addition, exercising rats that receive the BDNF blocker do not show the increase in CREB and synapsin I that vehicle treated exercising rats do, indicating that BDNF mediates downstream signaling that is affected by exercise (Vaynman et al., 2004). In one study, while fourteen days of voluntary exercise did not significantly increase BDNF expression in rat hippocampi, cyclic AMP response

element binding protein (CREB) was increased (Chen and Russo-Neustadt, 2005). The abrupt termination of voluntary wheel running decreases BDNF mRNA expression as well as its TrkB receptor in the hippocampus five days later (Widenfalk et al., 1999). In heterozygous BDNF knockout mice, CPP to a midrange dose of cocaine is lost (Hall et al., 2003). The changes in expression of signaling proteins such as CREB and TrkB after chronic exercise indicate that long-term physical activity affects plasticity, thus altering the capacity for learning. In regards to the CPP paradigm, future studies may focus on elucidating changes in signaling proteins that mediate BDNF expression and examine these changes in relation to performance on the CPP task.

Preference for the sucrose solution over water was not enhanced in AW rats in the present study. However, AW rats drank more sucrose solution and water than SED rats. It is important to note that rats were not deprived of water at any point during the experiment, so the appetitive properties of water should not have been greater than those of the sucrose solution. Future investigations should attempt to control for the bodily loss of water resulting from wheel running which may increase water consumption in AW rats, thereby decreasing preference for sucrose in the presence of water. The results of the current study, however, do not indicate a significant difference in sucrose preference for AW and SED rats. This finding suggests that chronic and voluntary exercise did not enhance the appetitive properties of sucrose solution.

Overall, the current study reveals that chronic and voluntary exercise benefits learning as evidenced by increased time spent per entry in and decreased number of entries into the morphine-paired chamber by AW rats. CPP to morphine was dose-dependent, with animals receiving 5 mg/kg morphine during conditioning exhibiting the highest degree of CPP to morphine. Exercise did not affect sucrose preference. A more robust effect of exercise on the magnitude of CPP to morphine may have been compromised by cross-tolerance to opioid agonists induced by exercise. To further determine if exercise enhances degree of or associative learning in CPP, a drug or stimuli that do not act on the opioid system may be used in place of morphine. In addition, the learning effect may be further explored by a more salient CPP paradigm. It may be helpful to determine if the learning benefits of exercise are more evident after fewer or more conditioning sessions than employed in the current study.

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