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Wheel running can accelerate or delay extinction of conditioned place preference for cocaine in male C57BL/6J mice, depending on timing of wheel access

Martina L. Mustroph,[1] Derrick J. Stobaugh,[2] Daniel S. Miller,[2] Erin K. DeYoung,[2] and Justin S. Rhodes[2]
[1]Neuroscience Program, The Beckman Institute, University of Illinois at Urbana-Champaign, Urbana, IL, USA
[2]Department of Psychology, The Beckman Institute, University of Illinois at Urbana-Champaign, Urbana, IL, USA

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Abstract

Aerobic exercise may represent a useful intervention for drug abuse in predisposed individuals. Exercise increases plasticity in the brain that could be used to reverse learned drug associations. Previous studies have reported that exposing mice to a complex environment including running wheels after drug conditioning abolishes conditioned place preference (CPP) for cocaine, whereas running can enhance CPP when administered before conditioning. The purpose of the present study was to test the hypothesis that timing of exercise relative to conditioning has opposing effects on cocaine CPP. Male C57BL/6J mice experienced 30 days of running or sedentary treatments either before or after cocaine conditioning. Control animals always received saline and never cocaine, but otherwise underwent the same conditioning and exercise treatments. Animals were given bromodeoxyuridine injections at the onset of conditioning or exercise, and euthanized at the end of the study to quantify survival of new neurons in the hippocampus as a marker of plasticity. Wheel running accelerated extinction of CPP when running occurred entirely after drug conditioning, whereas running delayed extinction when administered before conditioning. A single conditioning day after running was sufficient to abolish the accelerated extinction observed when all conditioning preceded running. Running approximately doubled adult hippocampal neurogenesis, whereas cocaine had no effect. These results suggest that exercise-induced plasticity can facilitate learning that context is no longer associated with drug. However, if drug exposure occurs after exercise, running-induced plasticity may strengthen drug associations. The results provide insights into the interaction between exercise and drug conditioning that could have implications for drug abuse treatments.

Introduction

Additional treatments for cocaine abuse are urgently needed. Compliance with current treatment programs is weak, and abstinence is typically ephemeral. For example, in a recent study, 52% of cocaine users dropped out of a National Institute for Drug Abuse treatment trial within 3 months (Ghitza *et al.*, 2010). Studies have suggested that aerobic exercise could be useful as an intervention for maintaining abstinence in individuals willing to substitute exercise for drug reward (Sinyor *et al.*, 1982). Exercise promotes brain plasticity and activates some of the same brain structures as those involved in reward and addiction. In rodents, the neural plasticity marker ΔFosB is upregulated in the striatum and nucleus accumbens after wheel running to a comparable degree as after chronic cocaine exposure (Brene *et al.*, 2007). In addition, mice bred for high levels of voluntary wheel running display increased levels of the brain activity marker c-Fos in brain structures implicated in reward. Among these are the lateral hypothalamus, medial frontal cortex, and striatum (Rhodes *et al.*, 2003). Taken together, these findings suggest that exercise may serve as a substitute reward, but whether substituting exercise for drugs helps to ameliorate addiction is still unknown.

Plasticity resulting from exercise could be used to modify associations between drug reward and contextual cues. For example, exercise increases adult hippocampal neurogenesis, and new neurons have been hypothesized to display greater ability to be molded in response to new experiences than pre-established neurons (van Praag *et al.*, 1999a). In addition to being a site where exercise exerts effects on the brain, the hippocampus may influence reward circuitry because of its anatomical connections. The hippocampus receives input from the nucleus accumbens and ventral tegmental area, and sends output to the nucleus accumbens (Eisch & Harburg, 2006). Manipulations of hippocampal dentate gyrus granule cells have been shown to influence dopaminergic signaling in the nucleus accumbens and ventral tegmental area, areas that are important for drug reward (Eisch & Harburg, 2006).

Consistent with the hypothesis that exercise can enhance plasticity and facilitate extinction of conditioned place preference (CPP), a recent study on C57BL/6J mice found that environmental enrichment including running wheels completely abolished CPP for cocaine when the enrichment was administered after conditioning (Solinas *et al.*, 2008). The same group found that rearing C57BL/6J mice from weaning in an enriched environment abolished cocaine CPP when the

Correspondence: M. L. Mustroph, as above.
E-mail: mmustr2@illinois.edu

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animals were conditioned and tested as adults (Solinas *et al.*, 2009). Similarly, in Lewis rats, forced treadmill running during adolescence weakened cocaine CPP when conditioning occurred after running (Thanos *et al.*, 2010). On the other hand, wheel running strengthened cocaine CPP when adult Long-Evans rats were given wheel access before conditioning (Smith *et al.*, 2008). To the best of our knowledge, no other studies have investigated the effects of running on cocaine CPP. The purpose of this study was to determine: (i) whether the influence of wheel running on cocaine CPP depends critically on the timing of wheel access relative to drug conditioning; and (ii) whether changes in CPP correlate with changes in adult hippocampal neurogenesis in response to the exercise and cocaine treatments.

Materials and methods

Animals

Ninety male C57BL/6J mice were obtained at 5 weeks of age (The Jackson Laboratory, Bar Harbor, ME, USA) and housed four per cage in a climate-controlled environment on a 12-h light/dark cycle (lights off at 09:00 h) for 12 days. The dimensions of cages without running wheels were $29 \times 19 \times 13$ cm (length × width × height). Animals were individually housed for 1 week before experimental procedures were started. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee, and adhered to NIH guidelines.

Drugs

Cocaine hydrochloride (Sigma Aldrich, St Louis, MO, USA) was dissolved in 0.9% saline and administered at a dose of 10 mg/kg via intraperitoneal injections in a volume of 10 mL/kg. The dose was chosen on the basis of the literature, and was prepared according to the salt form, not the base form (Zombeck *et al.*, 2008).

Place conditioning chambers

The place conditioning chambers were modeled after Cunningham *et al.* (2006) and were the same as in previous studies from our laboratory (Zombeck *et al.*, 2008; Johnson *et al.*, 2010). They consisted of 20 identical black acrylic boxes ($30 \times 15 \times 15$ cm), with removable clear plastic tops. The floors were interchangeable, and consisted of three types with distinct textures: HOLE, GRID, and HOLE/GRID. The HOLE/GRID floor consisted of half GRID and half HOLE. The distance traveled and location of mice within CPP boxes were recorded with TOPSCAN video tracking software (Clever Sys, Vienna, VA, USA) (Zombeck *et al.*, 2008; Johnson *et al.*, 2010).

CPP procedure

We followed the procedure of Cunningham *et al.* (2003, 2006). Runners and sedentary animals were counterbalanced with respect to the conditioned stimulus (CS), and experienced cocaine on GRID (CS+ GRID) or cocaine on HOLE (CS+ HOLE), and saline on the other texture. During testing, animals explored a chamber of the same size as during conditioning, except with the HOLE/GRID floor type. Hence, the animals were forced to spend time on either the HOLE or the GRID side, and the duration on HOLE was equivalent to the total duration of the test (30 min) minus the duration on GRID (see previous section). CPP was determined by comparing the duration spent on HOLE (or GRID; the statistics would be the same) between groups, CS+ HOLE vs. CS+ GRID. The design ensured that any difference in time spent on textures between groups (CS+ GRID vs. CS+ HOLE) was attributable to drug–context learning, as this was the only variable that differed between the two groups. Biases in baseline preference for textures could not produce false-positives with this method, because the time spent on one texture (HOLE or GRID) was compared between subgroups CS+ GRID and CS+ HOLE, both of which would be expected to display the bias if one developed. Hence, when the difference score was computed, any bias was subtracted out. The two groups were also matched for drug exposure, which is important because drug exposure itself could affect the development of biases in preference. Moreover, each group served as the other group's learning control, because both groups learned to associate one texture with cocaine and the other texture with saline. This is important because, as compared with using a control in which all animals receive saline on both textures, the experience of learning itself could bias preferences for the textures (Cunningham *et al.*, 2003, 2006).

On the days of CPP habituation, pretesting, conditioning, testing, and cocaine priming (see below), animals were moved to a testing room, where the lights were turned off at 09:00 h. Animals were kept in the room for 1 h before testing began. Between sessions, the chambers were cleaned with disinfectant. Animals were returned to home cages with or without wheels immediately after testing. Hence, runners had continuous access to running wheels throughout behavioral testing, except when in the conditioning chambers.

Habituation

To familiarize the animals with the place conditioning chambers, they were placed on a flat surface without a texture in the conditioning chambers in the morning (09:00 h; for 30 min) and in the afternoon (15:00 h; for 30 min) for 1 day without any injection treatment (Cunningham *et al.*, 2006).

Pretesting

To determine individual biases in preference for the textures prior to drug pairing, animals were weighed, given a 10 mL/kg saline injection, and immediately placed in the apparatus with the HOLE/GRID floor in the morning (09:00 h; for 30 min) and afternoon (15:00 h; for 30 min) (Cunningham *et al.*, 2006).

Conditioning

Four CS+ trials (i.e. cocaine paired with one floor texture: HOLE or GRID) and four CS− trials (i.e. vehicle paired with the other floor texture) were administered over 4 days. On each day, one CS+ trial and one CS− trial were administered in the morning and afternoon. The order of exposure to CS+ and CS− was counterbalanced within each group. Experimental animals were weighed, given an injection of 10 mg/kg cocaine (CS+ trial) or vehicle (CS− trial), and immediately placed on the appropriate floor texture. Control animals underwent an identical procedure, except that they always received vehicle (saline) on both floor textures.

Testing

Testing took place twice daily, in the morning (09:00 h; 30 min) and in the afternoon (15:00 h; 30 min), for four consecutive days. Prior to

each testing session, each animal was weighed, injected intraperitoneally with 10 mL/kg saline, and placed in the center of the HOLE/GRID conditioning chamber.

Cocaine priming

Cocaine priming began 2 days after the final CPP test session, and consisted of two daily (morning and afternoon) 30-min exposures to HOLE/GRID for four consecutive days. Experimental animals were weighed and injected with 10 mg/kg cocaine immediately before being place in the HOLE/GRID conditioning chamber, whereas control animals received a saline injection before all priming sessions.

Running wheels and sedentary treatment

The dimensions of running wheel cages were 36 × 20 × 14 cm (length × width × height), and a 23-cm diameter wheel was mounted in the cage top. Running wheel rotations were monitored continuously in 1-min increments throughout the experiments via magnetic switches interfaced to a computer. Animals assigned to the sedentary group were deliberately not housed in cages with locked wheels, because mice climb in locked wheels, and we intended to keep physical activity to a minimum in the sedentary group (Koteja *et al.*, 1999; Rhodes *et al.*, 2000, 2003).

Experimental design

Experiment 1

At 54 days of age, animals (n = 30) underwent habituation, pretesting, and cocaine CPP (n = 20, cocaine group) or CPP without cocaine (n = 10, control group), as detailed above (Fig. 1). During the four conditioning days, animals received daily injections of 50 mg/kg bromodeoxyuridine (BrdU) to label dividing cells. On the day after the four conditioning days, animals were placed individually in cages either without (sedentary, n = 10 cocaine and n = 5 control) or with (runner, n = 10 cocaine and n = 5 control) running wheels for 30 days. On the second day of sedentary or runner treatment, animals received one additional BrdU injection. After 30 days, animals underwent four consecutive days of CPP testing, and this was followed 1 day later by four consecutive days of CPP testing after cocaine priming. After testing on each day, animals were returned to their cages with or without running wheels, so that runners had continuous access to wheels throughout the testing period.

Experiment 2

At 54 days of age, animals underwent habituation and pretesting (n = 30). On the next day, animals were individually placed into cages either without (sedentary, n = 15) or with (runner, n = 15) running wheels for 30 days. During the first 10 days of sedentary or runner treatment, animals received daily injections of 50 mg/kg BrdU. After 30 days, animals underwent cocaine CPP (n = 20, cocaine group; 10 runners and 10 sedentary) or CPP without cocaine (n = 10, control group; five runners and five sedentary) for 4 days. Animals then underwent four consecutive days of CPP testing, followed 1 day later by four consecutive days of CPP testing after cocaine priming. After conditioning or testing on each day, animals were returned to their cages with or without running wheels, so that runners had continuous access to wheels throughout the testing period.

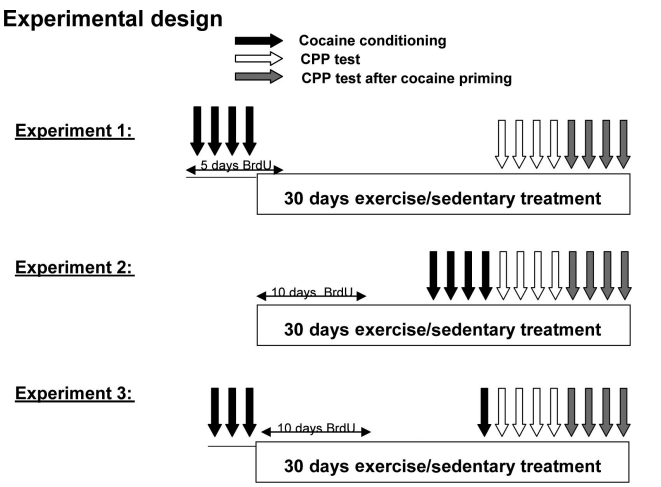

FIG. 1. Schematic diagram of the experimental design. The black arrows indicate when CPP conditioning sessions were administered. The white arrows indicate when CPP testing took place. The gray arrows indicate when CPP testing took place after a cocaine priming dose had been administered. The boxes indicate when the runner/sedentary conditions were administered relative to conditioning and CPP testing. Each experiment included 30 animals (20 conditioned with cocaine and 10 saline controls, divided equally into runner and sedentary groups). In all three experiments, animals experienced 1 day of habituation to reduce novelty effects, and 1 day of CPP pretesting to establish baseline texture preferences over the 2 days immediately preceding the conditioning (experiments 1 and 3) or runner/sedentary treatments (experiment 2). Animals in all three experiments experienced 30 days of uninterrupted running or sedentary treatment, and a total of 4 days of CPP conditioning. The three experiments differed in the timing of the four CPP conditioning sessions relative to the runner/sedentary treatment phase. Animals were returned to cages with or without running wheels immediately after conditioning and testing, to avoid the potential confound of animals experiencing withdrawal from running during the testing procedures.

Experiment 3

At 54 days of age, animals (n = 30) underwent habituation, pretesting and cocaine CPP (n = 20, cocaine group) or CPP without cocaine (n = 10, control group) for three consecutive days. On the next day, animals were placed individually in cages either without (sedentary, n = 10 cocaine and n = 5 control) or with (runner, n = 10 cocaine and n = 5 control) running wheels for 30 days. On the first 10 days of sedentary or runner treatment, animals received daily injections of 50 mg/kg BrdU. After 30 days, animals underwent one additional day of CPP conditioning to simulate a relapse episode, and this was followed by four consecutive days of CPP testing, and then, 1 day later, by four consecutive days of CPP testing with cocaine priming. After the last conditioning or testing session on each day, animals were returned to their cages with or without running wheels, so that runners had continuous access to wheels throughout the testing period.

Immunohistochemistry

Tissue preparation

Following behavioral testing, all animals (n = 90) were anesthetized with 100 mg/kg intraperitoneal sodium pentobarbital and then perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline (PBS) (0.287% sodium phosphate monobasic anhydrous, 1.102% sodium phosphate dibasic anhydrous, 0.9% sodium chloride in water). Brains were postfixed overnight, and then transferred to 30% sucrose in PBS. Brains were sectioned with a cryostat into 40-μm-thick coronal sections. Sections were placed into

tissue cryoprotectant (30% ethylene glycol, 25% glycerin, 45% PBS) in 24-well plates and stored at −20 °C. Two separate one-in-six series of these sections (i.e. series of sections throughout the rostrocaudal extent of the brain with 240-μm increments separating each section, approximately nine sections) were stained in the following way.

BrdU–diaminobenzidine (DAB)

Purpose: to detect BrdU-positive (newly divided) cells in the dentate gyrus. Free-floating sections were washed in tissue buffering solution (TBS) (1.3% Trizma hydrochloride, 0.19% Trizma base, 0.9% sodium chloride) and then treated with 0.6% hydrogen peroxide in TBS for 30 min. To denature DNA, sections were treated for 120 min with a solution of 50% de-ionized formamide and 2× SSC buffer, rinsed for 15 min in 2× SSC buffer, and then treated with 2 M hydrochloric acid for 30 min at 37 °C and with 0.1 M boric acid in TBS (pH 8.5) for 10 min at room temperature. Sections were then treated (blocked) with a solution of 0.3% Triton-X and 3% goat serum in TBS (TBS-X plus) for 30 min, and then incubated with primary antibody against BrdU raised in rat (catalog number OBT0030; AbD Serotec, Raleigh, NC, USA) at a dilution of 1 : 100 in TBS-X plus for 72 h at 4 °C. Sections were then washed in TBS, blocked with TBS-X plus for 30 min, and incubated in biotinylated secondary antibody against rat raised in goat (catalog number BA-9400; Vector, Burlingame, CA, USA) at 1 : 250 in TBS-X plus for 100 min at room temperature. Sections were then treated with the ABC system (catalog number PK-6100; Vector) and stained with a DAB kit (catalog number D4418-505ET; Sigma Aldrich).

Double-fluorescent label

Purpose: to determine the proportion of BrdU-positive cells in the dentate gyrus that differentiated into neurons. Sections were treated as for BrdU–DAB, except that a cocktail was used for the primary antibody step. Rat anti-BrdU (1 : 100, catalog number OBT0030; AbD Serotec) was combined with mouse anti-neuronal nuclear protein (NeuN) (1 : 50, catalog number MAB377; Millipore, Billerica, MA, USA) for 72 h at 4 °C. Secondary antibodies were conjugated with fluorescent markers (Cy2-green anti-mouse, and Cy3-red anti-rat, catalog numbers 115-225-166 and 112-165-167; Jackson ImmunoResearch, West Grove, PA, USA, respectively) at a dilution of 1 : 250, and also delivered as a cocktail.

Image analysis

BrdU–DAB

The entire granule layer (bilateral), represented in the one-in-six series, was photographed by systematically advancing the field of view of the Zeiss brightfield light microscope and taking multiple photographs, via a camera interfaced with a computer, under ×10 (total ×100) magnification. Positively labeled cells in these photographs were counted to generate estimates of the total number of labeled cells. The total volume of the dentate gyrus represented in the series was measured, so that the counts could be expressed per μm^3 dentate gyrus sampled.

Double label

A Leica SP2 laser scanning confocal microscope (×40 oil objective; pinhole size, 81.35 μm in diameter) was used to determine the proportion of dentate gyrus BrdU-positive cells that differentiated into neurons (NeuN+). Dentate gyrus BrdU-positive cells were identified as either co-expressing NeuN or not. Each BrdU-positive cell in the granular layer (represented in the one-in-six series) was analyzed by focusing through the tissue in the *z*-axis to establish co-labeling with NeuN. The number of new neurons per μm^3 per mouse was calculated as the number of BrdU cells per μm^3 multiplied by the average proportion of BrdU cells co-expressing NeuN for the designated group.

Statistical analysis

Data were analyzed with SAS (version 9.1) or R (version 2.7.2) statistical software. In all analyses, $P < 0.05$ was considered to be statistically significant. Each experiment (1, 2, and 3) was analyzed separately. CPP data were analyzed as follows. Control animals that never received cocaine were analyzed separately from cocaine-treated animals. First, the time spent on HOLE was analyzed by three-way repeated measures ANOVA, with CS (CS+ HOLE vs. CS+ GRID; between subjects), exercise history (runner vs. sedentary; between subjects), day of testing (1–4; within subjects) and all interactions entered as factors. Testing session, whether at 09:00 h or 15:00 h, was also included as a factor in initial models, but was never significant, and was therefore removed from the final linear models. In addition, the CPP data were analyzed separately within each group, using standard methods (Cunningham *et al.*, 2006). Within each group, the duration spent on HOLE was compared between the CS+ HOLE and CS+ GRID groups (Cunningham *et al.*, 2006). Numbers of new neurons in the granule layer of the dentate gyrus were analyzed by two-way ANOVA, with exercise history (runner vs. sedentary), cocaine treatment (cocaine vs. vehicle) and all interactions entered as factors. The correlation between distance traveled and number of new neurons was estimated by simple linear regression. The proportion of BrdU-labeled cells in the granule cell layer that co-expressed NeuN was analyzed by logistic regression, where proportion (binomial response) was modeled as a linear function of drug treatment (cocaine vs. saline), exercise group (runner vs. sedentary) and all interactions entered as factors.

Results

Baseline preference

During the pretest, before the animals had ever experienced cocaine, and before any of them ran on wheels, they spent approximately 50% of their time on each side, with an average of 15.1 min [± 0.32 standard error (SE)] on HOLE. Running for 30 days slightly changed the baseline preference of control animals, as was shown by CPP testing, when control animals that had never received cocaine but had run showed a bias towards HOLE that they had not displayed during the initial pretest. Sedentary control animals that never received cocaine spent approximately 50% of their time, an average of 14.8 min (± 0.74 SE), on HOLE, whereas runners spent approximately 60% of their time, 18.0 min (± 0.74 SE), on HOLE ($F_{1,246} = 10.8$, $P = 0.001$). This bias in preference observed in runners did not compromise the analysis of cocaine CPP, because the bias was present in both groups of runners (CS+ GRID and CS+ HOLE) being compared to establish CPP (see Materials and methods).

Locomotor activity in CPP chambers

During conditioning, animals given cocaine traveled an average of 56.7 m (± 1.26 SE) per conditioning session, whereas animals given saline traveled 35.0 m (± 1.26 SE) per conditioning session ($F_{1,413} = 617.5$, $P < 0.0001$). Runners moved a similar distance in the apparatus as sedentary animals after saline or cocaine administration.

Wheel running

Running increased from days 1 to 20, and then stayed at a plateau for the remaining days (for example, for experiment 1, day was significant; $F_{29,1311} = 23.7$, $P < 0.0001$) (Fig. 2). Cocaine had no influence on wheel running (Fig. 2). The average level of running across all animals in all experiments was 6.2 km (± 0.41 SE).

Experiment 1: Running after CPP

Sedentary animals displayed significantly stronger CPP than runners, as indicated by a significant interaction between texture group (whether conditioned with cocaine on HOLE or GRID) and exercise group (runner or sedentary) ($F_{1,128} = 7.7$, $P = 0.007$). In addition, CPP was extinguished faster in runners than in sedentary animals, as indicated by a significant interaction between exercise group and day ($F_{3,128} = 2.7$, $P = 0.046$) (Fig. 3A). *Post hoc* analyses revealed that sedentary animals displayed significant CPP on days 1–3 (all $P < 0.05$), whereas runners displayed significant CPP only on day 1 ($P < 0.05$). The strength of preference on day 1 was slightly lower in runners than in sedentary animals ($P = 0.08$).

During cocaine priming, sedentary animals displayed significantly stronger CPP than runners, as indicated by a significant interaction between exercise group and day ($F_{3,114} = 4.4$, $P = 0.006$) and a significant three-way interaction between exercise group, texture group, and day ($F_{3,114} = 3.0$, $P = 0.035$) (Fig. 3B). *Post hoc* analyses indicated that sedentary animals displayed significant CPP only on day 1 of cocaine priming ($P < 0.05$), whereas runners never displayed significant CPP during cocaine priming, and the strength of preference on day 1 was significantly lower in runners than in sedentary animals ($P = 0.03$).

Experiment 2: Running before CPP

Runners displayed significantly stronger CPP than sedentary animals, as indicated by a significant interaction between texture group and exercise group ($F_{1,128} = 10.3$, $P = 0.002$). In addition, CPP tended to be extinguished faster in sedentary animals than in runners, as suggested by an interaction between exercise group and day that approached significance ($F_{3,128} = 2.5$, $P = 0.066$) (Fig. 3C). *Post hoc* analyses revealed that runners displayed significant CPP on days 1–4 (all *P*-values < 0.05), whereas sedentary animals displayed significant CPP only on days 1–2 ($P < 0.05$), and the strength of preference on day 2 was significantly lower in sedentary animals than in runners ($P = 0.027$).

During cocaine priming, runners displayed significantly stronger CPP than sedentary animals, as indicated by a significant interaction between exercise group and day ($F_{3,128} = 5.2$, $P = 0.002$) and a significant interaction between texture group and day ($F_{3,128} = 5.8$, $P = 0.0009$) (Fig. 3D). *Post hoc* analyses indicated that runners displayed significant CPP on days 1–4 of cocaine priming ($P < 0.05$), whereas sedentary animals displayed significant CPP only on the first day of cocaine priming, and the strength of preference on day 1 tended to be lower in sedentary animals than in runners ($P = 0.07$).

Experiment 3: Running before CPP with simulated relapse episode

Runners and sedentary animals displayed significant CPP on all testing days, as indicated by a significant effect of texture group ($F_{1,128} = 46.1$, $P < 0.0001$) but no significant effects of exercise group, day, or any interactions (Fig. 3E). During cocaine priming, runners displayed significantly stronger CPP than sedentary animals, as indicated by a significant interaction between exercise group and day ($F_{3,128} = 5.0$, $P = 0.003$) (Fig. 3F). *Post hoc* analyses indicated that runners displayed significant CPP on days 1–4 of cocaine priming ($P < 0.05$), whereas sedentary animals displayed significant CPP only on the first day of cocaine priming, and the strength of preference on day 1 tended to be lower in sedentary animals than in runners ($P = 0.07$).

Hippocampal neurogenesis

Runners displayed an approximately twofold increase in the number of new neurons (BrdU cells co-labeled with NeuN) as compared with sedentary animals in each of the three experiments (experiment 1, $F_{1,24} = 29.7$, $P < 0.0001$; Experiment 2, $F_{1,26} = 31.3$, $P < 0.0001$; experiment 3, $F_{1,32} = 23.0$, $P < 0.0001$) (Fig. 4). The average distance traveled in the wheel over the 30 days by runners was significantly correlated with the number of new neurons ($F_{1,43} = 7.9$, $P = 0.007$). Cocaine had no effect on neurogenesis ($P > 0.05$). Analysis of logistic regression revealed that running significantly increased the proportion of BrdU cells that differentiated into neurons in all three experiments (deviance = 4.6, $P = 0.03$). The percentage of BrdU cells that differentiated into neurons as indicated by co-labeling with NeuN was 92 ± 1.2% SE in sedentary animals and 94 ± 0.8% SE in runners. Cocaine had no effect on proportion of BrdU cells that differentiated into neurons, and no interactions with cocaine or experiment were significant ($P > 0.05$). Given the high percentage of BrdU cells that differentiated into neurons, the results were similar when the total number of BrdU cells was analyzed instead of the total number of new neurons (i.e. BrdU cells co-labeled with NeuN). Running approximately doubled the number of BrdU cells in the granule layer in each of the three experiments (experiment 1, $F_{1,24} = 16.7$, $P = 0.0004$; experiment 2, $F_{1,26} = 22.6$, $P < 0.0001$; experiment 3, $F_{1,32} = 32.1$, $P < 0.0001$).

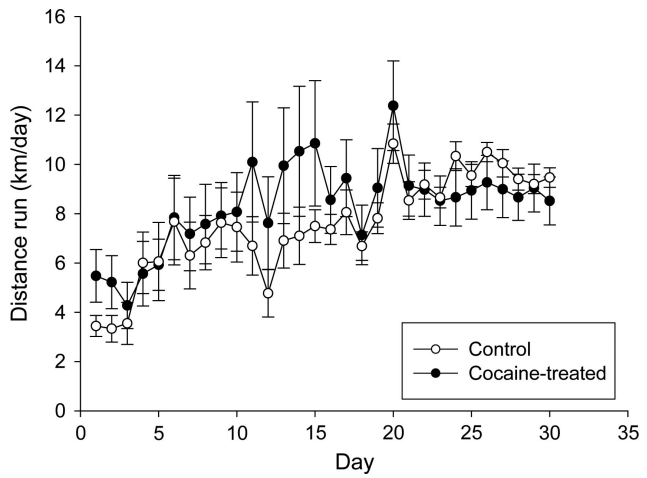

FIG. 2. Wheel running over the course of the study. The average distance run (km/day) (± SE) for a representative experiment (experiment 1) is shown separately for cocaine-treated animals ($n = 10$, solid circles) and control animals ($n = 5$, open circles) that never experienced cocaine. Wheel running data for experiments 2 and 3 were similar (data not shown). Increased wheel running over the first 20 days is typical for C57BL/6J mice.

Discussion

The main finding from the study is that wheel running has opposing effects on cocaine CPP, depending on when running is administered

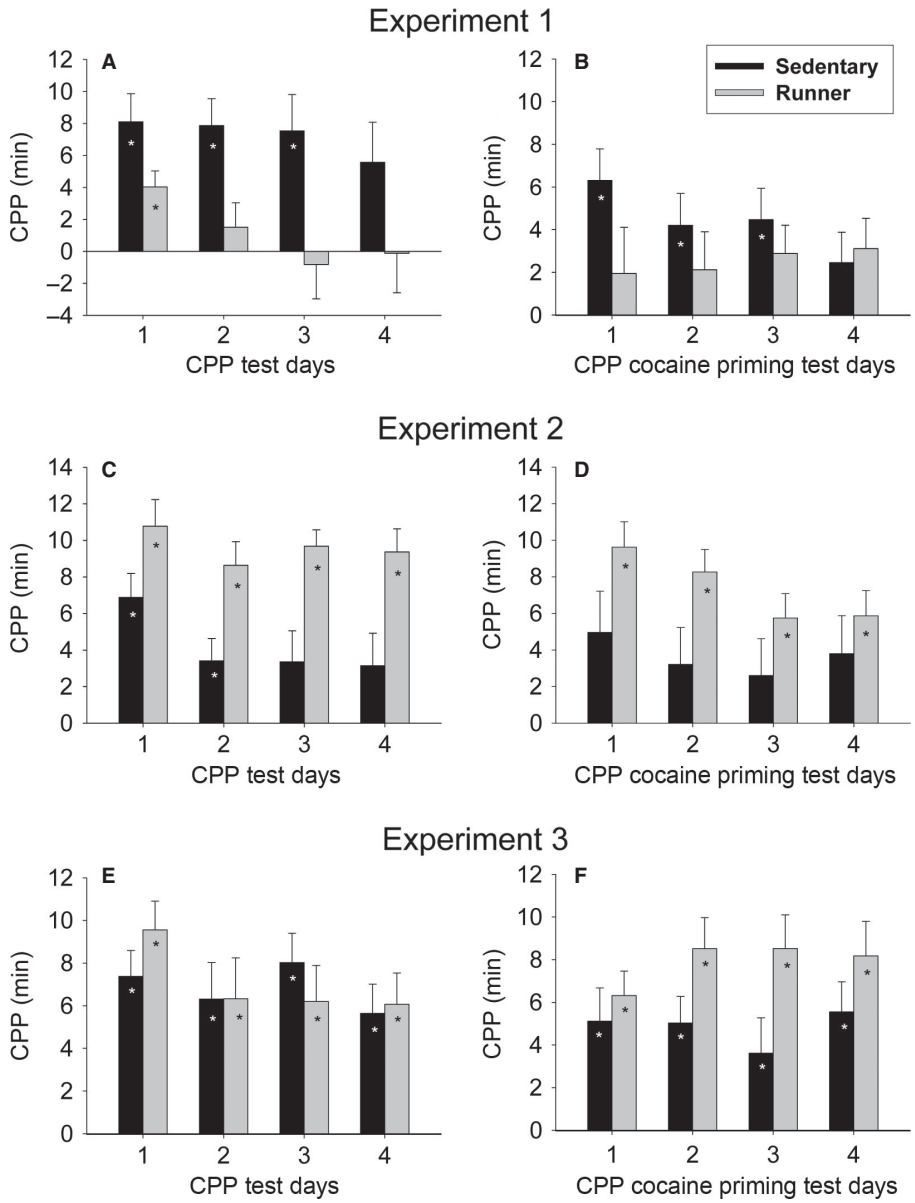

FIG. 3. CPP for cocaine during testing and cocaine priming. The mean difference in duration (min) ± SE spent on HOLE between animals receiving cocaine on HOLE (CS+ HOLE) and animals receiving cocaine on GRID (CS+ GRID) are plotted separately for runners and sedentary animals. Each bar represents data for 10 animals (n = 5, CS+ HOLE animals; n = 5, CS+ GRID animals). A, C and E show testing data for experiments 1, 2, and 3, respectively. B, D and F show cocaine priming data for experiments 1, 2, and 3, respectively. The stars indicate significant place preference at $P < 0.05$.

relative to drug conditioning (Fig. 3). The results have implications for drug rehabilitation programs that consider exercise as an intervention for maintaining abstinence (Sinyor *et al.*, 1982). We speculate that when exercise is administered before drug conditioning, the plasticity engendered from running strengthens the learning of the drug–context association, such that, when tested, the runners are resistant to extinction of this association. This interpretation is consistent with many previous reports that running enhances learning and memory (e.g. van Praag *et al.*, 1999b; Colcombe & Kramer, 2003; Eisenstein & Holmes, 2007; Clark *et al.*, 2008; Griffin *et al.*, 2009; Creer *et al.*, 2010). On the other hand, when exercise is administered after drug conditioning, the plasticity engendered from running cannot strengthen drug learning, because the plasticity is increased after the drug learning has taken place. However, the plasticity resulting from running could be recruited later to facilitate the new learning that the context is no longer associated with the drug, and hence accelerated extinction of CPP was observed in runners. Taken together, the results suggest that exercise could be a useful intervention to facilitate extinction of conditioned drug associations during abstinence. However, the benefit of exercise could be reversed if a relapse episode occurred after running had primed the brain for plasticity.

One of the important findings from the study was that running after drug conditioning no longer accelerated extinction of CPP for cocaine when a single conditioning session was administered after running (Fig. 3E). The single additional conditioning trial abolished the benefits of exercise in facilitating extinction, despite the fact that 75% of conditioning occurred prior to exercise exposure, when no new plasticity generated from running could help in the acquisition of cocaine–context learning. In accordance with the study of Smith *et al.* (2008), our results showed that even one drug conditioning day after

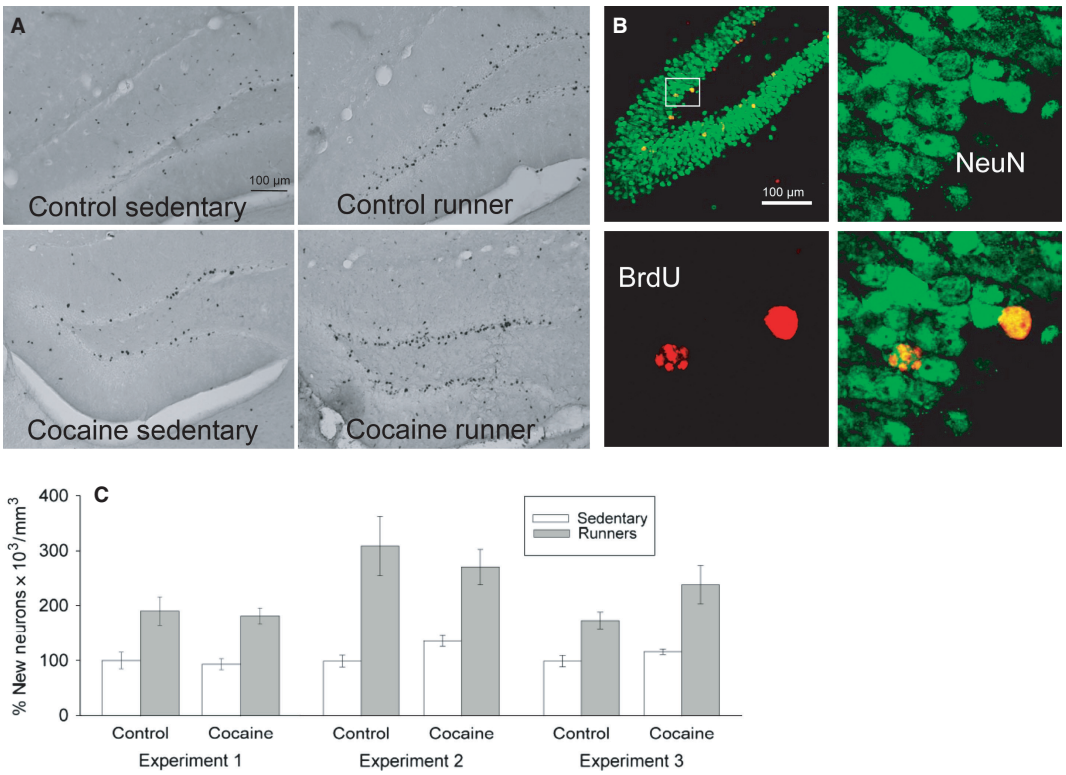

FIG. 4. Adult hippocampal neurogenesis. (A) Photographs of the dentate gyrus stained for BrdU–DAB, showing representative sections from each of the four groups. Black dots are nuclei stained positive for BrdU, indicating newly divided cells. (B) Photographs of a representative section through the dentate gyrus of a runner mouse double-stained green for NeuN (mature neuronal marker) and red for BrdU. The panels to the right show the tissue illuminated for each color separately and combined, zoomed in around the BrdU cells, indicating two episodes of neurogenesis. (C) Average number of new neurons per volume dentate gyrus, shown as a percentage of those in the average sedentary control animal. Runners are shown as gray bars, and sedentary animals as white bars. Data are shown separately for control animals (never treated with cocaine; $n = 5$ sedentary animals and $n = 5$ runners) and cocaine-treated animals ($n = 10$ sedentary animals and $n = 10$ runners) for each experiment. SE bars are shown. For interpretation of color references in figure legend, please refer to the Web version of this article.

exercise results in delayed CPP extinction, as compared to the results seen in studies where running combined with a complex environment preceded drug conditioning (Solinas *et al.*, 2008; Chauvet *et al.*, 2011). Moreover, the priming dose of cocaine elicited significant CPP across all cocaine priming days only in the animals that had run prior to cocaine conditioning (Fig. 3D and F). The fact that these animals' CPP was never extinguished, even during cocaine priming, suggests that their drug–context learning was especially strong, possibly because of the enhanced plasticity that was present at the time of drug conditioning, for example regarding brain-derived neurotrophic factor, insulin-like growth factor-1, angiogenesis, and synaptogenesis (Neeper *et al.*, 1995; Carro *et al.*, 2001; Dietrich *et al.*, 2008; Gomez-Pinilla *et al.*, 2008; Clark *et al.*, 2009).

When administered before conditioning, exercise strengthened cocaine CPP and delayed extinction (Fig. 3C). As discussed above, this result is consistent with a vast literature demonstrating pro-cognitive effects of exercise on many different forms of learning and memory (e.g., van Praag *et al.*, 1999b; Colcombe & Kramer, 2003; Eisenstein & Holmes, 2007; Clark *et al.*, 2008; Griffin *et al.*, 2009; Creer *et al.*, 2010). Although there are relatively few studies examining the effects of exercise on CPP for drugs of abuse, there is no *a priori* reason to believe that the effects of exercise or enrichment would be any different for drug learning than for other forms of associative learning. Consistent with this proposition and our results (Fig. 3C), early studies found that housing Sprague–Dawley rats in an enriched environment (without running wheels) from weaning until adulthood strengthened CPP for amphetamine (Bowling & Bardo, 1994; Bardo *et al.*, 1995), and a more recent study found that wheel running administered before conditioning strengthened CPP for cocaine in Long-Evans rats (Smith *et al.*, 2008). However, two other studies found the exact opposite (Solinas *et al.*, 2009; Thanos *et al.*, 2010). Solinas *et al.* (2009) recently reported that housing C57BL/6J mice from weaning until 2–3 months of age with toys and running wheels completely abolished cocaine CPP when conditioning and testing were administered after the enriched housing, as compared with mice housed in standard conditions, which displayed significant CPP. The study of Solinas *et al.* (2009) is very similar to ours, with the same strain of mice and running wheels. The only difference between the Solinas *et al.* (2009) study and ours was that their mice were housed in cages with toys and running wheels from weaning to adulthood, whereas our animals were housed only with running wheels, starting in adulthood. However, we would have expected the longer duration of enrichment to produce greater enhancement in CPP learning and retention, and certainly not to abolish cocaine CPP. Solinas *et al.* (2009) argued that enrichment abolished CPP not by altering learning but by reducing the rewarding effects of cocaine, which may be a possibility. The other study that found exercise before conditioning to attenuate CPP for cocaine (Thanos *et al.*, 2010) was conducted in adolescent rats, and used forced treadmill running as the form of exercise. Hence, this study has numerous differences from our study, the most important of which is probably the forced running, which can induce stress, and is not considered to be a rewarding form of exercise in animals (Greenwood *et al.*, 2011).

Our speculation that plasticity resulting from running facilitated learning in our experiments is consistent with our observation of increased adult hippocampal neurogenesis in runners as compared with sedentary animals in all three experiments (Fig. 4). A role for the hippocampus in associative learning has been established (Ferbinteanu & McDonald, 2001; Fuchs *et al.*, 2005; Rudy & Matus-Amat, 2005). Moreover, several papers have provided direct evidence that new neurons can enhance associative learning (Winocur *et al.*, 2006; Wojtowicz *et al.*, 2008; Hernandez-Rabaza *et al.*, 2009; Drew *et al.*, 2010). New neurons have been hypothesized to display greater ability to be molded in response to new experiences than older-established neurons, because their processes and connections are not yet solidified (van Praag *et al.*, 1999b). Hence, it is conceivable that, in the case where exercise is administered before conditioning, new neurons generated from running could be recruited during drug learning and lead to strengthening of associative learning, making the behavior more difficult to extinguish. Moreover, it is also conceivable that, when exercise is administered after conditioning, new neurons could be recruited during extinction, allowing more rapid acquisition of the new association that the context is no longer paired with drug. However, at present, the connections between neurogenesis and the CPP outcomes are correlations only. Many other changes occur in the brain as a result of exercise, including synaptogenesis, increases in trophic factors, growth factors, neurotransmitter concentrations, angiogenesis, and changes in dendritic morphology, among others that could account for our findings (Meeusen & De Meirleir, 1995; Neeper *et al.*, 1995; Carro *et al.*, 2001; Dietrich *et al.*, 2008; Gomez-Pinilla *et al.*, 2008; Clark *et al.*, 2009). Moreover, exercise is known to promote resistance to stress, which could contribute to the behavioral outcomes, depending on how stressful the CPP testing was perceived to be by the animals (Greenwood & Fleshner, 2008). One way to directly test the role of new neurons in the behavioral outcomes is to repeat the study with animals that are unable to generate new neurons from exercise, through the use of irradiation or transgenic mouse models (Saxe *et al.*, 2006; Clark *et al.*, 2008; Dupret *et al.*, 2008; Deng *et al.*, 2009).

Previous literature suggests that cocaine exposure reduces adult hippocampal neurogenesis (Noonan *et al.*, 2010; Sudai *et al.*, 2011). However, we did not observe such a result in any of the three experiments (Fig. 4). One explanation is the difference in duration of cocaine exposure between studies. In our study, animals were exposed to cocaine for a total of 8 days, 4 days during conditioning and 4 during cocaine priming, whereas cocaine exposure of up to 15 days is characteristic of studies that have found a significant cocaine-induced reduction in hippocampal neurogenesis. Moreover, most of the studies reporting a decrease in neurogenesis caused by cocaine employed operant conditioning methods, where the rats have to perform a lever press to receive an intravenous infusion of drug, as opposed to the classical conditioning methods used here, where animals are given intraperitoneal injections (Noonan *et al.*, 2010; Sudai *et al.*, 2011).

The present study is the first to show opposite effects of wheel running on cocaine CPP that depend critically on the timing of exercise relative to drug conditioning. The mechanisms by which running facilitates or extinguishes CPP are not known. One possible explanation is that plasticity generated from running, including new hippocampal units, could facilitate learning of drug–context associations or learning that drug is no longer associated with context. A better understanding of the mechanisms that mediate the effects of exercise on drug–context learning could lead to improved treatments for drug addiction.

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Abbreviations

BrdU, bromodeoxyuridine; CPP, conditioned place preference; CS, conditioned stimulus; DAB, diaminobenzidine; NeuN, neuronal nuclear protein; PBS, phosphate-buffered saline; SE, standard error; TBS, tissue buffering solution; TBS-X, 0.3% Triton-X and 3% goat serum in tissue buffering solution.

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