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Increased adult hippocampal neurogenesis is not necessary for wheel running to abolish conditioned place preference for cocaine in mice

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Abstract

Recent evidence suggests that wheel running can abolish conditioned place preference (CPP) for cocaine in mice. Running significantly increases the number of new neurons in the hippocampus, and new neurons have been hypothesised to enhance plasticity and behavioral flexibility. Therefore, we tested the hypothesis that increased neurogenesis was necessary for exercise to abolish cocaine CPP. Male nestin–thymidine kinase transgenic mice were conditioned with cocaine, and then housed with or without running wheels for 32 days. Half of the mice were fed chow containing valganciclovir to induce apoptosis in newly divided neurons, and the other half were fed standard chow. For the first 10 days, mice received daily injections of bromodeoxyuridine (BrdU) to label dividing cells. On the last 4 days, mice were tested for CPP, and then euthanized for measurement of adult hippocampal neurogenesis by counting the number of BrdU-positive neurons in the dentate gyrus. Levels of running were similar in mice fed valganciclovir-containing chow and normal chow. Valganciclovir significantly reduced the numbers of neurons (BrdU-positive/NeuN-positive) in the dentate gyrus of both sedentary mice and runner mice. Valganciclovir-fed runner mice showed similar levels of neurogenesis as sedentary, normal-fed controls. However, valganciclovir-fed runner mice showed the same abolishment of CPP as runner mice with intact neurogenesis. The results demonstrate that elevated adult hippocampal neurogenesis resulting from running is not necessary for running to abolish cocaine CPP in mice.

Introduction

Relapse is a major obstacle to recovery from drug abuse, and is triggered, in part, by exposure to drug-associated cues (O'Brien *et al.*, 1998; Volkow *et al.*, 2006). In order to attenuate relapse, it would be useful to find interventions that extinguish drug–context associations. Recent evidence suggests that exercise can reduce the incidence of relapse to drug use in humans (Brown *et al.*, 2010). Rodent work supports the idea that exercise reduces the strength of drug–context associations (Thanos *et al.*, 2010; Mustroph *et al.*, 2011). Taken together, human and rodent evidence suggests that exercise may reduce relapse by facilitating extinction of drug–context associations, but the neurobiological mechanisms are unknown.

The hippocampus is a site in the brain where the effects of exercise, associative learning and extinction converge. First, the hippocampus, specifically the dentate gyrus, plays a critical role in binding information from different sensory modalities into unique memories (Meyers *et al.*, 2003; Hernandez-Rabaza *et al.*, 2008; Johnson *et al.*, 2010; Chauvet *et al.*, 2011). Functional magnetic resonance imaging studies have demonstrated that drug users show bilateral activation of the hippocampus when shown images of drug paraphernalia (Michaelides *et al.*, 2012; Ames *et al.*, 2013). The hippocampus also plays an important role in extinction learning (Quirk & Mueller, 2008).

In addition to its role in associative learning and extinction, the hippocampus is a major site of activation during an acute bout of exercise (Clark *et al.*, 2011), and a center for plasticity in response to exercise (Neeper *et al.*, 1995; Dietrich *et al.*, 2008; Gomez-Pinilla *et al.*, 2008; Clark *et al.*, 2009). Moreover, exercise-induced morphological changes in the hippocampus correlate with improvements in cognitive functions (Colcombe & Kramer, 2003; Griffin *et al.*, 2009; O'Callaghan *et al.*, 2009; Creer *et al.*, 2010). One of the most remarkable features of hippocampal remodeling in response to exercise is the generation of new granule neurons in the dentate gyrus. New neurons may enhance associative learning (Winocur *et al.*, 2006; Hernández-Rabaza *et al.*, 2009; Drew *et al.*, 2010), because they are highly plastic units that are not yet fully integrated into existing circuitry (van Praag *et al.*, 1999). When exercise is implemented after conditioning in the conditioned place preference (CPP) paradigm, new neurons could be recruited during extinction and allow animals to rapidly acquire the new association, presented

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during testing, that context is no longer associated with drug (Mustroph *et al.*, 2011). Computational modeling supports the idea that acquisition of a task in which novel aspects arise in a familiar context (i.e. learning that a previously drug-paired texture is no longer associated with drug) is enhanced by hippocampal neurogenesis (Wiskott *et al.*, 2006; Garthe *et al.*, 2009). Hence, it makes sense that new neurons might contribute to CPP extinction.

To the best of our knowledge, no previous study has directly tested the causal role of new neurons in extinction of CPP behavior. Therefore, the goal of this study was to directly test the hypothesis that intact neurogenesis is required for exercise to abolish cocaine CPP by using the nestin–thymidine kinase (TK) transgenic mouse model, in which neurogenesis is selectively reduced.

Materials and methods

Animals

Eighty-two male nestin–TK transgenic mice were used in this experiment. Nestin–TK transgenic mice were originally obtained from S. G. Kernie (Columbia University, Department of Pathology and Cell Biology). The nestin–TK mice express a modified version of the herpes simplex virus TK driven by the *nestin* promoter and its second intron regulatory element (Yu *et al.*, 2008). This transgenic mouse line was generated by pronuclear injection into fertilised murine eggs in a C57BL/6J genetic background (Yu *et al.*, 2008). The mouse line allows temporally regulated, inducible ablation of early-dividing neural progenitors by systemic administration of the pro-drug ganciclovir (Yu *et al.*, 2008). Mice were bred in the Beckman Institute's animal facility, where a colony has been established. The genotype of each mouse was verified by tail snip followed by DNA extraction, polymerase chain reaction, and gel electrophoresis (with the glyceraldehyde-3-phosphate dehydrogenase gene used as a control gene). Mice were weaned at 3 weeks, and housed four per cage in a climate-controlled environment on a 12-h light/dark cycle (lights off at 09:00 h) for 4 weeks. The dimensions of cages without running wheels were as follows: length, 29 cm; width, 19 cm; and height, 13 cm (Harlan Teklad, Madison, WI, USA). Mice were individually housed for 2 weeks before the experimental procedures were started, and remained singly housed throughout the experiment. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee, and adhered to NIH guidelines. All measures were taken to minimize the number of mice used as well as the pain and suffering of the animals.

Experimental design

At ~65 days of age (range: 59–71 days), all mice underwent habituation, pretesting, and cocaine CPP conditioning (see 'CPP' below and Fig. 1). During the first 10 days after conditioning, all mice received daily intraperitoneal (i.p.) injections of 50 mg/kg bromodeoxyuridine (BrdU) to label dividing cells. On the day after the four conditioning days, mice were placed in cages either without (sedentary) or with (runner) running wheels, and received either a diet of normal (control) or valganciclovir (Val)-infused rodent chow for 28 days. Sample sizes were as follows: sedentary control, $n = 26$; runner control, $n = 18$; sedentary Val, $n = 20$; and runner Val, $n = 18$. After 28 days, mice underwent four consecutive days of CPP testing. Runner mice had continuous access to wheels, and mice continued to receive their designated diets during the days of testing.

Running wheels and sedentary treatment

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

Valganciclovir administration

After the conditioning phase of the experiment had been completed (see 'CPP' below), mice were switched from a diet of standard rodent chow (Harlan Teklad) provided *ad libitum* to a diet of rodent chow infused with Val (900 mg/kg) (Custom Animal Diets, Bangor, PA, USA), a valine ester pro-drug of ganciclovir (Pescovitz *et al.*, 2000), or control chow (Custom Animal Diets), also provided *ad libitum*. Upon ingestion, the vast majority of Val is rapidly converted to ganciclovir by hydrolysis (Jung & Dorr, 1999). Val was used in this study because its bioavailability is 10-fold higher than that of ganciclovir (Pescovitz *et al.*, 2000). Chow (Val-infused and control) was weighed every 7 days and replenished to maintain an *ad libitum* supply that would allow a suggested target Val consumption of 200 mg/kg (Blaiss *et al.*, 2011).

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 boxes indicate when the runner/sedentary conditions and Val-infused/normal chow were administered relative to conditioning and CPP testing. The experiment was performed on 82 mice (20 sedentary mice receiving Val-infused chow, 18 runner mice receiving Val-infused chow, 26 sedentary mice receiving normal chow, and 18 runner mice receiving normal chow). Mice experienced 1 day of habituation to reduce novelty effects and, subsequently, 1 day of CPP pretesting to establish baseline texture preferences over the 2 days immediately preceding the conditioning. Mice experienced 28 days of uninterrupted running or sedentary treatment and a total of 4 days of CPP conditioning. Mice were returned to cages with or without running wheels immediately after conditioning and testing to avoid the potential confound of mice experiencing withdrawal from running during the testing procedures.

Drugs

Cocaine hydrochloride (Sigma Aldrich, St Louis, MO, USA) was dissolved in 0.9% saline and administered at a dose of 10 mg/kg i.p. in a volume of 10 mL/kg. The dose was chosen on the basis of the literature, and was prepared according to the salt not the base form (Zombeck *et al.*, 2008; Johnson *et al.*, 2010; Mustroph *et al.*, 2011).

CPP

We used the same procedure as previously published by our laboratory (Johnson *et al.*, 2010; Mustroph *et al.*, 2011), based on Cunningham's apparatus and experimental design (Cunningham *et al.*, 2006).

Habituation

To familiarise the mice with the place conditioning chambers, they were placed on a flat surface without a texture in the conditioning chambers in the morning (10:00 h; for 30 min) and in the afternoon (16:00 h; for 30 min) for 1 day without any injection treatment.

Pretesting

To determine individual biases in preference for the textures prior to drug pairing, mice were weighed, received a 10 mL/kg saline injection, and were immediately placed in the apparatus with a hole/grid floor in the morning (10:00 h; for 30 min) and afternoon (16:00 h; for 30 min).

Conditioning

Four conditioned stimulus (CS+) trials (i.e. cocaine paired with one floor texture: hole or grid) and four CS– trials (i.e. vehicle paired with the alternate floor texture) were administered over a period of 4 days. The assignment to hole or grid was counterbalanced in each group. Each day, one CS+ trial and one CS– trial was administered in the morning and afternoon. The order of exposure to CS+ and CS– was counterbalanced within each group. Mice were weighed, received an injection of 10 mg/kg cocaine (CS+ trial) or vehicle (CS– trial), and were immediately placed on the appropriate floor texture in the morning (10:00 h; for 30 min) and afternoon (16:00 h; for 30 min).

Testing

Testing took place daily (morning and afternoon; 30 min) on days 29–32 after the last conditioning session. Prior to each testing session, each mouse was weighed, injected i.p. with 10 mL/kg saline, and placed into the center of the hole/grid conditioning chamber. All testing was conducted by experimenters blinded to the group assignment of the mice.

Immunohistochemistry

Tissue preparation

Following behavioral testing, all of the mice (n = 82) were anesthetised with 100 mg/kg i.p. sodium pentobarbital and then perfused transcardially with 4% paraformaldehyde in phosphate buffer solution (PBS) (0.287% sodium phosphate monobasic anhydrous, 1.102% sodium phosphate dibasic anhydrous and 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, and 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 rostro-caudal extent of the brain, with 240-μm increments separating each section; approximately nine sections) were stained in the following ways.

BrdU–3,3′-diaminobenzidine (DAB)

The purpose of this was 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, and 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-ionised formamide and 2X SSC buffer, rinsed for 15 min in 2X SSC buffer, treated with 2 M hydrochloric acid for 30 min at 37 °C, and then treated 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 incubated in primary antibody against BrdU raised in rat (AbD Serotec, Raleigh, NC, USA; catalog number OB0030) 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 (Vector, Burlingame, CA, USA; catalog number BA-9400) at a dilution of 1 : 250 in TBS-X plus for 100 min at room temperature. Sections were then treated with the ABC system (Vector; catalog number PK-6100), and stained with a DAB kit (Sigma Aldrich; catalog number D4418-505ET).

Double-fluorescent label

The purpose of this was 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; AbD Serotec; catalog number OBT0030) was combined with mouse anti-neuronal nuclear protein (NeuN) (1 : 250; Millipore, Billerica, MA, USA; catalog number MAB377) for 48 min at 4 °C. Secondary goat antibodies were conjugated with fluorescent markers (Cy2-green anti-mouse and Cy3-red anti-rat; Jackson ImmunoResearch, West Grove, PA, USA; catalog numbers 115-225-166 and 112-165-167, respectively) at a dilution of 1 : 250, and also delivered as a cocktail.

Image analysis

BrdU–DAB

All image analyses were conducted with experimenters blinded to the assignment group of the mice. 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 to 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 of dentate gyrus sampled.

Double-label

All image analyses were conducted with experimenters blinded to the assignment group of the mice. A Leica SP2 laser scanning confocal microscope (with a × 40 oil objective; pinhole of diameter 81.35 μm) was used to determine the proportion of dentate gyrus BrdU-positive cells that differentiated into neurons (NeuN-positive). Dentate gyrus BrdU-positive cells were identified as either co-expressing NeuN (mature neuronal nuclear marker) or not. Each BrdU-positive cell in the granule layer (represented in the one-in-six series) was analysed 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-positive cells per μm^3 multiplied by the average proportion of BrdU-positive cells co-expressing NeuN for the designated group.

Statistical analysis

Data were analysed with SAS (version 9.3) statistical software. In all analyses, $P < 0.05$ was considered to be statistically significant.

CPP

CPP data were analysed as follows. First, the duration spent on the hole texture was analysed by four-way repeated-measures ANOVA with CS (CS + hole vs. CS + grid; between subjects), exercise history (sedentary vs. runner; between subjects), diet (control vs. Val; between subjects), day of testing (1–4; within subjects) and all interactions entered as factors. Testing session, whether at 10:00 h or 16:00 h, was also included as a factor in initial models, but was never significant, and was therefore removed from the final linear models. Microanalysis of CPP on the initial test (day 1, a.m.) was analysed with a similar repeated-measures procedure, except that the within-subjects factor was bin number (1–6, consisting of duration on hole in 5-min bins over the 30-min test). *Post hoc* comparisons of CPP were conducted with unpaired *t*-tests comparing CS + hole with CS + grid within the different treatment groups.

Body mass, food consumption, and wheel running

These variables (except wheel running) were analysed by two-way ANOVA with exercise history (sedentary vs. runner), diet (control vs. Val) and all interactions entered as factors. Total wheel running distance traveled over the course of the study was analysed with an unpaired *t*-test comparing the control groups with the Val groups.

Neurogenesis

Total numbers of BrdU-positive cells in the granule layer and total numbers of new neurons (BrdU-positive cells co-labeled with NeuN) were analysed by two-way ANOVA with exercise history (sedentary vs. runner), diet (control vs. Val) and all interactions entered as factors. Total numbers of BrdU-positive cells and new neurons in runners were also analysed by analysis of covariance, with total distance run as the continuous predictor, and diet (control vs. val) as the categorical factor. The proportion of BrdU-positive cells in the granule cell layer that co-expressed NeuN was analysed by logistic regression, where proportion (binomial response) was modeled as a linear function of exercise history (sedentary vs. runner), diet (control vs. Val) and all interactions entered as factors.

Results

Body mass

Average body masses of the mice during CPP training (at the beginning of the experiment) and testing (at the end of the experiment) are shown in Fig. 1A. Body mass significantly increased over the duration of the experiment. However, runner mice gained significantly less weight than sedentary mice, the difference being ~1 g (Fig. 1A). This was indicated by a significant main effect of age (beginning vs. end of the experiment; $F_{1,82} = 102.23$, $P < 0.0001$) and a significant interaction between the exercise treatment (sedentary vs. runner) and age ($F_{1,82} = 11.31$, $P = 0.001$). No other main effects or interactions were significant.

Food consumption and dose of Val received

Sedentary mice ate 91% as much chow as runner mice ($F_{1,63} = 3.43$, $P = 0.0011$). There were no differences between consumption of Val-infused chow and that of normal chow (Fig. 2B). Because they ate more chow, runner mice received an average daily dose of 215 mg/kg/day ± 4.2 standard error (SE), whereas sedentary mice received an average daily dose of 187 mg/kg/day ± 5.4 SE from *ad libitum* chow consumption (Fig. 2C).

Wheel running

Mice increased their wheel running over the first 2–3 weeks, and thereafter maintained a plateau (Fig. 2D). No differences in running were observed between mice fed Val-infused chow and those fed normal chow. Runner mice receiving normal chow ran 4.3 km/day (±0.54 SE), and runner mice receiving Val-infused chow ran 4.2 km/day (±0.49 SE).

Hippocampal neurogenesis

Running increased neurogenesis, as indicated by a significant main effect of exercise on total numbers of BrdU-positive cells ($F_{1,32} = 22.5$, $P < 0.0001$) and total numbers of new neurons ($F_{1,32} = 19.1$, $P = 0.0001$). Val reduced neurogenesis, as indicated by a significant main effect of diet on total numbers of BrdU-positive cells ($F_{1,32} = 40.99$, $P < 0.0001$) and total numbers of new neurons ($F_{1,32} = 42.7$, $P < 0.0001$) (Fig. 3A and B). Significant interactions between diet and running for total numbers of BrdU-positive cells ($F_{1,32} = 6.12$, $P = 0.0189$) and total numbers of new neurons ($F_{1,32} = 5.9$, $P = 0.0209$) were observed (Fig. 3C). All *post hoc* pairwise differences between groups were significant ($P < 0.05$), except for sedentary control vs. runner Val, and sedentary Val vs. runner Val (Fig. 3C). Levels of neurogenesis were reduced by a proportionally greater amount in runner mice than in sedentary mice. Among sedentary mice, the Val-fed mice showed 42% as many BrdU-positive cells and 42% as many new neurons as normal chow-fed mice. Among runner mice, Val-fed mice showed 36% as many BrdU-positive cells and 35% as many new neurons as normal chow-fed mice.

A significant correlation between distance traveled and number of new neurons was observed in the runner control group. No correlation was observed in the runner Val group (Fig. 4D). This was indicated by significant effects of distance traveled ($F_{1,14} = 8.05$, $P = 0.01$) and diet ($F_{1,14} = 40.67$, $P < 0.0001$), and a significant interaction between distance traveled and diet ($F_{1,14} = 11.06$, $P = 0.005$) by analysis of covariance. The results were the same for

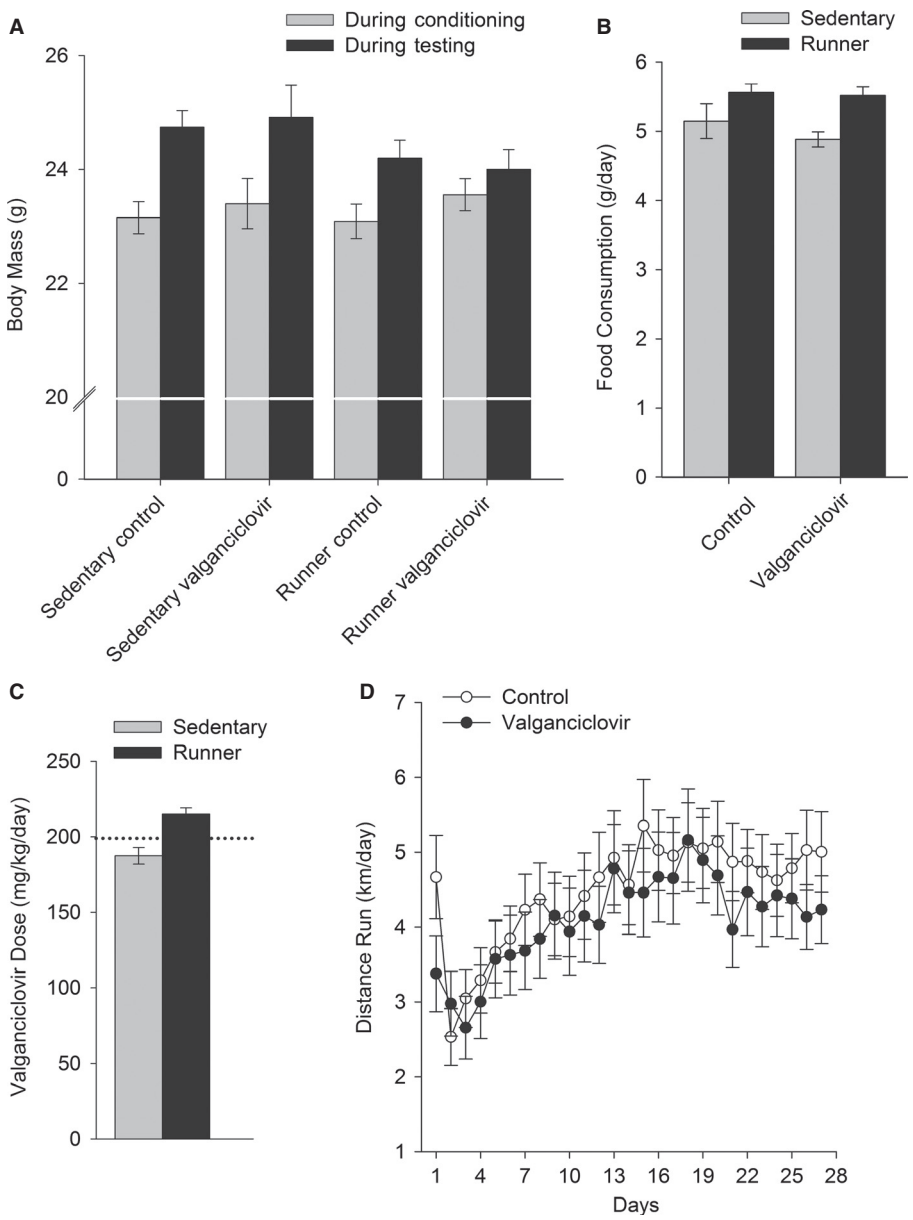

FIG. 2. Body mass, chow consumption, Val dose received and wheel running over the course of the study. (A) Average body mass ± SE shown separately for each group during conditioning (i.e. before runner/sedentary and Val-infused/normal chow assignments were made) and during testing (i.e. at the end of the runner/sedentary and Val-infused/normal chow phase of the experiment). Body weights slightly increased from conditioning to testing, and significantly more so for sedentary mice than for runner mice. (B) Average food consumption ± SE shown separately for each group. Food consumption was higher among runner mice. (C) Average Val dose received ± SE shown separately for sedentary mice and runner mice receiving Val-infused chow. Runner mice exceeded the desired dose of 200 mg/kg/day of Val. (D) Distance run ± SE shown separately for mice receiving normal chow and mice receiving Val-infused chow. Val did not affect running. Escalation of wheel running over the first 18 days is typical for mice.

BrdU-positive cells (distance traveled, $F_{1,14} = 8.03$, $P = 0.01$; diet, $F_{1,14} = 40.87$, $P < 0.0001$; interaction between distance and diet, $F_{1,14} = 11.08$, $P = 0.005$).

The percentages of BrdU-positive cells that differentiated into neurons, as indicated by co-expression of NeuN and BrdU, were: 83.5% (±2.6%), 81.3% (±3.1%), 87.8% (±2.3%), 88.0% (±2.3%) for the sedentary control, sedentary Val, runner control and runner Val groups, respectively. Analysis of logistic regression revealed that running significantly increased the proportion of BrdU-positive cells that differentiated into neurons (deviance = 4.4, $P = 0.04$). Diet and the interaction between diet and running were not significant.

Neither running nor diet had a significant effect on the volume of the dentate granule layer, nor were any significant interactions observed. The runner control group showed the largest volume (0.55 ± 0.020 mm^3), followed by the other groups: runner Val (0.50 ± 0.020 mm^3), sedentary control (0.50 ± 0.020 mm^3), and then sedentary Val (0.49 ± 0.020 mm^3).

CPP

Baseline preference

During the pretest, before the mice had ever experienced cocaine or Val, and before any of the mice ran on wheels, no significant difference in baseline preference was observed between any of the groups.

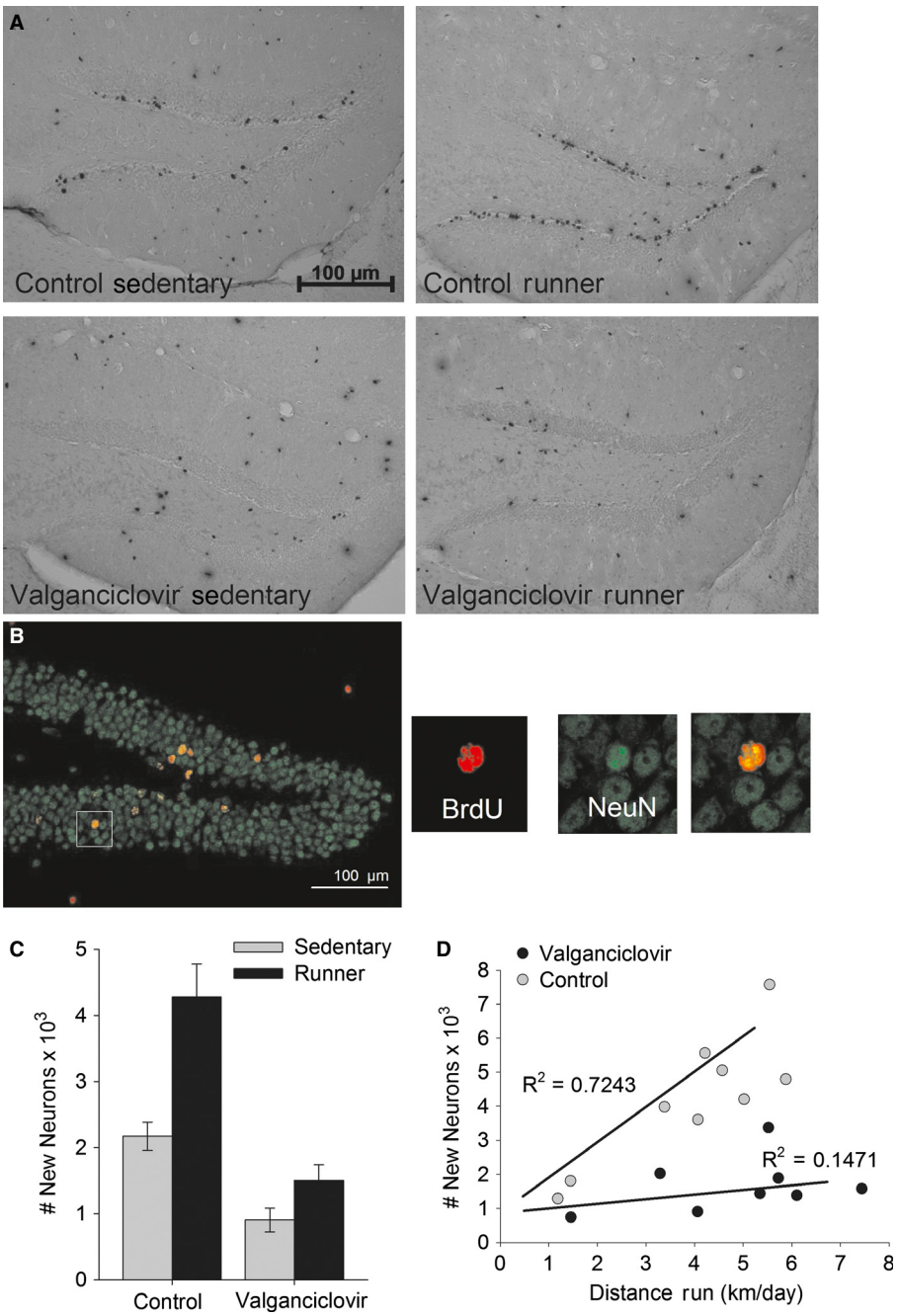

FIG. 3. Adult hippocampal neurogenesis. (A) Photographs of the dentate gyrus stained for BrdU with DAB as the chromogen, showing representative sections from each of the four groups. Black dots are nuclei staining positively for BrdU, indicating newly divided cells. (B) Photographs of a representative section through the dentate gyrus of a runner mouse receiving normal chow double-stained green for NeuN and red for BrdU. The panels on the right show the tissue illuminated for each color separately and combined zoomed in around the BrdU-positive cell, indicating an episode of neurogenesis. (C) Total numbers of new neurons ± SE shown separately for exercise (sedentary vs. runner) and diet treatment (control vs. Val). Neurogenesis was significantly reduced by Val in both runner mice and sedentary mice. Running significantly increased neurogenesis in the control group but not in the Val group. Importantly, runner mice treated with Val showed similar levels of neurogenesis as sedentary untreated mice. (D) Numbers of new neurons in runner mice plotted against average distance run (km/day) across the 28 days of uninterrupted running, plotted separately for runner mice receiving normal chow and runner mice receiving Val-infused chow. A positive correlation between distance traveled and number of new neurons was observed in control runner mice, whereas the correlation was absent in Val-treated runner mice.

Locomotor activity in CPP chambers

During testing, mice traveled an average of 24.4 m (±0.51 SE) per testing session. No differences between groups were observed.

CPP

Running completely abolished CPP in both mice fed normal chow and mice fed Val-infused chow, whereas sedentary mice fed either chow type showed significant CPP that was only extinguished by

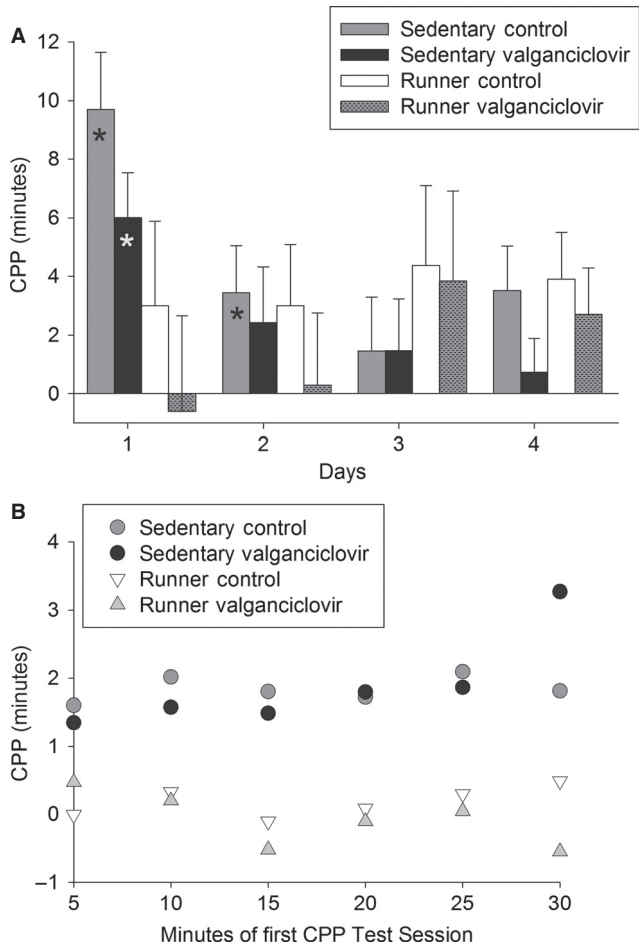

FIG. 4. CPP for cocaine during testing. (A) Mean difference in duration ± SE spent on the hole texture between mice receiving cocaine on the hole texture (CS + hole) and mice receiving cocaine on the grid texture (CS + grid) plotted separately for sedentary mice and runner mice receiving normal chow or Val-infused chow. Each bar represents data for the following mice: sedentary mice receiving normal chow (n = 13 CS + hole mice and n = 13 CS + grid mice); sedentary mice receiving Val-infused chow (n = 10 CS + hole mice and n = 10 CS + grid mice); runner mice receiving normal chow (n = 9 CS + hole mice and n = 9 CS + grid mice); and runner mice receiving Val-infused chow (n = 10 CS + hole mice and n = 8 CS + grid mice). The asterisks indicate significant place preference at $P < 0.05$. (B) Mean difference in duration ± SE spent on the hole texture between mice receiving cocaine on the hole texture (CS + hole) and mice receiving cocaine on the grid texture (CS + grid) during the first CPP testing session plotted in 5-min intervals separately for sedentary mice and runner mice receiving normal chow or Val-infused chow.

day 2 or 3 (Fig. 4A). This differential extinction of CPP, which was completely absent in runner mice but was present in sedentary mice, was indicated by a significant interaction between texture group (whether conditioned with cocaine on hole or grid), exercise history (runner or sedentary), and day of testing (1–4) ($F_{2,144}$ = 9.51, P = 0.0001). *Post hoc* analyses revealed that sedentary mice fed normal chow showed significant CPP on days 1 and 2 that was extinguished by day 3. Sedentary mice fed Val-infused chow showed significant CPP on day 1 that was extinguished by day 2. Runner mice never showed significant CPP on any day. There was no significant main effect of or any significant interactions involving diet (normal or Val-infused rodent chow) on CPP behavior.

Regarding within-session extinction on test day 1, microanalysis of the first CPP a.m. test session in 5-min bins showed that sedentary mice started out with significant CPP, whereas runner mice did not, and that the level of preference was maintained across the 30-min session, as was indicated by a significant main effect of exercise history (runner or sedentary) ($F_{1,20}$ = 106.04, $P < 0.0001$) and no effect of bin number or diet, or any interactions (Fig. 4B). This indicates that CPP was completely abolished in runner mice, rather than being extinguished during the first test.

Discussion

The main finding of the study is that elevated neurogenesis is not required for running to abolish cocaine CPP. This result is important, because the functional significance of new neurons in behavior is currently a highly contested topic, and the mechanisms underlying the benefits of exercise for reducing relapse to drug use are not well understood. With respect to the implications for drug abuse treatment regimens, the results suggest that increases in neurogenesis are not necessary for a treatment to diminish the rewarding value of contextual cues.

In addition to establishing that increased neurogenesis is not required for wheel running to abolish CPP, our study also demonstrates that intact neurogenesis is not required for expression or extinction of cocaine CPP in sedentary mice. This is because the sedentary Val group showed significantly reduced neurogenesis as compared with the sedentary control group (Fig. 3), but both showed similar CPP on day 1, and extinction of CPP on subsequent days (Fig. 4). Note that we believe it is wrong to conclude from these data that new neurons play no role in cocaine CPP. First, we did not completely eliminate neurogenesis, and it is possible that the remaining levels of neurogenesis were sufficient for expression and extinction of CPP. Also note that, even if new neurons are not required for expression and extinction, it is still possible that they are required for acquisition of CPP. In our study, Val was administered after CPP training, because our intention was to eliminate exercise-induced neurogenesis. In order to determine whether intact neurogenesis is required for acquisition of CPP, future studies would need to administer Val for a period of time before CPP training.

There are several alternative explanations for why eliminating exercise-induced neurogenesis had no significant influence on CPP behavior. We do not believe that the cumulative evidence necessarily favors one over the other, and not all explanations are mutually exclusive. The first possibility is that exercise-induced neurogenesis plays no direct role in abolishment of CPP; that is, wheel running affects CPP behavior via another mechanism. Alternative mechanisms include, but are not limited to, exercise altering the perception of the reward associated with the CS, or running causing the mice to forget about the drug–context association via mechanisms not involving neurogenesis. Another possibility is that, whereas new neurons are not required for wheel running to abolish CPP, they may be preferentially involved, similarly to the way in which the hippocampus is usually critically involved in contextual fear conditioning but is not absolutely required. It is well established that if the hippocampus is removed (lesioned) before contextual fear conditioning, the animal still can remember that the context was associated with the foot shock and freeze. However, if the hippocampus is removed after the animal has acquired the task but before the animal is tested, then the animal cannot remember the association of foot shock with context, and does not freeze. The interpretation is that the hippocampus is preferentially used if present, but that the brain can accomplish the task without the hippocampus if needed (Kim & Fanselow, 1992; Maren *et al.*, 1997; Cho *et al.*, 1999; Wiltgen *et al.*, 2006).

Demonstration of CPP in our model requires that the animal forms a positive (rewarding) association between the CS+ (texture) and US (cocaine) during conditioning, and that the animal remembers the association for 28 days between conditioning and testing. Therefore, the task encompasses both reward and memory components (Cunningham *et al.*, 2006). Exercise could have – independently of neurogenesis – altered the perception of the reward from cocaine-associated cues without affecting memory as such, or it could have affected memory without affecting reward, or some combination of both. After 28 days, the animal might remember that the cues were previously associated with cocaine, but these cues might now be perceived as less valuable, possibly because of the substitution of the exercise reward for the cocaine reward (Werme *et al.*, 2000; Ozburn *et al.*, 2008; Fontes-Ribeiro *et al.*, 2011; Lynch *et al.*, 2013). In that case, changes in the brain underlying the exercise-induced abolishment of cocaine CPP might occur in brain regions that comprise the natural reward circuit, such as the nucleus accumbens, lateral hypothalamus, extended amygdala, and ventral midbrain, and not necessarily the hippocampus (Werme *et al.*, 2000; De Chiara *et al.*, 2010; Olsen, 2011; Shapiro *et al.*, 2011; Zlebnik *et al.*, 2014).

In the current study, the idea that running acts as a substitute reward is supported because runner mice lacked CPP even on day 1 of testing (Fig. 4A), suggesting that running blunted the rewarding effect of the cocaine cue. Moreover, microanalysis of the first test indicated that CPP was absent in runner mice even from the very beginning of the first test (Fig. 4B). There are many reasons to suppose that exercise might have altered reward. In line with this theory are the findings that, in humans, exercise diminishes sensitivity to monetary rewards (Bothe *et al.*, 2013) and reduces neuronal responses in brain regions consistent with reduced pleasure being obtained from food (Evero *et al.*, 2012), findings that support the idea that exercise blunts the salience of alternative rewards in general. There is also biological evidence that exercise produces effects that substitute for drug reward. Exercise increases circulating endocannabinoid levels (Sparling *et al.*, 2003; Rola *et al.*, 2004; Heyman *et al.*, 2012; Raichlen *et al.*, 2013; Ferreira-Vieira *et al.*, 2014). Furthermore, running induces similar changes in the brain as cocaine. In Lewis rats, 30 days of running and 7 days of i.p. cocaine administration (10 mg/kg, identical to the dose used in our study) both upregulate mRNA of the endogenous opioid dynorphin in the medial caudate putamen, which is part of the brain reward pathway, to comparable levels (Werme *et al.*, 2000). This suggests a common mechanism of induction between running and cocaine, and the possibility of common neuronal adaptations in brain regions to running and cocaine (Werme *et al.*, 2000) that might allow running to substitute for drug reward.

The hypothesis that running serves as a substitute reward explains the abolished CPP that we observed from running in the present study. Evidence from our previous paper, however, favors the learning hypothesis over the reward substitution hypothesis. In our previous study, runner mice showed robust CPP on day 1 of testing, but CPP was then extinguished more rapidly than the CPP of sedentary mice (Mustroph *et al.*, 2011). The facilitated extinction of CPP from running in that study – rather than its complete absence from the start – suggests that an exercise-induced process promoted learning over the course of testing. Furthermore, in an experiment in which exercise was made available before conditioning, CPP was particularly robust, and was not extinguished even after 4 days, whereas CPP of sedentary animals was extinguished by day 2 (Fig. 4A) (Mustroph *et al.*, 2011). If exercise was a reward substitute, then running should have weakened CPP regardless of whether it had been implemented before or after conditioning. On the other hand, if exercise affects CPP by enhancing plasticity and learning, then it should strengthen CPP when it occurs before learning (conditioning), and facilitate CPP extinction when it occurs after learning, which is what we observed in our previous study (Mustroph *et al.*, 2011).

The behavioral data from the current study suggesting that running reduced the salience of the drug reward cue prompted us to closely examine the behavioral results of our previous study (Mustroph *et al.*, 2011). Microanalysis of the first CPP test session of that study revealed that CPP of runner mice started out at a higher magnitude than that of sedentary mice, but that CPP of runner mice was rapidly extinguished, whereas that of sedentary mice remained significant. To us, this is compelling evidence that exercise promoted learning, i.e. acquisition of the new drug–cue association during testing, which manifested as accelerated within-session CPP extinction in runner mice. This finding does not preclude the possibility that running in some situations can substitute for a drug reward. We speculate that, in the present study, exercise blunted the reward of the drug-associated cue on day 1 to a large enough degree that the effect of running on learning during the subsequent testing days, in the form of accelerated CPP extinction, was not evident to us because there was simply no significant CPP to begin with.

Given the central role of the hippocampus in the conditioned associations thought to underlie drug addiction (Everitt & Robbins, 2005), and the growing understanding that it is a major locus in the brain for change induced by exercise (Neeper *et al.*, 1995; Carro *et al.*, 2001; Dietrich *et al.*, 2008; Gomez-Pinilla *et al.*, 2008; Clark *et al.*, 2009), we believe that plasticity in the hippocampus contributes to the observed influences of exercise on CPP behavior. In addition to increasing adult neurogenesis, exercise increases extracellular brain-derived neurotrophic factor levels, insulin-like growth factor 1 levels, dendritic branching, and synaptogenesis, any of which could account for the differences that we observed between sedentary mice and runner mice in our study (Neeper *et al.*, 1995, 1996; Eadie *et al.*, 2005; Dietrich *et al.*, 2008).

Our results demonstrate that exercise can abolish cocaine CPP without increased adult hippocampal neurogenesis, but it is still possible that new hippocampal neurons are preferentially involved. Our study and the more recent studies using highly specific methods to reduce neurogenesis are finding that new neurons are not required for behavioral performance on a variety of hippocampus-involved tasks (Saxe *et al.*, 2006; Dupret *et al.*, 2008; Zhang *et al.*, 2008; Groves *et al.*, 2013). We believe that the data obtained in our study, along with meta-analysis of other work (Groves *et al.*, 2013), should be used to revise our thinking about the role of new neurons in behavioral performance. The revised view should consider the possibility that, when animals are learning hippocampus-dependent tasks, new neurons may be involved or even preferentially recruited if present, but, if new neurons are not available, older, established neurons can compensate and show sufficient plasticity for learning.

In a CPP test, many cues (e.g. the walls of the apparatus, the size of the textured areas, and the fact that the animals are moved into the testing room and handled immediately before the test, among many other features) are identical in all trials. In order to show CPP, the animals must be able to differentiate a subtle feature of the apparatus, i.e. the texture of the floor, suggesting that the CPP test, like other tests of contextual learning, may have a pattern separation component (Nakashiba *et al.*, 2008). This is important because the dentate gyrus plays a critical role in pattern separation (Groves *et al.*, 2013). Moreover, adult neurogenesis in the dentate gyrus has been hypothesised to support pattern separation (Clelland *et al.*, 2009; Scobie *et al.*, 2009; Tronel *et al.*, 2012). To the extent that

CPP involves pattern separation, our results are consistent with the recent study and meta-analysis of the literature suggesting that adult hippocampal neurogenesis is not essential for pattern separation in rodents (Groves *et al.*, 2013).

As with all approaches that intend to reduce neurogenesis by inducing the apoptosis of newly dividing cells, it is critical to consider side effects of our method and compensatory mechanisms when interpreting results. One of the primary advantages of our method for ablating neurogenesis in comparison with many others in the literature is the specificity and minimal level of invasiveness. We know from previous studies (Schloesser *et al.*, 2009) and our own unpublished work that Val alone fed to non-transgenic mice has no influence on neurogenesis or behavior. Previous work suggests that the toxicity of ganciclovir can be related to the way in which it is administered (Singer *et al.*, 2009). Hence, for the purposes of establishing an animal model for manipulating neurogenesis that is useful for behavioral testing, administering Val via chow appears to be the desired method, as opposed to i.p. or subcutaneous administration via minipump or intracerebroventricular administration, which can induce systemic toxicity or require implantation surgery, with the risk of surgical complications (Singer *et al.*, 2009). In our study, Val had no detectable influence on body mass (Fig. 2A), food intake (Fig. 2B), or wheel running behavior (Fig. 2D), and the mice showed normal levels of locomotor activity (data not shown) and CPP behavior (Fig. 4A).

Hence, the reduction in adult neurogenesis in our experiment was quite specific, and our experimental design was well-suited to determine the functional significance of the addition of new neurons from exercise in abolished CPP behavior. In our study, there were two possible outcomes: outcome 1 – runner mice with reduced neurogenesis show the same CPP as runner mice with intact neurogenesis; and outcome 2 – runner mice with reduced neurogenesis show different CPP from runner mice with intact neurogenesis. We observed outcome 1, which is the strongest possible result that we could have observed, because it establishes that the addition of new neurons as a result of running are not necessary for running to abolish cocaine CPP. Note that if we had observed outcome 2, i.e. different CPP in runner mice with reduced neurogenesis and runner mice with intact neurogenesis, then we could have only tentatively concluded that new neurons are necessary for the behavioral outcome. This is because possible unknown side effects or compensatory responses resulting from the treatment used to reduce neurogenesis, rather than the lost neurogenesis itself, could have contributed to the behavioral outcomes. We speculate that many of the previous studies that used systemic administration of antimitotic agents (Doetsch *et al.*, 1999; Shors *et al.*, 2002) or focal irradiation (Mizumatsu *et al.*, 2003) to reduce neurogenesis, including some of our own studies (Clark *et al.*, 2008), may have found reduced task performance in treated animals because of enduring side effects related to inflammation or toxicity of the treatment used to reduce neurogenesis rather than because of the loss of neurogenesis (Singer *et al.*, 2009).

Given the high incidence of relapse in drug abusers, effective treatment is probably going to require a large collection of life changes to be made. Nevertheless, any reduction in drug–cue strength may be helpful when an individual is recovering from drug use in an environment in which drug use occurred. Here, we provide evidence that exercise reduces drug–cue strength, and that it accomplishes this independently of new neurons. This finding is an important step in optimising treatment regimens relevant for drug dependence, as it shows that maximising neurogenesis resulting from running will probably provide no additional benefit to treatment outcomes.

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Abbreviations

BrdU, bromodeoxyuridine; CPP, conditioned place preference; CS, conditioned stimulus; DAB, 3,3′-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; TK, thymidine kinase; Val, valganciclovir.

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