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Voluntary Exercise Induces Anxiety-Like Behavior in Adult C57BL/6J Mice Correlating With Hippocampal Neurogenesis

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ABSTRACT: Several studies investigated the effect of physical exercise on emotional behaviors in rodents; resulting findings however remain controversial. Despite the accepted notion that voluntary exercise alters behavior in the same manners as antidepressant drugs, several studies reported opposite or no effects at all. In an attempt to evaluate the effect of physical exercise on emotional behaviors and brain plasticity, we individually housed C57BL/6J male mice in cages equipped with a running wheel. Three weeks after continuous voluntary running we assessed their anxiety- and depression-like behaviors. Tests included openfield, dark-light-box, elevated O-maze, learned helplessness, and forced swim test. We measured corticosterone metabolite levels in feces collected over a 24-h period and brain-derived neurotrophic factor (BDNF) in several brain regions. Furthermore, cell proliferation and adult hippocampal neurogenesis were assessed using Ki67 and Doublecortin. Voluntary wheel running induced increased anxiety in the openfield, elevated O-maze, and dark-light-box and higher levels of excreted corticosterone metabolites. We did not observe any antidepressant effect of running despite a significant increase of hippocampal neurogenesis and BDNF. These data are thus far the first to indicate that the effect of physical exercise in mice may be ambiguous. On one hand, the running-induced increase of neurogenesis and BDNF seems to be irrelevant in tests for depression-like behavior, at least in the present model where running activity exceeded previous reports. On the other hand, exercising mice display a more anxious phenotype and are exposed to higher levels of stress hormones such as corticosterone. Intriguingly, numbers of differentiating neurons correlate significantly with anxiety parameters in the openfield and dark-light-box. We therefore conclude that adult hippocampal neurogenesis is a crucial player in the genesis of anxiety. © **2009 Wiley-Liss, Inc.**

KEY WORDS: wheel running; brain-derived neurotrophic factor BDNF; corticosterone; hippocampus; depression

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Abbreviations used: BDNF, brain-derived neurotrophic factor; DCX, doublecortin.

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INTRODUCTION

The effect of physical activity on emotional behavior has been extensively studied in animals and has been suggested to alleviate symptoms of depression and anxiety (Greenwood et al., 2003; Duman et al., 2008; Leasure and Jones, 2008). Voluntary wheel running in rodents is also linked to health benefits in cardiovascular, metabolic, and neurodegenerative disturbances (Gielen et al., 2001; Nichol et al., 2007). Nonetheless, wheel running does not positively impact the mortality rate of male mice (Bronikowski et al., 2006).

Physical activity has also been associated with a plethora of cellular, molecular, and functional alterations within the brain. Wheel running for instance is reported to increase adult hippocampal neurogenesis in rodents (van Praag et al., 1999b). Running also alters the levels of neurotrophins and other growth factors including brain-derived neurotrophic factor (BDNF) (Neeper et al., 1995; Van Hoomissen et al., 2004; Duman et al., 2008; Greenwood et al., 2009). Increased synaptic plasticity and improved hippocampus-related cognitive performances induced by voluntary exercise may be based on such alterations (van Praag et al., 1999a; Nichol et al., 2007; Clark et al., 2008). Interestingly, physical activity also results in significant activation of the hypothalamic-pituitary adrenal (HPA) axis. For example, circulating corticosterone levels have been reported to increase significantly in mice engaged in 2 to 3 weeks of wheel running (Girard and Garland, 2002; Droste et al., 2003).

Despite a rather detrimental role of corticosteroids in depression, higher BDNF levels and increased neurogenesis evoked by wheel running in mice have been postulated to underlie the antidepressant effects of voluntary exercise (Duman et al., 2008). The role of adult neurogenesis in depression, particularly on its affective components, has however not yet been clarified. Nonetheless, a major impact of hippocampal neurogenesis on the cognitive impairments associated with depression was recently suggested on the basis of

TABLE 1.

Measures of Runners With and Without Blocked Wheels

		Runners	Blocked runners	*P* value	MDD
OF	Distance to walls (cm)	6.1 ± 0.8	6.4 ± 0.8	0.26	0.7
	Velocity (cm s^{-1})	6.8 ± 1.0	7.6 ± 1.6	0.20	1.1
	Total distance (m)	4.1 ± 0.6	4.5 ± 1.0	0.20	0.7
O-maze	Latency 1st exit (s)	110 ± 101	73 ± 98	0.41	82
	Total number of exits	3.6 ± 2.1	4.9 ± 3.0	0.27	2.1
	Number of full crosses	1.9 ± 1.9	1.2 ± 1.9	0.42	1.6
	Time on open arm (s)	38 ± 24	31 ± 24	0.45	19
DLB	Latency 1st exit (s)	164 ± 89	205 ± 69	0.27	65
	Endexploration (s)	221 ± 55	243 ± 57	0.38	46
	Number of exits	3.7 ± 2.2	3.4 ± 2.6	0.79	2.0
	Time in lit part (s)	24 ± 16	23 ± 18	0.99	14

MDD = Minimal detectable difference, i.e. the minimal difference for which a significant *P*-value would occur; values are presented as means ± standard deviation.

animal experiments (Sahay and Hen, 2007; Eisch et al., 2008; Perera et al., 2008).

The effect of voluntary exercise on anxiety-like behavior in mice is also not well understood. Current findings range from reduced anxiety (Greenwood et al., 2003; Duman et al., 2008), no effects on anxiety (Pietropaolo et al., 2006), to even more anxiety in rodents after voluntary exercise (Burghardt et al., 2004; Van Hoomissen et al., 2004; Leasure and Jones, 2008). Binder et al. (2004) reported both anxiogenic and anxiolytic effects of running in different tests in the same animal model. Several external factors might have contributed to these discrepancies, namely the level of physical activity, the housing conditions, and whether the running is voluntary or forced.

In an attempt to shed more light on a potential link between neurogenesis, neurotrophic factors, corticosterone, and anxiety-like behaviors, we subjected male C57BL/6J mice to 3 weeks of voluntary running and analyzed their behavior and brains subsequently. Tests for emotional behavior included openfield, dark-light-box, elevated O-maze, learned helplessness, and forced swim test. Our read-outs of neural plasticity comprised Ki67 and Doublecortin, endogenous markers for adult neurogenesis in the dentate gyrus, as well as BDNF protein levels in several brain regions. Moreover, we investigated stress hormone homeostasis by measuring fecal corticosterone metabolites in samples collected over a 24-h period. Correlational analyses served as a statistical means to describe the relation between the different parameters measured.

MATERIALS AND METHODS

Experimental Animals

Experiments were done with two cohorts of C57BL/6J male mice (Cohort 1: $n = 30$; Cohort 2: $n = 20$) obtained at the age of 8 weeks from Charles River (Sulzfeld, Germany). The behavioral and neurogenesis results of this study are based on Cohort 1. Cohort 2 was used to confirm our findings in the anxiety tests from Cohort 1, and to measure fecal corticosterone levels, BDNF levels in the brain, and the weight of adrenal glands and thymus. Upon their arrival, mice were single-housed in Macrolon Type III cages, in a temperature and humidity controlled room, on a 12-h light-dark cycle with lights on at 7 am. Water and food were available ad libitum. Handling and testing of the mice were done during the light phase of the light-dark cycle. All experimental procedures were approved by the German animal welfare authorities (Regierungspräsidium Karlsruhe).

Voluntary Wheel Running

Ten days after their arrival to our animal facilities, mice of the running groups (Cohort 1: $n = 20$; Cohort 2: $n = 10$) were given free access to a running wheel (diameter 11.5 cm) connected to a software-supported mechanical counter. The amount of running was measured and analyzed using the ClockLab software (Coulbourn, Whitehall, PA). To control any running-induced fatigue, we blocked the running wheels of one half of the runners in Cohort 1 ($n = 10$) 24 h before each behavioral test, while the remaining runners ($n = 10$) could run until the experiment started. We however pooled the data of all runners in Cohort 1 ($n = 20$) for statistical analyses since we found no behavioral changes in any of the tests after blocking the wheels of runners for 24 h (Table 1). All control animals ($n = 10$ in Cohorts 1 and 2) were supplied with a blocked running wheel during the whole period of the experiment, to equalize any possible enrichment effect due to the mere presence of a running wheel.

Behavioral Testing

Behavioral testing started 3 weeks after introducing the running wheels into the cages, and was performed during the light

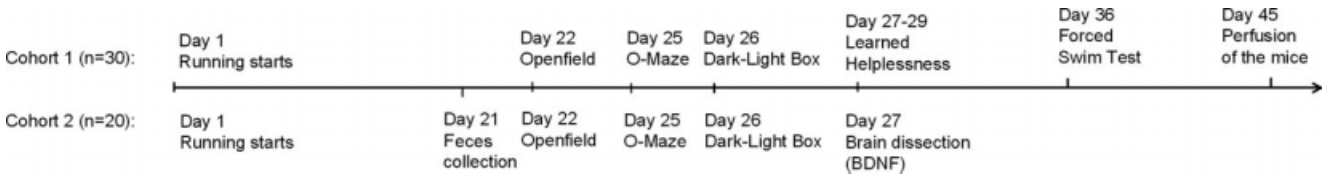

FIGURE 1. Timeline of the experimental design: The running groups performed exercise until they were sacrificed.

phase, i.e., in the animals' inactive phase, to keep experimental consistency with previous reports (Binder et al., 2004; Duman et al., 2008; Salam et al., 2009). Prior to each test, mice were acclimatized to the experimental room for at least 1 h. Tests were performed from the least to the most stressful based on post-test corticosterone elevations (Fig. 1; Chourbaji et al., 2008).

Openfield

Activity monitoring was conducted in a square shaped, white Openfield, measuring 50 × 50 cm^2 and illuminated from above with about 25 Lux. Mice were placed individually into the arena and monitored for 10 min by a video camera (Sony CCD IRIS). The resulting data were analyzed using the image processing system EthoVision 3.0 (Noldus Information Technology, Wageningen, The Netherlands). For each sample, the system recorded position, object area, and the status of defined events. Parameters assessed for the present study were total distance moved, velocity, and distance to walls. Furthermore the frequency of rearing (vertical movements of the animal) was recorded manually.

Elevated O-maze

The maze consisted of a gray plastic annular runway (width 6 cm, outer diameter 46 cm, 50 cm above ground level), covered with black cardboard paper to prevent mice from slipping off the maze. Two opposing sectors were protected by inner and outer walls of gray polyvinyl (height 10 cm). Animals were placed in one of the protected sectors and observed for 5 min. The maze was illuminated with 25 Lux. The following parameters were analyzed: latency to first exit, number of exits, full crosses (from one protected sector to the other), and total time spent in the open compartments.

Dark-light-box

The dark-light-box consisted of two plastic chambers, connected by a small tunnel. The dark chamber measured 20 × 15 cm^2 and was covered by a lid. The adjacent chamber, measuring 30 × 15 cm^2, was white and illuminated from above with 600 Lux. Mice were placed into the dark compartment and latency to first exit, number of exits, endexploration time (i.e., the latency until the mice reached the wall at the end of the bright compartment), and total time in the lit compartment were recorded for 5 min.

Learned helplessness

Mice were exposed to a transparent plexiglas shock chamber, equipped with a stainless steel grid floor (Coulbourn precision regulated animal shocker, Coulbourn Instruments, Düsseldorf, Germany), through which they received 360 unpredictable foot shocks (0.150 mA) with varying durations (1–3 s) and intervals (1–15 s), with one ~52 min session per day, for two consecutive days. Twenty-four hours after the second session, learned helplessness was assessed by testing two-way active avoidance performance. The shuttle box (Graphic State Notation, Coulbourn Instruments, Düsseldorf, Germany) consisted of two equal-sized compartments separated by a small gate. Each compartment contained a grid floor, through which current could be applied, and a signaling light at the top of both compartments. After 2-min habituation in the shuttle box, performance was analyzed according to the behavior during 30 shuttle escape trials. Each trial started with a conditioned light stimulus of 5 s, announcing a subsequent foot shock (intensity 0.15 mA) of maximum 10-s duration, with an intertrial interval of 30 s. The following behavioral reactions were defined: *avoidance* as adequate reaction to the light stimulus by moving to the other compartment before the shock occurs, *escapes* as shuttling to the other section as reaction to the electric shock, and *failures*, when no attempt to escape was made.

Hotplate test

To exclude altered pain sensitivity as a confounding factor for the learned helplessness, the mice were tested on the hotplate test (ATLab, Vendargues, France). Temperature was set at 53°C (±0.3°C) and a 45 s cut-off was determined to prevent injury of mice. Latency to first reaction, i.e., licking hind paws or jumping, was assessed.

Forced swim test

Mice were placed into a glass cylinder (23-cm height, 13-cm diameter), which was filled with water (21°C) up to a height of 12 cm. A testing period of 6 min was used to determine the onset and the percentage of time spent immobile. Mice were monitored by a video camera (Sony CCD IRIS). The resulting data were analyzed using the image processing system EthoVision 3.0 (Noldus Information Technology, Wageningen, The Netherlands). For each sample, the system recorded position, object area, and the status of defined events. Parameters assessed were latency to become immobile and the immobility time.

Immunohistochemistry

After 6 weeks of wheel running mice were anesthetized by i.p. injection of ketamine and xylazine, and perfused transcardially as described previously (Ben Abdallah et al., 2007). Brains were removed, postfixed for ~8 h in 4% paraformaldehyde, and kept in PBS overnight. Forty micrometer coronal sections were cut on a vibratome and kept at −20°C in antifreeze solution until further processing. To evaluate neurogenesis, we used a primary rabbit polyclonal anti-Ki67-antibody (1:5,000; NCL-Ki67p, Novocastra, Newcastle upon Tyne, UK) and a primary goat polyclonal anti-DCX-antibody (Doublecortin; 1:1,000; sc-8066, Santa Cruz Biotechnology, Santa Cruz, CA). Every sixth section was processed free-floating, as described previously (Ben Abdallah et al., 2007). Sections stained for DCX were counterstained with hematoxylin solution.

Hematoxylin Staining of Pyknotic Cells

Forty micrometer sections were mounted on glass slides and dried overnight at room temperature, then defatted in descending ethanol concentrations and rinsed in H_2O. Slides were then incubated in Mayer's Hematoxylin stock solution (Merck, Darmstadt, Germany) diluted 1:1 in H_2O for 5 min, differentiated in 1% acetic acid and 96% ethanol, dehydrated in 99% ethanol, cleared with xylol, and cover slipped.

Stereology and Morphology

Quantitative analyses were performed as described in Ben Abdallah et al. (2007). Briefly, Ki67-immunoreactive cells and pyknotic cells were counted in the subgranular zone of the dentate gyrus, using a 100× oil-immersion objective. Pyknotic cells were identified by their strongly and homogeneously stained nuclei. Cells in the uppermost focal plane of the section were excluded. Total cell number was calculated by multiplying the number of cells counted by the inverse of the section sampling fraction, i.e., 6. Total numbers of DCX-immunoreactive cells and granule cells were estimated using the optical fractionator [(West et al., 1991); StereoInvestigator 2000, Mircobrightfield, Williston, VT)] with a 100× oil-immersion objective. Counting frames (DCX: 45 × 35 μm^2; granule cells: 10 × 10 μm^2) were placed over the dentate gyrus at given intervals (DCX: 135 μm along the *x*-axis and 105 μm along the *y*-axis; granule cells: 240 μm along the *x*-axis and the *y*-axis). DCX-positive neurons, i.e., hematoxylin stained nuclei surrounded by a DCX-immunoreactive cytoplasm, and granule cells, i.e., hematoxylin stained nuclei, were counted throughout the section thickness but excluding cells in the uppermost focal plane.

Determination of BDNF Levels

After decapitation, hippocampus, frontal cortex, hypothalamus, and cerebellum were dissected and frozen on dry ice. Each specimen was homogenized by ultrasonication in 10–20 Vol. of lysing buffer containing 0.1 M Tris-HCl, pH 7.0, 0.4 M NaCl, 0.1% NaN_3, and a variety of protease inhibitors. BDNF protein levels were measured in the homogenates using commercial ELISA kits in principle according to the manufacturer's instructions (Promega) but adapted to the fluorometric technique also used for NGF determination as described in detail previously (Hellweg et al., 2003). The detection limit of the assay was 1 pg ml^{-1}.

Determination of Corticosterone Metabolites in the Feces

After 3 weeks of running and before starting the behavioral tests the mice of Cohort 2 were placed in fresh cages with normal bedding material for the sampling. All voided feces were collected 24 h later. Samples were analyzed for immunoreactive fecal corticosterone metabolites (FCM) using a 5α-pregnane-3β,11β,21-triol-20-one enzyme-immunoassay (EIA). Details regarding development, biochemical characteristics, and biological validation of this assay are described in Touma et al. (2003, 2004). Before EIA analysis, the fecal samples were homogenized and aliquots of 0.05 g were extracted with 1 ml of 80% methanol. The EIA used a double-antibody technique and was performed on antirabbit-IgG-coated microtitre plates. After overnight incubation (at 4°C) of standards (range: 0.8–200 pg/well) and samples with steroid antibody and biotinylated label, the plates were emptied, washed and blotted dry, before a streptavidin horseradish peroxidase conjugate was added. After 45-min incubation time, plates were emptied, washed, and blotted dry. The substrate (tetramethylbenzidine) was added and incubated for another 45 min at 4°C before the enzymatic reaction was stopped with 1 mol l^{-1} sulfuric acid. Then, the optical density (at 450 nm) was recorded with an automatic plate reader and the hormone concentrations were calculated. The intra- and interassay coefficients of variation were 8.8 and 13.4%, respectively.

Statistical Analysis

Statistical analysis was carried out using XLstat program Version 7.5, Addinsoft. Student *t* tests (two-tailed) were performed for the comparison between runners and controls. Results are reported as means ± S. E. M. Correlations between cell counts and behavioral measures were examined using Pearson's correlation coefficient. Significance was evaluated at a probability of 5% or less (<0.05).

RESULTS

Wheel Running Activity Increases Gradually Over Time

Mice ran around 3.5 km/dark phase on Day 1 and gradually increased their running activity until it reached a plateau on Day 10 with an average running distance of about 12 km/day.

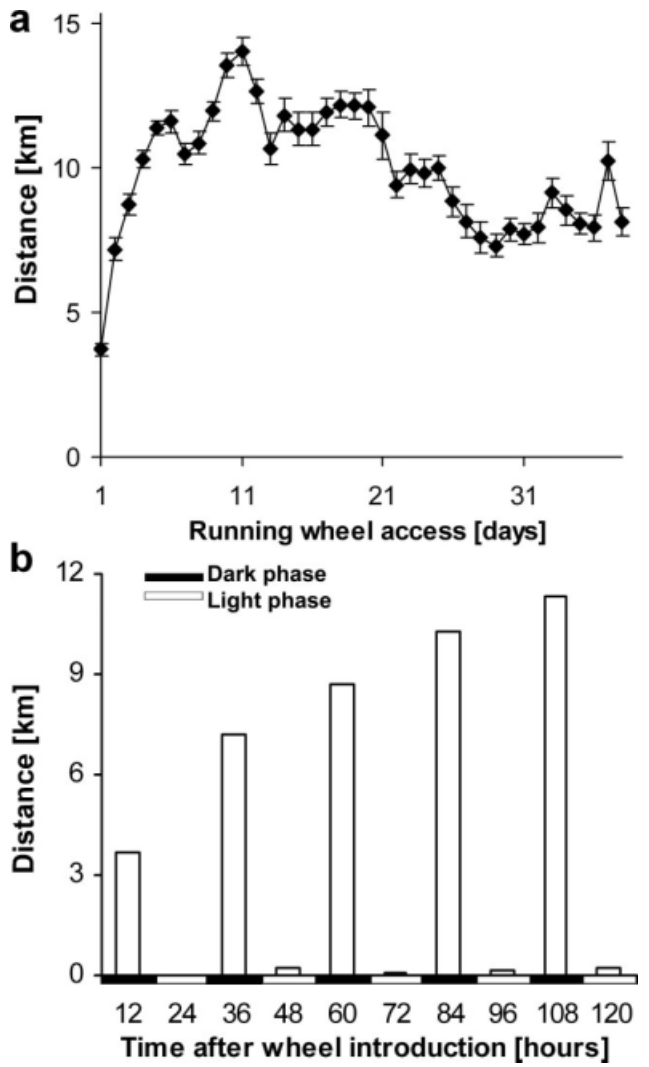

FIGURE 2. Exercising mice increase the daily running distance during their active phase. (a) The total running distance increased gradually until the behavioral tests started on day 21. (b) The mice performed their exercise almost only during their active phase when the lights were off.

When the behavioral testing began the mice reduced their daily running distance to about 9 km/day (Fig. 2a). During the inactive (light) phase mice ran on average only 150 m (Fig. 2b). All mice gained weight during the investigation period ($P < 0.001$), regardless of their running activity (Table 2).

Voluntary Wheel Running Reduces Exploration and Habituation

Openfield test

The Openfield test examines the locomotor and explorative behavior of an animal placed into an unknown open arena. In this test, the overall activity of the wheel runners was reduced (Fig. 3). After 3 weeks of voluntary running, mice traveled significantly shorter distances in the arena (4.3 m) compared to sedentary controls (5.2 m; $P = 0.005$; Fig. 3a) and moved with a lower velocity (runners = 7.18 cm s^{-1}; controls = 8.61 cm s^{-1}; $P = 0.005$; Fig. 3b). To control for possible fatigue-induced hypoactivity, we blocked the wheel in a subset of runners 24 h before behavioral testing (see Method section for details). However, we did not observe any difference in their openfield activity compared to runners with free wheel access until the test (Table 1), and analyzed them as one group.

Runners and controls did not differ in the average distance to walls during the first 5-min period of the Openfield test (runners = 6.12 cm; controls = 6.31 cm; Fig. 3c), but demonstrated a significant difference during the last 5 min of the test ($P = 0.001$), when controls increased the distance to walls compared to the first period of the test (7.26 cm; $P = 0.005$), while runners stayed close to the walls (6.39 cm; Fig. 3c). In line with these findings, the manually recorded rearing frequency revealed increased exploration behavior in the second half of the openfield test for the controls (first 5 min = 37.2; second 5 min = 52.3; $P < 0.001$) compared to runners (first 5 min = 28.3; second 5 min = 33.9). Additionally, the total number of rearings during the experiment were higher in controls (runners = 62.2; controls = 89.5; $P = 0.002$).

Elevated O-maze

The elevated O-maze imposes an approach-avoidance conflict on the mice, measuring anxiety by their aversion to enter the open sections of an elevated ring maze. In this test, runners exhibited significantly longer latencies for their first exit from the protected sector (runners = 91 s; controls = 39 s; $P = 0.050$; Fig. 3d). In addition, runners showed less total exits (runners = 4.3; controls = 7.3; $P = 0.014$; Fig. 3e) and less full crosses of an open, aversive sector (runners = 1.6; controls = 4.4; $P = 0.003$; Fig. 3f).

Dark-light-box

In the dark-light-box, the anxiety-like behavior of mice is analyzed by measuring their exploration of an aversive brightly lit compartment. Runners displayed higher latencies to enter the

TABLE 2.

Effects of Wheel Running on Metabolic Markers

	Runners	Controls	*P* value
BDNF (pg mg^{-1})			
Hippocampus	42.8	24.3	**0.017**
Frontal Cortex	10.8	16.7	0.182
Cerebellum	22.7	17.3	0.529
Hypothalamus	41.7	40.1	0.870
Weights (mg)			
Thymus	36.3	36.3	0.994
Adrenal gland	3.8	4.2	0.560
Bodyweights (g)			
Day 1	23.6	23.0	0.201
Day 35	24.9	24.9	0.976

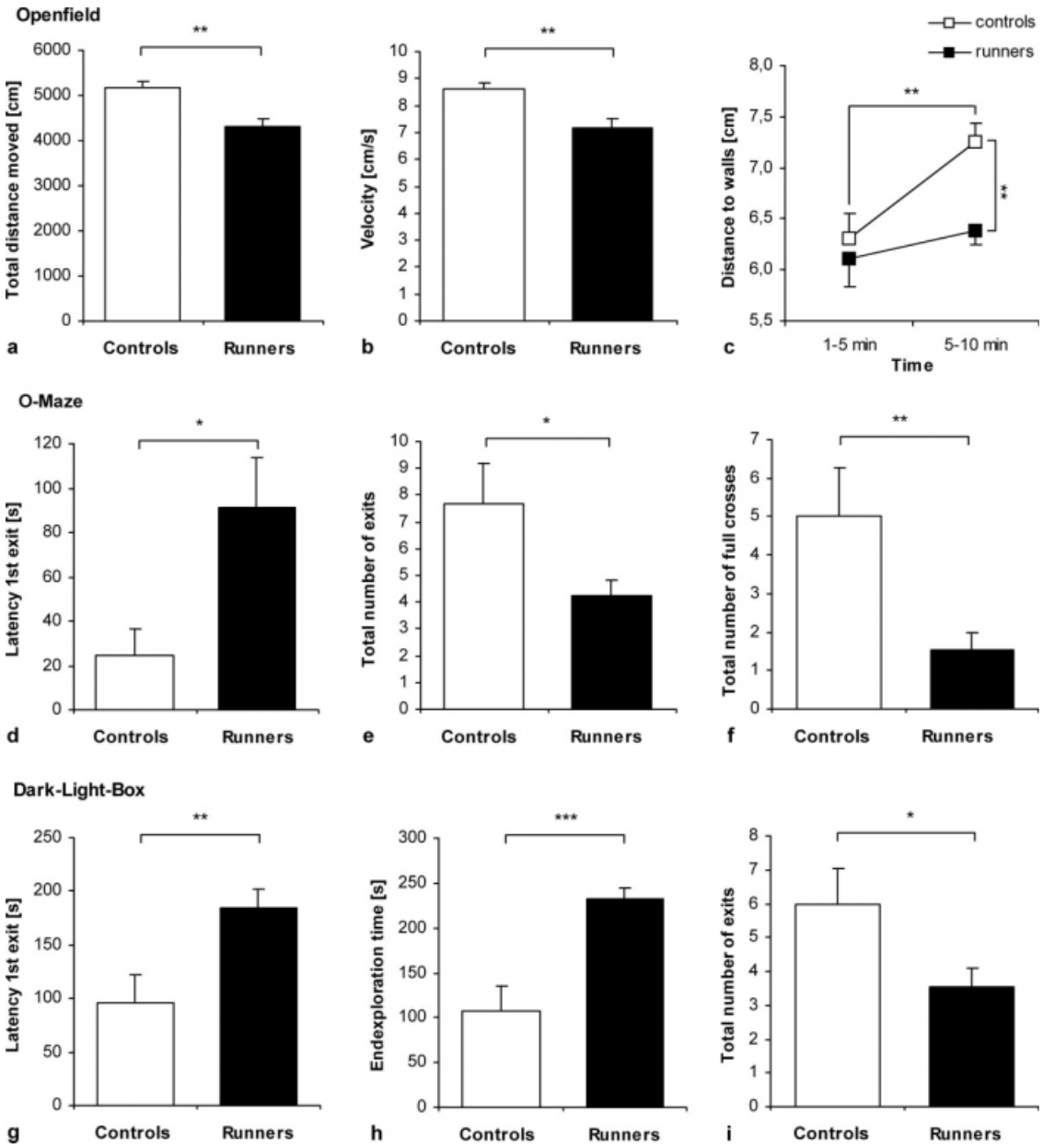

FIGURE 3. Running mice display increased anxiety-like behaviors in the Openfield, Elevated O-Maze, and Dark-Light-Box tests. Openfield test (a-c): Runners move shorter distances (a), at a slower velocity (b) compared to controls. (c) Runners also exhibit a lack of habituation in the distance to walls between the first and second half of the session. Elevated O-maze (d–f): In comparison with sedentary controls, the latency to make the first exit from protected sectors is significantly increased in runners (d), with also significantly less exits (e) and less full crosses from one protected sector to the other (f). Dark-light-box (g–i): Similarly to the O-maze data, runners have significantly higher latencies for a first exit from the dark compartment (g) and to explore the end of the aversive lit compartment (h). They also make significantly fewer total exits (i). Error bars correspond to standard error of mean (SEM). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

lit compartment of the arena (runners = 185 s; controls = 92 s; $P = 0.008$; Fig. 3g), and to make their first endexploration of the light compartment (runners = 232 s; controls = 104 s; $P < 0.001$; Fig. 3h). Furthermore, the runners showed less total exits from the dark compartment in the bright compartment (runners = 3.55; controls = 6; $P = 0.024$; Fig. 3i) and spent less time therein (runners = 23 s; controls = 37 s; $P = 0.076$).

Voluntary Wheel Running Does Not Affect Depression-Like Behavior

Learned helplessness

The learned helplessness test is a behavioral model of depression, which evaluates the coping capabilities of mice in an aversive test situation after 2 days of intense stress, evoked by exposure to a series of unpredictable and uncontrollable foot shocks

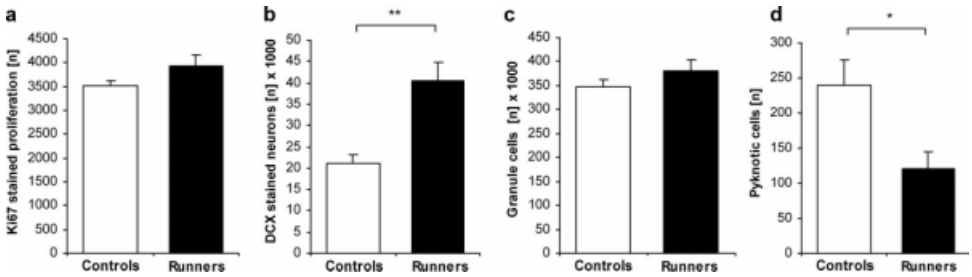

FIGURE 4. The level of adult hippocampal neurogenesis after 6 weeks of voluntary running was assessed using endogenous markers for cell proliferation (Ki67) and cell differentiation (DCX). (a) The number of Ki67-positive cells in the subgranular zone of the dentate gyrus are comparable between running and control mice, with only a slight tendency of more cells in runners. (b) DCX-positive cells are significantly increased by running, up to 100% compared to controls. (c) The total number of granule cells in the dentate gyrus is comparable between runners and controls. (d) Cell death measured by hematoxylin-stained pyknotic cells is decreased in runners. Error bars correspond to SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

(Chourbaji et al., 2005a). Three weeks of voluntary wheel running had no significant effect on the performance in the learned helplessness test. Runners showed similar escape latencies (runners = 2 s; controls = 2 s) and number of escapes (runners = 27; controls = 27) as nonrunning controls during the active avoidance session. Since altered pain sensitivity may represent a confounding factor for learned helplessness, the thermal pain sensitivity was measured with a hotplate test. However, there was no group difference between runners and controls in the latency to lick the hind paws (runners = 21 s; controls = 20 s) and to jump (runners = 31 s; controls = 31 s).

Forced swim test

The forced swim test represents a paradigm for the assessment of depressive despair behavior by measuring immobility scores in an inescapable aversive situation. In this test, runners and controls showed similar immobility times (runners = 212 s; controls = 209 s) and a comparable latency to start floating (runners = 15 s; controls = 19 s).

Voluntary Wheel Running Alters Neurogenesis and BDNF

Neural progenitor cell proliferation in the hippocampus was assessed by counting the number of Ki67-positive cells in the subgranular zone of the dentate gyrus. Ki67 is a marker protein expressed in all dividing cells during all phases of their cell cycle except G0 and early G1 phases (Scholzen and Gerdes, 2000; Kee et al., 2002). Runners and controls exhibited similar numbers of Ki67-positive cells (runners = 3939; controls = 3529; $P = 0.138$; Fig. 4a). Doublecortin (DCX) expression was used to estimate cycling precursors of neuronal lineage as well as maturing young granule cells (Couillard-Despres et al., 2005). Runners demonstrated a significant increase in the number of DCX-immunoreactive cells in the dentate gyrus (runners = 40488; controls = 21160; $P = 0.002$; Fig. 4b).

Pyknotic cells were identified by chromatin condensation and nuclear fragmentation. No changes were observed in the total number of granule cells in the dentate gyrus (runners = 378.809; controls = 341.506; $P = 0.258$; Fig. 4c). By contrast, a strong reduction of pyknotic cells was found in runners (-50%) when compared to controls (runners = 120; controls = 240; $P = 0.014$; Fig. 4d). We evaluated the ratio of pyknotic-to-proliferating cells, as a measure of new cells survival, and found a significant difference between our experimental groups (runners = 0.03; controls = 0.06; $P = 0.006$). When the data from the neurogenesis cell counts were correlated with the behavioral data from the anxiety tests we found strong correlations between neuronal differentiation stained with DCX and all anxiety parameters in dark-light-box and openfield test. Despite the fact that we found no significant difference between groups of runners and controls in Ki67-positive cells and total number of granule cells we found indeed correlations of cell numbers and anxiety parameters in dark-light-box and openfield (Table 3).

While neurogenesis is regarded as a cellular neuroplasticity marker, BDNF has been suggested as a molecular correlate for neural plasticity. Hippocampal BDNF levels were significantly

TABLE 3.

Correlations of Neurogenesis, Cell Proliferation, Granule Cells, and Anxiety

	Dark-light-box				Openfield		
	Lat	EndEx	Exits	Time	ToD	Rear	Velo
DCX	**	**	**	**	**	*	**
P value	0.004	0.002	0.008	0.006	0.001	0.011	0.001
r	0.71	0.76	−0.68	−0.70	−0.78	−0.67	−0.78
Ki67			*			*	
P value	0.110	0.073	0.039	0.137	0.065	0.019	0.065
r	0.43	0.48	−0.54	−0.40	−0.49	−0.60	−0.49
Granule cells							
P value	0.067	0.055	0.079	0.063	$P > 0.2$	$P > 0.2$	$P > 0.2$
r	0.49	0.51	−0.47	−0.49			

Lat = Latency 1st exit; EndEx = Endexploration; Time = Time in lit compartment; ToD = Total distance moved; Rear = All Rearings; Velo = Velocity; *r* = correlation coefficient;
*$P < 0.05$; **$0.001 < P < 0.01$.

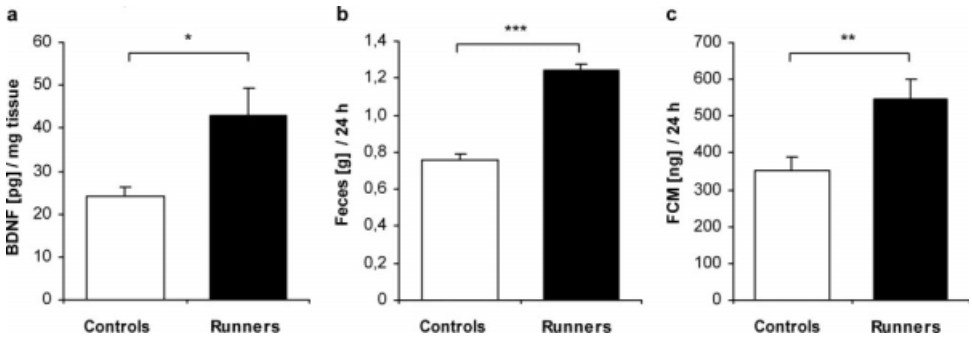

FIGURE 5. Voluntary running increases BDNF levels in the hippocampus and elevates fecal corticosterone metabolites. (a) After 4 weeks of exercise hippocampal BDNF content measured by ELISA is more pronounced in voluntary runners, with an ~1.7-fold increase compared to controls. (b) The measured weight of excreted feces shows an ~1.6-fold increase in running mice and (c) the quantification of immunoreactive fecal corticosterone metabolites (FCM) revealed higher levels in runners after 3 weeks of running compared to nonrunning mice. Error bars correspond to SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

increased in runners when compared with the control mice (Fig. 5a, Table 2). In contrast, BDNF levels in the frontal cortex, cerebellum, and hypothalamus were not altered by voluntary exercise (Table 2).

Voluntary Wheel Running Increases Corticosterone Excretion

In mice, corticosterone is the major glucocorticoid produced by the adrenals. The measurement of corticosterone metabolites in fecal samples is a noninvasive technique to monitor this stress hormone. The 24-h fecal sampling after 3 weeks of running revealed a significant increase in the total amount of excreted feces in runners (runners = 1.24 g; controls = 0.76 g; $P = 0.001$; Fig. 5b). Taking this difference into account, the absolute amount of fecal corticosterone metabolites (FCM) produced over 24 h was calculated instead of using FCM concentrations for comparing adrenocortical activity between runners and controls. Our enzyme immunoassay results revealed a significantly higher production of FCM in runners (runners = 545.5 ng; controls = 350.4 ng; $P = 0.008$; Fig. 5c). However, no running-induced differences in the weight of adrenal glands or of the thymus were observed (Table 2).

DISCUSSION

In the present study, we report two major findings: a significant increase in BDNF levels and neuronal differentiation in the hippocampus after 3–6 weeks of voluntary exercise, in parallel with elevations of fecal corticosterone metabolite levels, and a running-induced anxiety-like behavioral phenotype in novel and aversive environments.

Anxiety-Levels Increase After Long-Term Running

In our study, long-term voluntary running consistently reduced the exploration of aversive compartments in the open-field, dark-light-box, and O-maze tests, reflecting anxiety. Although previous studies related reduced exploration to immediate fatigue after running (Duman et al., 2008), blocking the wheels 24 h before testing our runners did not increase their exploratory behaviors compared to runners which wheels were not blocked, ruling out the possible influence of fatigue under our experimental conditions. This finding is also in agreement with a recent study reporting better motor capacities of exercising mice in the Rotarod test (Salam et al., 2009). In line with our findings, a previous study has reported similar observations in mice during the first 10 min of the openfield test after running voluntarily for 4 weeks (Binder et al., 2004).

In addition, we observed an impaired intrasession habituation in runners, when comparing distance to walls and number of rearings between first and second halftimes of the openfield. This impairment reflects alterations in adaptivity to new environments (Muller et al., 1994), confirming our interpretation that voluntary wheel running has anxiogenic effects.

Although behavioral alterations induced by voluntary exercise have been extensively studied, available reports are remarkably controversial. One possible reason might be the variable extent of wheel-activity between different studies. A comparison between several mouse strains suggested a genetic influence on daily wheel running, with an average running distance per day varying between 2 and 10 km (Harri et al., 1999; Droste et al., 2003; Binder et al., 2004; Lightfoot et al., 2004; Clark et al., 2008; Duman et al., 2008). We chose C57BL/6J mice because of their wide use in exercise and neurogenesis research. It is worth noting that the distance run by our mice averaged about 12 km/day during the first 3 weeks, exceeding that reported previously in the same strain. Several factors might underlie this varying activity levels in C57BL/6J mice strain, including housing conditions, age, gender, as well as the impact of pheromones on the motivation to run (Lightfoot et al., 2004).

Recently, it was argued that not only the distance run, but also the time spent running are important for inducing anxiety-like behavior (Leasure and Jones, 2008). As our mice spent remarkably more time running than animals from previous studies, we assume that this excessive and prolonged running

activity could also be one of the factors leading to a higher anxiety-like behavior.

Exercise-Induced Hippocampal Neurogenesis Correlates With Increased Levels of Anxiety

Voluntary exercise has been closely associated with increased adult hippocampal neurogenesis (van Praag et al., 1999b). For instance the number of proliferating cells detected by BrdU injections over 12 consecutive days increases significantly in runners compared to sedentary controls (van Praag et al., 1999b). In the present study however we observed only a slight increase of Ki67-positive cells in the runners, which was not statistically significant. Noteworthy is that Van Praag et al. (1999b) reported their findings in C57BL/6 females, housed in social groups, while our runners were single-housed males. Social housing has been suggested to further increase the running effect on cell proliferation compared to individual housing (Christie et al., 2008). Moreover, our mice were sacrificed during their light phase, a period during which it has been reported that running effect on cell proliferation is low to non-detectable, due to circadian changes of cell division (van der Borght et al., 2006).

We also observed ~100% increase of the total number of DCX-positive cells in the DG after 6 weeks of running indicating more neuronal differentiation in runners. This is also in line with the running study by Van Praag et al. (1999b) in which running induced significant increase of BrdU-positive cells colabeled with a neuronal marker. Additionally, the increase in neurogenesis in our data was paralleled by a decrease in cell death confirmed by the number of pyknotic cells in the subgranular zone. With these cellular changes one would expect more granule cells in the dentate gyrus. Running did not however induce significant changes in the total number of granule cells, which is in good agreement with previous reports (Koehl et al., 2008). It is worth noting that our morphological analysis was performed 6 weeks after the initiation of voluntary running, and after exposure to aversive environments (openfield, O-maze, and dark-light-box) and aversive stimuli (heat, electric foot shock, and swimming). This said, one must keep in mind that both experimental groups were treated and manipulated in similar manners, which should equalize any possible influence of behavior on neurogenesis in runners and controls. Therefore it should not change our interpretations that our runners bear higher neurogenesis, and that this correlates with their behavioral performances.

The role of adult neurogenesis in emotional behaviors is a matter of debate. To our knowledge we report here for the first time a strong correlation between anxiety and neurogenesis. Furthermore, we found as well correlations of anxiety parameters and total numbers of granule cells and correlations of anxious behavior and proliferation. These data underline a role of hippocampal plasticity in the development of anxiety. This hypothesis is in agreement with a recent study by Leasure and Jones (2008) who report significantly more BrdU-positive cells and increased anxiety-like behaviors in the Openfield test in forced running rats. Neurogenesis in the dentate gyrus is also positively correlated with running distance in outbred mice (Rhodes et al., 2003). Therefore excessive running in our model might have led to more pronounced neurogenesis, compared to previous models, which explain the discrepancy between reported findings regarding anxiety. Even in humans, anxiety is a (transient) side effect of SSRIs, which induce neurogenesis (Masand and Gupta, 1999).

Alterations in Corticosterone Homeostasis as a Consequence of Long-Term Wheel Running

Our results suggest an increase of corticosterone levels due to wheel running, detected by analysis of fecal corticosterone metabolites. This agrees with previous findings in plasma levels comparing high runners and low runners (Malisch et al., 2008), or runners and sedentary controls (Sellers et al., 1988), although others did not observe such plasma corticosterone changes (Kannangara et al., 2008). This is also in agreement with recent reports suggesting that engaging in learning tasks and enriched housing increase the levels of both corticosterone and hippocampal neurogenesis (Leuner et al., 2004; Kannangara et al., 2008). Moreover, physical activity induces a significant elevation in markers for adult neurogenesis (van Praag et al., 1999a,b) along with a strong activation of the HPA axis inducing a release of adrenocorticotropic hormone (ACTH) and corticosterone (Droste et al., 2003).

Previous studies have reported a positive correlation between risk assessment, anxiety-like behaviors and corticosterone levels in rodents (Rodgers et al., 1999). The assumption that voluntary running in mice is anxiogenic is therefore in line with corticosterone elevation. From these data and our own, we suggest that the beneficial effects of running on neurogenesis are not negatively influenced by the increased corticosterone levels. The next paragraphs should substantiate this view.

Role of Hippocampal BDNF in Anxiety, and its Link to Corticosterone and Neurogenesis

We observed a significant increase in BDNF levels in the hippocampus after long-term wheel running. It was recently shown that both, high levels (Govindarajan et al., 2006; Yee et al., 2007; Deltheil et al., 2008) as well as low levels (Chen et al., 2006) of BDNF may play an essential role in the genesis of anxiety in mice. Govindarajan et al. (2006) hypothesized that BDNF plays a crucial role in the signaling of stress-induced plasticity in the amygdala. But BDNF is also a mediator of hippocampal neurogenesis (Lee et al., 2002; Monteggia et al., 2004; Scharfman et al., 2005; Pinnock and Herbert, 2008) and is induced in the hippocampus by voluntary exercise as described here and elsewhere (Neeper et al., 1995; Van Hoomissen et al., 2004; Duman et al., 2008; Greenwood et al., 2009). Additionally, the effect of BDNF on cell proliferation and neurogenesis is highly corticosterone sensitive, as BDNF becomes ineffective in the absence of a diurnal rhythm of corticosterone (Pinnock and Herbert, 2008). We observed elevated

levels of fecal corticosterone metabolites in exercising mice, but could not make a statement with respect to the diurnal rhythm using this method. However, an altered daily rhythm of corticosterone in runners with higher peaks (e.g., at the beginning of the active phase) was reported by others (Droste et al., 2003). More corticosterone with a rhythmic pattern in runners might therefore facilitate the effect of BDNF on neurogenesis, which is indicated in other reports (Lee et al., 2002; Monteggia et al., 2004; Pinnock and Herbert, 2008). We therefore hypothesize that higher corticosterone peaks in runners could contribute to hippocampal plasticity with increased neurogenesis via BDNF.

Anxiety and the Hippocampus: A Potential Contribution of BDNF and Neurogenesis to Hippocampal Hyperactivation

One of the most sophisticated theories of anxiety is Gray and McNaughton's "The Neuropsychology of Anxiety" (Gray and McNaughton, 2000), in which the hippocampus is presented as a key player in the development of anxiety (McNaughton, 1997; Gray and McNaughton, 2000). Septo-hippocampal lesions were shown to induce anxiolytic effects on behavior. Moreover, anxiolytic drugs produce a specific impairment of the theta activity in the hippocampus, which is involved in initial learning and unexpected environmental changes (McNaughton et al., 2006; Jeewajee et al., 2008 no. 146). It was therefore suggested that a hyperactivity of the septo-hippocampal system is related to anxiety (McNaughton, 1997), and that the increased activation of the hippocampus in anxiety disorders might underlie an increase in hippocampal volume (Kalisch et al., 2006).

Despite the high comorbidity of depression and anxiety-related diseases, it has been shown that related anatomical changes, such as the hippocampal volume, can be different. In contrast to a possible loss of hippocampal volume in depression (Sheline et al., 1996; Videbech and Ravnkilde, 2004), a positive correlation of trait anxiety and hippocampal volume has been reported in humans (Rusch et al., 2001). The activity of the behavioral inhibition system, which is linked to anxiety (Gray and McNaughton, 2000) is also positively associated with hippocampal volume in humans (Barros-Loscertales et al., 2006; Cherbuin et al., 2008). However, there are also reports of no correlation of hippocampal volume and trait anxiety in humans (Woollett et al., 2008).

Nevertheless, the treatment of both anxiety and depression is quite similar. For instance SSRIs, which increase BDNF levels, are a common treatment for both (Balu et al., 2008). A selective downregulation of BDNF in the forebrain of female mice had anxiolytic and depression-like effects, while transgenic BDNF-overexpressing mice revealed simultaneously anxiogenic and antidepressant effects, further highlighting the dissociation between the two disorders (Govindarajan et al., 2006; Monteggia et al., 2007).

A link between BDNF, neurogenesis, and hippocampal volume has already been suggested (Lee et al., 2002). It is also known that running increases the volume of the rodent hippocampus after 8–10 weeks of voluntary exercise (Rhodes et al., 2003; Clark et al., 2008). An association of aerobic fitness and increased hippocampal volume has been already reported in elderly humans (Erickson et al., 2009).

Increases in hippocampal neurogenesis and BDNF levels might underlie the hippocampal volume increase after 8–10 weeks. This volume gain may reflect increased use/activity of the hippocampal system that contributes to the genesis of anxiety. Running induces both, long- and short-term potentiation in the dentate gyrus of adult rats (Farmer et al., 2004). Differences in the activity of the septo-hippocampal system can rise also from varying neurogenesis levels (Schaevitz and Berger-Sweeney, 2005). This said, our data suggest a contribution of exercise-induced hippocampal plasticity to the rise of anxiety.

As wheel running is strongly linked to cognitive improvements, the increase of hippocampal activity and volume could be as well interpreted as a cognitive gain. Although the relation of anxiety and cognition was not investigated in the present study, it was indeed hypothesized in previous reports (Rodgers et al., 1999; McNaughton and Corr, 2004), where a strong correlation between high corticosterone levels, increased risk assessment, and improved information processing was found.

Voluntary Running and Neurogenesis Have No Antidepressant Effect

Several studies found an antidepressant effect of voluntary running in mice and attributed it to an increase of adult hippocampal neurogenesis (Greenwood et al., 2003; Brene et al., 2007; Duman et al., 2008). To our surprise our model did not exhibit such changes after 3 weeks of voluntary running. Instead, both runners and controls performed comparably in both the learned helplessness and forced swim tests. The explanation for this discrepancy cannot be annexed to running-induced fatigue since baseline activity throughout the learned helplessness was not altered in runners compared to controls. Another explanation is that antidepressant effects of running could be masked by increased anxiety. This however seems unlikely since all previous observations from our laboratory suggest no statistical correlation between outcomes from anxiety and depression tests (unpublished data). On the other hand, the suggested antidepressant effect of voluntary running might be the consequence of environmental factors (Chourbaji et al., 2005b) such as the enriching nature of the running wheels, which were not discussed in previous reports (Duman et al., 2008). It is worth noting that the contribution of hippocampal neurogenesis in depression has been questioned also in humans where it was reported that cell proliferation is not decreased in postmortem tissue from patients with depression, in contrast to patients with schizophrenia (Reif et al., 2006).

Another hypothesis regarding the absence of antidepressant effects of exercise is that extreme elevation of neurogenesis following excessive running might have induced high anxiety and neutralized the antidepressant effect of voluntary exercise.

Concluding Remarks

In conclusion, our findings in mice with excessive voluntary wheel running reveal a contribution of neurogenesis in the development of anxiety disorders. While links between corticosterone, BDNF, hippocampal volume, and anxiety already existed, we found a new aspect regarding the relation between neurogenesis and anxiety. Since structural and pathophysiological alterations are different in the genesis of anxiety and depression, especially regarding the hippocampus, one should consider new treatments for anxiety disorders involving rather a restoration of BDNF and neurogenesis to normal levels instead of an excessive increase of these plasticity markers. Further studies are required in order to investigate and clarify the role of BDNF and neurogenesis in anxiety patients, which may be achieved in part with novel imaging techniques (Romer et al., 2008).

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