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Voluntary Wheel Running Reverses the Decrease in Subventricular Zone Neurogenesis Caused by Corticosterone

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Adult neurogenesis within the dentate gyrus (DG) of the hippocampus can be increased by voluntary exercise but is suppressed under stress, such as with corticosterone (CORT). However, the effects of exercise and CORT on the cell proliferation of the other traditional neurogenic site, the subventricular zone (SVZ), have been reported with controversial results. In addition, the cotreatment effects of voluntary exercise and CORT have not been investigated. This study aims to determine whether CORT can suppress cell proliferation in the SVZ and whether this can be reversed by voluntary exercise. In the present study, the effect of chronic (4 weeks) CORT treatment and wheel running simultaneously on the SVZ cell proliferation of adult Sprague–Dawley rats was examined. The results showed that cell proliferation indicated by bromodeoxyuridine (BrdU) was increased by voluntary wheel running, whereas it was decreased by CORT treatment within the SVZ of the rats without running. For the rats with both CORT treatment and wheel running, it was found that the number of BrdU-labeled cells was approximately at the same level as the vehicle control group. Furthermore, these proliferating cells expressed doublecortin (DCX), a migrating neuroblast marker. Wheel running increased the percentage of BrdU-labeled cells expressing DCX in the SVZ, whereas CORT treatment decreased this percentage. Thus, chronic injection of CORT can decrease the number of proliferating cells, while wheel running can reverse the decrease in cell proliferation within the SVZ to normal levels. In addition, CORT can suppress the cell differentiation within the SVZ, and this was alleviated by wheel running as indicated by the double labeling of BrdU and DCX.

Key words: Adult neurogenesis; Corticosterone (CORT); Subventricular zone (SVZ); Voluntary exercise; Wheel running

INTRODUCTION

Adult neurogenesis has been documented to occur in two major regions that have been regarded as the traditional neurogenic sites of the mammalian central nervous system (CNS). The two regions include the subgranular zone (SGZ) within the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricular wall, which lies in the forebrain[1]. Studies on adult neurogenesis within the traditional neurogenic sites are becoming increasingly important, as new cells produced from these two sites have been shown to be functionally integrated into several neuronal circuits[1,2]. For example, new cells

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generated from the SGZ migrate a short distance to the granular layer of the DG and then differentiate into mature neurons that are involved in hippocampal-dependent functions including spatial learning and memory[3,4]. The new cells generated in the SVZ migrate via the rostral migratory stream (RMS) pathway to the olfactory bulb for further interneuron differentiation, which plays a key role in olfactory cell differentiation[1,5].

Neurogenesis within the SGZ and SVZ can be modulated by many factors, such as environmental enrichment[6,7], psychotrophic agents[8], exercise[7,9,10], and stress[11,12]. Voluntary wheel running and using an enriched environment have been regarded as positive components for stimulating neurogenesis[13]. In contrast, stressful experiences have been found to alter the processes of adult neurogenesis and have been related to psychiatric illness including major depressive disorder[14,15]. It has been extensively studied that elevated stress hormone levels triggered by daily stressful experiences can cause pathophysiological changes in SGZ neurogenesis, which is expressed as a development of depressive disorder. However, studies on how stress can alter neurogenesis and the functional outcomes on another neurogenic region (the SVZ) have been lacking.

The SVZ is another region with continuous neurogenesis. The newly generated cells have been suggested to play a vital role for both olfactory functions and male rodents' sexual behavior under depressed condition[16]. In addition, enhanced SVZ neurogenesis by voluntary exercise under ischemic conditions has been suggested to have a neuroprotective role[17]. In relation to depressive disorders, few previous reports have shown that antidepressants can reverse the corticosterone (CORT)-induced decrease in cell proliferation[12,18]. However, prolonged chronic treatment with antidepressants can cause a decrease in SVZ cell proliferation[19]. The results of these studies suggest that alterations of neurogenesis in the SVZ are governed by a complex mechanism, which is not yet fully understood. Although different studies showed that CORT can decrease cell proliferation[18] whereas voluntary exercise can increase the cell proliferation of the SVZ[7], the findings were reported in separate studies, and there is a lack of investigation showing the cotreatment effects of CORT and voluntary exercise in the SVZ. In the present study, chronic treatment with CORT was applied to adult rats to induce a stress response in order to determine whether the cotreatment with voluntary wheel running can attenuate the adverse effect of CORT on cell proliferation within the SVZ.

MATERIALS AND METHODS

All experiments and procedures in this study were approved by the committee on the use of live animals in teaching and research (CULATR #2402-11) of the University of Hong Kong.

Experimental Design

Adult male Sprague–Dawley rats (Laboratory Animal Unit, The University of Hong Kong, HKSAR, P.R. China) weighing 250 ± 20 g were used for the experiments. Experimental procedures were carried out according to the guidelines of the University of Hong Kong. The rats were housed individually and kept under a 12/12 light–dark cycle with ad libitum access to food and water. The rats were administered with subcutaneous injections of either a vehicle (sesame oil) or 40 mg/kg CORT daily for 28 days after a 2-day adaptation period. Sesame oil alone was injected as a sham control to determine the effects of the vehicle[20,21]. The rats were divided into four groups: (1) vehicle-treated nonrunners (V_N), (2) vehicle-treated runners (V_R), (3) CORT-treated nonrunners (CORT_N), and (4) CORT-treated runners (CORT_R). The rats were assigned randomly to be either in the runner or in the nonrunner groups by keeping them in standard cages individually equipped with an unlocked or a locked wheel (diameter: 31.8 cm, width: 10 cm; Nalgene Nunc International, Rochester, NY, USA), respectively. The daily wheel revolutions were recorded using the Vital Viewer software (Mini-Mitter Company Inc, Bend, OR, USA). The running regime commenced on the first day of injection of either vehicle or CORT as described in the following.

Preparation and Administration of CORT

CORT with a dosage of 40 mg/kg (Catalog No. C2505; Sigma-Aldrich, St. Louis, MO, USA) was freshly prepared before use by vortexing with sesame oil (catalog No. S3547; Sigma-Aldrich) and sonicating for 60 min. The prepared CORT was injected subcutaneously daily into the back of the neck using a detailed protocol described by Hellsten et al.[22]. The rats in the vehicle control group were injected daily with only sesame oil as vehicle treatment.

Preparation and Administration of BrdU

Bromodeoxyuridine (BrdU) (catalog No. B5002; Sigma-Aldrich) was dissolved in 0.9% normal saline and was injected intraperitoneally (IP) during the last 3 days of treatment (day 28 to day 30). All the rats received two injections of BrdU per day (50 mg/kg) with an 8-h interval in between and a total of six injections for the whole treatment.

Perfusion and Tissue Preparation

The rats were anesthetized with sodium pentobarbital (Alfasan International B.V., Woerden, Holland) and perfused transcardially with 0.9% normal saline followed by 4% paraformaldehyde (PFA) (International Laboratory USA, San Francisco, CA, USA). The brains were then dissected out and postfixed overnight in 4% PFA at 4°C. They were cryoprotected in 30% sucrose in 0.1 M phosphate buffer for sectioning until they sank. Coronal

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sections were cut with a thickness of 30 μm, and the sections were collected and kept in the 12-well plate with cryoprotectant at 4°C until immunostaining.

BrdU Immunohistochemistry

The stored frozen sections were affixed onto gelatin-coated slides and air dried overnight. Antigen retrieval was performed by incubating slides in citric acid (pH 6.0) (catalog No. 13735; USB, Affymetrix, Cleveland, OH, USA) at 90°C for 30 min, and the sections were then washed using 0.01 M phosphate-buffered saline (PBS) (Sigma-Aldrich). The sections were incubated in 1 M hydrochloric acid for 30 min at 37°C and then incubated with borax buffer (pH 8.0) (Merck, Darmstadt, Germany) for 15 min for neutralization. The sections were incubated with the primary antibody (1:1,000 rats anti-BrdU; catalog No. ab6326; Abcam, Cambridge, UK) overnight at room temperature (RT). The sections were washed with PBS and incubated with the secondary antibody (1:200 biotinylated rabbit anti-rat; catalog No. E046801; Dako, Carpinteria, CA, USA) for 2 h. After the secondary antibody incubation, the sections were then incubated with avidin–biotin complex for amplification of signal (catalog No. PK6100; Vector Laboratories, Burlingame, CA, USA). BrdU immunoreactivity was visualized with 3,3′-diaminobenzidine (DAB) tetrahydrochloride (catalog No. 002014; Invitrogen, Carlsbad, CA, USA).

Fluorescence Immunohistochemistry

The sections were at first incubated in citric acid (pH 6.0) for 30 min for fluorescence double labeling of the BrdU and the DCX. They were then washed in 0.01 M PBS. The sections were incubated in 1 M hydrochloric acid for 30 min and were then incubated with borax buffer (pH 8.0) for 15 min for neutralization. The primary antibodies [1:1,000 rats anti-BrdU (catalog No. ab6326; Abcam) and 1:200 rabbit anti-DCX (catalog No. 4604S; Cell Signaling Technology, Beverly, MA, USA)] together with goat serum for blocking were incubated overnight at RT. The sections were washed with PBS and incubated with Alexa Fluor-conjugated secondary antibody [1:200 goat anti-rat, 568 nm red (catalog No. A11077) and 1:200 goat anti-rabbit, 488 nm green (catalog No. A11034); both from Invitrogen] for 2 h. The sections were then covered with a coverslip using mounting medium (Invitrogen) and kept in the dark.

Data Quantification and Statistical Analysis

The coronal brain sections with the BrdU-positive cells were analyzed by an experimenter blinded to the treatments. The brain sections containing the SVZ were from part of the brain located between the anterior to bregma 1.44 mm and posterior to bregma 0.36 mm (average four sections per animal, six animals per group) such that part of the brain between the appearance of the third ventricle to the disappearance of the anterior commissural fiber was included[23]. The SVZ was outlined by StereoInvestigator (MBF Bioscience, Williston, VT, USA) at 4× objective, followed by cell counting with 40× objective. The cell number was estimated by an optical fractionator such that a counting frame with size 60×60 μm was systemically random sampled along the outlined SVZ[24]. A guard zone height of 5 μm and dissector height of 10 μm were employed. The averaged coefficients of error of the outlined areas were less than 0.1, which is within the acceptable range for stereological analysis. Results were presented as the number of BrdU-positive cells per section ± SEM.

Neurogenesis within the SVZ was determined by double labeling the BrdU-positive cells with DCX. At least 40 BrdU-positive cells were randomly picked to determine the percentage of these proliferating cells that expressed DCX under a fluorescence microscope with 20× objective (MBF Bioscience). Data were presented as the average percentage of the proliferating BrdU-positive cells within SVZ with DCX expression ± SEM.

Two-way analysis of variance (ANOVA) on SPSS software version 13 (IBM, Armonk, NY, USA) was applied to determine the interaction effects between CORT and running on the number of BrdU-positive cells and the percentage of BrdU-positive cells expressing DCX. Furthermore, a priori analysis by two-tailed Student's t-test with subsequent Bonferroni's correction was applied for individual comparisons between specific groups based on planned hypotheses: (1) CORT can suppress SVZ neurogenesis and (2) running can enhance SVZ neurogenesis under both vehicle and CORT treatments. The three individual comparisons were (1) control vehicle-treated nonrunner (V_N) compared to vehicle-treated runner (V_R); (2) control vehicle-treated nonrunner (V_N) compared to CORT-treated nonrunner (CORT_N); and (3) CORT-treated nonrunner (CORT_N) compared to CORT-treated runner (CORT_R). Bonferroni's correction where the α level was adapted to 0.0167 for three individual comparisons (α': $0.05/3 = 0.0167$)[25–27] was applied. Two-tailed Student's *t*-test followed by Bonferroni's correction for the above three group comparisons was also applied to body weight and ratio of adrenal to body weight analysis.

RESULTS

Changes in Body and Adrenal Weights in Animals After 28 Days of CORT Injection

The changes in body weights of different treatment groups were measured throughout the 4 weeks of treatment (taken at days 1, 7, 14, and 28). At the start of the treatment, there were no differences in the average body weights among the animals. Student's *t*-test analysis revealed that significantly lower body weights were

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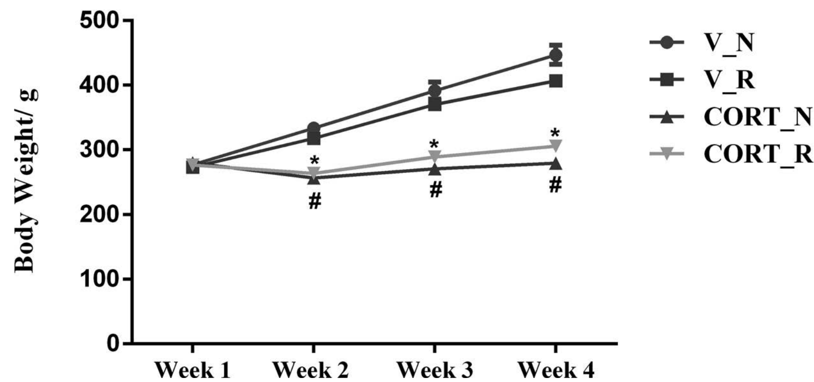

Figure 1. Changes in body weights throughout 28 days of treatments. The corticosterone-treated groups were found to have significant lower body weights than the vehicle-treated groups throughout weeks 2 to 4. The values are expressed in mean ± SEM and analyzed by Student's *t*-test with subsequent Bonferroni's correction. *$p < 0.01$, V_N compared to CORT_N group; #$p < 0.01$, V_N compared to CORT_R group. $n = 6$ per group. V, vehicle treated; CORT, corticosterone treated; N, nonrunner; R, runner.

found in both CORT-treated groups, either with or without running, compared respectively to the vehicle-treated control group starting from week 2 until week 4 as shown in Figure 1 ($p < 0.01$).

Adrenal glands were dissected out and weighed after sacrifice. The ratio of adrenal to body weight was measured to determine the degree of atrophy of the adrenal glands caused by CORT injection[28]. Both CORT-treated groups, with or without running, were found to have obvious adrenal atrophy when compared to the vehicle-treated control group ($p < 0.01$) (Fig. 2).

Suppression of the Proliferation of BrdU-Positive Cells in SVZ by CORT and Reversal by Voluntary Wheel Running

Vehicle-treated runners (V_R) (Fig. 3A) exhibited a significant increase in the estimated number of BrdU-positive cells in the SVZ (1,062.05 ± 103.97 cells) when compared to the vehicle-treated nonrunner group (V_N) (669.42 ± 58.55 cells) (Fig. 3B). Nonrunning animals that received a high dosage of CORT (CORT_N) (Fig. 3C) had a significant decrease in BrdU-positive cells (423.42 ± 29.02 cells) when compared to the control V_N group (Fig. 3E). Simultaneous treatment of wheel running and CORT (CORT_R) (Fig. 3D) was able to reverse the decrease in the proliferating cells caused by CORT with a significant increase in BrdU-positive cells (908.92 ± 98.95 cells) (Fig. 3E). No interaction effects were found between CORT and running treatment on the BrdU-positive cell quantification by two-way ANOVA [$F(1,20) = 0.946$, $p = 0.342$].

Enhancement of the Differentiation of Proliferating BrdU-Positive Cells in SVZ by Voluntary Wheel Running

Doublecortin (DCX) is a microtubule-binding protein that is associated with the migration and differentiation of neuroblasts. The double labeling of the cell proliferation markers, BrdU and DCX (Fig. 3F), indicates that these proliferating cells in the SVZ have differentiated into migrating neuroblasts, which may be recruited by the RMS pathway to the olfactory bulb. The percentage of BrdU-positive cells expressing DCX was increased by wheel running as indicated in the vehicle-treated runner group (V_R) (58.33 ± 2.39%) compared to the vehicle-treated nonrunner group (V_N) (40.0 ± 3.00%) (Fig. 3G). A high dosage of CORT suppressed neuronal differentiation within the SVZ such that a significant decrease in the percentage of BrdU-positive cells expressing DCX (26.67 ± 2.11%) was found. The above suppression

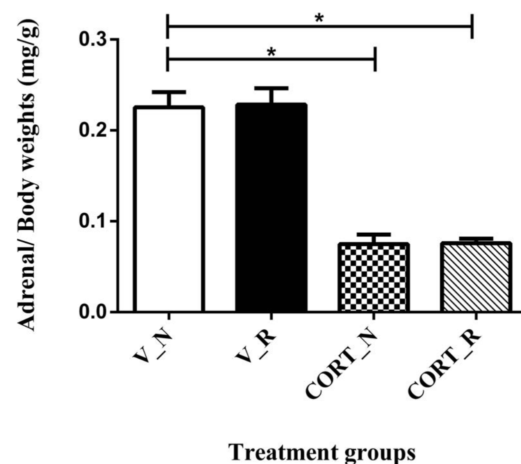

Figure 2. Adrenal-to-body weight ratio. Corticosterone treatment caused a significant adrenal gland atrophy compared to the vehicle-treated nonrunner group (V_N). The values are expressed as mean ± SEM and analyzed by Student's *t*-test followed by Bonferroni's corrections. *$p < 0.01$, $n = 6$ per group. V, vehicle treated; CORT, corticosterone treated; N, nonrunner; R, runner.

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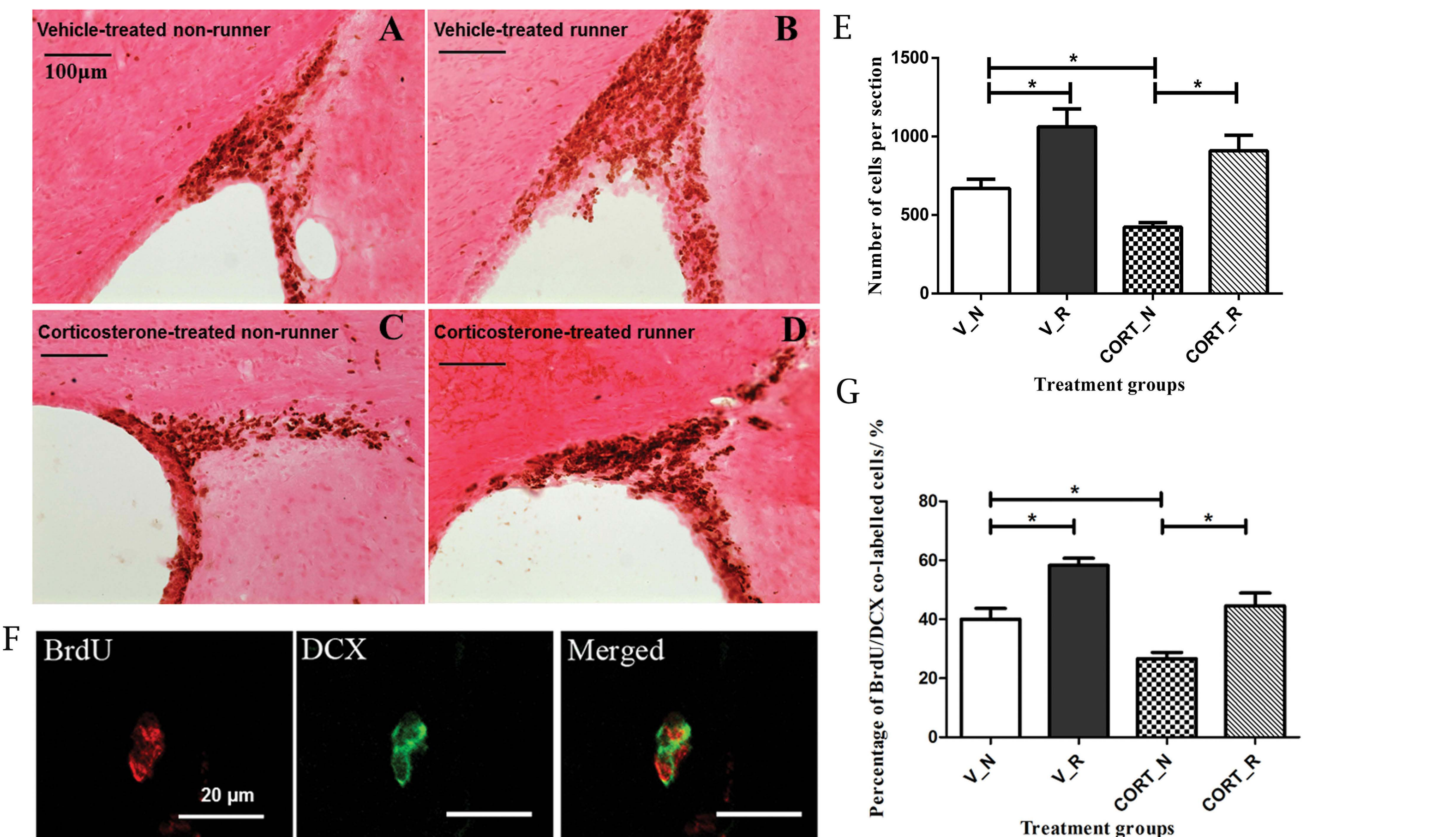

Figure 3. (A) Representative photomicrographs of BrdU-positive cells (newly proliferated cells) in the dorsal horn of the SVZ in the control vehicle-treated nonrunner group. There is a noticeable increase in the BrdU expression in (B) of the vehicle-treated runner group. (C) A representative photomicrograph of the corticosterone-treated nonrunner group, which has a noticeable decrease in BrdU expression. (D) Runner group with corticosterone treatment. (E) Statistical analysis, Student's *t*-test followed by Bonferroni's correction, of the BrdU-positive cells within SVZ. Vehicle-treated runners have a high number of BrdU-positive cells compared to the vehicle-treated nonrunners. BrdU-positive cells are shown to increase in number in the corticosterone-treated runner group when compared to the corticosterone-treated nonrunner group ($n = 5$–6 per group). (F) Representative fluorescence photomicrograph of the double labeling of BrdU (red) and DCX (green), a migrating neuroblast marker. (G) Statistical analysis of the percentage of BrdU-positive cells expressing DCX. The vehicle-treated runner group has a higher percentage of double labeling of the two types of markers compared to the vehicle-treated nonrunner group. The corticosterone-treated runner group has a significant increase in percentage of double labeling of $BrdU^+/DCX^+$ when compared to the corticosterone-treated nonrunner group. *$p < 0.0167$, significant as corrected by Bonferroni's correction for a set of three contrasts. V, vehicle treated; CORT, corticosterone treated; N, nonrunner; R, runner; SVZ, subventricular zone; BrdU, bromodeoxyuridine; DCX, doublecortin.

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effects caused by CORT could be reversed by wheel running (CORT_R) (44.58 ± 4.30%) (Fig. 3G). No interaction effects were found between CORT and running on the percentage of DCX/BrdU by two-way ANOVA analysis [$F(1,18) = 0.004$, $p = 0.950$].

DISCUSSION

The present study demonstrated that CORT treatment could suppress cell proliferation in the SVZ, and voluntary wheel running could reverse this suppression. According to the previous studies as summarized in Table 1, CORT could significantly decrease SVZ neurogenesis similar to its effects on the hippocampal neurogenesis. However, controversial findings on the effects of voluntary running on the two neurogenic sites have been observed. Furthermore, although the effects of CORT and wheel running on neurogenesis in SVZ have previously been investigated, they have not been investigated simultaneously. To our knowledge, this is the first report demonstrating the cotreatment effects of CORT and wheel running on SVZ cell proliferation.

Recently, Chae et al. reported that chronic swimming exercise could have a long-term survival effect on new neurons found in the SVZ[29]. It has also been suggested that physical exercise may exert positive effects on the SVZ only with an extended treatment period as SVZ neurogenesis has a late onset[19]. Previous reports from our group have demonstrated that subcutaneous injections with 40 mg/kg CORT for 14 days could decrease adult neurogenesis in both the SVZ[12] and the hippocampus[30]. The present study has extended the treatment period up to 28 days and found that the rats injected with CORT only have the lowest level of neurogenesis found in the SVZ, whereas the addition of wheel running can significantly increase the proliferation rate back to normal. In addition, the rats with wheel running only also had enhanced neurogenesis within the SVZ when compared to the nonrunner group under normal condition. Therefore, the present results indicate that stress not only modulates cell proliferation in the hippocampus but also has an effect on the SVZ. Also, SVZ cell proliferation may be regulated by physical activity.

The findings of this study are different from those of the previous ones that reported that physical activity has no effect on SVZ cell proliferation[23]. This may be due to (1) different regions of the SVZ being investigated. It has been suggested by Blackmore et al. that voluntary wheel running could induce neurogenesis more robustly in the rostral region of the SVZ[31]. The brain sections for cell quantification in this study also covered the rostral coordinates as suggested by Blackmore et al. Future investigations are needed to further validate if an obvious difference can be identified between different coordinates of SVZ in our current animal models. (2) Different strains of animals were used. Previous reports used adult mice for investigation[23], whereas this study used adult Sprague–Dawley rats. A report has previously shown that there were differences in the process of adult neurogenesis between rats and mice in that a faster maturing of neurons was found in rats than in mice within the hippocampus[32]. Thus, the process of SVZ proliferation may also be different among different animal species. (3) Differences in BrdU administration for the identification of proliferating cells within the SVZ. In this study, two doses per day of BrdU were injected continuously for 3 days before sacrificing the rats, whereas a different treatment regime of BrdU was reported from different studies in terms of dosages and the duration of treatment[33,34]. The results may be affected by a different course of BrdU treatment since a single injection labels fewer cells and thus may cause difficulty in detecting any differences. Alternatively, multiple injections of BrdU can give robust labeling of the proliferating cells, and the changes in the number of proliferating cells may be diluted and thus may also be difficult to detect differences between treatment groups[35].

Although this study showed that wheel running can promote SVZ neurogenesis under stress condition, the functional significance of this increase still remains unclear. Counteracting the decrease in SVZ neurogenesis was

Table 1. Summary of Previous Findings on the Effects of Stress and Running on the Two Neurogenic Sites

Region Investigated	Animal Strain	Stress on Neurogenesis	Running on Neurogenesis	Treatment Period	References
SGZ	Sprague–Dawley rat	Decreased	Promoted	2 weeks	30
SVZ	Sprague–Dawley rat	Decreased	–	2 weeks	12
SGZ and SVZ	CD1 mice	–	SGZ: promoted SVZ: promoted	6 weeks	7
SGZ and SVZ	C57BL/6 mice	–	SGZ: promoted SVZ: no effects	12 days	23

–, not applicable.

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previously found to be correlated to the improvement in the male rat's sexual performance[36], as well as suggesting a reduction in sexual dysfunction of human depressed patients[37,38]. Besides relating to sexual behaviors, Negoias et al. have reported that a decrease in olfactory bulb volume was found in patients with acute major depression and speculated that the reduction in neurogenesis contributed to this reduction[39]. Alterations in olfactory bulb volume not only affect olfactory sensitivity but can also induce fear and sadness emotions via disinhibition of the amygdala projected from the olfactory bulb[40]. Neurogenesis in the SVZ has been suggested to be involved in neuroprotective mechanisms, as the new cells have been shown to migrate to areas other than the olfactory bulb when challenged with brain damage. They have been seen to migrate to the cortex or striatum after a stroke to modulate existing synaptic connections within brain regions[13,41].

Chronic treatment with fluoxetine was previously shown to decrease neurogenesis in the SVZ with less neurons formed within the olfactory bulb[19], suggesting undesirable side effects of antidepressants. According to clinical studies, some antidepressants could have the side effect of altering the sexual performance of depressed patients[42,43], whereas sexual dysfunctions could be reduced by physical exercise[44]. Our current findings have provided evidence that wheel running can reverse the decrease of SVZ neurogenesis under stress conditions. Moreover, this study further demonstrates that physical exercise may provide a more natural and noninvasive approach to counteract stress in depression. Physical exercise could possibly be prescribed for therapeutic use for stress-related disorders or other conditions related to the SVZ in the future.

LIMITATIONS

The current study lacks behavioral readouts on determining the possible functional role of enhancing neurogenesis in the SVZ by exercise under a depressed condition. On the basis of the results that we have obtained from this study, we will further investigate the possible therapeutic role of exercise in alleviating sexual dysfunction under a depressed condition via modulating SVZ neurogenesis.

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