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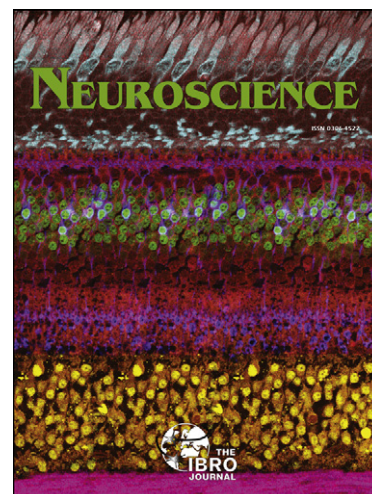
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**EFFECTS OF VOLUNTARY RUNNING ON PLASMA LEVELS OF
NEUROTROPHINS, HIPPOCAMPAL CELL PROLIFERATION AND
LEARNING AND MEMORY IN STRESSED RATS**

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Abstract

Previous studies have shown that a two-week treatment with 40 mg/kg corticosterone (CORT) in rats suppresses hippocampal neurogenesis and decreases hippocampal brain derived neurotrophic factor (BDNF) levels and impairs spatial learning, all of which could be counteracted by voluntary wheel running. BDNF and insulin-like growth factor (IGF-1) have been suggested to mediate physical exercise-enhanced hippocampal neurogenesis and cognition. Here we examined whether such running-elicited benefits were accompanied by corresponding changes of peripheral BDNF and IGF-1 levels in a rat model of stress. We examined the effects of acute (5 days) and chronic (4 weeks) treatment with CORT and/or wheel running on (1) hippocampal cell proliferation, (2) spatial learning and memory and (3) plasma levels of BDNF and IGF-1. Acute CORT treatment improved spatial learning without altered cell proliferation compared to vehicle treatment. Acute CORT-treated non-runners showed an increased trend in plasma BDNF levels together with a significant increase in hippocampal BDNF levels. Acute running showed no effect on cognition, cell proliferation and peripheral BDNF and IGF-1 levels. Conversely, chronic CORT treatment in non-runners significantly impaired spatial learning and suppressed cell proliferation in association with a decreased trend in plasma BDNF level and a significant increase in hippocampal BDNF levels. Running counteracted cognitive deficit and restored hippocampal cell proliferation following chronic CORT treatment; but without corresponding changes in plasma BDNF and IGF-1 levels. The results suggest that the beneficial effects of acute stress on cognitive improvement may be mediated by BDNF-enhanced synaptic plasticity that is hippocampal cell proliferation-independent, whereas chronic stress may impair cognition by decreasing hippocampal cell proliferation and BDNF levels. Furthermore, the results indicate a

trend in changes of plasma BDNF levels associated with a significant alteration in hippocampal levels, suggesting that treatment with running/CORT for four weeks may induce a change in central levels of hippocampal BDNF level, which may not lead to a significant change in peripheral levels.

Key words: corticosterone, BDNF, IGF-1, hippocampal cell proliferation, voluntary running, stress

Physical exercise enhances learning and memory and exerts antidepressive effects (Ernst et al., 2006, Yau et al., 2011a). Animal studies have shown that free wheel running improves spatial learning in concurrence with changes in hippocampal plasticity, including increased neurogenesis, enhanced long-term potentiation (LTP) (van Praag et al., 1999a, van Praag et al., 1999b), and elevated expression of brain-derived neurotrophic factors (BDNF) (Cotman and Berchtold, 2002, Farmer et al., 2004, Cotman et al., 2007).

Enhanced hippocampal neurogenesis from physical exercise (van Praag et al., 1999b) is putatively regarded a basis for improving cognitive function (van Praag et al., 1999a, van Praag et al., 2005) and counteracting depression (Ernst et al., 2006). In contrast, reduced neurogenesis by chronic stress or by high doses of glucocorticoid treatment (Gould et al., 1992, Gould et al., 1998) has been associated with cognitive deficits and linked to the psychopathology of clinical depression (Duman et al., 1997, Ernst et al., 2006). Ablation of neurogenesis in animal studies further indicates that hippocampal neurogenesis potentially mediates the beneficial effects of exercise on cognitive performance and depressive disorders (Clark et al., 2008, Lafenetre et al., 2010, Yau et al., 2011b).

Several neurotrophic factors, such as BDNF and insulin-like growth factor (IGF-1) have been suggested to be potential mediators for the exercise-derived hippocampal plasticity (Cotman and Berchtold, 2002, Cotman et al., 2007). Increased neurogenesis and improved cognitive function by exercise are in parallel with BDNF up-regulation in the brain (Cotman and Berchtold, 2002, Vaynman et al., 2004, Zheng et al., 2006), possibly caused by the exercise-triggered uptake of IGF-1 into the brain (Carro et al., 2000). In a study conducted by Trejo *et al.* mutant mice with low

levels of serum IGF-I showed reduced hippocampal neurogenesis together with impaired spatial learning which could not be reversed by running (Trejo et al., 2008). This suggests that exercise-induced neurogenesis may be mediated by the entry of peripheral IGF-1 into the brain (Trejo et al., 2001). Thus, peripheral BDNF and IGF-1 levels are increasingly seen as potential biomarkers for evaluating the exercise-elicited cognitive improvement in studies involving human subjects.

Clinical observation has demonstrated a decrease in blood BDNF level in depressive patients and an increase in serum BDNF level after antidepressant treatment (Martinowich et al., 2007, Molendijk et al., 2011). Since it is not feasible to directly measure the alteration of hippocampal neurogenesis in live human brain tissues, clinical studies have most exclusively relied on examining the serum or plasma levels of neurotrophins following interventions. It has recently been shown that the alteration in blood BDNF level can reflect its release from the brain (Klein et al., 2011) where contributes to the increase in plasma BDNF in response to exercise (Rasmussen et al., 2009). Given the positive association between hippocampal neurogenesis, levels of neurotrophins (e.g. BDNF and IGF-1) and cognitive improvement after exercise intervention suggested by other studies, it would be interesting to investigate whether alterations of peripheral neurotrophins are accompanied by the corresponding changes of hippocampal cell proliferation. A positive correlation would justify the rational to apply such peripheral biomarkers in evaluating cognitive state following exercise for translational studies on human subjects.

In a rat model of stress using repeated CORT injections, we have previously shown that voluntary wheel running for 14 days could restore the decrease in hippocampal

cell proliferation and attenuate learning impairment together with enhanced expression of BDNF protein levels (Yau et al., 2011b). However, it is unclear whether peripheral changes of BDNF and IGF-1 correspond to alteration in hippocampal cell proliferation. Hence, we investigated the effects of acute and chronic CORT treatments, with or without running on hippocampal-dependent learning and memory, cell proliferation in the hippocampus, and peripheral levels of BDNF and IGF-1.

EXPERIMENTAL PROCEDURES

Animals and housing conditions

All experimental procedures were approved and followed the guidelines of the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong. Adult male Sprague-Dawley rats (250 ± 20 g) were housed individually with unlocked running wheels for runners or locked wheels for non-runners. Animals were divided into several groups including: the vehicle-treated non-runners (CtrlN) and runners (CtrlR), the 40mg/kg CORT-treated non-runners (40mgN) and runners (40mgR). These were then further subdivided into either acute (5 days) or chronic (28 days) treatment groups ($n=4-6$ per group). All rats were kept on a 12 h light-dark cycle with *ad libitum* access to food and water.

Animal treatments

After adaptation in the cages with locked wheels for two days, the rats received daily treatments with sesame oil or 40 mg/kg CORT and subjected to unlocked or locked wheels for 5 days (acute treatment) or 28 days (chronic treatment). The treatment was started on Day 3 and ended on Day 7 for acute treatment or on Day 30 for chronic treatment. Corticosterone (Sigma-Aldrich, St Louis, MO) was prepared and injected subcutaneously according to the method of Hellsten *et al.* (Hellsten *et al.*, 2002). Bromodeoxyuridine (BrdU, 50mg/kg body weight) was dissolved in normal saline and administered intraperitoneally in 12-hr intervals during the last 3 days of the treatments for labeling proliferating cells. This was followed by the Morris water maze task. Changes in body weight was monitored weekly, and the adrenal weight was measured after sacrificing the animals (see Figure 1).

Exercise training

Runners were housed singly in cages equipped with running wheels (diameter 31.8 cm; width 10 cm; Nalgene Nunc International, Rochester, NY). Rats were allowed to run freely for 5 or 28 days and wheels were then locked during the water maze task. Wheel revolutions were recorded using the VitalViewer software (Mini Mitter Company, Inc, Bend, OR). Non-runners were housed individually in identical cages with locked wheels.

Morris water maze task

Spatial learning and memory was examined using the water maze task with a hidden platform 0.5 cm below the water surface. The water was made opaque by adding non-toxic ink. The test was conducted for five consecutive days with four trials per day with the platform located in the same position. Animals were brought into the testing room 30 min prior to the test for adaptation. The starting points were randomly assigned to each animal every day. The latency for the animals to reach the platform was recorded by a trained observer. If the animals did not find the platform after a maximum of 60 s, it was brought to the platform for 20 s and assigned a score of 60 s. The animals were then returned to the cage and given a 4 min inter-trial rest.

On the sixth day, a probe trial was performed with the platform removed from the tank. Time spent in the target area in the probe trial was analyzed using SmartJunior software (Panlab, Spain). The target area was defined as a circular area with the diameter of 20 cm measured from the centre of the platform as previously applied (Yau et al., 2011b).

Sucrose preference test

The test was performed after three or four weeks of CORT treatments. The rats were firstly supplied with 1 % sucrose in place of water for 24 hr. They were then offered simultaneously one bottle containing 1 % sucrose and one bottle with tap water for 24 hr. The position of the two bottles was swapped after 12 hr. The consumption of water and sucrose was recorded by weighing the bottles before and after the test. The sucrose preference was presented as the percentage of total amount of liquid consumption.

Tissue preparation

Twenty-four hours after the end of the water maze task, animals were anesthetized with a mixture (2:1, v/v) of ketamine 200 mg/ml and xylazine and transcardially perfused with 0.9 % saline solution, followed by 4 % paraformaldehyde for 20 min. Brains were removed from the skull and posted fixed overnight at 4 °C. After that, the brains were placed in 30 % sucrose solution until they sank. Twelve series of 40 µm coronal sections were cut with a freezing sliding microtome (Model 860; American Optical, Buffalo, NY) and the brain slices were stored in cryoprotectant until use. The cryoprotectant consists of 200 ml 0.1 M phosphate buffered saline (PBS), 150 g sucrose and 150 ml ethylene glycol, with the final volume adjusted to 500 ml with 0.1 M PBS.

Immunohistochemistry and immunofluorescence

BrdU labeling and double staining were performed as previously described (Lau et al., 2007, Yau et al., 2011b). Briefly, the sections were boiled in 0.01 M citric acid (pH = 6.0) at 95 °C for 30 min, followed by three rinses of 0.01M PBS. The sections were then incubated with 2 N HCl at 37 °C for 30 min in an oven, and subsequently

neutralized with boric acid buffer (pH = 8.5) for 15 min at room temperature. After three rinses with PBS, the sections were incubated with mouse anti-BrdU antibody (1:1000, Roche Molecular Biochemicals, Essex, UK) overnight at room temperature. The sections were then washed and incubated with biotinylated goat anti-mouse antibody (1:200, Vector Laboratories, Inc Burlingame, CA). This was followed by incubation with an avidin-biotin complex (Vector Laboratories), and finally the sections were visualized with diaminobenzidine. For co-labeling, the sections were incubated with primary antibodies: rat anti-BrdU antibody (1:1000, Abcam, Cambridge, MA) and rabbit anti-doublecortin (DCX, 1:200, Cell Signaling, Danvers MA) overnight in blocking diluent. Sections were then rinsed and incubated with Alexa Fluor 488-conjugated goat anti-rabbit and Alexa Fluor 568-conjugated goat anti-rat IgG (1:200, Molecular Probes, Eugene, OR) overnight at room temperature. After rinsing, sections were coverslipped with mounting medium.

Quantification of BrdU-labeled cells and neuronal differentiation

Brain sections were counted in one-in-twelve series of coronal sections as reported previously (Yau et al., 2011b). All BrdU positive cells were counted under 40X objective using the StereoInvestigator (MicroBrightField, Williston, VT), and only those within the granular cell layer (GCL) and two cell diameters below the GCL were counted. The resulting numbers were then multiplied by 12 to obtain the total number of BrdU positive cells in the dentate gyrus.

For phenotypic analysis, thirty BrdU positive cells per animal were randomly selected from the dorsoventral axis of the dentate gyrus and analyzed for the co-expression of BrdU and DCX using fluorescence microscopy (Axioplan, Zeiss, Oberkochen, Germany) with 40X magnification. A cell with a BrdU-positive nucleus stained together with a DCX positive surrounding soma was determined as a double labeled

cell. The proportion of DCX positive cells over the thirty BrdU positive cells was obtained as the indicator for neuronal differentiation. The counting was performed in a blinded manner.

Plasma BDNF and IGF-1 measurements

Blood samples were collected 1-hr after injection of CORT on the last day of the treatment as previously performed (Yau et al., 2011b). In brief, animals were anaesthetized using the mixture of ketamine and xylazine (2:1 ratio) and blood was collected from the tail vein within 3 min using a heparinized needle. Blood samples were then centrifuged (Eppendorf centrifuge 5417R, USA) at 1000 x g for 20 min at 4 °C. Plasma aliquots were then stored at -80 °C until use. The BDNF and IGF-1 levels were determined using commercially available ELISA kits (BDNF: Chemikine BDNF Sandwich ELISA Kit, Millipore, Billerica, CA; Mouse/Rat IGF-1 Quantikine ELISA Kit, R & D system, Minneapolis, MN). Sample neurotrophin levels were calculated from the standard curve transforming the absorbance into neurotrophin concentrations, which presented in the unit of ng/ml.

BDNF enzyme-linked immunosorbent assay

Briefly, the BDNF standards, plasma samples (200-fold dilution) or 50µg hippocampal protein sample brought to 100 µl using sample diluent were added to 96-well plate and incubated overnight with shaking at 4 °C. After washing the plate with wash buffer, the diluted biotinylated mouse anti-BDNF monoclonal antibody was added to the plate with incubated at room temperature for three hours. After washing, the diluted streptavidin-HRP conjugate solution was added and incubated for one hour at room temperature. TMB/E substrate was incubated for 15 min, followed by stop solution. The absorbance was immediately measured at 450 nm.

IGF-1 enzyme-linked immunosorbent assay

The IGF-1 standard and samples (1000-fold dilution) were added to 96-well plate and incubated with 150 μ l assay diluent for two hours at 4 °C. After washing, the plate was incubated with cold IGF-1 conjugate for one hour at 4 °C, followed by washings and 30-min incubation with substrate solution at room temperature. The reaction was stopped by adding stop solution and absorbance was measured at 450 nm and corrected by subtracting reading at 570 nm.

Statistical analysis

Two-way ANOVA with running and CORT treatment as the between-subject factors was performed with post-hoc Fisher's LSD test. The data of body weight change and spatial learning were analyzed using repeated measures ANOVA with duration as the within-factor, and exercise and CORT treatment as the between-subject factors. Student's t-test was applied to compare two sets of data. Values were presented as mean \pm SEM.

RESULTS

Changes in body weight and adrenal weight by CORT treatment and running

The adrenal to body weight ratio was measured to assess the efficacy of CORT injection. Acute CORT treatment for 5 days significantly decreased adrenal weight to body weight ratio when compared with the vehicle treatment (effect of CORT: $F(1,22)=9.956$, $P=0.00037$; Fig 2a.). Running did not affect the adrenal weight to body weight ratio (effect of running: $F(1,22)=0.169$, $P=0.686$) and there was no interaction between CORT and running (CORT X running: $F(1,22)=0.0004$, $P=0.983$).

Chronic CORT treatment also significantly decreased the adrenal weight to body weight ratio (effect of CORT: $F(3,21)=16.8$, $P=0.00687$; Fig 2b.). In contrast, chronic running significantly increased the adrenal weight to body weight ratio (effect of running: $F(3,21)=66.253$, $P=0.000288$; interaction CORT X running: $F(3,21)=64.732$, $P=0.000338$). Post hoc test indicated that the CtrlR group showed a significant increase in the ratio compared with the CtrlN group ($P < 0.005$), indicating that running increased the adrenal weight in the normal rats. CORT treatment significantly decreased the ratio in both non-runners and runners when compared to the vehicle-treated non-runners and runners, respectively ($P < 0.005$). All rats showed an increase in the body weight gain during the 28-day treatment (repeated measure ANOVA; effect of day: $F(4,92)=236.315$, $P < 0.005$). Running did not affect the body weight gain, but CORT significantly decreased body weight gain (effect of running: $F(1,23)=0.007$, $P=0.936$; effect of CORT: $F(1,23)=72.283$, $P < 0.005$; Running X CORT : $F(1,23)=6.626$, $P=0.019$). Pos hoc test showed that CORT treatment significantly decreased body weight gain compared with the vehicle treatment (Fig 2c. Day 1, $F(3,23)=0.434$, $P=0.731$; Day 2, $F(3,23)=10.275$, $P < 0.005$; Day 3,

$F(3,23)=21.276$, $P<0.005$; Day 4, $F(3,23)=35.516$, $P<0.005$; Day 5, $F(3,23)=37.887$, $P<0.005$).

Depression-like phenotype of the CORT-treated rats

We have previously validated the stress level and depression-like behavior of the CORT-treated rats, where the result indicates a significant elevation in CORT level and an increase in immobility time in the forced swim test following 14-day CORT injection (Yau et al., 2011b). In the present study, we assessed the behavior of anhedonia, a hallmark symptom of depression, which is characterized by a decrease in sucrose preference compared to water in the sucrose preference test. The rats with 3-week and 4-week CORT treatment showed a decrease in sucrose preference compared with the vehicle-treated rats (Fig 2d. Student's t-test, $P<0.05$), indicating that chronic CORT treatment induced depression-like behavior in the animals.

Running activity in the vehicle-treated and CORT-treated rats

Both the vehicle-treated and CORT-treated rats gradually increased their running distance during the first week in acute and chronic treatment. Rats with acute CORT treatment showed a higher running activity relative to the vehicle-treated rats, with significant differences observed on Day 4 and Day 5 (Fig 3a. $P<0.05$). The total running distance was significantly higher in the CORT-treated rats relative to the vehicle-treated rats. (Fig 3b, Student's t-test, $P<0.05$). Rats with chronic CORT treatment displayed a higher initial running activity during the first two weeks compared to vehicle-treated rats (Fig 3c. $P<0.05$). However, there was no significant difference in the total running distance in the vehicle treatment group compared to the CORT treatment group (Fig 3d. $P>0.05$).

Effect of acute and chronic treatment with CORT and running on hippocampal cell proliferation

Neither running nor CORT treatment for 5 days affected hippocampal cell proliferation in the rats (Fig 4a. effect of running: $F(3,20)=1.372$, $P=0.257$); effect of CORT: $F(3,20)=1.504$, $P=0.237$). The data indicated that acute treatment with CORT and running showed no effect on modulating the proliferating rate of hippocampal progenitor cells.

Chronic running significantly increased the number of BrdU positive cells in the dentate gyrus compared with the non-runners (Fig 4c. effect of running: $F(3,19)=10.649$, $P=0.00517$). In contrast, chronic treatment with CORT significantly suppressed hippocampal cell proliferation (effect of CORT, $F(3,19)=9.318$, $P=0.00806$). Post hoc analysis showed a significant increase in the number of BrdU positive cells in the CtrlR group compared with the CtrlN group ($P=0.023$), indicating that running for 28 days significantly increased hippocampal cell proliferation in normal rats. Conversely, the 40mgN group showed a significant decrease in the number of BrdU positive cells in the dentate gyrus compared to the CtrlN group ($P=0.046$), indicating that CORT suppressed hippocampal cell proliferation. The 40mgR group exhibited a significant increase in BrdU positive cells compared to the 40mgN group ($P=0.05$), indicating running counteracted the suppressive effect of CORT treatment on hippocampal cell proliferation.

Effect of acute and chronic treatment on hippocampal neuronal differentiation

We examined the neuronal differentiation by quantifying the ratio of BrdU positive cells expressing the immature neuronal marker: doublecortin (DCX). Acute treatment with CORT or running showed no effect on the ratio of DCX to BrdU positive cells,

suggesting that neuronal differentiation was affected by neither acute CORT treatment nor running (Table 1 and Fig 5a. effect of running: $F(3,18)=0.465$, $P=0.506$; effect of CORT: $F(3,18)=0.00786$, $P=0.931$). In contrast, chronic running significantly increased neuronal differentiation as indicated by a significant increase in the ratio of DCX to BrdU positive cells in the runners compared with the non-runners (Table 1 and Fig 5b. effect of running: $F(1,20)=5.740$, $P=0.031$) whereas chronic treatment with CORT suppressed neuronal differentiation (effect of CORT: $F(1,20)=34.77$, $P=0.000389$). Post hoc analysis showed that running significantly increased neuronal differentiation in the vehicle-treated rats compared with the non-runner counterparts ($P=0.039$). The 40mgN and 40mgR groups both showed a significant decrease in the ratio compared with the CtrlN group ($P<0.05$).

Acute CORT treatment improved spatial learning, but not memory consolidation

Two-way ANOVA with repeated measures showed that all rats learned to locate the hidden platform during the learning phase (Fig 6a. effect of day: $F(4, 24)=85.575$, $P=0.000230$). A significant interaction between day and CORT treatment was observed (day X CORT: $F(4,24)=4.836$, $P=0.00158$), but without a main effect of running (effect of running: $F(3,24)=0.586$, $P=0.454$). However, acute treatment with CORT showed a main effect on spatial learning (Fig 6a. effect of CORT: $F(3,24)=5.136$, $P=0.0353$). Post hoc analysis confirmed that the 40mgN and 40mgR groups both showed a significant decrease in the escape latency compared with the CtrlN group ($P=0.013$ and $P=0.026$ respectively). However, in the probe trial test, acute treatment with CORT or running did not affect time spent in the target area, indicating that memory consolidation was not affected by acute running or CORT treatment (Fig 6b).

Running reversed chronic CORT-impaired spatial learning

Animals with chronic treatment showed that all rats learnt to locate the position of the hidden platform during the 5-day training period (Fig 6c. effect of day: $F(4,24)=29.541$, $P=0.00087$). Two-way ANOVA indicated a significant difference between the non-runners and runners (effect of running: $F(3,24)=6.076$, $P=0.033$). The 40mgN group showed a significant increase in the escape latency compared with the CtrlN or CtrlR groups, respectively (Day 1: $P=0.024$ & $P=0.035$ and Day 2: $P=0.030$ & $P=0.015$, respectively), indicating impaired spatial learning after chronic treatment with CORT. In contrast, running decreased escape latency as evidenced by the decreased escape latency in the CtrlR group compared with the 40mgN group (Day 4, $P=0.014$). Running also reversed CORT-impaired spatial learning in the 40mgN group as evidenced by a significant decrease in escape latency in the 40mgR group compared with the 40mgN group (Day 2, $P=0.009$). The data indicated that running significantly counteracted the impairment in spatial learning induced by CORT treatment.

In the probe trial, two-way ANOVA indicated insignificant effects of running or CORT treatment on the time spent in the target area (Fig 6d. effect of running: $F(3,24)=1.923$, $P=0.182$; effect of CORT: $F(3,24)=0.002$; $P=0.961$), suggesting that chronic running and CORT treatment did not affect memory consolidation.

Peripheral and central levels of BDNF after acute and chronic treatment

We next investigated the linkage between cognitive enhancement and increase in peripheral BDNF and IGF-1 levels in the rats with running. We measured the plasma BDNF level since it has been reported that the cortex and hippocampus are the major sources for the increased plasma BDNF level in response to physical exercise (Rasmussen et al., 2009). Two-way ANOVA indicated a significant main effect of

CORT on plasma BDNF level in acute treatment (Fig 7a. effect of CORT: $F(3, 20)=4.726$, $P=0.042$). There was a significant increase in plasma BDNF level in the 40mgN group relative to the CtrlR group ($P=0.011$) following acute treatment. There was an increased trend in plasma BDNF level in CORT-treated non-runners compared to the vehicle-treated non-runners, and this trend was accompanied with a significant elevation in hippocampal BDNF levels (Fig 7c. Student's test, $P<0.05$ compared to the vehicle-treated non-runners).

In contrast, there was no significant change in plasma BDNF level following chronic treatment with CORT or running (effect of running: $F(3,18)=0.602$, $P=0.580$; effect of CORT: $F(3,18)=0.024$, $P=0.903$). However, there was a decreased trend in plasma BDNF level in the CORT-treated non-runners, and this decrease was associated with a significant decrease in hippocampal BDNF level (Fig 7d. Student's t-test, $P<0.05$ compared to the vehicle-treated non-runners).

Plasma level of IGF-1 after acute and chronic treatment

There was no significant changes in plasma levels of IGF-1 following acute treatment with running and/or CORT (Fig 7b. acute: effect of running: $F(3,21)=0.307$, $P=0.587$; effect of CORT: $F(3,21)=3.009$, $P=0.101$), or following the chronic treatment with running and/or CORT (Fig 7b. effect of running: $F(3, 18)=2.564$, $P=0.132$; effect of CORT: $F(3, 18)=2.648$, $P=0.126$).

DISCUSSION

The present study investigated the effects of acute and chronic running on cognitive improvement and the correspondent changes of peripheral BDNF and IGF-1 levels in

the CORT-injected animal model of stress. To the best of our knowledge, this is the first study examining the linkage between peripheral BDNF and IGF-1 levels and changes in hippocampal cell proliferation in rats after running. Consistent with our previous findings (Yau et al., 2011b), the result showed that chronic running reversed the suppression of hippocampal cell proliferation and impaired spatial learning by CORT treatment. However, no significant alterations of peripheral BDNF and IGF-1 levels after running for 28 days were observed. Interestingly, our data showed that acute CORT treatment increased spatial learning without altering hippocampal cell proliferation, which was accompanied by a significant increase in hippocampal BDNF levels, whereas chronic treatment with CORT impaired spatial learning and memory, and decreased hippocampal cell proliferation in association with a significant decrease in hippocampal BDNF level, but with no significant changes of peripheral BDNF and IGF-1 levels.

The animal model of stress mimicked by subcutaneous CORT injection

The CORT injected animal model of stress has been repeatedly reported to significantly increase plasma CORT level and cause depression-like behavior in a dose-dependent manner (Johnson et al., 2006, Yau et al., 2011b). The depressionogenic effects of the CORT injection paradigm are known to be time- and dose-dependent. Treatment with 40 mg/kg CORT for 14 or 21 days considerably increases depression-like behavior, whereas neither 20 mg/kg CORT nor 10 mg/kg CORT treatment produces significant increase in depression-like behavior (Gregus et al., 2005, Johnson et al., 2006). Our previous report has shown that treatment with 20 mg/kg CORT for 14 days exerts a beneficial effect on hippocampal plasticity (Yau et al., 2011c). It has also been reported that acute CORT treatment could produce

antidepressant-like effect (Dunn and Swiergiel, 2008, Stone and Lin, 2008) whereas chronic CORT treatment for 3 weeks or longer significantly increased depression-like behavior (Zhao et al., 2008, Zhao et al., 2009). The present study showed that the rats treated with CORT for 3 or 4 weeks demonstrated a behavior of anhedonia as evidenced by decreased sucrose preference. Our data also showed that acute (5 days) treatment with CORT significantly improved hippocampal-dependent learning whereas chronic (28 days) treatment with CORT led to learning impairment, in agreement with results reported by others that short-term and long-term CORT treatments differentially lead to positive and negative effects on animal behavior (Zhao et al., 2009). With reproducible findings from the same animal model, the protocol of repeated treatment with CORT has been validated as a reliable method for inducing stress in rodents in a controllable manner (Sterner and Kalynchuk, 2010).

Effects of acute treatment on hippocampal cell proliferation, neurotrophins and spatial learning and memory

Our results demonstrated the differential effects of acute and chronic treatment with 40 mg/kg CORT on hippocampal plasticity. The acute treatment with CORT improved spatial learning that associated with a higher plasma BDNF levels and a significant increase in hippocampal BDNF levels, but did not affect hippocampal cell proliferation, suggesting that the positive influence of acute stress on spatial learning may not necessarily involve an enhancement in hippocampal cell proliferation under acute stress condition. It is known that acute stress is able to promote memory formation (Shors, 2001) and enhances acquisition up to 2 days after stressor cessation (Servatius and Shors, 1994). Acute exposure to stress has also been clinically shown to facilitate spatial navigation performance (Duncko et al., 2007). These

improvements in spatial learning triggered by acute stress might be due to the transient activation of NMDA receptor (Shors and Mathew, 1998) or increased BDNF level. In fact, the association between the increase in hippocampal BDNF levels and improvement in spatial learning following acute CORT treatment in the present study suggests that the acute effect of stress on improving cognition may possibly be mediated by BDNF-enhanced synaptic plasticity. Our result echoed other reports that mRNA level of BDNF in the hippocampus can be rapidly increased by a short-time exposure to immobilization stress, suggesting this rapid change in BDNF expression may be a form of neuronal plasticity for coping with new stimuli (Marmigere et al., 2003) as BDNF is well-known for its critical involvement in the induction of LTP and synaptic plasticity (Kovalchuk et al., 2002).

The present data also showed that a higher level of peripheral BDNF corresponded to significant increase in hippocampal BDNF level and improvement in spatial learning following acute CORT treatment. However, there was no significant change in plasma BDNF level following 5-day running. A human study has shown that plasma BDNF level was increased after rowing exercise for 4 hr and then returned to resting levels after recovery for 1 hr (Rasmussen et al., 2009), indicating the transient increase in plasma BDNF in response to acute exercise. Acute treatment (5 days) in the present study may not be able to trigger substantial changes in peripheral levels of neurotrophins.

There was no change in plasma IGF-1 levels after the acute treatment with running and/or CORT. The responses of peripheral IGF-1 to interventions (e.g. exercise) are inconsistent among studies with some reporting no alteration in serum IGF-1 levels (Banfi et al., 1994, Griffin et al., 2011), while others reported an increase in IGF-1

levels after intensive exercise training in human subjects (Hartmann and Singrun, 1968, Schwarz et al., 1996). It has been reported that rats with one hour treadmill running displayed increased uptake of IGF-1 into the brain without affecting basal level of serum IGF-1 (Carro et al., 2000). It is possible that regulations of IGF-1 level in response to intervention may depend on the duration and intensity (Griffin et al., 2011), and a significant change in peripheral IGF-1 may only be observed after intensive interventions.

Effect of chronic treatment on hippocampal cell proliferation, neurotrophins and spatial learning and memory

Our previous study has reported that running counteracted CORT-impaired spatial learning and neurogenesis following two weeks of treatment (Yau et al., 2011b). The beneficial effect of running on promoting hippocampal neurogenesis has also been shown in a genetically depressed rat model (Bjornebekk et al., 2005). We here confirmed our previous observation that treatment with 40 mg/kg CORT over 4-weeks significantly decreased hippocampal cell proliferation and caused behavioral deficit in the water maze task that was reversed by running. From phenotypic analysis of newly proliferating cells, we showed that treatment with CORT over 4 weeks suppressed the ratio of DCX/BrdU co-labeled cells, indicating reduced neuronal differentiation by chronic treatment with CORT. The results echoed the finding by Wong and Herbert that high levels of glucocorticoid decrease neuronal differentiation of the surviving cells (Wong and Herbert, 2006). The suppressive effect of CORT on cell proliferation may result from the decreased hippocampal BDNF levels, since BDNF is an important neurotrophic factor for promoting neuronal survival and growth (Barde, 1994) the effects of which could be inhibited by high levels of CORT

(Rasmusson et al., 2002, Jacobsen and Mork, 2006). In fact, we have observed a significant decrease in hippocampal BDNF level in rats with chronic CORT treatment. Furthermore, our previous study has shown that running restored hippocampal cell proliferation in association with restored hippocampal BDNF level following a 2-week CORT treatment (Yau et al., 2011b). As a result, the number of newborn neurons that can functionally integrate into neuronal circuitry of the hippocampus was decreased, leading to impairment in hippocampal function, and consequent behavioral deficits under stress condition (Yau et al., 2011b).

Prolonged elevation of corticosteroid reduces the availability of hippocampal BDNF level (Rasmusson et al., 2002, Jacobsen and Mork, 2006) and suppresses the synthesis of IGF-1 in the liver (the main source of peripheral IGF-1) (Yakar et al., 1999). In the present study, we demonstrated that chronic CORT treatment significantly impaired spatial learning, suppressed cell proliferation and decreased hippocampal BDNF level, together with relative lower peripheral BDNF and IGF-1 levels compared to the vehicle-treated non-runners. Furthermore, running promoted hippocampal cell proliferation in absence of changes in peripheral BDNF and IGF-1 levels in the vehicle-treated and CORT-treated rats. Our results indicated a trend in changes of peripheral BDNF levels together with a significant alteration in hippocampal cell proliferation following chronic treatment with CORT. However, there are studies showing that animals with 4-week exposure to stressful event displayed decrease in serum and hippocampal BDNF concentrations in association with morphological changes that presented in depressive disorder (Sheline et al., 1999, Duman and Monteggia, 2006), such as reduced hippocampal volume and neurogenesis, CA3 dendritic retractions and decreased spine density (Blugeot et al., 2011). It has been reported that changes in blood BDNF level reflect the changes in the hippocampus

(Blugeot et al., 2011). We observed a decrease in hippocampal cell proliferation, and impairment in spatial learning together with a trend towards lower levels of plasma BDNF and IGF-1 in the non-runners with chronic CORT treatment. It is possible that the change in hippocampal BDNF levels was too small to be reflected in plasma levels in our CORT-treated rats. The changes of central BDNF might not be detectable in peripheral samples, though hippocampal BDNF levels have been significantly decreased following the 4-week CORT treatment. Furthermore, we have found that rats treated with 50 mg/kg CORT for two weeks showed a considerable decrease of BDNF levels in both hippocampus (Yau et al., 2011b) and plasma (Yau et al., Unpublished data). It is possible that the changes in brain BDNF levels in our 40 mg/kg CORT-treated rats may be too small to be reflected in the peripheral levels.

The results of studies reporting changes in peripheral levels of BDNF and IGF-1 after interventions are controversial. It has been reported that serum BDNF levels were up-regulated after exercise intervention in humans (Tang et al., 2008, Knaepen et al., 2010), whereas those of IGF-1 varied after exercise (Kraemer et al., 1995, Abellan et al., 2006). However, there are also reports showing no change in the basal BDNF level in healthy subjects with a 5-week aerobic exercise training (Griffin et al., 2011). Furthermore, there is no change in serum BDNF level in depressive patients reflecting the therapeutic effects of physical exercise after 12-week training (Toups et al., 2011). Oppositely, it has been reported that aerobic exercise increased the size of hippocampus in association with increase serum BDNF level in older subjects (Erickson et al., 2011). It is possible that interventions triggering substantial changes in brain levels of neurotrophins may be critical to induce significant changes to the peripheral levels.

CONCLUSION

Our results suggest that the beneficial effects of acute stress on cognitive improvement may be mediated by BDNF-enhanced synaptic plasticity in a new cells-independent manner, whereas the detrimental effects of chronic stress on cognitive impairment may be due to suppressed hippocampal cell proliferation and BDNF levels. Enhancement in spatial learning and elevation in hippocampal BDNF levels were accompanied by a corresponding increased trend in the plasma BDNF level following acute CORT treatment. Conversely, impairment in spatial learning, and suppression in hippocampal cell proliferation and decrease in hippocampal BDNF levels were not associated with significant peripheral changes of BDNF levels following chronic CORT treatment. Furthermore, the beneficial effects of running on hippocampal cell proliferation in normal and CORT-treated rats were in absence of significant alteration in peripheral levels of neurotrophins. As discussed above, it is possible that mild change in central levels of neurotrophins after 4-week treatment with running or CORT may not be reflected in peripheral levels. Since responses of peripheral BDNF and IGF-1 to exercise training are variable and may depend on duration and intensities as suggested by Griffin et al., (Griffin et al., 2011), conditions triggering substantial changes in the brain which can be reflected in peripheral neurotrophin levels warrant further investigation in attempt to show BDNF or IGF-1 as biomarkers for the effects of exercise on cognitive improvement.

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FIGURE LEGENDS

Fig. 1. Schematic diagram showing the experimental procedures and treatment timeline. After two days of adaptation, rats were treated with either vehicle or CORT and were housed with either unlocked or locked wheels for 5 or 28 days. BrdU was intraperitoneally injected during the last three days of the treatment. Spatial learning and memory was evaluated using the water maze task one day later and then the animals were sacrificed 24 hr after the test.

Fig. 2. Change of body weight and adrenal weight after treatment. (a) Acute treatment with CORT significantly decreased adrenal to body weight ratio compared with the vehicle treatment. (b) Chronic treatment with CORT significantly decreased the ratio compared with the vehicle treatment. Running increased adrenal weight in the normal rats. # $P < 0.005$ compared to the CtrlN group and CtrlR group, respectively; * $P < 0.005$ compared to the CtrlN group; ** $P < 0.005$ main effect of CORT. (c) The CORT-treated rats gained less body weight than the vehicle-treated rats during the treatment period. * $P < 0.005$ when compared to the 40mgN group and 40mgR group, respectively. (d) CORT treatment for 3 or 4 weeks significantly decreased sucrose consumption compared with the vehicle treatment. * $P < 0.05$ compared to the vehicle-treated control group. CtrlN or CtrlR: the vehicle-treated non-runners or runners; 40mgN or 40mgR: 40mg/kg CORT-treated non-runners or runners.

Fig. 3. Running activity of the rats with vehicle or CORT treatment. (a & b) Acute treatment with CORT increased the running distance when compared with the vehicle-treated rats with a significant difference observed on Day 4 and Day 5. Acute CORT treatment increased total running activity compared to the vehicle treatment. (c & b) The CORT-treated rats showed higher running activity relative to vehicle-treated

rats during the first 2-weeks of running. However, there was no difference in total running distance between the vehicle and chronic CORT treatment. * $P < 0.05$ compared to the vehicle-treated rats.

Fig. 4. Effect of acute and chronic treatments on hippocampal cell proliferation. (a) Representative images showing immunohistochemical staining of BrdU positive cells in the dentate gyrus of the hippocampus. (b) Acute treatment with CORT or running did not affect the number of BrdU positive cells in the dentate gyrus. (c) Representative images of BrdU positive cells after chronic treatment. (d) Chronic running (CtrlR) significantly increased the number of BrdU positive cells in the dentate gyrus when compared with the normal non-runners (CtrlN). CORT treatment without running (40mgN) robustly decreased the number of BrdU positive cells, whereas running restored the number of BrdU positive cells in rats treated with 40 mg/kg CORT (40mgR). * $P < 0.05$, ** $P < 0.005$ compared to the CtrlN group. # $P < 0.05$ to the 40mgN group. Scale bar: 10X : 100 μ m; 40X: 30 μ m. CtrlN or CtrlR: the vehicle-treated non-runners or runners; 40mgN or 40mgR: 40mg/kg CORT-treated non-runners or runners.

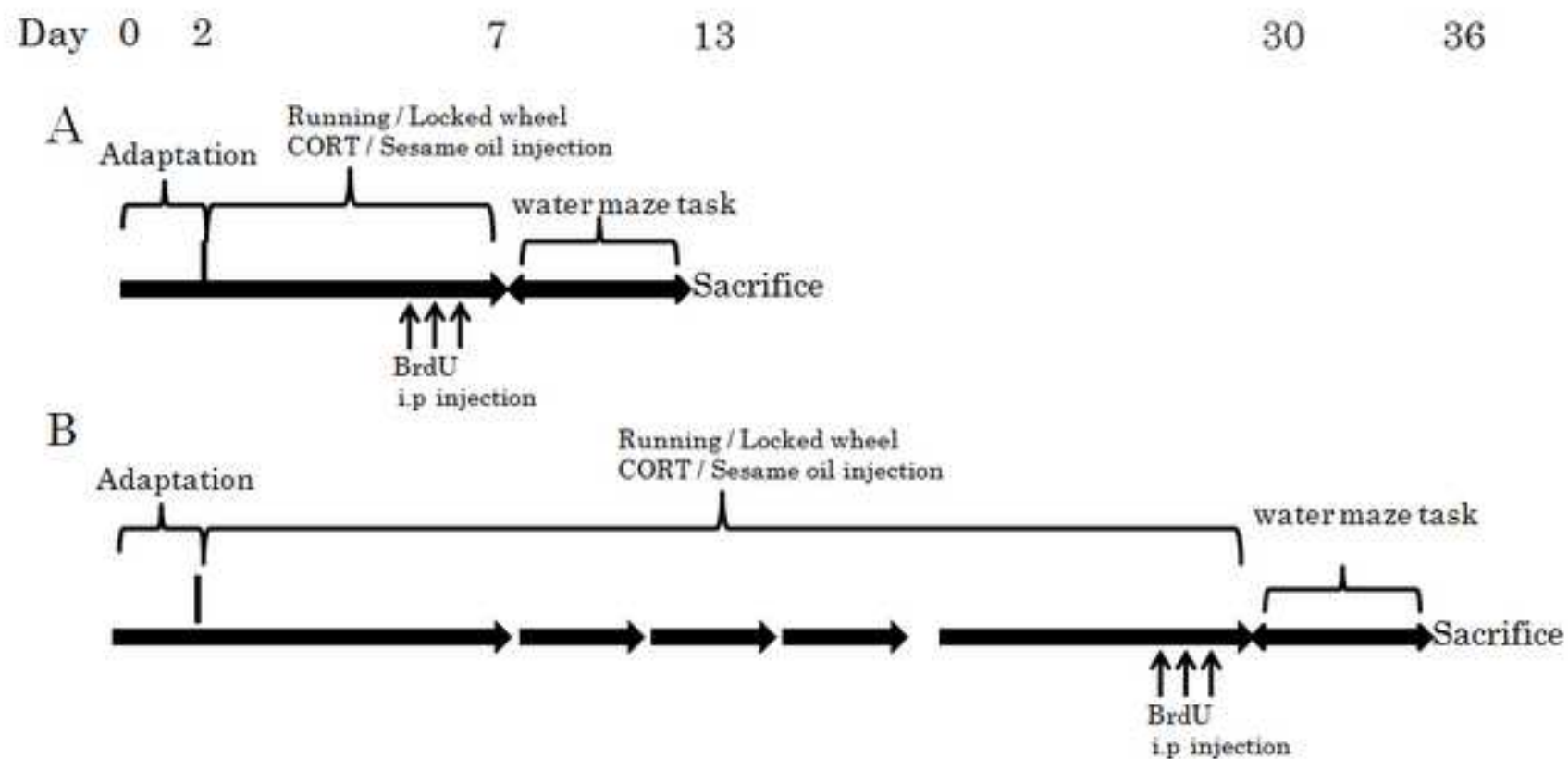
Fig. 5. Acute and chronic treatment on neuronal differentiation. (a-c) Representative images of co-labeled cells. (d) Acute treatment with CORT or running did not affect the ratio of DCX to BrdU positive cells. (e) Chronic running in the vehicle-treated rats significantly increased the ratio of DCX to BrdU positive cells, whereas chronic treatment with CORT significantly decreased the ratio. ### $P < 0.005$ compared to the CtrlN group. # $P < 0.005$; * $P < 0.05$ compared to the CtrlN and CtrlR groups, respectively. Scale bar = 50 μ m. DCX: doublecortin; BrdU: 5-bromo-3-deoxyuridine for labeling proliferating cells; DCX: immature neuronal marker, doublecortin. CtrlN

or Ctrl R: the vehicle-treated non-runners or runners; 40mgN or 40mgR: the 40 mg/kg CORT-treated non-runners or runners.

Fig. 6. Effects of acute and chronic treatments on spatial learning and memory **(a)** Acute treatment with CORT significantly decreased escape latency when compared with the vehicle treatment. Main effect of CORT: * $P < 0.05$. **(b)** The time spent in the target area was not affected by running or CORT treatment. **(c)** CORT treatment in the non-runners significantly increased the escape latency compared with the CtrlN, CtrlR and 40mgR groups. * $P < 0.05$ compared to the CtrlN and CtrlR groups on Day 1 and Day 2; # $P < 0.05$ compared to the 40mgR group on Day 2. ** $P < 0.05$ compared to the CtrlR group on Day 4. **(d)** Running showed no effects on time spent in the target area in rats treated with either vehicle or 40 mg/kg CORT. * $P < 0.05$. CtrlN or Ctrl R: the vehicle-treated non-runners or runners; 40mgN or 40mgR: the 40 mg/kg CORT-treated non-runners or runners. Scale bar: 100 μm .

Fig. 7. Plasma levels of BDNF and IGF-1 after acute or chronic treatment. **(a)** Acute treatment with CORT significantly increased the plasma level of BDNF. Main effect of CORT: * $P < 0.05$; # $P < 0.05$ compared to the CORT-treated non-runners. There was no significant change in BDNF levels following chronic treatment. **(b)** Acute treatment with CORT and/or running did not alter plasma level of IGF. CtrlN or Ctrl R: the vehicle-treated non-runners or runners; 40mgN or 40mgR: the 40 mg/kg CORT-treated non-runners or runners. **(c)** Acute CORT treatment significantly increased hippocampal BDNF levels. **(d)** Chronic CORT treatment significantly decreased hippocampal BDNF levels

Figure 1



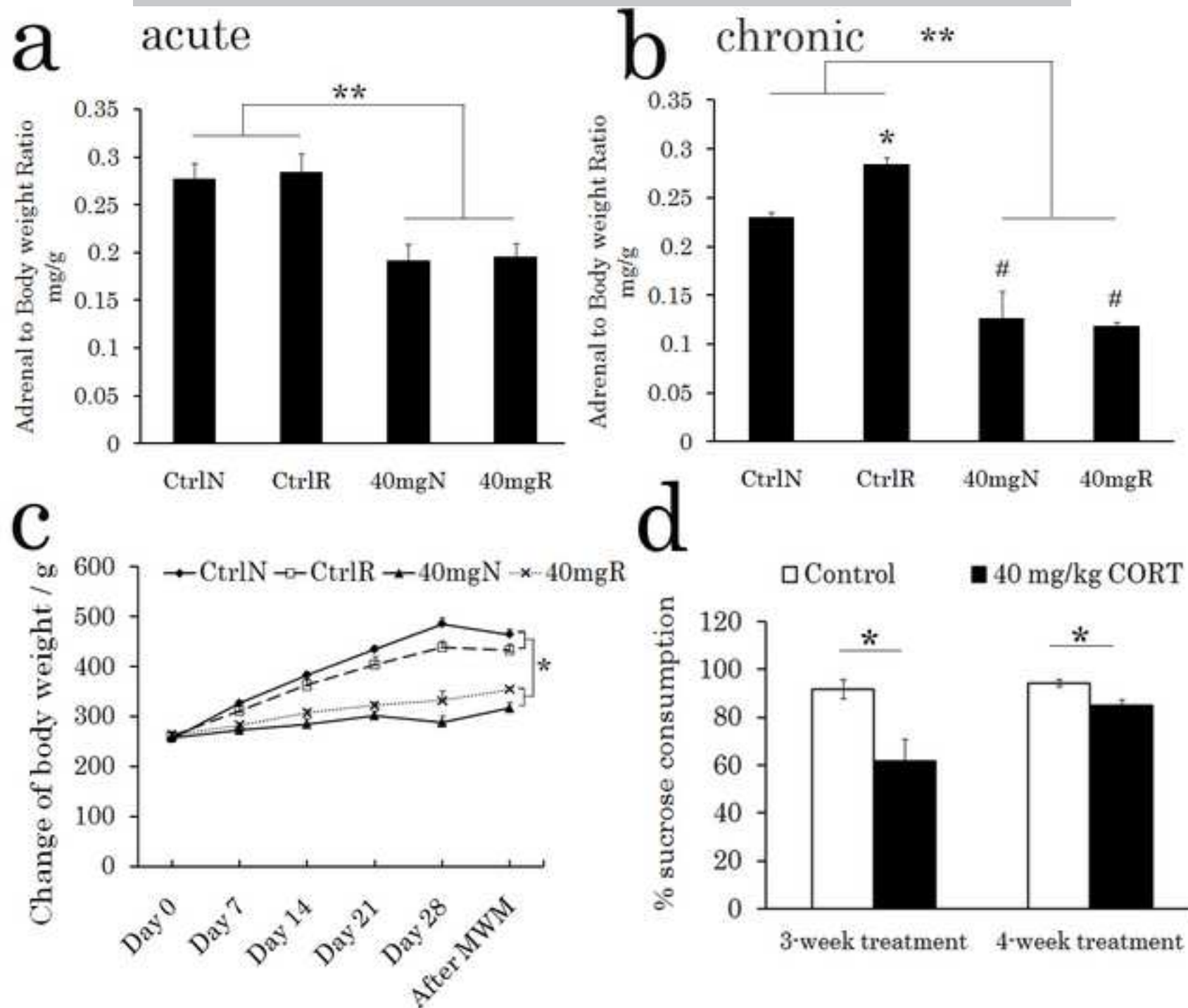
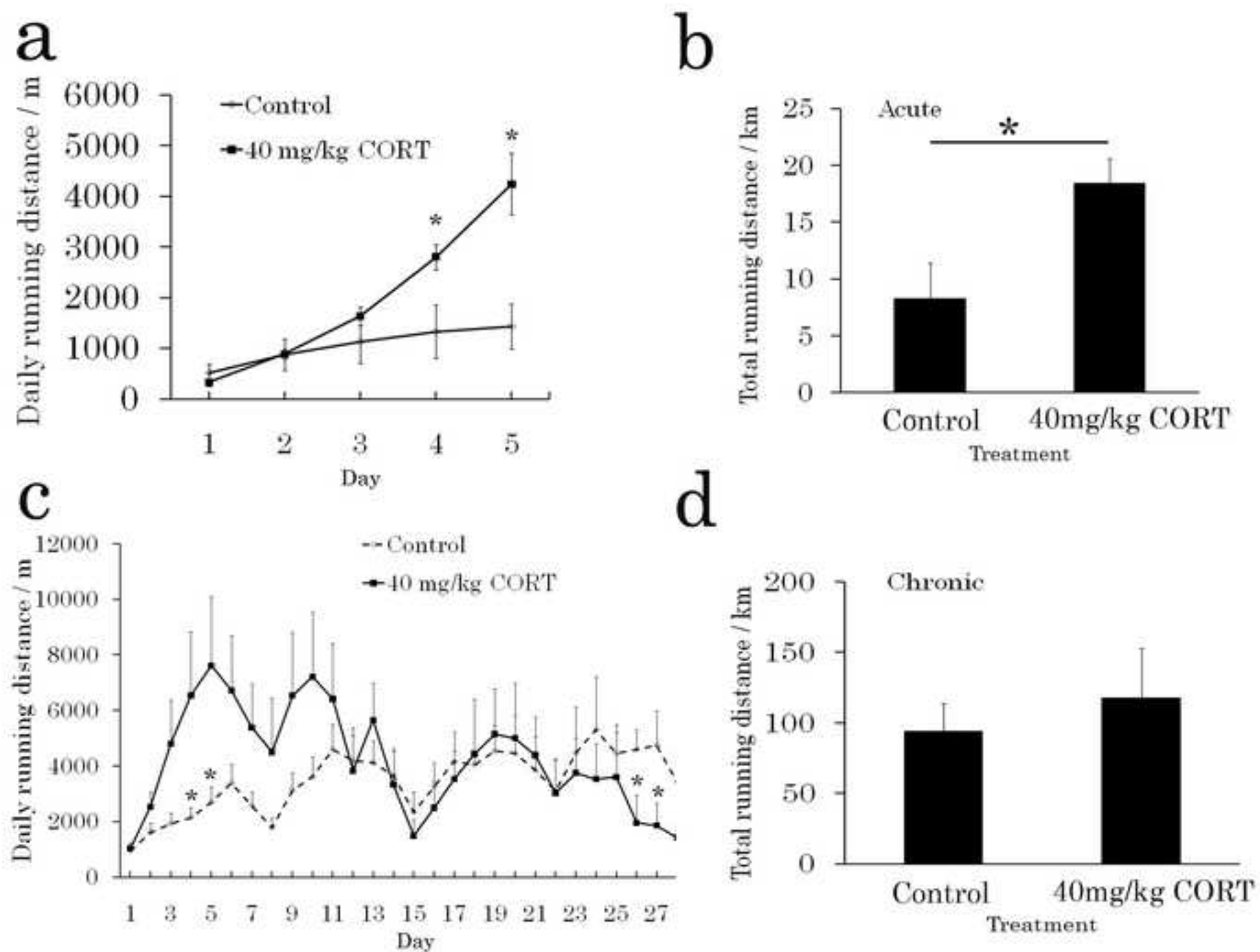
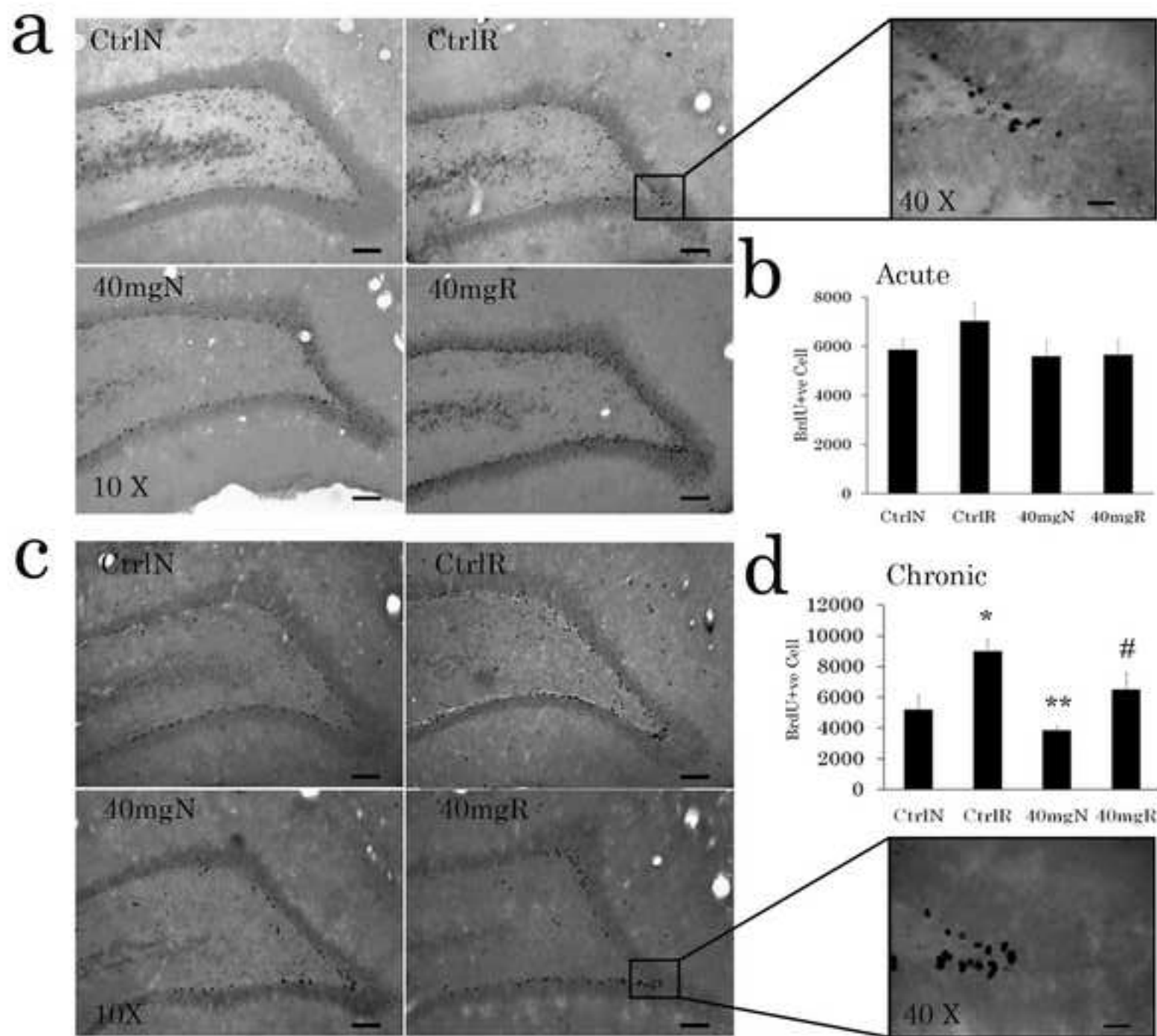
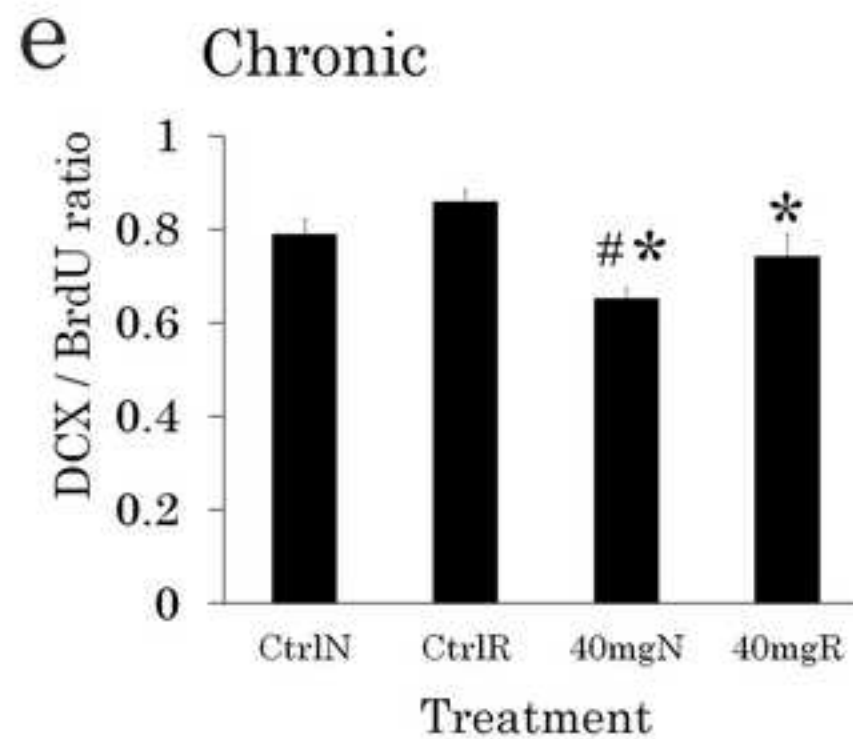
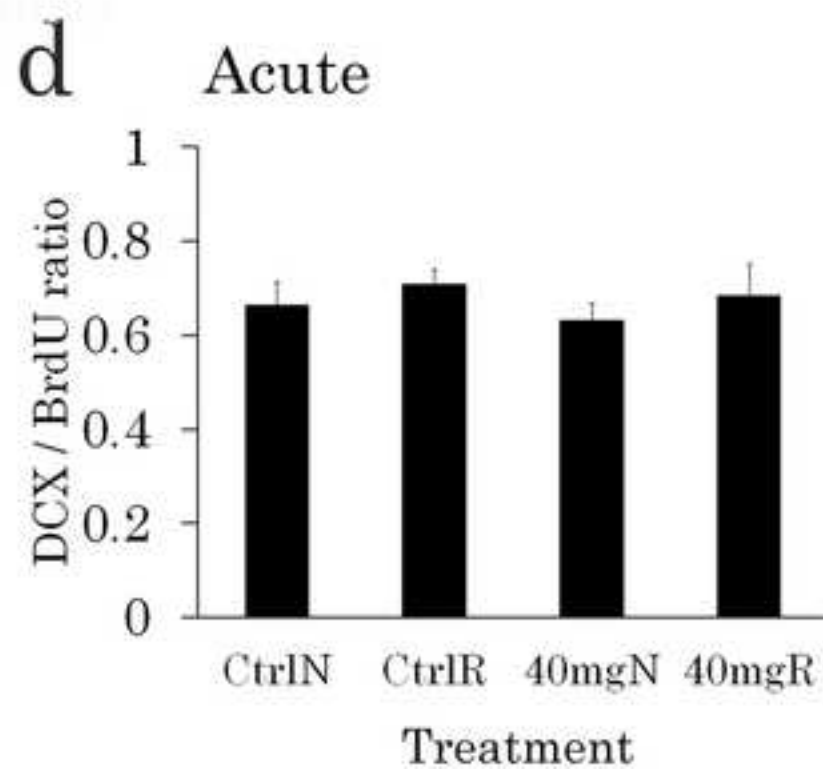
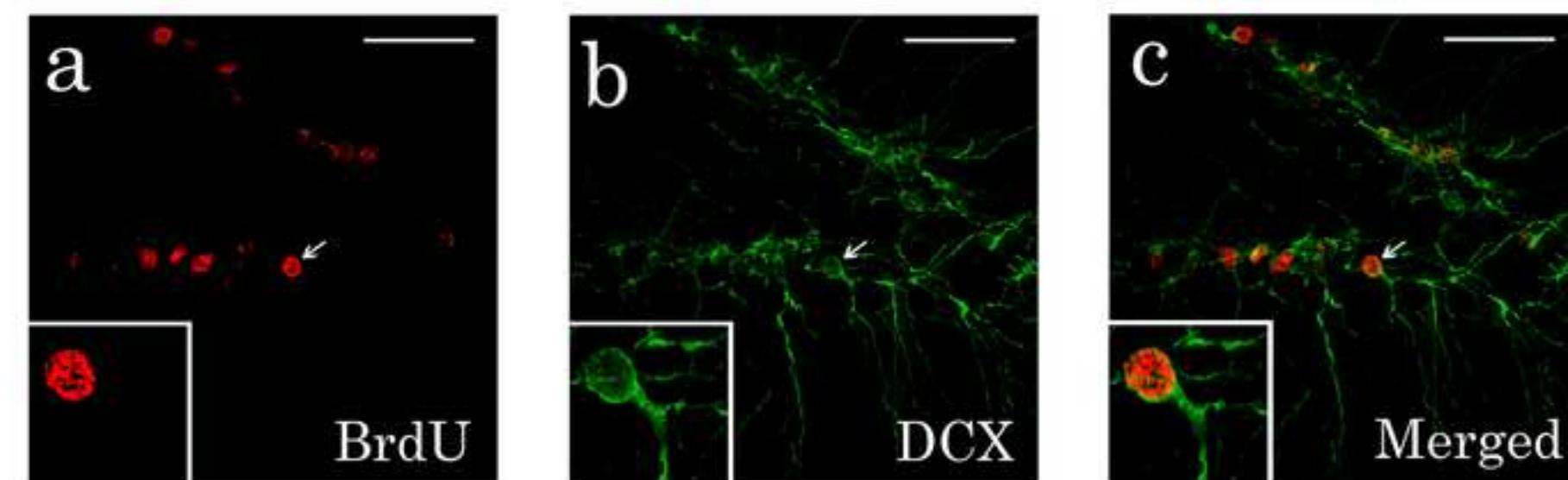
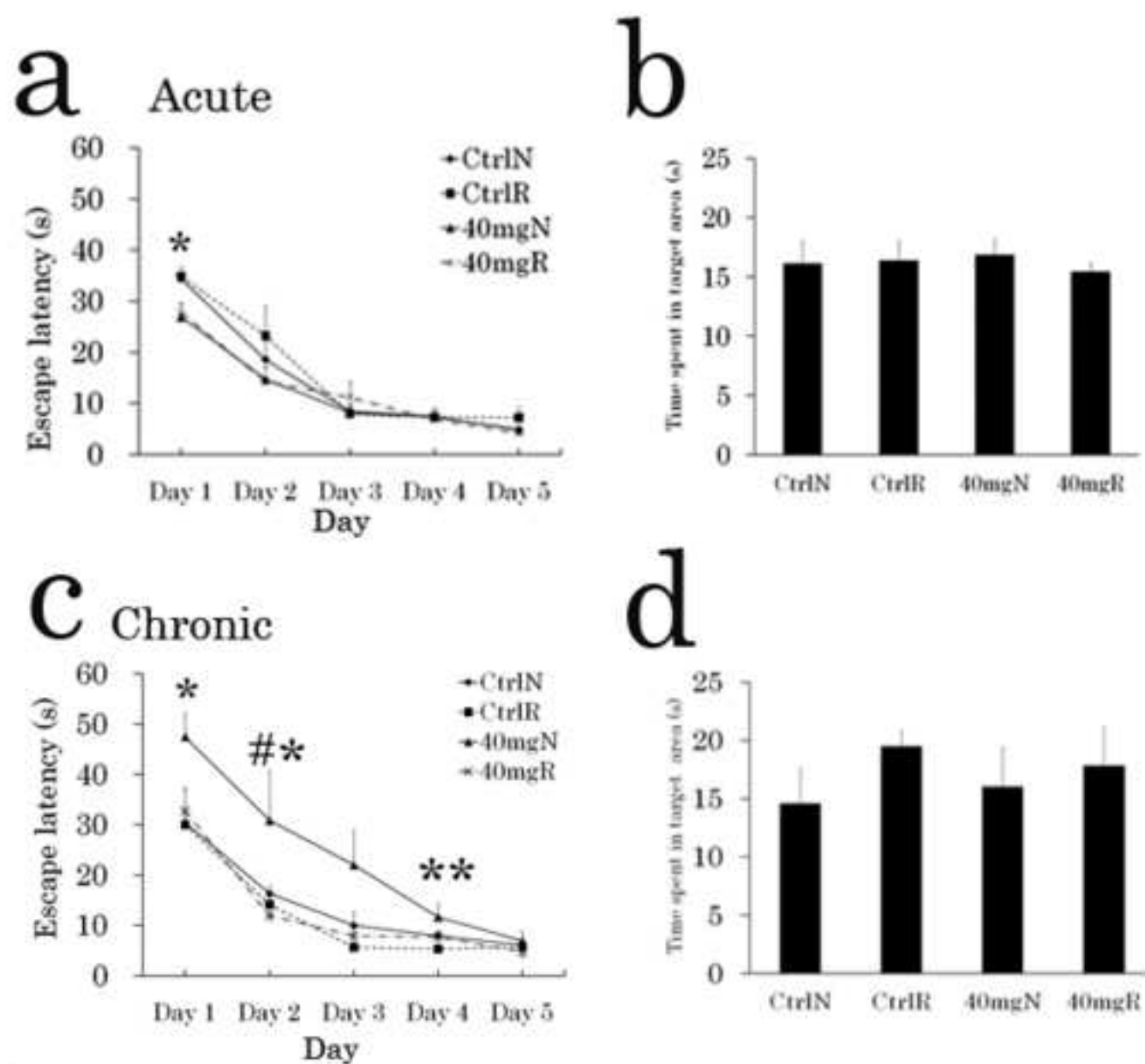


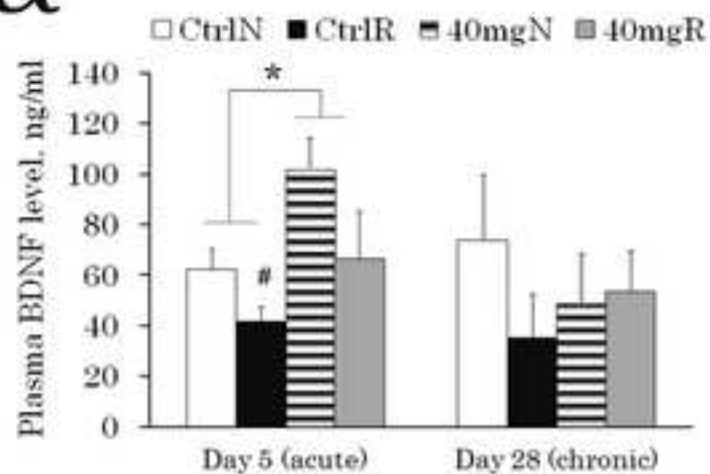
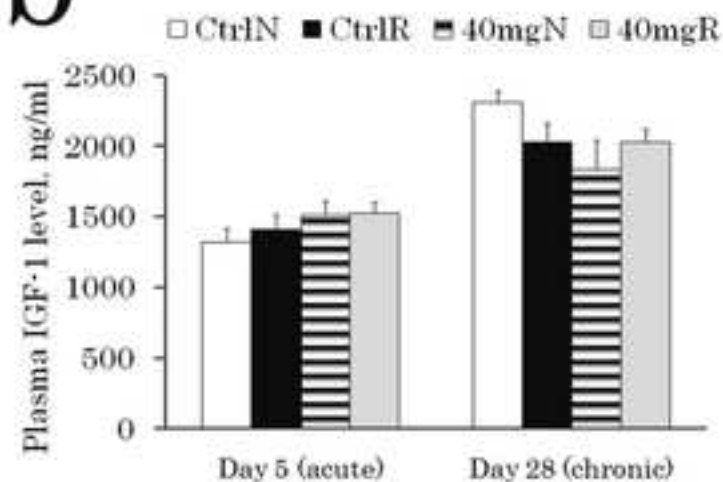
Figure 3



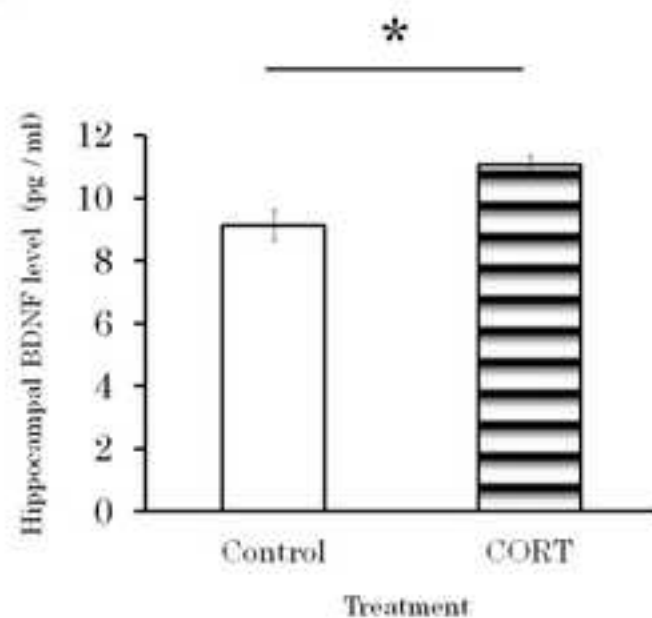






a**b****c**

Acute

**d**

Chronic

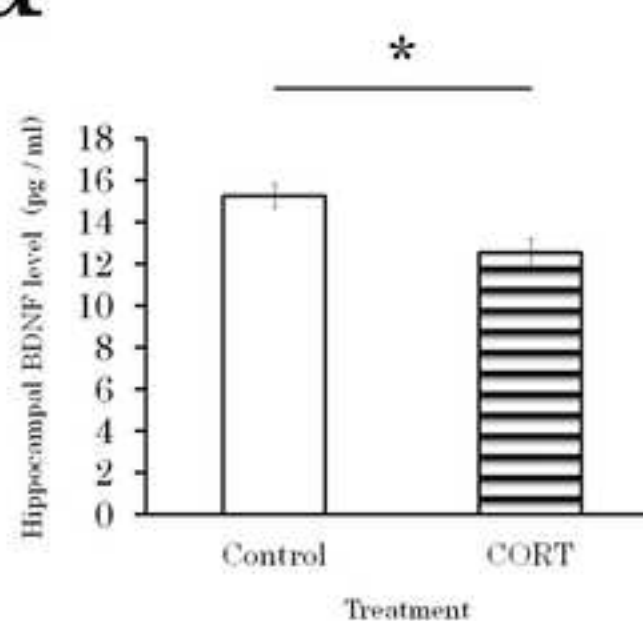


Table 1: Neuronal differentiation of proliferating cells in the dentate gyrus

	Acute		Chronic	
	Number of BrdU+ and DCX+ cells	Co-labeled ratio	Number of BrdU+ and DCX+ cells	Co-labeled ratio
CtrlN	20 ± 1.56	0.66 ± 0.050	25 ± 0.51	0.79±0.031
CtrlR	20 ± 1.69	0.71 ± 0.030	26 ± 0.63 ##	0.88±0.021 ##
40mgN	19 ± 3.41	0.62 ± 0.041	21 ± 1.55 #	0.65±0.022 #
40mgR	19 ± 1.89	0.64 ± 0.052	22 ± 1.54 *	0.70±0.021*

P<0.005 compared to the CtrlN group. # P<0.005; * P<0.05 compared to the CtrlN and CtrlR groups, respectively.

CtrlN or Ctrl R: the vehicle-treated non-runners or runners; 40mgN or 40mgR: the 40 mg/kg CORT-treated non-runners or runners.

Research Highlights

- We tested changes in blood BDNF, IGF-1, cognition and neurogenesis in stressed rats with running
- Acute stress enhanced cognition, blood and brain BDNF levels
- Chronic stress impaired cognition, neurogenesis and lowered brain BDNF level
- Running restored cognition and neurogenesis, did not alter blood neurotrophins
- Running-improved cognition could not be reflected by blood BDNF and IGF-1 levels