Neuroscience 308 (2015) 180–193

SYNERGISTIC EFFECTS OF DIET AND EXERCISE ON HIPPOCAMPAL FUNCTION IN CHRONICALLY STRESSED MICE

C. P. HUTTON,[a†] N. DÉRY,[a†] E. ROSA,[b] J. A. LEMON,[c] C. D. ROLLO,[d] D. R. BOREHAM,[c] M. FAHNESTOCK,[b] D. DECATANZARO,[a] J. M. WOJTOWICZ[e] AND S. BECKER[a*]

[a] *Department of Psychology, Neuroscience and Behaviour, McMaster University, Hamilton, ON L8S 4K1, Canada*

[b] *Department of Psychiatry and Behavioural Neurosciences, McMaster University, Hamilton, ON L8S 4K1, Canada*

[c] *Department of Medical Physics and Applied Radiation Sciences, McMaster University, Hamilton, ON L8S 4K1, Canada*

[d] *Department of Biology, McMaster University, Hamilton, ON L8S 4K1, Canada*

[e] *Department of Physiology, University of Toronto, Toronto, ON M5S 1A8, Canada*

Abstract—Severe chronic stress can have a profoundly negative impact on the brain, affecting plasticity, neurogenesis, memory and mood. On the other hand, there are factors that upregulate neurogenesis, which include dietary antioxidants and physical activity. These factors are associated with biochemical processes that are also altered in age-related cognitive decline and dementia, such as neurotrophin expression, oxidative stress and inflammation. We exposed mice to an unpredictable series of stressors or left them undisturbed (controls). Subsets of stressed and control mice were concurrently given (1) no additional treatment, (2) a complex dietary supplement (CDS) designed to ameliorate inflammation, oxidative stress, mitochondrial dysfunction, insulin resistance and membrane integrity, (3) a running wheel in each of their home cages that permitted them to exercise, or (4) both the CDS and the running wheel for exercise. Four weeks of unpredictable stress reduced the animals' preference for saccharin, increased their adrenal weights and abolished the exercise-induced upregulation of neurogenesis that was observed in non-stressed animals. Unexpectedly, stress did not reduce hippocampal size, brain-derived neurotrophic factor (BDNF), or neurogenesis. The combination of dietary supplementation and exercise had multiple beneficial effects, as reflected in the number of doublecortin (DCX)-positive immature neurons in the dentate gyrus (DG), the sectional area of the DG and hippocampal CA1, as well as increased hippocampal BDNF messenger ribonucleic acid (mRNA) and serum vascular endothelial growth factor (VEGF) levels. In contrast, these benefits were not observed in chronically stressed animals exposed to either dietary supplementation or exercise alone. These findings could have important clinical implications for those suffering from chronic stress-related disorders such as major depression. © **2015 Published by Elsevier Ltd. on behalf of IBRO.**

Key words: stress, neurogenesis, hippocampus, exercise, dietary supplements, psychological depression.

INTRODUCTION

Chronic stress can have a profoundly negative impact on the brain and contributes to a number of psychological disorders including major depression (McEwen, 2003; Miller and Hen, 2015). Evidence from rodents and primates links severe and prolonged elevation of glucocorticoids (corticosterone, cortisol) to hippocampal damage (Sapolsky, 1985; Sapolsky et al., 1995). This damage includes synaptic atrophy (Watanabe et al., 1992; Magariños et al., 1997) and reduced neurogenesis (Watanabe et al., 1992; Gould et al., 1992, 1997, 1998). Glucocorticoids bind extensively in the healthy hippocampus to both glucocorticoid and mineralocorticoid receptors (Reul and De Kloet, 1985; Aronsson et al., 1988), and chronic stress can reduce the number of hippocampal mineralocorticoid receptors (López et al., 1998). Normally, the hypothalamic–pituitary–adrenal (HPA) axis shows habituation to repeated exposure to a stressor, such that glucocorticoid elevation following the stressor diminishes, but the reduction in hippocampal mineralocorticoid receptors can lead to impairment of this habituation (Cole et al., 2000).

These detrimental effects on the hippocampus correlate with behavioral signs of major depressive disorder. Evidence indicates that the effects of serotonergic antidepressant medications rely upon intact adult hippocampal neurogenesis (Malberg et al., 2000; Santarelli et al., 2003; Sahay et al., 2011). For example, social isolation stress induced anhedonia and depression-like behavior in monkeys; this was alleviated by fluoxetine treatment, which also upregulated neurogenesis (Perera et al., 2011). However fluoxetine's behavioral antidepressant effect was abolished by focal hippocampal X-irradiation (Perera et al., 2011), which is highly toxic to immature neurons (Snyder et al., 2001; Winocur et al., 2006). Similarly, chronic unpredictable mild stress induced

*Corresponding author. Tel: +1-905-525-9140x23020; fax: +1-905-529-6225.
E-mail address: becker@mcmaster.ca (S. Becker).
† These authors contributed equally to this work.
Abbreviations: BDNF, brain-derived neurotrophic factor; CDS, complex dietary supplement; CUS, chronic unpredictable stress; DCX, doublecortin; DG, dentate gyrus; HPA, hypothalamic–pituitary–adrenal; IGF-1, insulin-like growth factor-1; mRNA, messenger ribonucleic acid; VEGF, vascular endothelial growth factor.

http://dx.doi.org/10.1016/j.neuroscience.2015.09.005
0306-4522/

anhedonic behavior in mice, accompanied by a 30% reduction in neurogenesis and habituation of the hippocampal inhibitory influence on the HPA axis (Surget et al., 2011). This habituation was evident in a reduction in the number of newly born neurons in the dentate gyrus (DG) activated by dexamethasone, a synthetic glucocorticoid. Moreover, disruption of neurogenesis by irradiation impaired the ability of fluoxetine to restore hippocampal modulation of HPA activity during chronic stress, suggesting that this modulation may depend upon neurogenesis. Although it is challenging to study human neurogenesis, a number of studies have associated the number and duration of depressive episodes with loss of hippocampal volume and memory function (see e.g. Sheline et al., 1999; MacQueen et al., 2003).

Two factors that can enhance neurogenesis and offset stress and depression are dietary antioxidants (Lau et al., 2005; Valente et al., 2009) and long-term aerobic exercise (van Praag et al., 1999, 2005; Creer et al., 2010; Déry et al., 2013; Winocur et al., 2014). Voluntary aerobic exercise enhanced neurogenesis in rodents for up to 9 months (Merkley et al., 2014). In humans, exercise was found to increase serum brain-derived neurotrophic factor (BDNF; Erickson et al., 2011), DG blood volume (indicative of angiogenesis; Pereira et al., 2007) and memory scores on a behavioral test of pattern separation (Déry et al., 2013). Diet and exercise affect biochemical processes and signaling pathways that are also altered in age-related cognitive decline. These include neurotrophin expression (Fahnestock et al., 2012), cellular oxidative stress (Valente et al., 2009), inflammation (Goshen et al., 2008) and mTOR regulation (Ota et al., 2014). In rodents, a complex dietary supplement (CDS) greatly ameliorated age-related physiological and cognitive decline in transgenic growth hormone mice (a model of accelerated aging) and aged wild-type controls (Lemon et al., 2003). When aged mice received the same supplement from weaning onward, they performed as well as young mice on the hidden platform version of the Morris water maze (Aksenov et al., 2013) – a test on which younger mice typically outperform older ones. Aged mice who received the CDS also had larger brains than age-matched non-supplemented controls. The same CDS also protected mice from radiation-induced DNA damage and immunological apoptosis (Lemon et al., 2008a,b).

The CDS (Table 1) was designed to target five major mechanisms associated with aging: inflammation, oxidative stress, mitochondrial dysfunction, insulin resistance and membrane integrity. Although this approach may not identify contributions of any one ingredient, mounting evidence supports the potent neuroprotective effects of CDSs exhibiting some overlap in ingredients or physiological targets (Milgram et al., 2002; Parachikova et al., 2010). Broad-spectrum, antioxidant-rich micronutrient supplementation also shows promise in treatment of mood disorders, while single nutrient supplements generally produce weak results (Rucklidge and Kaplan, 2013; Popper, 2014). For example, pre-partum micronutrient supplementation lessens the risk and severity of postpartum depression (Leung et al., 2013).

Table 1. Ingredients included in the complex dietary supplement

Ingredient	Daily dose for a 35 g mouse
Acetyl-L-Carnitine	14.4 mg
Acetylsalicylic Acid	2.5 mg
Alpha-Lipoic Acid	0.72 mg
β-Carotene	50 IU
Bioflavonoids	4.32 mg
Chromium picolinate	1.44 μg
Cod Liver Oil	5.04 IU
Coenzyme Q10	0.44 mg
DHEA	0.15 mg
Flax Seed oil	21.6 mg
Folic Acid	0.01 mg
Garlic	26.6 μg
Ginger	7.2 mg
Gingko Biloba	1.44 mg
Ginseng	8.64 mg
Green Tea Extract	7.2 mg
L-Glutathione	0.36 mg
Magnesium	0.72 mg
Melatonin	0.01 mg
N-Acetyl Cysteine	7.2 mg
Potassium	0.36 mg
Rutin	0.72 mg
Selenium	1.08 μg
Vitamin B1	0.72 mg
Vitamin B3	0.72 mg
Vitamin B6	0.72 mg
Vitamin B12	0.72 μg
Vitamin C	3.6 mg
Vitamin D	2.5 IU
Vitamin E	1.44 IU
Zinc	0.14 mg

Although both exercise and nutraceuticals can enhance hippocampal volume (Erickson et al., 2011) and neurogenesis (Lau et al., 2005), the two together may produce greater effects. The combination of an antioxidant-fortified diet and environmental enrichment reduced age-related cognitive impairment and increased BDNF levels in dogs more than did either treatment alone (Fahnestock et al., 2012). Beneficial interactions between combinations of dietary supplementation and environmental enrichment or exercise have also been observed in investigations of Alzheimer's disease (Pop et al., 2010) and synaptic plasticity (Wu et al., 2008). Although environmental enrichment and exercise affect neurogenesis via distinct pathways (neuronal survival and proliferation respectively; Olson et al., 2006), they are difficult to dissociate experimentally because environmental enrichment protocols typically have an exercise component. Conversely, exercise protocols enrich the animal's environment by affording it access to novel complex objects with which it can interact meaningfully.

We exposed mice to a complex series of unpredictable stressors while concurrently giving some of them the CDS, a running wheel in the home cage, or both. We predicted that dietary supplementation and aerobic exercise would synergistically mitigate the impact of chronic stress on the hippocampus. Specifically, we hypothesized that the combination of CDS and exercise would prevent depression-like

behavior and normalize hippocampal volume, neurogenesis and BDNF levels.

EXPERIMENTAL PROCEDURES

Subjects

The subjects were 154 male C57BL/6 mice (Charles River, St. Constant, Quebec, Canada), initially aged 6 weeks and weighing 20–25 g. Upon arrival, animals were divided into one of eight treatment groups, but no experimental procedures were conducted until animals had acclimated to the facility for approximately two weeks. However, the CDS or plain bagel chip was provided to the animals during this time. Animals were individually housed with a reversed 12:12-h light/dark cycle (lights off during the day, lights on at night) and constant temperature (~22 °C ±0.5 °C) and humidity. Mice were provided Harlan™ Teklad 22/5 Rodent Diet chow and water *ad libitum* except as required for some of the stress manipulations described below. Animals were also given the CDS and saccharin-flavored water (0.1%) depending on which experimental group they had been assigned to. Following the two-week acclimation period, animals were divided into and left in their experimental cages for 4 weeks. We employed a 3-factor $2 \times 2 \times 2$ design: exercise (wheel running or not), diet (CDS or not) and stress (chronic unpredictable stressors or not). Because mice exercise by running on the cage walls and ceiling in wire cages we prevented this via Plexiglas barriers. All mice were caged with woodchip bedding and provided with standard ABS plastic tubes in which to hide/sleep.

As described below, half of the animals were designated for immunohistochemical assays ($n = 77$), while the other half were designated for hippocampal BDNF assays ($n = 77$). All mice were delivered isoflurane via nosecone and it was confirmed they were in a plane of anesthesia via toe pinch. Blood was then collected via cardiac puncture of the right atrium for later analysis of serum BDNF, insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF). Thus, all animals, whether destined for immunohistochemical or neurotrophic assays, were euthanized via exsanguination. The mice designated for immunohistochemistry were administered intracardiac perfusion using paraformaldehyde (PFA) and brains were extracted. For those mice that were not perfused, cerebral cortex and hippocampi were immediately dissected and flash frozen using liquid nitrogen and then adrenal glands were dissected and subsequently weighed. All procedures were conducted in accordance with Canadian Council for Animal Care and the McMaster University Animal Research Ethics Board guidelines.

CDS

The dosages, preparation and administration of the CDS were previously described (Lemon et al., 2003). Briefly, ingredients were included based on human tolerability and doses were based on human recommendations with adjustments for differences in body size and metabolic rate. The CDS was prepared in aqueous solution, absorbed onto a small piece of bagel and left to dry. These bagel chips were given daily to mice in the supplemented group halfway through the photoperiod (at the beginning of their awake cycle) and were usually consumed within minutes. All animals who were not assigned to a supplemented group were provided with similarly sized bagel pieces that did not contain the CDS. The dietary supplement was provided to animals during the two-week acclimation period. Thus, there was a brief pre-load phase with the CDS prior to the onset of stress. See Table 1 for CDS ingredients.

Chronic unpredictable stress (CUS)

This procedure involved a novel combination of stressors, each validated in other studies (Harkin et al., 2002; Elizalde et al., 2008; Strekalova and Steinbusch, 2010). Stressors were selected based on their likelihood of increasing circulating stress hormones without compromising the animals' health in other ways. During the four-week protocol, animals were pseudo-randomly exposed to 3 of 13 of the following stressors on each day (see Table 2):

Elevated pillar. This consisted of a small, cloth-covered platform approximately 10.1 cm wide by 20.3 cm long and approximately 1.2 m off the ground (Thorpe et al., 2014). When on this platform a mouse had to be vigilant to avoid falling off. The elevated pillar stress lasted 1 h and was administered during the animal's normal awake period (i.e., dark phase).

Lights off/cage tilt overnight. During the animal's sleep cycle, its cage was placed on a 45° tilt for a period of 12 h with lights turned off. This can disturb the circadian rhythm of the mouse.

Cage tilt overnight. During the animal's sleep period, its cage was placed on a 45° tilt for a period of 12 h.

Lights on during the day. Mice are most active in the dark so turning the lights on for 6 h during the waking period can disrupt the circadian rhythm, sleep cycle and associated hormones and neurotransmitters (Harkin et al., 2002; Elizalde et al., 2008).

Strobe light during the day. Similar to the "lights on during the day" stressor, a bright, blinking strobe during the animal's waking (dark) period can impact sleep (Harkin et al., 2002; Elizalde et al., 2008; Schmidt and Duman, 2010) and also represents a direct stress. The strobe light was always delivered concurrently with one of predator odor or ultrasonic noise.

Predator odor. Mice were exposed to cat hair during their normal awake cycle (lights off) (McEuen et al., 2008; Schmidt and Duman, 2010; d'Audiffret et al., 2010), in combination with a strobe light turned on (during the animals' awake period).

Ultrasonic noise. Sound was emitted in the 32–62-kHz frequency range from small electrical outlet-mounted speakers. This frequency range is outside the audible

Table 2. The chronic unpredictable stress (CUS) paradigm used to simulate the chronic stressors and sleep disturbances thought to underlie major depression in humans

Day #	Day	Morning	Afternoon	Overnight
1	Monday	water deprivation (3 h)	lights on (6 h)	lights off/cage tilt (12 h)
2	Tuesday	restraint (30 min)	predator odor/strobe (6 h)	ultrasonic noise (12 h)
3	Wednesday	handling (10 min)	predator exposure (3 h)	cage tilt (12 h)
4	Thursday	elevated pillar (1 h)	ultrasonic noise (6 h)	wire cage (12 h)
5	Friday	ultrasonic noise/strobe (6 h)	restraint (30 min)	wire cage (12 h)
6	Saturday	predator exposure (6 h)	lights on (6 h)	food dep./cage tilt (12 h)
7	Sunday	water deprivation (3 h)	elevated pillar (1 h)	cage tilt (12 h)
8	Monday	water deprivation (3 h)	lights on (6 h)	lights off/cage tilt (12 h)
9	Tuesday	restraint (30 min)	predator odor/strobe (6 h)	ultrasonic noise (12 h)
10	Wednesday	handling (10 min)	predator exposure (3 h)	cage tilt (12 h)
11	Thursday	elevated pillar (1 h)	ultrasonic noise (6 h)	wire cage (12 h)
12	Friday	ultrasonic noise/strobe (6 h)	restraint (30 min)	wire cage (12 h)
13	Saturday	predator exposure (6 h)	lights on (6 h)	food dep./cage tilt (12 h)
14	Sunday	water deprivation (3 h)	elevated pillar (1 h)	cage tilt (12 h)
15	Monday	water deprivation (3 h)	lights on (6 h)	lights off/cage tilt (12 h)
16	Tuesday	restraint (30 min)	predator odor/strobe (6 h)	ultrasonic noise (12 h)
17	Wednesday	handling (10 min)	predator exposure (3 h)	cage tilt (12 h)
18	Thursday	elevated pillar (1 h)	ultrasonic noise (6 h)	wire cage (12 h)
19	Friday	ultrasonic noise/strobe (6 h)	restraint (30 min)	wire cage (12 h)
20	Saturday	predator exposure (6 h)	lights on (6 h)	food dep./cage tilt (12 h)
21	Sunday	water deprivation (3 h)	elevated pillar (1 h)	cage tilt (12 h)
22	Monday	water deprivation (3 h)	lights on (6 h)	lights off/cage tilt (12 h)
23	Tuesday	restraint (30 min)	predator odor/strobe (6 h)	ultrasonic noise (12 h)
24	Wednesday	handling (10 min)	predator exposure (3 h)	cage tilt (12 h)
25	Thursday	elevated pillar (1 h)	ultrasonic noise (6 h)	wire cage (12 h)
26	Friday	ultrasonic noise/strobe (6 h)	restraint (30 min)	wire cage (12 h)
27	Saturday	predator exposure (6 h)	lights on (6 h)	food dep./cage tilt (12 h)
28	Sunday	water deprivation (3 h)	elevated pillar (1 h)	cage tilt (12 h)

bandwidth for human hearing but is detectable to rodents. High-frequency sound waves were emitted at a rate of 80 oscillations per second, which is claimed to be irritating to rodents who will actively try to escape the sound. Since our mice were kept in cages that would render the sound inescapable, these high-frequency sound waves should activate the animal's stress response while not causing any other sort of physiological damage. The ultrasonic emitters are commercially available and were purchased from Victor®, a pest control company. The Victor® ultrasonic rodent repeller was only turned on periodically and for no longer than 12 h, alone or in combination with a strobe light turned on (during the animal's awake cycle).

Water deprivation. Mice were deprived of water for periods of no longer than 3 h. The anticipation of a prolonged water deprivation event is stressful and also impacts hydration and feeding (Mantella et al., 2005; Elizalde et al., 2008). Water deprivation did not occur on the same day as, and did not immediately precede, saccharin preference testing.

Food deprivation/cage tilt. Mice were deprived for 12 h during their normal sleep period, in combination with cage tilt, which may have made it slightly more difficult for them to regulate body weight (Harkin et al., 2002; Mantella et al., 2005; Schmidt and Duman, 2010).

Handling. During the mouse's awake period (lights off), the experimenter placed the mouse on a table and held it by the base of the tail for 10 min. The mouse was then returned to its home cage. Restraint is a commonly applied stress for rodents (e.g. Magariños et al., 1997).

Restraint. Restraint is a stressor that is commonly implemented in the rodent literature (e.g. Snyder et al., 2011). The restraint system that we used was DecapiCone (Braintree Scientific, Braintree, MA, USA). These restraint cones are made from a tapered plastic film. One must simply hold the plastic cone with one hand and place the mouse into the cone using the other hand. Once the mouse has been placed inside of the DecapiCone, with their head through one end to allow for breathing and the other end tied off to create a snug fit, they are rendered immobile. Otherwise, the mice are completely unharmed. Each animal was not restrained for more than 30 min per session.

Predator exposure. Mice in our stressed group were wheeled into an adjacent room that houses rats. Each mouse was then placed into one side of a specially constructed cage that has been partitioned into two halves, while a rat was placed into the other half. The partition is a metal wire screen with small openings, which prevented the animals from being able to harm one another. However, this type of screening would still allow for enough contact between the two animals to effectively initiate the mouse's stress response. Mice and rats were left in these cages for either 3 or 6 h periods, with 2 or 3 days between each exposure. Following each exposure, mice were returned back to their original cages [and their holding room].

Deprived housing. For durations of 12 h, stressed mice were placed into specially constructed cages with wire mesh floors and no bedding material. However, they still had free access to food and water. The lack of bedding material or enrichment devices made for a type of impoverished housing that was mildly stressful for the animals, but did not otherwise cause any type of bodily harm. Following each deprivation period, mice were placed back into their original cages with adequate bedding material.

Aerobic exercise

Animals in the exercise condition were provided with a running wheel in their home cage. Daily observations and an ergometer attached to a subset of running wheels (Mouse Igloo Fast-Trac, Bioserv) verified that, in most cases, mice repeatedly used the running wheels. For sedentary groups Plexiglas liners minimized physical activity.

Behavioral measures

Saccharin preference during the waking period was used as a non-invasive behavioral measure of anxiety and stress (Willner et al., 1992). Reduced preference for saccharin is a sign of anhedonia, a core symptom of major depression in humans. Saccharin preference was measured by the proportion of saccharin-flavored water consumed relative to unflavored water. Saccharin is an artificial sweetener that was used in place of sucrose to avoid any physiological changes associated with the increased intake of sucrose. Mice were always provided with two water bottles fixed to their cages. On two days of the week one of these contained saccharin-flavored water, while the other contained regular, unflavored water. Every other day both water bottles contained unflavored water, to maintain the hedonic value of saccharin over time. The position of the saccharin and unflavored water bottles was swapped upon each administration, so that the animal could not learn the position of, and become biased toward drinking from the bottle containing saccharin-flavored water. Each test began 1 h before the animals' awake period (7:00 h) and ended 1 h after initiation of the sleep period (21:00 h). Body weight was measured at the start of the experiment, once a week during CUS and the day before sacrifice.

Immunohistochemistry

Animals were anaesthetized with isoflurane and euthanized via transcardial perfusion with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA). Following perfusion, whole brains were extracted and post-fixed in PFA for 24 h, after which they were transferred to a 0.1% sodium azide (in PBS) solution and stored at 4 °C until sectioning. The right hemisphere of each brain was dissected and sectioned from anterior to posterior in the coronal plane in a PBS-filled well on a Leica VT1000S Vibratome (Heidelberg, Germany) into 40-μm slices that were transferred to a 48-well plate (four sections per well) filled with 0.1% sodium azide solution at 4 °C. Nine sections containing the DG were sampled from each animal using immunohistochemistry methods described previously (Wojtowicz and Kee, 2006).

Doublecortin (DCX) was assayed as a marker of neurogenesis. DCX is a microtubule binding protein expressed transiently in proliferating neural precursor cells and migrating neuroblasts, but not in mature neurons. This is a recognized biomarker of immature, adult-born neurons (Brown et al., 2003; McDonald and Wojtowicz, 2005). All sections were first incubated with anti-DCX antibody ab18723 (Abcam, Toronto, ON, Canada) and then Alexa Fluor 568 Donkey Anti-Rabbit IgG antibody (Life Technology). Antibodies were suspended in a phosphate-buffered saline solution containing 0.3% Triton X-100 detergent, which enabled them to penetrate cell membranes. Sections were washed (on a rotomixer) in PBS 3 times (5 min each) before and after each antibody incubation period. After the final wash, sections were mounted on labeled glass slides using a paintbrush and distilled water, permitted to air dry in a shaded area (to limit light exposure), coated with PermaFluor™ aqueous mounting medium (Thermo Scientific, Mississauga, ON, Canada), covered with a glass coverslip, and then stored in a slide folder at 4 °C before being transferred to a slide box for storage.

Cell counts and hippocampal substructure area measurements

Immunolabeled cells in the subgranular zone (SGZ) of the DG were counted using a 40× objective lens (Nikon, OPTIPHOT-2 fluorescence microscope). Cells were counted exhaustively throughout the entire 40-μm thickness of each section by gradually adjusting the focus of the microscope but excluding the upper and lower edges of the sections. The counting procedure was verified by at least two independent investigators who were blinded with respect to the animal's group assignment. The SGZ was defined as a two-cell diameter wide (or approximately 20 μm) zone beneath the granule cell layer. Black and white images of each section, captured using a Sensicam CCD camera and SensiControl v4.02 software at 4× magnification, were then used to obtain length measurements (mm) of the SGZ on the upper and lower blades of the DG using Image J software (http://rsb.info.nih.gov/ij/). Cell counts and length measurements were then used to calculate the number of DCX + cells per mm SGZ, averaged across sections in each region (dorsal, medial and ventral) and throughout the DG. Additional 4× magnification images of the same sections were also used with Image J software to obtain surface area measurements of the DG and CA1 as indicators of hippocampal size.

Quantification of hippocampal BDNF messenger ribonucleic acid (mRNA) using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Following sacrifice of animals by exsanguination under isoflurane anesthesia, the hippocampi were dissected

and immersed in liquid nitrogen, then stored at −80 °C until use. The methods employed for the subsequent isolation of RNA, reverse transcription and quantification of BDNF using qRT-PCR followed protocols similar to those reported previously (Fahnestock et al., 2012). Briefly, hippocampal tissue samples were first weighed then sonicated for 3–5 s in cold Trizol™ solution (Life Technologies, Inc., Gaithersburg, MD, USA). RNA was isolated from the homogenate by centrifugation and extraction using a 70% ethanol solution, RNeasy™ spin column (Qiagen, Mississauga, ON, Canada), DNase treatment and elution as per the manufacturer's instructions.

RNA concentrations and integrity were determined by spectrophotometry and agarose gel electrophoresis, respectively. Only samples with A260/A280 ratios greater than 1.7 were processed further. One microgram of each RNA sample was then reverse transcribed and 50 ng of the resulting cDNA was amplified in the Stratagene MX3000p machine as previously described (Fahnestock et al., 2012) using qPCR SuperMix (Invitrogen), forward and reverse primers [BDNF: 5′ GCG-GCA-GAT-AAA-AAG-ACT-GC 3′ (forward) and 5′ CTT-ATG-AAT-CGC-CAG-CCA-AT 3′ (reverse); β-actin: 5′ AGC-CAT-GTA-CGT-AGC-CAT-CC 3′ (forward) and 5′ CTC-TCA-GCT-GTG-GTG-GTG-AA 3′ (reverse)], ROX reference dye (Invitrogen), and cDNA or reference standard for absolute quantification. A "no template" control lacking cDNA was included. Only those qRT-PCR runs with efficiencies greater than 90% and R^2 greater than 0.99 were included in subsequent statistical comparisons. BDNF copy numbers for each animal determined with MXP Pro v3.0 software were normalized to copy numbers of β-actin (Fahnestock et al., 2012).

Enzyme-linked Immmunosorbent Assays (ELISA)

Animals were anaesthetized with isoflurane, blood was collected via cardiac puncture and then immediately placed on ice in labeled 1.5 mL microfuge tubes. After remaining on ice for 1–1.5 h to permit coagulation, blood samples were centrifuged for 15 min at 3000 rpm at 4 °C. The supernatant (serum) was then transferred to 1.5 mL cryotubes and stored at −80 °C until use. Serum was analyzed using a sandwich BDNF ELISA kit (BDNF ELISA kit: Human, Rat, Mouse; Biosensis) according to the manufacturer's instructions. Each sample was tested in duplicate on separate plates, and the measurements from the two plates were averaged prior to the statistical analyses described below. VEGF and IGF-1 were analyzed using the same procedures with kits from Biosensis®.

Statistical analyses

Correlation analyses were performed on all measures, in addition to an omnibus 2 × 2 × 2 (stress, exercise and diet) factorial ANOVA. All measures were also tested using a linear contrast (Fox, 2008) for the a priori hypothesis that the combination of diet and exercise, but neither intervention alone, would reveal benefits in animals exposed to the CUS paradigm (stress = stress × diet = stress × exercise < stress × exercise × diet, hereafter referred to as the (S = S × D = S × E) < (S × D × E) contrast. An alpha level of 0.05 was used to determine significance on all statistical tests.

RESULTS

Saccharin preference and adrenal weight

To determine the extent to which animals in stress groups were affected by CUS exposure, we recorded the ratio of saccharin-flavored water to regular water consumed by the animals twice per week during acclimation as well as each of the four weeks of the experiment. The ratio of the adrenal gland to body weight at the time of euthanasia was also calculated. Saccharin preference is a measure of anhedonia, while adrenal weight is a physiological indicator of allostatic load. Separate 2 × 2 × 2 ANOVAs applied to the saccharin preference data revealed a reduced saccharin preference among stress groups beginning in the first week of stress exposure ($F_{1,61} = 44.6$, $p < 0.0001$) that continued for the duration of the experiment (see Fig. 1).

The 2 × 2 × 2 ANOVA results for the post mortem adrenal weight measurements revealed significantly enlarged adrenal glands in the stress groups (corrected to body weight, main effect of stress, $F_{1,68} = 90.74$, $p < 0.0001$; Fig. 2). There was also a significant negative correlation between saccharin preference (any of weeks 1–4) and adrenal weight/body weight ($r = -0.498$, $p = 0.002$). We verified that there were no group differences in saccharin preference prior to stress exposure (week 0 or baseline, $F_{7,61} = 1.5$, $p = 0.18$). In addition to the main effect of stress, there was also an interaction effect between exercise and adrenal mass ($F_{1,68} = 14.001$, $p = 0.0004$). Evaluation of our a priori hypothesis that the combination of diet and exercise would counteract the effects of CUS, using the (S = S × D = S × E) < (S × D × E) contrast, revealed that by the 4th week of CUS exposure the combination of diet and exercise (but neither alone) did partially restore saccharin preference ($t_{1,61} = 5.1$, $p = 0.0001$, not significant for data from weeks 0–3), but did not affect adrenal gland size. These data show that mice were strongly impacted by stress manipulations but this impact was partially reversed with a combination of the CDS and exercise (at least for hedonic status).

DG and CA1 size

To determine the effects of stress, exercise and diet on the overall structure of the hippocampus, we measured the area (mm^2) of both the DG and CA1, which were clearly visible in photographs of the sampled DCX-immunolabeled sections. These were also used for quantification of adult born neurons in the DG (e.g., Fig. 3A). Area measurements are shown in Table 3. ANOVAs for the DG area measurements revealed diet by stress ($F_{1,64} = 5.169$, $p = 0.026$) and exercise × stress ($F_{1,64} = 4.406$, $p = 0.0398$) interactions. The (S = S × D = S × E < S × E × D) contrast was also significant ($t_{1,64} = 3.218$, $p = 0.002$). These effects were also observed for the sectional CA1 area data

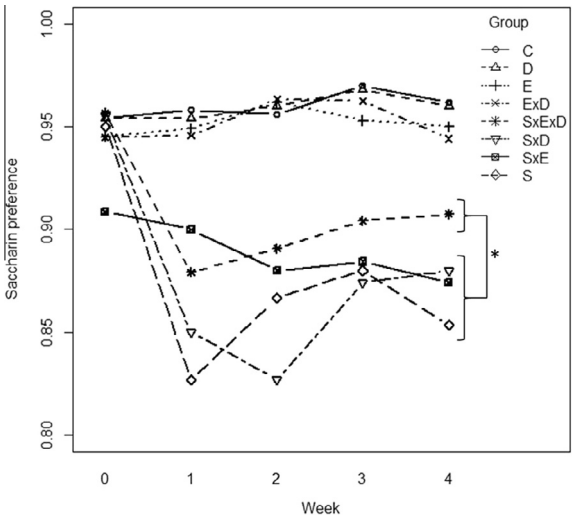

Fig. 1. Saccharin preference (proportion of saccharin to plain water consumed) over time. Chronically stressed animals showed a persistent reduction in their preference for saccharin flavored water over unsweetened water ($2 \times 2 \times 2$ ANOVA, $p < .0001$). By the fourth week of CUS exposure, only those animals which had also exercised and received dietary supplementation exhibited an amelioration of this anhedonic behavior ($S = S \times D = S \times E$) < ($S \times D \times E$) planned contrast; $^{*}p = 0.0001$). (C = Control; S = Stress; D = Diet; E = Exercise).

(diet × stress, $F_{1,64} = 6.23$, $p = 0.015$; $F_{1,64} = 6.71$, $p = 0.012$; ($S = S \times D = S \times E < S \times E \times D$) contrast: $t_{1,64} = 2.806$, $p = 0.007$). ANOVAs of the CA1 area data revealed a strong trend (significant at the 0.10 level) suggesting a negative effect of stress ($F_{1,64} = 3.8897$, $p = 0.053$). Comparison between stress groups for both measures can be seen in Fig. 3. The similarity in results between DG and CA1 measures suggests an overall hippocampal structural modification by the combination of exercise and the CDS.

Neurogenesis

Estimates of neurogenesis were obtained by measuring the number of DCX-positive immunolabeled cells per 40-μm coronal section of DG (sectional quantity) averaged across nine sampled sections (Fig. 4A, B). A $2 \times 2 \times 2$ ANOVA showed a robust main effect of exercise on neurogenesis ($F_{1,64} = 7.272$, $p = 0.009$) and a diet by stress by exercise interaction ($F_{1,64} = 4.156$, $p = 0.0456$). The ($S = S \times D = S \times E < S \times E \times D$) contrast was also significant (groups $t_{1,64} = 2.887$, $p = 0.0053$), suggesting that the main effect of exercise was driven by differences in animals that had not been exposed to chronic stress, while also showing the effectiveness of the diet × exercise combination in increasing neurogenesis in animals exposed to chronic stress. Considering that we observed similar effects in the DG and CA1 sectional area data, we also applied the same analysis to the DCX-positive cell counts after first correcting for the length (mm) of the subgranular zone (SGZ), to obtain neurogenesis density measurements. This correction for SGZ length attenuated the effect of diet and exercise in stress groups (diet × exercise × stress interaction no longer significant, $F_{1,64} = 2.28$, $p = 0.135$; ($S = S \times D = S \times E$) < ($S \times E \times D$) contrast, $t_{1,64} = 1.837$, $p = 0.07$), but not that of exercise alone ($F_{1,64} = 9.756$, $p = 0.003$), although it revealed a trend toward an effect of stress in reducing the density of adult born neurons ($F_{1,64} = 3.39$, $p = 0.07$). This effect of stress was marginally significant when examined using a directional *t*-test (controls > stress) based on the a priori prediction of a negative effect of stress on neurogenesis ($t_{1,64} = 1.837$, $p = 0.054$).

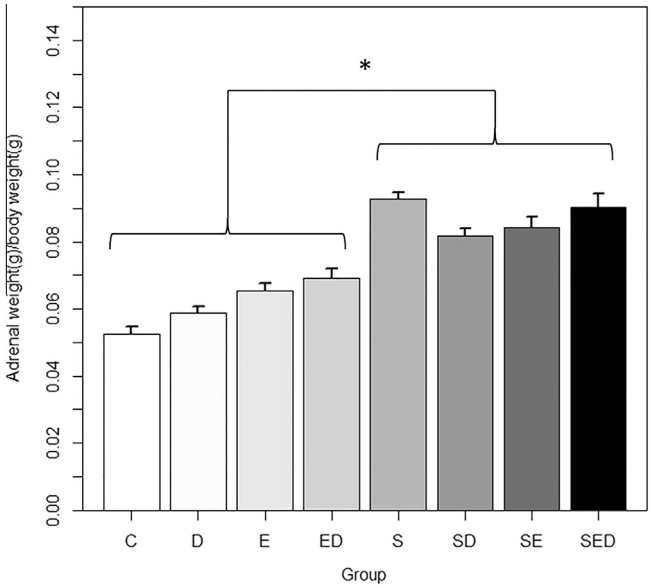

Fig. 2. Adrenal weight (corrected to body weight) in stressed vs. non-stressed animals. Chronically stressed animals showed enlarged adrenal glands ($2 \times 2 \times 2$ ANOVA, $^{*}p < .0001$). Mice in exercise groups also showed increased adrenal mass ($2 \times 2 \times 2$ ANOVA, $p = 0.0004$), demonstrating that both chronic stress and exercise can induce adrenal gland growth. (C = Control; S = Stress; D = Diet; E = Exercise).

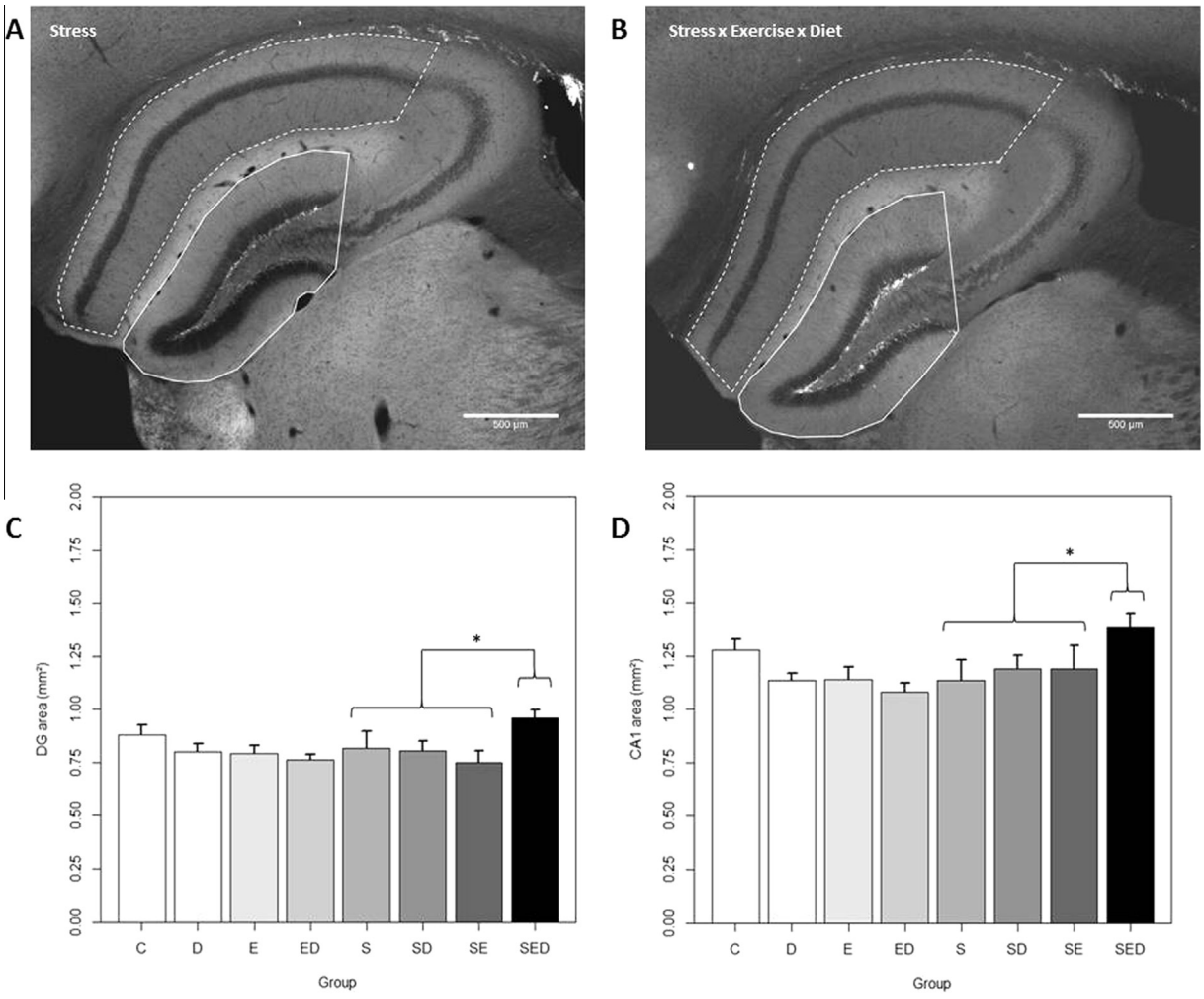

Fig. 3. Effect of diet and exercise on hippocampal area in stressed mice. (A, B) Fluorescence micrographs (4× magnification) showing the surface area of the DG (solid outline) and CA1 (dashed outline) in samples from the stress-only (A) and stress × diet × exercise (B) groups. (C, D) Surface area measurements (mean ± SE, mm^2) of DG (**C**) and CA1 (D) per 40-μm section of the hippocampus for each group reveal that the among chronically stressed animals in the current study, hypertrophy of the hippocampus is only induced by the use of diet and exercise in combination (S = S × D = S × E < S × E × D contrast; $^*p = 0.002$ for DG; $^*p = 0.007$ for CA1). (C = Control; S = Stress; D = Diet; E = Exercise).

Table 3. Average area measurements (mm^2) for each group ± standard error

Group	Structure	
	DG	CA1
Control	0.88 ± 0.03	1.28 ± 0.03
Diet	0.80 ± 0.02	1.13 ± 0.02
Exercise	0.79 ± 0.03	1.14 ± 0.04
Diet × Exercise	0.76 ± 0.02	1.08 ± 0.03
Stress	0.82 ± 0.06	1.14 ± 0.07
Stress × Diet	0.80 ± 0.03	1.21 ± 0.05
Stress × Exercise	0.77 ± 0.04	1.12 ± 0.08
Stress × Exercise × Diet	0.96 ± 0.03*	1.39 ± 0.05*

* Diet × exercise interaction effect, significantly different from the stress-only group.

Hippocampal BDNF expression

We evaluated alterations due to stress, exercise or diet in the expression of the key neurotrophic factor, BDNF (which regulates changes in synaptic density, neurogenesis and hippocampal volume). We used qRT-PCR to quantify BDNF mRNA (normalized to the level of β-actin, a housekeeping gene) in hippocampal tissue homogenates from animals that had not been used for immunohistochemistry (Fig. 5). The $2 \times 2 \times 2$ ANOVA revealed a strong trend toward a main effect of stress: $F_{1,57} = 3.049$, $p = 0.0862$, while the (S = S × D = S × E < S × E × D) contrast indicated that the combination of exercise and diet significantly increased hippocampal BDNF levels in chronically stressed animals ($t_{1,57} = 2.226$, $p = 0.03$).

Serum BDNF, IGF-1 and VEGF

Levels of BDNF, IGF-1 and VEGF in the peripheral circulation were quantified using ELISA in serum samples collected from all animals, to determine if the changes observed in the CNS extended to peripheral factors. Application of the same statistical methods as used above, a $2 \times 2 \times 2$ ANOVA and planned (S = S × E = S × D < S × E × D) contrast, revealed

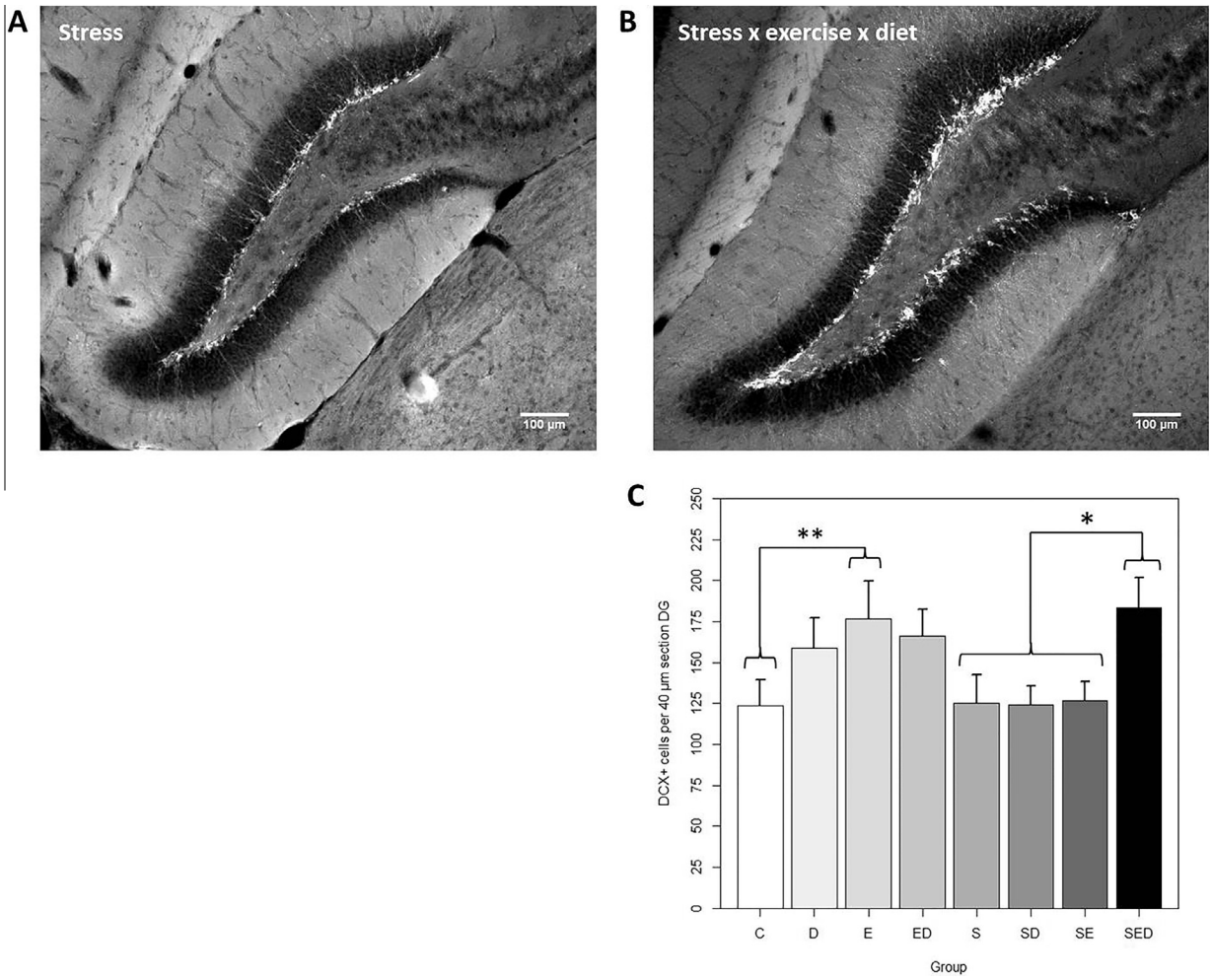

Fig. 4. Effect of stress, exercise and diet on neurogenesis. (A, B) Fluorescence micrographs (10× magnification) showing doublecortin (DCX) labeled adult born neurons in the DG of subjects from the stress-only (A) and stress × exercise × diet (B) groups. (C) Quantity of DCX-positive cells (mean ± SE) per 40-μm section of the DG for each group demonstrating that exercise (but not diet) is sufficient to enhance neurogenesis in non-stressed animals ($2 \times 2 \times 2$ ANOVA, $^{**}p = 0.009$). Among stressed animals neither exercise nor diet alone increases neurogenesis, but the combination does (S = S × D = S × E < S × E × D contrast, $^{*}p = 0.0053$), demonstrating a synergistic effect of diet and exercise under conditions of chronic stress. (C = Control; S = Stress; D = Diet; E = Exercise).

no significant changes in serum BDNF or IGF-1 across groups. However, in the case of VEGF, the omnibus ANOVA revealed a main effect of stress ($F_{1,42} = 2.125$, $p = 0.029$; Fig. 6) and a trend toward a diet × stress interaction ($F_{1,42} = 3.03$, $p = 0.089$), suggesting that the CDS affected VEGF differently in stress group animals compared to controls.

DISCUSSION

These results demonstrate that a combination of aerobic exercise and supplementation with a complex nutraceutical formulation exerted potent neurotrophic effects in stressed rodents evident across diverse biomarkers relevant to hippocampal physiology. Although mice in all stressed groups were strongly affected in measures of depressive-like behavior (saccharin preference) and allostatic burden (adrenal gland size), by the final week of testing some recovery of saccharin preference was seen in stressed animals that exercised and received the CDS. The adrenal glands were also enlarged in exercising but otherwise non-stressed animals. Such an effect of exercise alone has been previously reported (Song et al., 1973; Droste et al., 2003), but this effect does not result in increased corticosteroid binding in the hippocampus (Droste et al., 2003). While others have noted a larger effect of chronic stress on hippocampal structure and plasticity than reported here (Watanabe et al., 1992; Gould et al., 1997, 1998), we did consistently observe trends toward atrophic effects of stress across a wide variety of measures including hippocampal CA1 and DG size, neurogenesis and BDNF mRNA. The fact that these results did not reach significance may have been due to the specific stress protocol used here. In the literature, chronic stress has widely varying effects, ranging from mild to severe pathological changes in the hippocampus. This may reflect the wide range of stress paradigms used in these studies. A study comparing CUS to chronic immobilization stress (CIS) found that the former more robustly impacted

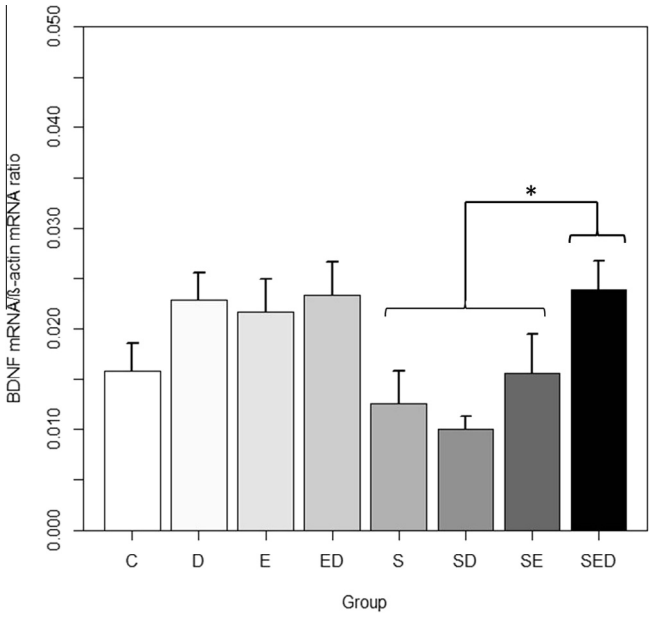

Fig. 5. Effect of stress, exercise and diet on hippocampal BDNF mRNA. qRT-PCR measurements of BDNF mRNA copy numbers normalized to β-actin copy numbers in hippocampal tissue. Under conditions of chronic stress, BDNF expression in the hippocampus is upregulated by a combination of diet and exercise (S = S × D = S × E < S × E × D contrast, $^{*}p = 0.03$), but not by exercise or diet alone. (C = Control; S = Stress; D = Diet; E = Exercise).

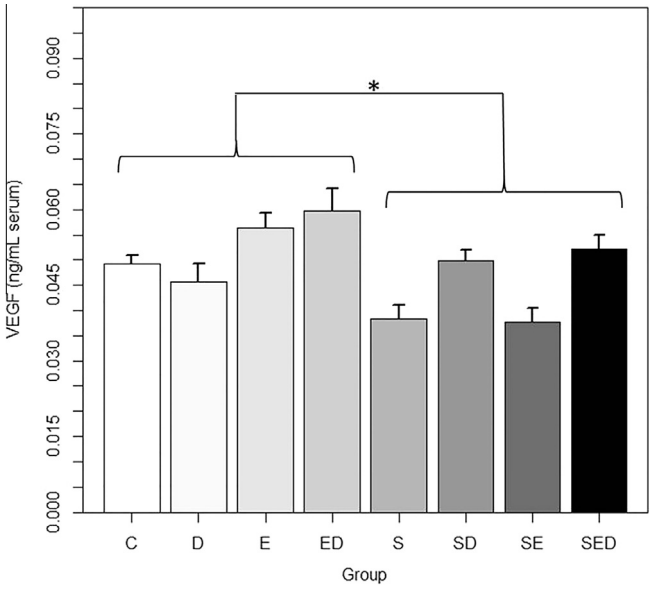

Fig. 6. Influence of stress, exercise and diet on VEGF in peripheral circulation (serum). ELISA measurements of serum VEGF revealed reduced levels in mice exposed to chronic stress (2 × 2 × 2 ANOVA, $^{*}p = 0.029$). A trend toward a diet × stress interaction (2 × 2 × 2 ANOVA, $p = 0.089$) was also detected, suggesting that the effect of the CDS on VEGF may differ in stress group animals compared to controls (Fig. 6). (C = Control; S = Stress; D = Diet; E = Exercise).

the amygdala, while the latter was more detrimental to the hippocampus and behavioral measures of anxiety (elevated plus maze; Vyas et al., 2002). Thus, the use of CIS may be more effective in future studies on hippocampal physiology. Furthermore, the disparity between the behavioral and neurological results in the present study suggests that the former are more overtly altered by CUS.

We also found a potent enhancement of neurogenesis by a prolonged period of voluntary aerobic exercise in non-stressed control groups, which was abolished by chronic stress. The positive effect of exercise on neurogenesis is consistent with previous studies (van Praag et al., 1999, 2005). In contrast to the benefits of exercise, we did not observe a statistically significant benefit of diet in healthy animals. The dietary supplement

might not be expected to have an impact in healthy, young adult control animals given that they might not exhibit markedly elevated levels of oxidative stress, inflammation or other mechanisms targeted by the CDS. The dietary supplement alone also failed to affect most of our measures significantly in stressed groups, consistent with the work of Fahnestock et al. (2012) examining the effects of diet and environmental enrichment in a canine model of age-related cognitive decline. The supplement was originally designed to ameliorate aging (Lemon et al., 2003). Thus, supplementation over longer periods of time or in older animals, as in previous studies (Aksenov et al., 2013), would likely produce much more robust effects in both healthy animals and those that are chronically stressed (which can accelerate cognitive decline, see Lupien et al., 2009).

The finding that the combination of diet and exercise (but neither one alone) partially restored hedonic behavior and exerted multiple trophic effects on the hippocampus underscores the potential for potent synergistic effects between the CDS and aerobic exercise in the treatment of stress-related psychiatric disorders in humans. To our knowledge this is the first report of a successful attempt at disrupting the trophic effects of exercise on neurogenesis using chronic stress and then reinstating it through dietary supplementation. Future research could examine whether similar synergistic interactions are observed when combining other interventions known to affect hippocampus-dependent cognitive functions, such as environmental enrichment (Kempermann et al., 1997; Fahnestock et al., 2012) or cognitive training (Madore et al., 2014), with a similar complex nutraceutical supplement.

It is remarkable that benefits of the combined intervention were observed across nearly all measures included in this study: anhedonia, hippocampal BDNF, DG and CA1 volume, neurogenesis and serum VEGF, suggesting that the effects of diet and exercise in mitigating stress were not specific to a single physiological process or biochemical pathway. It is also noteworthy that the neurogenesis and hippocampal area measurements were conducted in a separate cohort of animals from the hippocampal BDNF assays, yet parallel effects of exercise and diet were observed in both groups. Moreover, the amelioration of anhedonia was observed in both groups of stressed animals receiving diet and exercise. Future studies could investigate whether other measures of neuroplasticity such as dendritic spine density, arborization and synapse morphology would be similarly affected, in light of the changes we observed in the CA1 and DG sectional areas, and previous evidence suggesting that stress-induced hippocampal volume loss is due to synaptic degeneration rather than cellular apoptosis (Watanabe et al., 1992; Magariños et al., 1997; Lucassen et al., 2001). In support of this it has been found that, with the exception of newly born neurons in the subgranular and subventricular zones (Linnarsson et al., 2000), BDNF is not a survival factor for postnatal CNS neurons but rather regulates dendritic complexity and synaptic plasticity (Rauskolb et al., 2010). This insight in particular hints at the possibility that the neurotoxic effects of chronic stress, when observed, may be reversible given the correct combination of nutraceutical and exercise based interventions. Moreover, comparable or even greater effects of diet and exercise in animals exposed to stress may be seen in other brain structures, such as the dorsolateral and dorsomedial prefrontal cortices, which are implicated in stress-induced depression (Larrieu et al., 2014).

While it is impossible to determine the mechanism by which diet and exercise exerted their neuroprotective benefits based on the design of the current study and the complexity of the dietary supplement, the data did provide some clues. Notably, the large effect of exercise, but not diet, on neurogenesis in non-stress groups hints at the possibility that chronic upregulation of the HPA axis interferes with the agonistic action of exercise on neurogenesis, which is known to depend upon the intact signaling of neurotrophic factors, including BDNF (Li et al., 2008), VEGF (Fabel et al., 2003) and IGF-1 (Vivar et al., 2013).

While BDNF features most prominently in the neurophysiological influence of exercise on neurogenesis, our results for VEGF protein levels in serum are especially informative. Given that we observed a trend toward a selective effect of the CDS, but not exercise on serum VEGF (diet $\times$ stress interaction) in animals exposed to chronic stress, it is conceivable that the potential restoration of exercise-mediated neurotrophic effects are driven by VEGF. This is supported by research implicating glucocorticoid receptor signal transduction in the suppression of VEGF and BDNF expression (Smith et al., 1995; Koedam et al., 2002; Kawashima et al., 2010). Alternatively, given the established effects of the CDS on mitochondrial activity (Aksenov et al., 2013), perhaps chronic stress exposure reduced the amount of running in the animals who exercised but did not receive the CDS. On the other hand, physical activity may have been restored by the metabolic effects of the diet. These alternatives could easily be examined in future studies by inhibiting VEGF signaling and/or recording the amount of running of exercise group animals. Lack of detailed records on the amount, duration and speed of running in exercising animals is a limitation in the current study. Regardless, if these hypotheses prove to be correct, it seems reasonable to conclude that the synergistic effects of exercise and diet depend, at least in part, on the upregulation of BDNF and/or VEGF.

A limitation of this study is the exclusive use of male mice, as is common in studies that investigate neurogenesis because female circulating hormones impact neurogenesis levels (Ormerod and Galea, 2001). Nonetheless, considering the relatively high incidence of depression and anxiety disorders in women (Altemus, 2006), it is critically important for future work to include both sexes. Overall, our findings have important clinical implications for those suffering chronic stress-related psychiatric disorders such as major depression. Our results clearly demonstrate the potential of combining CDSs and exercise as an alternative to the pharmacological treatment of mood and anxiety disorders associated with

chronic stress. In addition, the ineffectiveness of either exercise or dietary supplementation alone suggests that their effects are synergistic and recommends against future attempts at monotherapeutic treatment.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

Acknowledgments—We recognize the substantial contributions of A. LoGuidice in assistance with the qRT-PCR analysis of BDNF mRNA from hippocampal tissue samples and B. Michalski for technical support with neurotrophic assays. We also thank M.E. Cybulski and L. Stoa for help with preparation and administration of the dietary supplement and stressor administration, and J. Thorpe for her assistance in pilot testing and stressor administration. Finally, we thank S. Alikanzadeh, C. Leung and Y. Zhou for their assistance with the DCX immunochemistry and Y.F. Tan for her technical support with the immunohistochemical assays. This work was supported by NSERC discovery grants to S. Becker, D.R. Boreham, D. deCatanzaro and J.M. Wojtowicz, a CIHR operating grant (MOP: 119271) to J.M. Wojtowicz, and a grant from the Alzheimer's Society of Canada to M. Fahnestock.

REFERENCES

Aksenov V, Long J, Liu J, Szechtman H, Khanna P, Matravadia S, Rollo CD (2013) A complex dietary supplement augments spatial learning, brain mass, and mitochondrial electron transport chain activity in aging mice. Age (Dordr) 35:23–33.

Altemus M (2006) Sex differences in depression and anxiety disorders: potential biological determinants. Horm Behav 50:534–538.

Aronsson M, Fuxe K, Dong Y, Agnati LF, Okret S, Gustafsson JA (1988) Localization of glucocorticoid receptor mRNA in the male rat brain by in situ hybridization. Proc Natl Acad Sci U S A 85:9331–9335.

Brown JP, Couillard-Després S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG (2003) Transient expression of doublecortin during adult neurogenesis. J Comp Neurol 467:1–10.

Cole MA, Kalman BA, Pace TWW, Topczewski F, Lowrey MJ, Spencer RL (2000) Selective blockade of the mineralocorticoid receptor impairs hypothalamic–pituitary–adrenal axis expression of habituation. J Neuroendocrinol 12:1034–1042.

Creer DJ, Romberg C, Saksida LM, van Praag H, Bussey TJ (2010) Running enhances spatial pattern separation in mice. Proc Natl Acad Sci U S A 107:2367–2372.

d'Audiffret AC, Frisbee SJ, Stapleton PA, Goodwill AG, Isingrini E, Frisbee JC (2010) Depressive behavior and vascular dysfunction: a link between clinical depression and vascular disease? J Appl Physiol 108:1041–1051.

Déry N, Pilgrim M, Gibala M, Gillen J, Wojtowicz JM, Macqueen G, Becker S (2013) Adult hippocampal neurogenesis reduces memory interference in humans: opposing effects of aerobic exercise and depression. Front Neurosci 7:66.

Droste SK, Gesing A, Ulbricht S, Müller MB, Linthorst ACE, Reul JMHM (2003) Effects of long-term voluntary exercise on the mouse hypothalamic–pituitary–adrenocortical axis. Endocrinology 144:3012–3023.

Elizalde N, Gil-Bea FJ, Ramírez MJ, Aisa B, Lasheras B, Del Rio J, Tordera RM (2008) Long-lasting behavioral effects and recognition memory deficit induced by chronic mild stress in mice: effect of antidepressant treatment. Psychopharmacology 199:1–14.

Erickson KI, Voss MW, Prakash RS, Basak C, Szabo A, Chaddock L, Kim JS, Heo S, Alves H, White SM, Wojcicki TR, Mailey E, Vieira VJ, Martin SA, Pence BD, Woods JA, McAuley E, Kramer AF (2011) Exercise training increases size of hippocampus and improves memory. Proc Natl Acad Sci U S A 108:3017–3022.

Fabel K, Fabel K, Tam B, Kaufer D, Baiker A, Simmons N, Kuo CJ, Palmer TD (2003) VEGF is necessary for exercise-induced adult hippocampal neurogenesis. Eur J Neurosci 18:2803–2812.

Fahnestock M, Marchese M, Head E, Pop V, Michalski B, Milgram WN, Cotman CW (2012) BDNF increases with behavioral enrichment and an antioxidant diet in the aged dog. Neurobiol Aging 33:546–554.

Fox J (2008) Applied regression analysis and generalized linear models, second ed., pp 176–186. Thousand Oaks, CA: Sage Publications.

Goshen I, Kreisel T, Ben-Menachem-Zidon O, Licht T, Weidenfeld J, Ben-Hur T, Yirmiya R (2008) Brain interleukin-1 mediates chronic stress-induced depression in mice via adrenocortical activation and hippocampal neurogenesis suppression. Mol Psychiatry 13:717–728.

Gould E, Cameron HA, Daniels DC, Woolley CS, McEwen BS (1992) Adrenal hormones suppress cell division in the adult rat dentate gyrus. J Neurosci 12:3642–3650.

Gould E, McEwen BS, Tanapat P, Galea LA, Fuchs E (1997) Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. J Neurosci 17:2492–2498.

Gould E, Tanapat P, McEwen BS, Flügge G, Fuchs E (1998) Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. Proc Natl Acad Sci U S A 95:3168–3171.

Harkin A, Houlihan DD, Kelly JP (2002) Reduction in preference for saccharin by repeated unpredictable stress in mice and its prevention by imipramine. J Psychopharmacol 16:115–123.

Kawashima H, Numakawa T, Kumamaru E, Adachi N, Mizuno H, Ninomiya M, Kunugi H, Hashido K (2010) Glucocorticoid attenuates brain-derived neurotrophic factor-dependent upregulation of glutamate receptors via the suppression of microRNA-132 expression. Neuroscience 165:1301–1311.

Kempermann G, Kuhn HG, Gage FH (1997) More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493–495.

Koedam JA, Smink JJ, van Buul-Offers SC (2002) Glucocorticoids inhibit vascular endothelial growth factor expression in growth plate chondrocytes. Mol Cell Endocrinol 197:35–44.

Larrieu T, Hilal LM, Fourrier C, De Smedt-Peyrusse V, Sans N, Capuron L, Layé S (2014) Nutritional omega-3 modulates neuronal morphology in the prefrontal cortex along with depression-related behaviour through corticosterone secretion. Transl Psychiatry 4:e437.

Lau FC, Shukitt-Hale B, Joseph JA (2005) The beneficial effects of fruit polyphenols on brain aging. Neurobiol Aging 26(Suppl 1):128–132.

Lemon JA, Boreham DR, Rollo CD (2003) A dietary supplement abolishes age-related cognitive decline in transgenic mice expressing elevated free radical processes. Exp Biol Med 228:800–810.

Lemon JA, Rollo CD, Boreham DR (2008a) Elevated DNA damage in a mouse model of oxidative stress: impacts of ionizing radiation and a protective dietary supplement. Mutagenesis 23:473–482.

Lemon JA, Rollo CD, McFarlane NM, Boreham DR (2008b) Radiation-induced apoptosis in mouse lymphocytes is modified by a complex dietary supplement: the effect of genotype and gender. Mutagenesis 23:465–472.

Leung BMY, Kaplan BJ, Field CJ, Tough S, Eliasziw M, Gomez MF, McCargar LJ, Gagnon L (2013) Prenatal micronutrient supplementation and postpartum depressive symptoms in a pregnancy cohort. BMC Pregnancy Childbirth 13:2.

Li Y, Luikart BW, Birnbaum S, Chen J, Kwon CH, Kernie SG, Bassel-Duby R, Parada LF (2008) TrkB regulates hippocampal neurogenesis and governs sensitivity to antidepressive treatment. Neuron 59:399–412.

López JF, Chalmers DT, Little KY, Watson SJ (1998) Regulation of serotonin(1A), glucocorticoid, and mineralocorticoid receptor in rat and human hippocampus: implications for the neurobiology of depression. Biol Psychiatry 43:547–573.
Linnarsson S, Willson CA, Ernfors P (2000) Cell death in regenerating populations of neurons in BDNF mutant mice. Mol Brain Res 75:61–69.
Lucassen PJ, Vollmann-Honsdorf GK, Gleisberg M, Czéh B, De Kloet ER, Fuchs E (2001) Chronic psychosocial stress differentially affects apoptosis in hippocampal subregions and cortex of the adult tree shrew. Eur J Neurosci 14:161–166.
Lupien SJ, McEwen BS, Gunnar MR, Heim C (2009) Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat Rev Neurosci 10:434–445.
MacQueen GM, Campbell S, McEwen BS, Macdonald K, Amano S, Joffe RT, Nahmias C, Young LT (2003) Course of illness, hippocampal function, and hippocampal volume in major depression. Proc Natl Acad Sci U S A 100:1387–1392.
Madore KP, Gaesser B, Schacter DL (2014) Constructive episodic simulation: dissociable effects of a specificity induction on remembering, imagining, and describing in young and older adults. J Exp Psychol Learn Mem Cogn 40:609–622.
Magariños AM, Verdugo JM, McEwen BS (1997) Chronic stress alters synaptic terminal structure in hippocampus. Proc Natl Acad Sci U S A 94:14002–14008.
Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci 20:9104–9110.
Mantella RC, Vollmer RR, Amico JA (2005) Corticosterone release is heightened in food or water deprived oxytocin deficient male mice. Brain Res 1058:56–61.
McEwen BS (2003) Mood disorders and allostatic load. Biol Psychiatry 54:200–207.
McEuen JG, Beck SG, Bale TL (2008) Failure to mount adaptive responses to stress results in dysregulation and cell death in the midbrain raphe. J Neurosci 28:8169–8177.
McDonald HY, Wojtowicz JM (2005) Dynamics of neurogenesis in the dentate gyrus of adult rats. Neurosci Lett 385:70–75.
Merkley CM, Jian C, Mosa A, Tan YF, Wojtowicz MM (2014) Homeostatic regulation of adult hippocampal neurogenesis in aging rats: long-term effects of early exercise. Front Neurosci 8:1–12.
Milgram NW, Zicker SC, Head E, Muggenburg BA, Murphey H, Ikeda-Douglas CJ, Cotman CW (2002) Dietary enrichment counteracts age-associated cognitive dysfunction in canines. Neurobiol Aging 23:737–745.
Miller BR, Hen R (2015) The current state of the neurogenic theory of depression and anxiety. Curr Opin Neurobiol 30:51–58.
Olson AK, Eadie BD, Ernst C, Christie BR (2006) Environmental enrichment and voluntary exercise massively increase neurogenesis in the adult hippocampus via dissociable pathways. Hippocampus 16:250–260.
Ormerod BK, Galea LAM (2001) Reproductive status influences cell proliferation and cell survival in the dentate gyrus of adult female meadow voles: a possible regulatory role for estradiol. Neuroscience 102:369–379.
Ota KT, Liu R, Voleti B, Maldonado-Aviles JG, Duric V, Iwata M, Dutheil S, Duman C, Boikess S, Lewis DA, Stockmeier CA, DiLeone RJ, Rex C, Aghajanian GK, Duman RS (2014) REDD1 is essential for stress-induced synaptic loss and depressive behavior. Nat Med 20:531–535.
Parachikova A, Green KN, Hendrix C, LaFerla FM (2010) Formulation of a medical food cocktail for Alzheimer's disease: beneficial effects on cognition and neuropathology in a mouse model of the disease. PLoS One 5:e14015.
Perera TD, Dwork AJ, Keegan KA, Thirumangalakudi L, Lipira CM, Joyce N, Lange C, Higley JD, Rosoklija G, Hen R, Sackeim HA, Coplan JD (2011) Necessity of hippocampal neurogenesis for the therapeutic action of antidepressants in adult Nonhuman primates. PLoS One 6:1–13.
Pereira AC, Huddleston DE, Brickman AM, Sosunov AA, Hen R, McKhann GM, Sloan R, Gage FH, Brown TR, Small SA (2007) An in vivo correlate of exercise-induced neurogenesis in the adult dentate gyrus. Proc Natl Acad Sci U S A 104:5638–5643.
Pop V, Head E, Hill M-A, Gillen D, Berchtold NC, Muggenburg BA, Milgram NW, Murphy MP, Cotman CW (2010) Synergistic effects of long-term antioxidant diet and behavioral enrichment on beta-amyloid load and non-amyloidogenic processing in aged canines. J Neurosci 30:9831–9839.
Popper CW (2014) Single-micronutrient and broad-spectrum micronutrient approaches for treating mood disorders in youth and adults. Child Adolesc Psychiatr Clin N Am 23:591–672.
Rauskolb S, Zagrebelsky M, Dreznjak A, Deogracias R, Matsumoto T, Wiese S, Erne B, Sendtner M, Schaeren-Wiemers N, Korte M, Barde Y-A (2010) Global deprivation of brain-derived neurotrophic factor in the CNS reveals an area-specific requirement for dendritic growth. J Neurosci 30:1739–1749.
Reul JMHM, De Kloet ER (1985) Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117:2505–2511.
Rucklidge JJ, Kaplan BJ (2013) Broad-spectrum micronutrient formulas for the treatment of psychiatric symptoms: a systematic review. Expert Rev Neurother 13:49–73.
Sahay A, Scobie KN, Hill AS, O'Carroll CM, Kheirbek MA, Burghardt NS, Fenton AA, Dranovsky A, Hen R (2011) Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. Nature 472:466–470.
Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301:805–809.
Sapolsky R (1985) A possible mechanism for glucocorticoid toxicity in the hippocampus: increased vulnerability of neurons to metabolic insults. J Neurosci 5:1228–1232.
Sapolsky RM, Uno H, Rebert CS, Finch CE (1995) Hippocampal damage associated with prolonged glucocorticoid exposure in primates. J Neurosci 10:2897–2902.
Schmidt HD, Duman RS (2010) Peripheral BDNF produces antidepressant-like effects in cellular and behavioral models. Neuropsychopharmacology 35:2378–2391.
Sheline YI, Sanghavi M, Mintun MA, Gado MH (1999) Depression duration but not age predicts hippocampal volume loss in medically healthy women with recurrent major depression. J Neurosci 19:5034–5043.
Smith MA, Makino S, Kvetnansky R, Post RM (1995) Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. J Neurosci 15:1768–1777.
Snyder JS, Kee N, Wojtowicz JM (2001) Effects of adult neurogenesis on synaptic plasticity in the rat dentate gyrus. J Neurophysiol 85:2423–2431.
Snyder JS, Soumier A, Brewer M, Pickel J, Cameron HA (2011) Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. Nature 476:458–461.
Song MK, Ianuzzo CD, Saubert IV CW, Gollnick PD (1973) The mode of adrenal gland enlargement in the rat in response to exercise training. Pflugers Arch Eur J Physiol 339:59–68.
Strekalova T, Steinbusch HWM (2010) Measuring behavior in mice with chronic stress depression paradigm. Prog Neuro-Psychopharmacology Biol Psychiatry 34:348–361.
Surget A, Tanti A, Leonardo ED, Laugeray A, Rainer Q, Touma C, Palme R, Griebel G, Ibarguen-Vargas Y, Hen R, Belzung C (2011) Antidepressants recruit new neurons to improve stress response regulation. Mol Psychiatry 16:1177–1188.
Thorpe JB, Gould KE, Borman ED, deCatanzaro D (2014) Circulating and urinary adrenal corticosterone, progesterone, and estradiol in response to acute stress in female mice (*Mus musculus*). Horm Metab Res 46:211–218.
Valente T, Hidalgo J, Bolea I, Ramirez B, Anglés N, Reguant J, Morelló JR, Gutiérrez C, Boada M, Unzeta M (2009) A diet enriched in polyphenols and polyunsaturated fatty acids, LMN

diet, induces neurogenesis in the subventricular zone and hippocampus of adult mouse brain. J Alzheimers Dis 18:849–865.
van Praag H, Kempermann G, Gage FH (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 2:266–270.
van Praag H, Shubert T, Zhao C, Gage FH (2005) Exercise enhances learning and hippocampal neurogenesis in aged mice. J Neurosci 25:8680–8685.
Vivar C, Potter MC, van Praag H (2013) All about running: synaptic plasticity, growth factors and adult hippocampal neurogenesis Carmen. Curr Top Behav Neurosci 15:289–320.
Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S (2002) Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. J Neurosci 22:6810–6818.
Watanabe Y, Gould E, McEwen BS (1992) Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons. Brain Res 588:341–345.
Willner P, Muscat R, Papp M (1992) Chronic mild stress-induced anhedonia: a realistic animal model of depression. Neurosci Biobehav Rev 16:525–534.
Winocur G, Wojtowicz JM, Huang J, Tannock IF (2014) Physical exercise prevents suppression of hippocampal neurogenesis and reduces cognitive impairment in chemotherapy-treated rats. Psychopharmacology 231:2311–2320.
Winocur G, Wojtowicz JM, Sekeres M, Snyder JS, Wang S (2006) Inhibition of neurogenesis interferes with hippocampus-dependent memory function. Hippocampus 16:296–304.
Wojtowicz JM, Kee N (2006) BrdU assay for neurogenesis in rodents. Nat Protoc 1:1399–1405.
Wu A, Ying Z, Gomez-Pinilla F (2008) Docosahexaenoic acid dietary supplementation enhances the effects of exercise on synaptic plasticity and cognition. Neuroscience 155:751–759.

(Accepted 2 September 2015)
(Available online 7 September 2015)