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Skeletal myofiber VEGF is required for the exercise training-induced increase in dentate gyrus neuronal precursor cells

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Key Points Summary

1. Peripheral vascular endothelial growth factor (VEGF) is necessary for exercise to stimulate hippocampal neurogenesis.
2. Here we report that skeletal myofiber VEGF directly or indirectly regulates exercise-signaled proliferation of neuronal precursor cells
3. Our results found skeletal myofiber VEGF to be necessary for maintaining blood flow through hippocampal regions independent of exercise training state.
4. This study demonstrates that skeletal myofiber VEGF is required for the hippocampal VEGF response to acute exercise.
5. These results help us establish the mechanisms by which exercise, through skeletal myofiber VEGF, affect the hippocampus.

Abstract

Exercise signals neurogenesis in the dentate gyrus of the hippocampus. This phenomenon requires vascular endothelial growth factor (VEGF) originating from outside the blood brain barrier, but no cellular source has been identified. Thus, we hypothesized that VEGF produced by skeletal myofibers plays a role in regulating hippocampal neuronal precursor cell proliferation following exercise training. This was tested in adult conditional skeletal myofiber-specific VEGF gene-ablated mice ($VEGF^{HSA-/-}$) by providing $VEGF^{HSA-/-}$ and non-ablated ($VEGF^{f/f}$) littermates with running wheels for 14 days. Following this training period, hippocampal cerebral blood flow (CBF) was measured by functional magnetic resonance imaging (fMRI) and neuronal precursor cells (BrDU+/Nestin+) were detected by immunofluorescence. The $VEGF^{f/f}$ trained group showed improvements in both speed and endurance capacity in acute treadmill running tests ($p<0.05$). The $VEGF^{HSA-/-}$ group did not. The number of proliferating neuronal precursor cells was increased with training in $VEGF^{f/f}$ ($p<0.05$) but not in $VEGF^{HSA-/-}$ mice. Endothelial cell (CD31+) number did not change in this region with exercise training or skeletal myofiber VEGF gene deletion. However, resting blood flow through the hippocampal region was lower in $VEGF^{HSA-/-}$ mice, both untrained and trained, than untrained $VEGF^{f/f}$ mice ($p<0.05$). An acute hypoxic challenge decreased CBF ($p<0.05$) in untrained $VEGF^{f/f}$, untrained $VEGF^{HSA-/-}$, and trained $VEGF^{HSA-/-}$, but not trained $VEGF^{f/f}$ mice. $VEGF^{f/f}$, but not $VEGF^{HSA-/-}$ mice, were able to acutely run on a treadmill at an intensity sufficient to increase hippocampus VEGF levels. These data suggest that VEGF expressed by skeletal myofibers may directly or indirectly regulate both hippocampal blood flow and neurogenesis.

Abbreviations BrdU, 5-Bromo-2'deoxyuridine; BW, body weight; CASL, continuous arterial spin labeling; CBF, cerebral blood flow; Cre, Cre recombinase; VEGF, vascular endothelial growth factor; $VEGF^{HSA-/-}$, skeletal myofiber VEGF gene deletion mouse**;** $VEGF^{f/f}$, mice whose VEGF gene is floxed with LoxP sites without CRE gene

Introduction

Exercise is well known to have many benefits for overall metabolism and locomotion. Exercise is also thought to improve memory and spatial learning and assuage the symptoms of anxiety and depression (Snyder *et al.*, 2011; Kiuchi *et al.*, 2012; Marlatt *et al.*, 2012). Improvements in brain function stem from an ability to increase or maintain the number of new neurons in hippocampal regions (van Praag *et al.*, 1999). This seems to be particularly important during aging, and exercise has been shown to slow the loss of neuronal stem cells and promote their commitment to a neuronal specific lineage (Yang *et al.*, 2015). New neurons within the dentate gyrus of the hippocampus reside in a highly vascularized environment in contact with endothelial cells (Shen *et al.*, 2004). Thus,

neuronal precursor cells are well positioned to continuously sense and respond to changes in circulating levels of oxygen, nutrients and neurotrophic factors.

The signals produced by exercise training, which may directly or indirectly regulate the number of neuronal precursor cells in the hippocampus, have not been well defined. In skeletal muscle, exercise enhances the ability to provide and utilize oxygen and nutrients by augmenting the number of capillaries that perfuse muscle, oxidative metabolic enzymes activities and mitochondrial biogenesis (Yan *et al.*, 2011; Summermatter *et al.*, 2013). Agonists of metabolic regulators, AMPK and PPARδ, have been shown to induce neurogenesis in the adult mouse dentate gyrus within a similar time frame as exercise training (Kobilo *et al.*, 2011). However, exercise training may also increase the availability of growth factors produced in the periphery that circulate and enter the cerebral vascular niche. For instance, elegant studies using heterochronic parabiotic mice demonstrate that blood supplied by young mice can induce vascular remodeling that promotes neurogenesis in the dentate gyrus. In particular GDF11, a TGFß family member, in young blood was shown to mediate this formation of new neurons (Katsimpardi *et al.*, 2014). Interestingly, GDF11 has also been reported to rejuvenate the stem cell population in skeletal muscle (Sinha *et al.*, 2014).

An additional exercise-responsive growth factor, thought to play a role in both cerebral and peripheral neural function, is vascular endothelial growth factor (VEGF) (Oosthuyse *et al.*, 2001; Sun *et al.*, 2003; Tang *et al.*, 2010). In skeletal muscle VEGF expression is rapidly and transiently increased in locomotor muscle following an acute, exhaustive exercise bout (Breen *et al.*, 1996; Tang *et al.*, 2010). VEGF expressed by skeletal myofibers is essential for improving exercise capacity and augmenting skeletal muscle capillary number with exercise training (Delavar *et al.*, 2014). This latter finding is accompanied by a coordinated increase in Nes+/Pax7+ stem cells associated with capillary endothelial cells (Christov *et al.*, 2007; Shefer *et al.*, 2013). VEGF produced in the periphery has also been shown to play a role in exercise induced cerebral neurogenesis. In a study by Fabel *et* al. blocking antibodies against VEGF were introduced into the peripheral circulation, and shown to completely abolish hippocampal neurogenesis induced by running exercise (Fabel *et al.*, 2003). However, the peripheral source of the circulating VEGF necessary for exercise induced cerebral neurogenesis has not been identified.

Most (60-90 percent) of the VEGF content in adult skeletal muscle is produced by skeletal myofibers (Delavar *et al.*, 2014; Knapp *et al.*, 2016). Therefore, we hypothesized that skeletal myofiber VEGF may either be 1. a source of VEGF for the brain or 2. May indirectly modify other exercise signals that regulate the fate of neuronal stem cells in the dentate gyrus. Thus, in this study the number of neuronal precursor cells in the dentate gyrus of the hippocampus was measured in four groups of mice: mice with normal skeletal myofiber VEGF production that were a) exercise-trained or b) remained untrained (cage-confined) and mice with reduced levels of skeletal myofiber VEGF that were c) trained or d) remained untrained. One important factor, which decreases with age and can be accentuated by exercise training is cerebral blood flow. Changes in hippocampal blood flow were also evaluated to assess regulation of vasculature function

as a VEGF-mediated signaling mechanism and a hypoxic challenge was performed to evaluate the autoregulatory capabilities of the cerebral vasculature. The ability to acutely increase hippocampal VEGF levels with an exhaustive exercise bout was also tested.

Methods

Animals

All animal protocols were reviewed and approved by the University of California, San Diego, Animal Care and Use Committee and conducted in accordance to guidelines outlined by the Guide for the Care and Use of Laboratory Animals (National Research Council). Seventy-two male mice on a C57BIL/6J background (Jackson Laboratory) were used for this study. Our methods comply with the animal ethics checklist outlined in the *Journal of Physiology* (Grundy, 2015). Mice were housed under standard laboratory conditions under a 12-h dark/light cycle with water and food *ad libidum*. Body mass was measured on day 0 (before induction with tamoxifen), day 21 (following tamoxifen-induced gene deletion) and day 36 (after the two-week exercise-training or cage confinement period).

VEGF Deletion Strategy

We used a strategy of tamoxifen-driven conditional skeletal myofiber-specific VEGF gene deletion in adult mice. Thus, this was not a lifelong gene deletion, nor was the VEGF gene deleted in any of the other cells residing in the muscle. Seventy-two male mice, 30 positive and 42 negative for the HSA-CRE-ER^{T2} gene, all on a homozygous Nes-GFP/VEGF*LoxP* background (Gerber *et al.*, 1999; Yamaguchi *et al.*, 2000; Schuler *et al.*, 2005) were maintained for this study. Presence of the HSA-CRE-ER^{T2} gene was determined using PCR analysis of tail DNA. All mice at 4 months of age were administered tamoxifen (1 mg/mouse, i.p.) for five consecutive days (days 0-5 of the experiment) to induce ablation of the VEGF gene specifically in myofibers of mice that expressed the HSA-CRE-ER^{T2} transgene as previously described (Schuler *et al.*, 2005; Delavar *et al.*, 2014; Knapp *et al.*, 2016). VEGF protein levels were measured in the plantaris muscle to ensure efficient ablation of the VEGF gene in VEGF$^{HSA-/-}$ mice.

Exercise Training and Testing

Three weeks after initiating VEGF gene deletion with tamoxifen, a time when VEGF protein levels were previously found to be reduced by over 90% in skeletal muscle (Knapp *et al.*, 2016), mice were randomly separated into cages without (untrained, groups a and c) or with (trained, groups b and d) running wheels for two weeks (days 21 to 35) and housed under the same standard laboratory conditions described previously. Trained mice had 24-hour voluntary access to an individual running wheel (wheel circumference: 35.8 cm) attached to a bike pedometer, which was used as a magnetic revolution counter (Sigma BC 906, St, Charles, IL). Measurements (distance, time, and average speed) were recorded every 24 hours.

Exercise testing was performed to elucidate physiological differences in the training response. Prior to group separation, two treadmill (Model Cl-4, Omnitech, Columbus,

OH) protocols were used to measure submaximal endurance and maximal speed. These tests were repeated after the two-week period in which groups a) and c) remained confined to standard mouse cages while groups b) and d) were trained by being given access to a running wheel. The maximal speed test was performed the morning before the endurance capacity test. Maximal speed was measured on a treadmill at a 10° incline with speeds increasing 3-4 cm/s every minute until exhaustion. Endurance capacity was measured as time to exhaustion on a treadmill at a 10° incline at 33 cm/s (Billat *et al.*, 2005). Mice were encouraged to run using an electric shock grid (≤ 4 milliamps) and manual air jets. Mice were deemed exhausted when they spent 10 continuous seconds on the shock grid.

Cerebral Blood Flow Measurement

Cerebral blood flow was measured to assess functional changes in hippocampal vasculature. Cerebral blood flow in the hippocampus was non-invasively measured using an arterial spin labeling MRI technique (Lei *et al.*, 2011). Mice were naïve to isoflurane prior to the cerebral blood flow measurements (Wegener & Wong, 2008). Mice were anesthetized with 2% isoflurane and then maintained lightly anesthetized with 0.8% isoflurane. Physiological parameters of body temperature (37°C), O_2 saturation, respiration rate and heart rate were monitored throughout the fMRI protocol. Mice were imaged in a 7T small animal magnet (Bruker Biosciences Corp., Billerica, MA). The imaging coil system was a 7.2 cm transmit coil with a 1 cm receive-only surface coil. In the magnet, mice were initially maintained on 21% O_2. An anatomical scan was performed to locate the coronal section through the hippocampus. The anatomical scan was followed by a 6-minute T1 relaxation scan and two 5-minute CASL (continuous arterial spin labeling) scans. Next, the mice underwent a hypoxic challenge by lowering the inspired gas to 10% O_2. Once physiological parameters were stabilized one 5-minute CASL scan was performed at 10% O_2. CASL scans were analyzed by a radiologist (M.S.) who was blinded to the mouse genotype, training condition and inspired O_2 level during the scan. Blood flow was quantified from the CASL scan using MATLAB (MathWorks, Natick, MA) to evaluate regions of interest (ROI) that encompassed the left and right sections of the hippocampus. Cerebral blood flow is reported as the average cerebral blood flow in ml/min.

Detection of Proliferating Neuronal Precursor and Endothelial Cells

To assess the potential initiation of a hippocampal neurogenesis and angiogenesis, 5-Bromo-2’deoxyuridine (BrdU, B5002 Sigma) was administered intraperitoneal on 8 of the days of wheel running or cage confinement (days 22-25 and 29-32 after the initial tamoxifen injection) at a dose of 50 mg per kg of body mass. On day 37 the mice were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). Unresponsiveness to toe pinch was used to ensure the mice were adequately anesthetized. Hind limb muscles were removed for analysis and measurement of VEGF protein levels in the plantaris. The animals were then sacrificed and perfused with 120 ml of PBS followed by 40 ml of 4% paraformaldehyde. The brain was removed and stored in 2% PFA at 4°C for 24-48 hours before being transferred to a solution consisting of 30% sucrose and 0.1% PFA at 4°C. The brain was divided into the left and right hippocampus and frontal cortex before being frozen on a cork in isopentane and protected in freezing

medium. Consecutive 16 um coronal sections were prepared with a cryostat, placed on slides and stored at -20°C. Neuronal precursor cells were detected by immunohistochemistry using antibodies specific for proliferating cells and the *in vivo* Nes-GFP signal was amplified with a rabbit antibody against GFP (Encinas & Enikolopov, 2008). Endothelial cell proliferation was detected using immunohistochemistry for CD31, an endothelial cell marker, to investigate changes in the hippocampal angiogenesis as a potential mechanism utilized by skeletal myofiber VEGF to up-regulate neurogenesis in this region. All immunohistochemistry steps were performed at room temperature unless noted otherwise. Cerebral sections on slides were washed in PBS (3x5 minutes), permeabilized with 0.3% Triton X-100 for 15 minutes, incubated in 2M HCl for 30 minutes and neutralized in 0.1M borate buffer (pH=8.5) for 10 minutes. The sections were then washed in PBS with 0.5% Tween 20 (3x5 minutes) before a 30-minute incubation in blocking solution (3% BSA, 0.1% Triton X-100 in PBS). Primary antibodies were applied overnight at 4°C. Primary antibodies used were anti-BrdU (3:1000, B35128, Invitrogen, Molecular Probes), anti-GFP (1:1000, G10362, Invitrogen, Molecular Probes) and anti-CD31 (1:200, Cat. No. 550274 BD Pharmingen). Signals were detected with AlexaFluor secondary antibodies (Molecular Probes, Invitrogen) at a 1:1000 dilution. Fluorescence was preserved with Pro-long Gold reagent with DAPI. The entire dentate gyrus of one section was imaged using confocal microscopy. Co-localization was assessed and quantified with ImageJ. BrdU+/Nes+ co-localization was quantified and standardized to the total number of nestin positive cells (GFP+) and to the total number of cells (DAPI+). CD31+ cells were co-localized with BrdU and standardized to the total number of CD31+ cells and to the total number of cells (DAPI+).

Hippocampal VEGF Levels following an Acute Exercise Bout

An acute exercise bout was utilized to determine the effect of skeletal muscle VEGF ablation on hippocampal VEGF levels during training. On day 21 of the experiment 41 additional untrained mice (27 $VEGF^{f/f}$ and 14 $VEGF^{HSA-/-}$) were subjected to a one-hour treadmill bout or rest (control). The treadmill was set to a 10° incline and mice were randomized to a speed of 33 cm/s or 40 cm/s. Immediately following the acute exercise bout the mice were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg), the animals were sacrificed and the brain was removed, and the hippocampus isolated for measurement of VEGF protein levels.

VEGF Protein Levels

VEGF protein levels in the plantaris from all groups of mice and hippocampus from the acutely exercised mice were measured with an ELISA (VEGF Mouse ELISA, R&D Systems, La Jolla, CA) and standardized to total protein levels (Bio-Rad DC protein assay).

Statistics

A two-way ANOVA was used to detect differences between the genotypes and exercise conditions. A Tukey post-hoc test was used to identify specific differences between the groups. Cerebral blood flow measurements in mice breathing 21% oxygen and then

switched to a 10% oxygen gas mixture were analyzed with a two-way, repeated measure ANOVA and Fisher's LSD post-hoc test. $P \leq 0.05$ was considered significant for all tests.

Results

Body mass

In the untrained $VEGF^{f/f}$ there was a trend for body mass to increase over the time frame of the experiment (Table 1). No difference in body mass was detected between groups prior to training. Main effects of time ($p<0.05$) and mouse group ($p<0.01$) were detected with no interaction. Following the period of exercise training or cage-confinement without running wheels the untrained $VEGF^{HSA-/-}$ mice weighed less than the untrained $VEGF^{f/f}$ mice ($p<0.05$).

Conditional ablation of skeletal myofiber VEGF gene

To confirm efficient VEGF gene deletion, VEGF levels (Figure 1) were measured in a representative skeletal muscle. VEGF levels (Figure 1) were reduced 61.5% in the plantaris of $VEGF^{HSA-/-}$ mice relative to $VEGF^{f/f}$ (VEGF/Total Protein: $VEGF^{f/f}$, 52.3 ± 4.9 pg/μg; $VEGF_{HSA}^{-/-}$, 20.2 ± 6.7 pg/μg. genotype effect, $p=0.001$). Plantaris VEGF levels measured at the end of the two-week voluntary training period were not increased in either genotype (VEGF/Total Protein: untrained $VEGF^{f/f}$, 43.9 ± 6.9 pg/μg; trained $VEGF^{f/f}$, 59.1 ± 5.7 pg/μg; untrained $VEGF^{HSA-/-}$, 20.6 ± 3.1 pg/μg; trained $VEGF^{HSA-/-}$, 19.7 ± 5.9 pg/μg).

Exercise training

No difference in daily voluntary wheel running distance, time or speed (Figure 2) was observed between the $VEGF^{f/f}$ and $VEGF^{HSA-/-}$ mice over the two-week exercise training protocol. The average time, distance and speed ran per day was not different between the groups over the two-week period. The average time ran per day was 120 ± 46 min. for trained $VEGF^{f/f}$ mice and 185 ± 28 min. for trained $VEGF^{HSA-/-}$ mice. The average distance ran per day was 3.88 ± 1.71 km for trained $VEGF^{f/f}$ mice and 5.46 ± 1.02 km for trained $VEGF^{HSA-/-}$ mice. The average speed ran per day was 40.4 ± 9.7 cm/s for trained $VEGF^{f/f}$ mice and 44.7 ± 6.5 cm/s for trained $VEGF^{HSA-/-}$ mice. Total distances and times ran over the two weeks were also not different between $VEGF^{f/f}$ and $VEGF^{HSA-/-}$ groups. The total time ran was 1680 ± 637 min. for trained $VEGF^{f/f}$ mice and 2595 ± 393 min. for trained $VEGF^{HSA-/-}$ mice. The total distance ran was 54.3 ± 24.0 km for trained $VEGF^{f/f}$ mice and 76.4 ± 14.3 km for trained $VEGF^{HSA-/-}$ mice.

Treadmill exercise testing

There was no difference in maximum treadmill speed or endurance (time to exhaustion at 33 cm/s) between genotype or training groups prior to running wheel training (Figure 3). The trained $VEGF^{f/f}$ mice showed a 9% increase in maximal speed ($p<0.05$) and a 65% increase in time to exhaustion ($p<0.05$). The trained $VEGF^{HSA-/-}$ mice did not significantly improve speed or endurance after running wheel training. Untrained $VEGF^{f/f}$ and $VEGF^{HSA-/-}$ mice showed no changes in speed or endurance relative to baseline after two weeks of rest.

Functional changes in hippocampal vasculature

Hippocampal blood flow was measured under normoxic and hypoxic conditions to assess functional changes in hippocampal vasculature. Normoxic, resting blood flow through the hippocampal region was 36% lower in untrained $VEGF^{HSA-/-}$ mice ($p=0.05$) and 40% lower in trained $VEGF^{HSA-/-}$ ($p=0.04$) than untrained $VEGF^{f/f}$ mice (Figure 4). The O_2 saturation was maintained in the magnet at rest (O_2 saturation: untrained $VEGF^{f/f}$, 95.9 ± 0.6%; trained $VEGF^{f/f}$, 93.6 ± 0.9%; untrained $VEGF^{HSA-/-}$, 93.5 ± 1.2%; trained $VEGF^{HSA-/-}$, 94.2 ± 0.7%). An acute hypoxic challenge with 10% inspired O_2 decreased blood flow. Compared to each group's normoxic levels, flow fell by 58% in untrained $VEGF^{f/f}$ ($p=0.003$), 73% in untrained $VEGF^{HSA-/-}$ ($p=0.02$), and 80% in trained $VEGF^{HSA-/-}$ ($p=0.02$). There was a trend for an acute hypoxic challenge to lower cerebral blood flow in trained $VEGF^{f/f}$ mice ($p=0.08$). The mice were unable to maintain O_2 saturation during the hypoxic challenge (O_2 saturation: untrained $VEGF^{f/f}$, 77.2 ± 3.4%; trained $VEGF^{f/f}$, 79.2 ± 4.2%; untrained $VEGF^{HSA-/-}$, 79.1 ± 2.4%; trained $VEGF^{HSA-/-}$, 80.6 ± 3.0%).

Confocal analysis of proliferating neuronal precursor cells

In order to measure the potential for exercise and skeletal muscle VEGF to stimulate hippocampal neurogenesis, proliferating neuronal precursor cells were quantified. The $VEGF^{f/f}$ mice showed a significant increase (Figure 5) in proliferating neuronal precursor cells with training in both means of quantification: Nes+/BrdU+ co-localized cells per total Nes+ cells and Nes+/BrdU+ co-localized cells per total DAPI+ cells ($p<0.05$). The proliferating neuronal precursor cell training response was absent in $VEGF^{HSA-/-}$ mice and both genotypes of untrained mice.

Endothelial changes in hippocampal vasculature

In addition to analyzing functional blood flow changes, vasculature changes were assessed by measuring the number of proliferating endothelial cells. Training did not reveal a change in endothelial cell proliferation in the $VEGF^{f/f}$ groups. Likewise, there was no training response in the $VEGF^{HSA-/-}$ mice and endothelial cell proliferation was not different between genotypes (Figure 6).

Hippocampal VEGF levels in response to an acute exercise bout in untrained mice

Although neurogenesis is a phenomenon that occurs with chronic exercise, we sought to document the effect of skeletal myofiber VEGF ablation on hippocampal VEGF levels at rest and in response to an exercise bout. $VEGF^{f/f}$ mice were run at 33 cm/sec or 40 cm/sec for one hour. $VEGF^{HSA-/-}$ mice could not maintain a speed of 40 cm/sec for the one-hour period and as a result hippocampal VEGF levels at only 33 cm/s were collected in the $VEGF^{HSA-/-}$ mice (Figure 7). The $VEGF^{f/f}$ mice ran at 40 cm/sec showed an increase ($p<0.05$) in hippocampus VEGF levels (19.70 ± 1.55 pg/μg) above the $VEGF^{f/f}$ rest group (11.45 ± 1.56 pg/μg). Neither the $VEGF^{HSA-/-}$ mice (14.62 ± 2.50 pg/μg) nor the $VEGF^{f/f}$ mice (14.05 ± 1.23 pg/μg) ran acutely at 33 cm/s showed an increase in hippocampal VEGF from their genotype respective resting group (Rest $VEGF^{HSA-/-}$, 11.11 ± 1.77 pg/μg).

Discussion

In this study we found, as reported by several other laboratories (van Praag *et al.*, 1999; Fabel *et al.*, 2003; Yang *et al.*, 2015), that voluntary wheel running for two weeks increases the number of neuronal precursor cells in the dentate gyrus of the hippocampus. The new findings in the present study are 1. That VEGF expressed by peripheral skeletal myofibers is necessary for this initial step in the mechanism to enhance the production of new neuronal cells in response to exercise; 2. Inhibition of VEGF expression in skeletal myofibers is accompanied by reduced blood flow to the hippocampus and this effect is similar in both untrained and trained mice; 3. Blood flow changes cannot be attributed to a change in the number of vessels in this region (based on our CD31+ results); 4. Resting VEGF levels in the hippocampus are not affected by induced VEGF knockout in skeletal myofibers; 5. A bout of acute exercise increases hippocampal VEGF levels in $VEGF^{f/f}$ mice, but not $VEGF^{HSA-/-}$ mice; 6. A hypoxic challenge with 10% inspired O_2 lowers cerebral blood flow in trained and untrained $VEGF^{HSA-/-}$ mice and trained $VEGF^{f/f}$ mice from normoxia.

Thus, this study supports the work of Fabel et al suggesting the peripheral VEGF originating from outside the blood brain barrier is essential for the cerebral neurogenesis in response to exercise training (Fabel *et al.*, 2003), and expands upon it by showing that skeletal myofiber VEGF appears to be the key source.

Skeletal myofiber VEGF is required to signal neuronal precursor cell proliferation

The main finding of this study is that VEGF expressed by skeletal myofibers is essential for increasing the number of neuronal precursor cells, as defined by the proliferation marker, bromo-deoxyuridine, and the neuronal precursor cell marker, nestin. VEGF is 1. a short lived protein (~ 1 h) (Levy *et al.*, 1996), 2. transiently increased with an exhaustive exercise bout (Breen *et al.*, 1996; Tang *et al.*, 2010), and 3. is not differentially expressed in the hippocampus following chronic exercise training (Inoue *et al.*, 2015). These findings support the possibility that bioactive VEGF may be produced by skeletal myofiber, enter the circulation and act to stimulate proliferation of neuronal precursor cells in the hippocampus. Alternatively, skeletal myofiber expressed VEGF may be important for the release or activation of additional exercise-responsive factors, metabolites or signaling of skeletal muscle afferent nerve activity that indirectly play a role in signaling cerebral neurogenesis. These direct and indirect mechanisms would both be in agreement with the study of Fabel et al. in which VEGF blocking antibodies administered peripherally, and thought to be too large to cross the blood-brain barrier, prevented exercise induced-neurogenesis (Fabel *et al.*, 2003). However, it is yet to be determined whether skeletal myofiber VEGF is transported across the blood-brain barrier and directly interacts with the neuronal precursor cell or has an indirect effect on blood flow or metabolism to signal cell proliferation. Alternatively, integrated functions signaled by VEGF in exercising skeletal myofiber may play a role in allowing exercise tolerance levels to achieve a threshold that stimulates cerebral VEGF expression or other factors that promote neuronal precursor cell proliferation in the hippocampus. $VEGF^{HSA-/-}$ mice could not acutely exercise at a sufficient intensity to increase VEGF expression in the hippocampus. Given the transient half-life of the VEGF protein, it will take further labeling studies to distinguish these possibilities. Furthermore, whether the mechanism of

increased neuronal precursor cells in response to exercise begins at a neural stem cell or progenitor cell is not known (Overall *et al.*, 2016) and future studies will be necessary to determine how many neuronal precursor cells commit to a neuronal cell linage and form functional neurons (Laplagne *et al.*, 2006; Vivar *et al.*, 2012; Deshpande *et al.*, 2013). Peripheral VEGF originating from outside the blood brain barrier is essential for the cerebral neurogenesis in response to exercise training (Fabel *et al.*, 2003), and the present study expands upon this observation by showing that skeletal myofiber VEGF may be the key source.

Voluntary wheel running parameters similar among groups

In this study two weeks of voluntary wheel running was used to exercise train the mice (van Praag *et al.*, 1999; Fabel *et al.*, 2003). Angiogenesis and neurogenesis have been reported to occur in the skeletal muscle and hippocampus, respectively, after just 7 days (Fabel *et al.*, 2003; Waters *et al.*, 2004). Furthermore, voluntary exercise does not necessarily stimulate stress or the release of stress hormones that could influence cerebral plasticity as has been reported for forced treadmill exercise (Arida *et al.*, 2004). One initial concern with the voluntary mode of exercise is that the experimental mouse groups may not run the same amount and thus receive different exercise stimuli than the control group. Mice were monitored for the daily and cumulative time, duration and speed that they spent on the running wheels. The daily and cumulative average for all parameters in the myofiber VEGF knockout mice were not different than the $VEGF^{f/f}$ mice, therefore the $VEGF^{HSA-/-}$ mice received at least a comparable training dose as the $VEGF^{f/f}$ mice.

Integrated cerebral and skeletal muscle adaptations to exercise training

Although the voluntary training parameters of running distance, time and speed between mice that do or do not express normal skeletal myofiber VEGF levels were not different and even tended to be greater in skeletal myofiber VEGF deficient mice, the training adaptations were clearly different. In tests for maximal running speed and endurance on a treadmill, the control group ($VEGF^{f/f}$) showed improvements. In contrast skeletal myofiber VEGF deficient mice were not able to improve their exercise performance. These findings are similar to previous studies in our laboratory in which mice were exercise trained using forced treadmill exercise (Delavar *et al.*, 2014). Thus, while the mice were not limited in their daily activity and frequently went on and off the running wheels, when pushed to their limits a difference in exercise tolerance was revealed. Whether this is due to an insufficient central or peripheral blood flow in response to exercise or some other possibly neurogenesis limiting factor is unknown. The neuronal importance of an exercise training response is highlighted in findings by Nokia et al (Nokia *et al.*, 2016) that demonstrate that hippocampal neurogenesis after exercise training is increased in rats selectively bred for a high exercise performance response to aerobic training. Koblio et al (Kobilo *et al.*, 2011) reported that when factors known to be enhanced with endurance exercise, peroxisome proliferator-activated receptor delta and AMPK kinase, are activated with the peripheral agonists, GW501516 and AICAR, respectively, in rodents that spatial memory can be improved and neurogenesis detected in the dentate gyrus. The latter finding is especially true for the metabolic regulator,

AICAR. In skeletal muscle peroxisome proliferator-activated receptor delta and AMPK regulate VEGF expression at the transcriptional and post-transcriptional level, respectively, and as a result VEGF from skeletal muscle could be an intermediate in the cerebral changes induced by these metabolic agonists (Ouchi *et al.*, 2005; Wang *et al.*, 2006). Whether changes in cellular metabolism are taking place in both the peripheral skeletal myofibers and cells located in the dentate gyrus or these are indirect effects of changing muscle metabolism will require further investigations.

Cerebral blood flow requirement for exercise-induced cerebral neurogenesis

An additional adaptation to exercise training, with respect to the oxygen transport system, is to augment vascular structure and function. Skeletal muscle angiogenesis allows more O_2 and nutrients to be available for an increased number of mitochondria in trained muscle and this is accompanied by greater speed and endurance exercise capacities, and appears to be VEGF-dependent (Olfert *et al.*, 2009; Delavar *et al.*, 2014; Tang *et al.*, 2015). Regional blood flow is also known to increase in the dentate gyrus, CA3 region and hippocampal fimbria with treadmill training (Holschneider *et al.*, 2007). VEGF-dependent changes in vascular function that allow enhanced flow through arteries and perfusion of the muscle capillary bed during exercise may occur through nitric oxide or other metabolic signaling pathways (Tschakovsky *et al.*, 1996; Thomas *et al.*, 2003; Lee-Young *et al.*, 2009). In the present study VEGF-dependent control of perfusion through capillary beds appears to extend beyond locomotor skeletal muscles (Knapp *et al.*, 2016) and influence or regulate perfusion of the hippocampus. Blood flow through the hippocampal region at rest under normoxic conditions was 36% and 40% lower in untrained and trained myofiber VEGF null mice relative to untrained $VEGF^{f/f}$ mice. This finding of reduced hippocampal perfusion in myofiber VEGF null mice suggests that poor perfusion at rest or during exercise, may be a contributing factor in the unresponsiveness of mice with conditional, skeletal myofiber VEGF gene deletion to signal neurogenesis in response to exercise training. In the current study this change in blood flow is not accompanied by a change in the number of new endothelial cells (CD31+/BrdU+), a marker of angiogenesis (Zhang *et al.*, 2014), in the dentate gyrus with skeletal myofiber VEGF gene deletion or exercise training. However, previous studies have found hippocampal vascular density to increase with exercise training for a longer duration (Clark *et al.*, 2009). Furthermore, it has been reported that mice with HIF-1α gene inactivation exhibit both regression of cerebral vasculature and loss of the nestin-expressing neural stem cells (Li *et al.*, 2014). The present study suggests that adequate skeletal myofiber VEGF is necessary to maintain resting dentate gyrus blood flow. Further studies will be required to assess the precise changes in hippocampal regional perfusion during an acute exercise bout.

A hypoxic challenge was used to stimulate an additional metabolic stress that could potentially occur during exercise. Untrained $VEGF^{f/f}$ mice and both untrained and trained $VEGF^{HSA-/-}$ mouse groups had significant, substantial decreases in blood flow that ranged from 57 to 80 percent with a hypoxic challenge. This reduction in cerebral blood flow under the time-period of low oxygen in our protocol is a predicted consequence of the fall in pCO_2 or hypocapnia (Duong, 2007). Interestingly, the trained $VEGF^{f/f}$ group did not

show a significant decrease in cerebral blood flow with the hypoxic challenge. Animal and human studies have found VEGF levels to be increased in the brain in response to chronic hypoxia and exercise (Dombrowski *et al.*, 2008; Tang *et al.*, 2010; Yang *et al.*, 2016). Thus, additional training effects from skeletal myofiber VEGF may attenuate this decrement in cerebral blood flow under hypoxic conditions.

Summary

This study has established that VEGF expressed by skeletal muscle myofibers is important for increasing the number of neuronal precursor cells in the hippocampus in response to exercise training. This has many implications for chronic diseases that exhibit impaired cognitive function. While there are many metabolites and growth factors that could play a role in the exercise response, this study suggests that adequate blood flow to the hippocampus is associated with the neurogenesis response. It also provides support for developing therapeutic strategies in which neurogenesis factors delivered through a peripheral or myofiber route work in conjunction with exercise to enhance neurogenesis and spatial memory. Identifying the precise factors that are diminished with age or altered with specific neurodegenerative disease would facilitate such an approach.

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Competing Interests

The authors have no competing interests.

Author Contributions

BR exercise trained the mice, collected the fMRI, histology and gene expression measurements and prepared the manuscript. MS performed and analyzed the fMRI measurements of cerebral blood flow. MY provided the Nestin-GFP mice. PDW assisted with the research design and manuscript preparation. ECB designed the experiments, assisted in data collection and manuscript preparation.

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Tables

Table 1. Body Weights

Group	Day 0 (g)	Day 21 (g)	Day 36 (g)
Untrained $VEGF^{f/f}$	22.8 ± 0.6	23.5 ± 0.5	25.3 ± 1.6
Trained $VEGF^{f/f}$	24.0 ± 0.7	25.3 ± 0.7	24.6 ± 0.5
Untrained $VEGF^{HSA-/-}$	20.7 ± 0.7	22.0 ± 0.8	22.0 ± 0.7*
Trained $VEGF^{HSA-/-}$	22.8 ± 0.7	24.5 ± 0.6	24.5 ± 0.6

Data is mean ± SEM *denotes difference from untrained $VEGF^{f/f}$ condition on day 36 ($p<0.05$)

Figure Legends

Figure 1. Skeletal muscle VEGF levels in skeletal myofiber VEGF gene deleted mice are not recovered by exercise training. VEGF levels in the plantaris were measured by ELISA. Data are expressed as the mean ± SEM, n=4-5.

Figure 2. Control and skeletal myofiber VEGF-deficient mice voluntarily run for similar daily distance and time over a two-week period. The distance (km), time (min.) and speed (cm/s) that each mouse in the $VEGF^{f/f}$ or $VEGF^{HSA-/-}$ group voluntary ran on running wheels was recorded each day. No differences were observed between the groups. Data are expressed as the daily mean ± SEM, n=5-7 mice per group.

Figure 3. Skeletal myofiber VEGF is essential for improving endurance capacity in response to voluntary exercise training. Mice were given access to running wheels (**Trained**) or cage-confined without wheels (**Untrained**) for two weeks. Endurance and maximum speed was measured on a treadmill before (**Pre-Training**) and after the training period (**Post-Training**). The endurance data are presented as the time (in minutes) before the mice reached exhaustion when run at 33 cm/s. Maximum speed data is presented as the highest speed (cm/s) the mice achieved. Data are expressed as the mean ± SEM, n=4-8. The effects of genotype group, time (two-week interval) and interaction were measured with a two-way ANOVA. *Significant effect of training in the $VEGF^{f/f}$ mice compared to the other trained (Post-Training) groups, $p<0.01$.

Figure 4. Cerebral blood flow (CBF) to the hippocampus is decreased by inhibition of skeletal myofiber expressed VEGF independent of the exercise training state. *(A-D)* Representative images of cerebral blood flow generated with MRI detected by continuous arterial spin labeling in a coronal section in *(A)* untrained $VEGF^{f/f}$, *(B)* trained $VEGF^{f/f}$, *(C)* untrained $VEGF^{HSA-/-}$ or *(D)* trained $VEGF^{HSA-/-}$ anesthetized mice breathing 21% oxygen. Scale is 0-250 ml/min. *(E)* Blood flow in the hippocampal region computed

as ml/min averaged over 6 minutes in anesthetized mice spontaneously breathing 21% oxygen followed by 10% oxygen. Data are expressed as the mean ± SEM, n=4-8. *indicates a difference with 21% O_2 within the same group, $p<0.05$. # indicates a difference from untrained $VEGF^{f/f}$ under 21% oxygen, $p<0.05$.

Figure 5. Increased number of nestin positive (Nes+) cells in $VEGF^{f/f}$, but not $VEGF^{HSA-/-}$ mice, following two weeks of voluntary exercise training. (*A-H*) Confocal images of the dentate gyrus sections revealing DAPI, BrdU and Nestin positive cells. (*I*) BrDU+/Nes+ cells per number of Nes+ cells. (*J*) BrDU+/Nes+ cells per total DAPI labeled cells. Data are expressed as the mean ± SEM, n=6-8. * Indicates a significant difference between $VEGF^{f/f}$-trained and $VEGF^{HSA-/-}$ trained, $p<0.05$. # Indicates a significant difference between $VEGF^{f/f}$-trained and the other three groups, $p<0.01$. (*A-D*) scale bar = 50 µm. (*E-H*) scale bar = 10 µm.

Figure 6. Skeletal myofiber VEGF gene deletion or exercise training does not alter the number of CD31 positive capillaries (*A-D*) Confocal images of the dentate gyrus sections revealing DAPI, BrdU and CD31 positive cells. (*E*) BrDU+/CD31+ cells per number of CD31+ cells. (*F*) BrDU+/CD31+ cells per total DAPI labeled cells. Data are expressed as the mean ± SEM, n=5-8. No statistical differences were observed between the experimental groups. Scale bar = 50 µm.

Figure 7. VEGF levels in the hippocampus in response to an acute exercise bout on the treadmill. $VEGF^{f/f}$ and $VEGF^{HSA-/-}$ mice were subjected to a 1-hour exercise session on the treadmill. The $VEGF^{f/f}$ could easily maintain a speed of 33 cm/sec for 1 hour. The $VEGF^{HSA-/-}$ had trouble completing the test. An additional group of $VEGF^{f/f}$ mice were run at a higher speed of 40 cm/sec (approximately 60% of the average maximal speed for this group). VEGF levels were measured in the hippocampus dissected one hour after the exercise session. Data are expressed as the mean ± SEM, n=6-14. * indicates a difference between $VEGF^{f/f}$ mice run at 40 cm/sec and the $VEGF^{f/f}$ rest group, $p<0.05$.