Experimental Gerontology 87 (2017) 57–66

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Impact of β-hydroxy β-methylbutyrate (HMB) on age-related functional deficits in mice

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ARTICLE INFO

Article history:
Received 27 June 2016
Received in revised form 10 November 2016
Accepted 21 November 2016
Available online 23 November 2016

Section Editor: Christiaan Leeuwenburgh

Keywords:
Aging
Mesenchymal stem cells
Nutrition
Cognition
Neurogenesis

ABSTRACT

β-Hydroxy β-methylbutyrate (HMB) is a metabolite of the essential amino acid leucine. Recent studies demonstrate a decline in plasma HMB concentrations in humans across the lifespan, and HMB supplementation may be able to preserve muscle mass and strength in older adults. However, the impact of HMB supplementation on hippocampal neurogenesis and cognition remains largely unexplored. The purpose of this study was to simultaneously evaluate the impact of HMB on muscle strength, neurogenesis and cognition in young and aged mice. In addition, we evaluated the influence of HMB on muscle-resident mesenchymal stem/stromal cell (Sca-1^+CD45^-; mMSC) function to address these cells potential to regulate physiological outcomes. Three month-old ($n = 20$) and 24 month-old ($n = 18$) female C57BL/6 mice were provided with either Ca-HMB or Ca-Lactate in a sucrose solution twice per day for 5.5 weeks at a dose of 450 mg/kg body weight. Significant decreases in relative peak and mean force, balance, and neurogenesis were observed in aged mice compared to young (age main effects, $p \leq 0.05$). Short-term HMB supplementation did not alter activity, balance, neurogenesis, or cognitive function in young or aged mice, yet HMB preserved relative peak force in aged mice. mMSC gene expression was significantly reduced with age, but HMB supplementation was able to recover expression of select growth factors known to stimulate muscle repair (HGF, LIF). Overall, our findings demonstrate that while short-term HMB supplementation does not appear to affect neurogenesis or cognitive function in young or aged mice, HMB may maintain muscle strength in aged mice in a manner dependent on mMSC function.

1. Introduction

Cognitive impairment is frequently observed in older adults, and the occurrence of neurodegenerative disease increases dramatically with age. As of 2010, approximately 2.3 million people between the ages of 75–84 are estimated to suffer from Alzheimer's Disease with that number expected to double by 2030 (Herbert et al., 2013). The mechanisms behind age-related cognitive impairment are varied including increased neuroinflammation, significant reductions to hippocampal volume, and a lack of new neuron formation in late adulthood (Barrientos et al., 2010; Kuhn et al., 1996; Lucassen et al., 2010; Wynne et al., 2009). Concurrent with neurodegeneration is a substantial age-related loss of skeletal muscle mass and strength (sarcopenia), which is estimated to affect upwards of 50% of individuals 80 years or older (von Haehling et al., 2010). The onset of these physical and cognitive impairments with age can significantly decrease an individual's independence and quality of life (Janssen et al., 2004). Therefore, novel rehabilitation or nutritional strategies must be identified and implemented to treat and prevent age-related disabilities.

β-Hydroxy β-methylbutyrate (HMB), a metabolite of the essential amino acid leucine, can attenuate the loss of skeletal muscle mass associated with prolonged bedrest or immobilization in older adults and aged rats (Alway et al., 2013; Deutz et al., 2013). HMB also prevents dexamethasone-induced myotube atrophy *in vitro* (Aversa et al., 2012) as well as attenuates the decrease in protein synthesis observed with cancer cachexia in mice (Eley et al., 2007). HMB supplementation can preserve lean body mass and improve muscle function in both older adults and aged rodents (Stout et al., 2013; Vallejo et al., 2016; Vukovich et al., 2001; Wilson et al., 2012; Wilson et al., 2014). Overall, the majority of studies suggest at least some benefits of HMB supplementation in the preservation of muscle function and/or lean body mass across the lifespan (Rowlands & Thomson, 2009; Wu et al., 2015). While the impact of HMB on skeletal muscle is most often investigated, minimal information exists regarding the effect of HMB on neurogenesis and cognition. Recently Salto et al. (2015) demonstrated the ability for HMB to increase neurite outgrowth in mouse neuroblastoma Neuro2a

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http://dx.doi.org/10.1016/j.exger.2016.11.010
0531-5565/

cells *in vitro*, and work by Kougias et al. (2016) demonstrated that long-term HMB supplementation preserves basilar dendritic size in the medial prefrontal cortex of aged rats, which may account for improvements in working memory (Hankosky et al., 2016). Further studies are necessary to investigate the impact of HMB supplementation on cognitive function in the context of aging, particularly given recent results that demonstrate a decline in endogenous plasma HMB concentration with age (Shreeram et al., 2016).

Mesenchymal stem/stromal cells (MSCs) reside in the interstitium of multiple tissues throughout the body, including skeletal muscle. Our lab has identified a specific mononuclear cell fraction in muscle (Sca-1$^+$CD45$^-$) that possess an MSC signature (CD90$^+$CD105$^+$-CD31$^-$CD34$^-$) as well as a capacity for multi-lineage differentiation (Valero et al., 2012). MSCs derived from muscle (mMSCs) are largely non-myogenic based on a lack of expression for Pax7, MyoD, and Myf5, and provide an important stromal role in satellite cell activation and myofiber repair (Valero et al., 2012; Zou et al., 2015). Despite the established role for mMSCs in the positive regulation of muscle repair and function in young mice, minimal information exists with regard to mMSC stromal capacity in aged mice. Our lab recently demonstrated that collagen significantly reduces mMSC capacity for growth factor synthesis and satellite cell activation *in vitro* (De Lisio et al., 2014). Therefore, it is reasonable to speculate that mMSC function may be compromised as a result of the collagen accumulation observed in the aged skeletal muscle niche (Alnaqeeb et al., 1984), and that these events may adversely impact the capacity for aged skeletal muscle to repair and grow in response to physiological stimuli.

The purpose of the current study was to simultaneously evaluate the impact of HMB supplementation on muscle strength, neurogenesis, and cognition in young and aged mice. The specific impact of HMB on mMSC function was assessed both *in vivo* and *in vitro*. We hypothesized that HMB would beneficially impact both muscle strength and cognition in mice, and that the observed changes would correlate with improvements in mMSC function.

2. Methods

2.1. Animals

Three month-old (Young) and 24 month-old (Aged) female C57BL/6 mice were obtained from Charles River and the National Institute on Aging (NIA), respectively. Animals were fed standard laboratory chow and had access to water *ad libitum*. Animals were housed in pairs (2 mice per cage) in a pathogen-free animal room under controlled conditions (12-hour light/dark cycle, 25 °C). Animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Urbana-Champaign, and all National Institute of Health guidelines for the care and use of animals were followed.

2.2. HMB supplementation

The experimental group was supplemented with a 10% sucrose solution containing Ca-HMB (Abbott Industries, Champaign, IL). The control group was supplemented with a 10% sucrose solution containing an equal concentration of calcium in the form of Ca-Lactate (Fisher Scientific, Pittsburgh, PA). Supplementation occurred twice per day (at the beginning and end of the light cycle) for approximately 5.5 weeks. A 5.5 week experimental duration was chosen in order to accommodate the time frame for new neuron formation and integration within the adult brain (Aimone et al., 2014), allowing for simultaneous evaluation of the impact of HMB supplementation on neurogenesis, cognition, and skeletal muscle function. Animals received an appropriate volume of solution for a final dosing concentration of 450 mg/kg body weight/dose of Ca-HMB or Ca-Lactate (Baxter et al., 2005; Wilson et al., 2012). The solution was delivered *via* a modified 10 mL serological pipette fitted with a double ball bearing sipper tube, as described previously (Bulwa et al., 2011). Mice were divided into four groups based on body weight prior to the start of the experiment: Young + Control (Y + C, n = 10), Young + HMB (Y + H, n = 10), Aged + Control (A + C, n = 9), Aged + HMB (A + H, n = 9).

2.3. Body weight and grip strength measures

Each animal was weighed prior to the experiment and weight was recorded weekly thereafter. Four-limb grip strength was measured weekly using a Digital Grip Strength Meter (Columbus Instruments, Columbus, OH). For grip strength measures, mice were placed upon a metal mesh grid connected to a force transducer and were allowed to grip with all four limbs. After gripping was confirmed, the researcher gently pulled the mouse's tail posteriorly, parallel to the grid at a constant rate until volitional release. Force was recorded (gram-force) for three trials. Weight measures obtained immediately after each test was used to express peak and mean grip strength relative to weight (gram force (g)/gram body weight (g)). The same tester performed all trials.

2.4. Home cage activity monitoring

As described previously (Zombeck et al., 2011), animals were transferred from their regular housing into clear acrylic areas (18.5 × 33.5 × 16.0 cm) covered with a clear lid for video tracking over 5 days. Food and water was provided on the side of the cages to allow the mouse to remain visible in all areas of the cage. Ceiling mounted video cameras interfaced with two computers using TopScan video tracking software (Clever Sys Inc., Reston, VA), and continuously monitored the distance traveled within the cage (red light was used during the dark cycle for continuous video tracking). Total distance (meters) traveled per day was recorded.

2.5. Rotarod

An automated rotarod unit (AccuRotor RotaRod Tall Unit, 63 cm fall height, 30 mm diameter rotating dowel; Accuscan, Columbus, OH) was used to assess balance and coordination. Mice were placed on the stationary dowel which then began acceleration at a rate of 60 rpm/min. Latency to fall was recorded using a photobeam which stopped the timer automatically when the falling mouse broke the plane of the photobeam. Each mouse performed four consecutive trials for three consecutive days.

2.6. Novel object recognition

Mice were placed in the testing arena (50 × 38 × 20 cm) for 10 min of habituation on Day 1. On Day 2, mice were placed in the arena with two identical objects and allowed to explore the objects for 30 s before being returned to their home cages. On Day 3, mice were placed in the arena with one familiar object and one completely novel object. The mice had 5 min to explore the arena. Mice were video monitored using TopScan software to measure time spent sniffing each object.

2.7. Passive avoidance

On Day 1 (acquisition), mice were placed in the illuminated compartment of a Gemini™ Avoidance System (SD Instruments, San Diego, CA) and allowed to explore both illuminated and non-illuminated compartments *via* a raised guillotine door. Once the animal completely entered the non-illuminated compartment, the door was automatically closed and an electric foot shock was delivered (0.5 mA, 4 s duration). After 10 s, the chamber was illuminated and the guillotine door was raised allowing the mouse access to the non-illuminated chamber. Trials continue until the animal avoids the non-illuminated compartment for 120 s. On Day 2 (retention), mice were placed in the

illuminated compartment and the latency time for entering the non-illuminated compartment was recorded (maximum latency time of 300 s).

2.8. Tissue collection and preservation

Prior to euthanasia, puromycin dihydrochloride (EMD Millipore, Billerica, MA) was injected intraperitoneally (0.04 μmol/g body weight) to assess protein synthesis (Goodman et al., 2011). Thirty minutes post-injection, mice were fully anesthetized using isoflurane, and gastrocnemius-soleus muscle complexes were excised and weighed. One complex was immediately frozen in precooled isopentane, while the other complex was immersed in 1× PBS plus penicillin/streptomycin, and used immediately for mMSC isolation. Following excision of gastrocnemius-soleus complexes, animals were taken off isoflurane and anesthetized with 200 mg sodium pentobarbital/kg body weight. Brains were subsequently perfused transcardially with 4% paraformaldehyde in 1× PBS and fixed overnight. Fixed brains were transferred to a 30% sucrose (in 1× PBS) solution and frozen for further histological analysis.

2.9. Sca-1$^+$CD45$^-$ cell isolation and FACS

Sca-1$^+$CD45$^-$ cell isolation was performed as previously described (Huntsman et al., 2013; Valero et al., 2012). Briefly, muscle tissue was minced and enzymatically digested for 45–60 min at 37 °C. The enzyme solution consisted of 0.2–0.3% collagenase Type 2 (Worthington, Biochemical Corp., Lakewood, NJ), 60 U/mL DNase (Sigma-Aldrich, St. Louis, MO) and 2.5 mM $CaCl_2$ in a 1× PBS solution. Mononuclear cells were isolated *via* filtration using 70 μm filters (Fisher Scientific). Filtered samples were then incubated for 10 min on ice with anti-mouse CD16/CD32 (eBioscience, San Diego, CA) to block Fc-mediated nonspecific interactions. Following blocking, cells were incubated with a mix of monoclonal anti-mouse antibodies, Sca-1-phyocerythrin (PE) and CD45-allophycoctanin (APC) (anti-Sca-1-PE, 600 ng/10^6 cells and anti-CD45-APC, 300 ng/10^6 cells, eBioscience), diluted in filtered 2% FBS in 1× PBS for 1 h on ice. Following antibody staining, cells were washed in 2% FBS and filtered through a 40 μm filter before fluorescence-activated cell sorting (FACS). Negative and single-stained controls were used to establish gates for FACS, which was performed using an iCyt Reflection System located at Carle Hospital's Biomedical Research Center (Urbana, IL). Sca-1$^+$CD45$^-$ cells were either collected in mMSC-specific growth media (high glucose Dulbecco's Modified Eagle's Medium (DMEM), 10% FBS, 5 μg/mL gentamycin) for *in vitro* experiments or collected and lysed with Buffer RLT (Qiagen, Valencia, CA). Lysed samples were stored at −80° to be analyzed for relative mRNA expression.

2.10. In vitro incubation of MSCs with HMB

Sca-1$^+$CD45$^-$ cells were extracted and sorted (as described above) from 5 week-old SJL/C57BL6 mixed background wild-type mice from our own breeding colony. Post-sorting, cells were seeded on culture dishes (2.5×10^4 cells/cm^2) and incubated at 37 °C and 5% CO_2 with growth media which was changed every 3–4 days. Upon reaching confluency, cells were dissociated using Accutase (ThermoFisher, Waltham, MA), then seeded on 6-well plates at a density of 100,000 cells/well. Once confluent, cells were incubated with serum-free growth media (DMEM) for 3 h. Media was removed and cells were incubated with 0, 50, or 100 μg HMB/mL DMEM for 3 h. HMB was supplied in the form of β-hydroxisovaleric acid from Sigma Aldrich (Saint Louis, MO). At the end of each time point, cells were lysed with Buffer RLT (Qiagen) and lysates were snap frozen in liquid nitrogen to be analyzed for relative mRNA expression.

2.11. RNA isolation and cDNA synthesis

RNA was extracted from cell lysates using RNeasy Micro Kit (Qiagen), following the manufacturer's instruction. Quantity of isolated RNA was assessed in duplicate on a Take-3 application plate using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT). Starting RNA concentration of at least 15 ng (and up to 250 ng) was used to perform reverse transcription *via* the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY) per manufacturer's instructions.

2.12. High throughput microfluidics qPCR using Fluidigm Biomark™ HD

High throughput microfluidic quantitative PCR (qPCR) of synthesized cDNA was run on a 96 × 96 Dynamic Array Integrated Fluidic Circuit (IFC) (Fluidigm, San Francisco, CA) by the Functional Genomics Unit at the Roy J. Carver Biotechnology Center (Urbana, IL). This qPCR system results in a more accurate and repeatable analysis of multiple genes within multiple samples (up to 96 genes analyzed in up to 96 samples). Diluted cDNA samples and primers were mixed together on a Dynamic Array Chip using the IFC system. The chip was then loaded into the Fluidigm Biomark™ HD for qPCR analysis. Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as the housekeeping gene, and relative mRNA expression was expressed relative to the Y + C group using the ΔΔCt method.

2.13. cDNA preamplification and quantitative PCR

Preamplification of cDNA was completed using TaqMan PreAmp Master Mix Kit (Life Technologies). The primer pool was composed of inventoried TaqMan primers, which were diluted in Tris-EDTA buffer to a final concentration of 0.2×. PreAmp reagent was mixed with the primer pool and sample cDNA in a thin-walled 0.2 mL PCR tube. Each reaction was amplified for 14 cycles using a thermocycler (ABI Geneamp 9700, Life Technologies) and then diluted in diethylpyrocarbonate (DEPC) RNase-free water. qPCR was performed using the 7900HT Fast Real-Time PCR System with TaqMan Universal PCR Master Mix (Applied Biosystems, Grand Island, NY). All genes were normalized to HPRT for *in vivo* analyses or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for *in vitro* analyses, and expressed relative to corresponding control condition. Gene expression data are presented using the ΔΔCt method with cycle threshold (Ct) replicate values within 0.5 Ct units. Inventoried TaqMan primers were purchased from Applied Biosystems. Primer information and gene expression assay ID numbers used in this study are provided in Supplementary Table 1.

2.14. Evaluation of protein expression

Muscle protein was manually homogenized in ice-cold Triton X extraction buffer (20 mM Hepes, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol (DTT), 1 mM sodium vanadate, 1% Triton X-100, 10% Glycerol) supplemented with 10 μM leupeptin, 3 mM benzamidine, 5 μm pepstatin A, 10 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate solution was further disrupted by occasional vortexing for 30 min on ice. Samples were then centrifuged at 15000 rpm for 15 min at 4 °C, and the supernatant was removed. Protein concentrations were quantified using the Bradford protein array with bovine serum albumin (BSA) used for the standard curve. Equal amounts of protein (30–50 μg) were separated by SDS-PAGE using 8–10% acrylamide gels and transferred onto nitrocellulose membranes. Ponceau S staining was used to verify equal loading of protein. Membranes were blocked in Tris-buffered saline (pH 7.8) with 0.1% Tween-20 (TBS-T) containing 5% BSA for 1 h, and incubated overnight at 4 °C with the following primary antibodies purchased from Cell Signaling Technology (Beverly, MA): phospho-Akt^{Ser473} (#4058S), Akt (#9272S), phospho-mTOR^{Ser2448} (#2971), mTOR (#2972), phospho-

p70S6 Kinase^{Thr389} (#9205), p70S6 Kinase (#9202). Following overnight incubation, membranes were rinsed with TBS-T and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Cell Signaling Technologies, Inc., Beverly, MA). Bands were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo-Scientific, Rockford, IL) and a Bio-Rad ChemiDoc XRS system (Bio-Rad, Hercules, CA). Quantity One software (Bio-Rad) was used to complete the quantification of bands.

2.15. Immunohistochemistry

Perfused brain were sectioned by cryostat into 40 μm-thick coronal sections and stored in 24-well plates containing tissue cryoprotectant (30% ethylene glycol, 25% glycerin, 45% PBS) at −20 °C. To identify immature neurons within the granular layer of the dentate gyrus, a one-in-six-series of rostral to caudal sections (separated by 240 μm increments) from each mouse were stained for DCX-DAB. Briefly, free-floating sections were treated with goat anti-DCX (1:1000, Santa Cruz Biotechnology, TX) primary antibody for 48 h at 4 °C. Following primary antibody incubation, sections were then incubated in biotinylated secondary antibody donkey anti-goat (1:200, Santa Cruz Biotechnology, TX) for 90 min. Post-secondary incubation, sections were washed and then treated with the ABC system for 60 min (Vector Laboratories, Burlingame, CA) and stained with a DAB kit (Sigma Aldrich). DCX-DAB images were acquired using a Zeiss AxioCam digital camera and Axiovision software (Zeiss, Thornwood, NY). To evaluate neurogenesis, the total number of DCX^+ cells per coronal section of the granular layer of the dentate gyrus was calculated using ImageJ software.

2.16. Statistical analysis

All data is presented as mean ± SEM. Two-way ANOVA followed by LSD post-hoc analysis was performed to determine HMB × Age interactions and main effects of age or HMB for all *in vivo* measures. Weekly grip strength and daily activity monitoring measures were assessed using repeated measures ANOVA followed by LSD post-hoc analysis. Spearman's rho was used to determine the correlation between gene expression/relative muscle weight and grip strength *in vivo*. For *in vitro* experiments, one-way ANOVA followed by LSD post-hoc analysis was used to determine HMB main effects on mMSC gene expression. Data was transformed to correct non-normal data when appropriate. All statistical analyses were performed with SPSS Ver. 22 (IBM, Chicago, IL). Differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Effect of HMB and age on body weight, muscle weight, and grip strength

Body weight was increased in aged mice compared to young (age main effect, $p < 0.01$) with no effect of HMB on body weight in young or aged cohorts (Fig. 1A). Absolute (data not shown) and relative gastrocnemius-soleus muscle weight was not affected by age or HMB supplementation (Fig. 1B). Relative peak grip strength showed a significant Time × Age interaction over the course of the study ($p = 0.04$) (Fig. 1C). Relative peak grip strength was significantly reduced in aged mice compared to young (age main effect, $p < 0.001$), but HMB prevented this reduction in relative peak force after 5.5 weeks of supplementation ($p = 0.05$) (Fig. 1D). Relative mean grip strength demonstrated a significant Time × Age ($p = 0.03$) and Time × HMB interaction ($p = 0.05$) (Fig. 1E). Relative mean grip strength was significantly reduced with age (age main effect, $p < 0.05$) and a trend was observed for HMB to increase mean force at the end of the supplementation period ($p = 0.06$) (Fig. 1F). The hypertrophic signaling pathway, including AKT (Fig. 2A), mTOR (Fig. 2B), and $p70^{S6K}$ (Fig. 2C) phosphorylation, total protein, and the subsequent ratio was evaluated, but no differences were detected between groups. Similarly, no changes in puromycin incorporation were observed with age or HMB supplementation (data not shown).

3.2. Effect of HMB on cognitive function in aged mice

The experimental design for supplementation and behavioral testing is presented in Fig. 3A. After 2 weeks of HMB supplementation, cognitive testing began and lasted for approximately 3.5 weeks with continued supplementation. To assess physical activity, locomotion was tracked daily over 5 days. The total distance traveled each day was not significantly different between groups (Fig. 3B). Cerebellar control of muscular balance and coordination was assessed using rotarod testing. The average time spent on the rotarod over the 3 days of testing was significantly reduced with age (age main effect, $p < 0.05$) and no recovery was observed with HMB supplementation (Fig. 3C). Neither fear-conditioned learning (passive avoidance) nor recognition-based learning (novel object recognition) was altered by age or HMB supplementation. Latency to cross during the passive avoidance test and percent time with novel object during the novel object test were unaffected by age or treatment (Fig. 3D–E). The total number of newly formed (immature) neurons, as measured by doublecortin positive (DCX^+) cells, was significantly reduced in the dentate gyrus of the hippocampus with age (age main effect, $p < 0.05$), but not affected by HMB (Fig. 3F).

3.3. mMSC function is blunted with age and partially restored with HMB supplementation

mMSC gene expression was evaluated using a Fluidigm system (37 genes). Table 1 presents mMSC relative mRNA expression in all groups and the respective statistical results. Cell surface marker gene expression was altered such that CD90 (MSC) and neural-glial antigen 2 (NG2; pericyte), gene expression was decreased with age, but HMB had no effect on relative expression. Additionally, a trend towards a decrease in platelet-derived growth factor α (PDGFRα) expression was noted with age ($p = 0.07$). Growth and neurotrophic factor gene expression was significantly downregulated with age, specifically the expression of LIF, HGF, IGF-1, VEGFa, EGF, BDNF, NGF, and FNDC5. Interestingly, an Age × HMB interaction was detected for HGF ($p = 0.02$), and HMB recovered LIF and HGF gene expression in aged mice. Several factors associated with remodeling of the extracellular matrix were also downregulated with age, including matrix metalloproteinase 2 and 14 (MMP-2 and MMP-14), collagen type I alpha-1 (Col1α1), plasminogen activator inhibitor-1 (PAI-1), and fibroblast-specific protein 1 (FSP-1), none of which were recovered with HMB. Lysyl oxidase (LOX) gene expression was increased with HMB supplementation in both young and aged mice. The proinflammatory cytokine tumor necrosis factor alpha (TNFα) gene expression was increased with age, while the anti-inflammatory protein RPL13a (Basu et al., 2014) was reduced with age.

A significant correlation was detected between relative peak force and HGF gene expression ($r = 0.46$, $p = 0.02$), but no correlation was observed between relative peak force and LIF gene expression ($r = 0.20$, $p = 0.35$) nor relative peak force and relative muscle weight ($r = 0.08$, $p = 0.63$).

3.4. HMB alters the mMSC gene expression profile in vitro

mMSCs isolated from skeletal muscle of young mice were incubated with HMB *in vitro* to determine the immediate response to HMB exposure. Thirty five different genes were evaluated and data are presented based on relevance to *in vivo* results (LIF, HGF, NGF) and/or putative involvement in muscle health (nerve repair, vascularization, immune function). LIF expression was significantly decreased with direct exposure to HMB ($p < 0.001$), whereas HGF and NGF remained unaltered

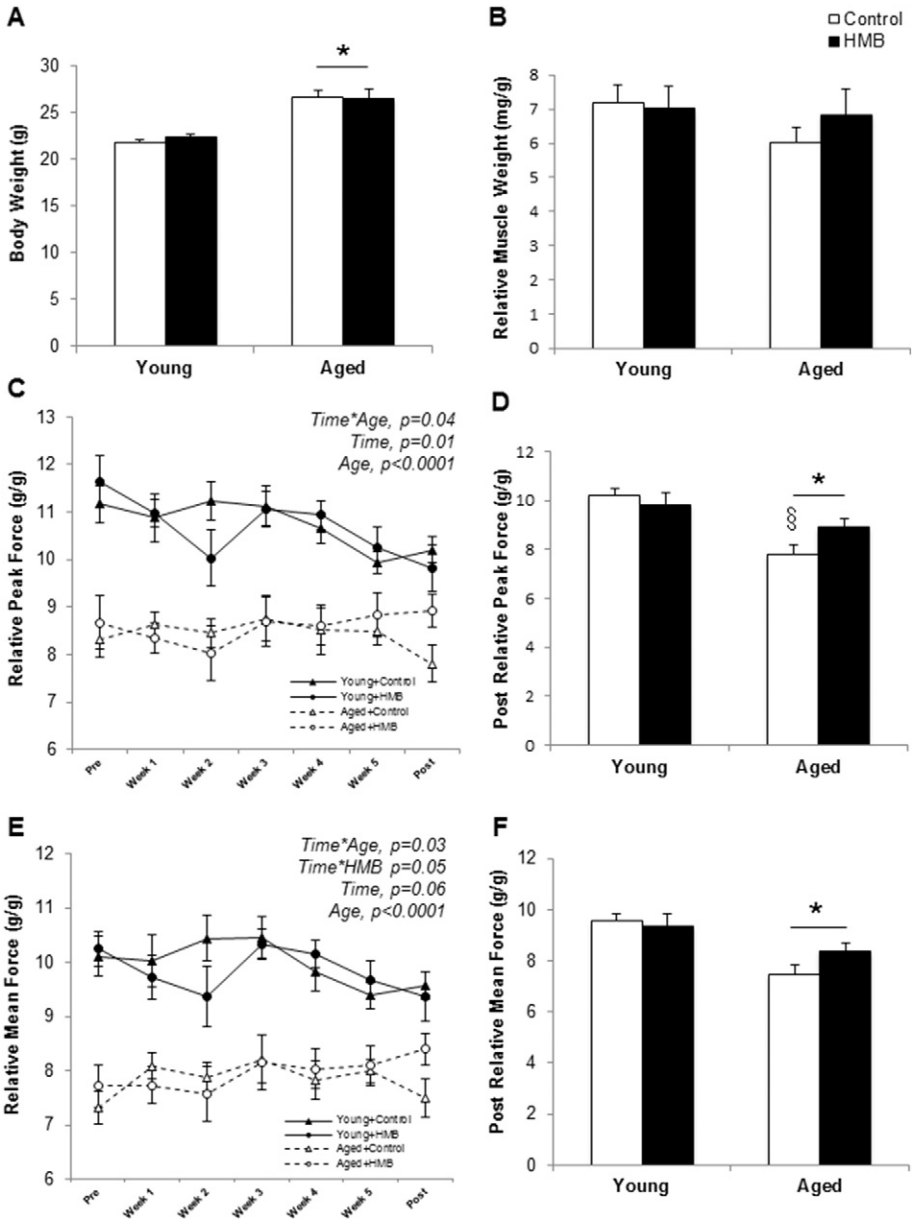

Fig. 1. Acute HMB supplementation does not affect body or muscle weight, but restores grip strength in aged mice. Body weight was increased in aged mice after 5.5-weeks of supplementation with no increase in gastrocnemius-soleus muscle weight (A and B). Both relative peak and mean grip strength were reduced in aged mice compared to young mice with HMB supplementation preserving strength in aged mice (C—F). Values are mean ± SEM. *$p < 0.05$, age main effect; §$p < 0.05$ compared to all other groups. n = 9–10 per group.

(Fig. 4A–C). Relative mRNA expression of EGF and neurotrophin-3 (NTF-3) was increased with HMB treatment *in vitro* ($p < 0.002$ and $p < 0.01$, respectively) (Fig. 4D–E), while the expression of angiogenin appeared to increase in a dose-dependent manner (not statistically significant) (Fig. 4F). In contrast to *in vivo* results, CCL2 gene expression was significantly decreased with HMB ($p < 0.01$), and a trend towards a decrease was noted for granulocyte macrophage colony-stimulating factor (GM-CSF) ($p = 0.12$) (Fig. 4G–H).

4. Discussion

The purpose of the current study was to evaluate the impact of HMB supplementation on muscle strength, neurogenesis, and cognition in young and aged mice. Reductions in strength, balance, and neurogenesis were detected in aged mice compared to young; however, the behavioral tests performed to evaluate cognition did not reveal any age-related impairment. While HMB supplementation did not provide any benefit to young mice nor mitigate the age-related declines in balance or neurogenesis, HMB did attenuate the small, but significant reduction to relative peak force observed in aged mice. Given the potential for muscle resident stem/stromal cells (mMSCs) to influence skeletal muscle repair and function (Huntsman et al., 2013; Valero et al., 2012; Zou et al., 2015), we evaluated the effect of HMB on mMSC gene expression both *in vivo* and *in vitro*. We demonstrate for the first time that mMSC gene expression is altered with age in a manner that suggests a marked decline in the ability for mMSCs to synthesize factors necessary to support skeletal muscle repair, maintenance, and turnover in aged mice. HMB supplementation did not substantially reverse the age-related changes to mMSC gene expression. However, preservation of LIF and HGF gene expression, as well as a positive correlation between HGF expression and relative peak force in aged mice supplemented with HMB, suggest the potential for HMB to improve nerve function or prime the muscle for repair in the event of injury. Thus, this data provide evidence of a novel role for mMSCs in initiating the health benefits of HMB.

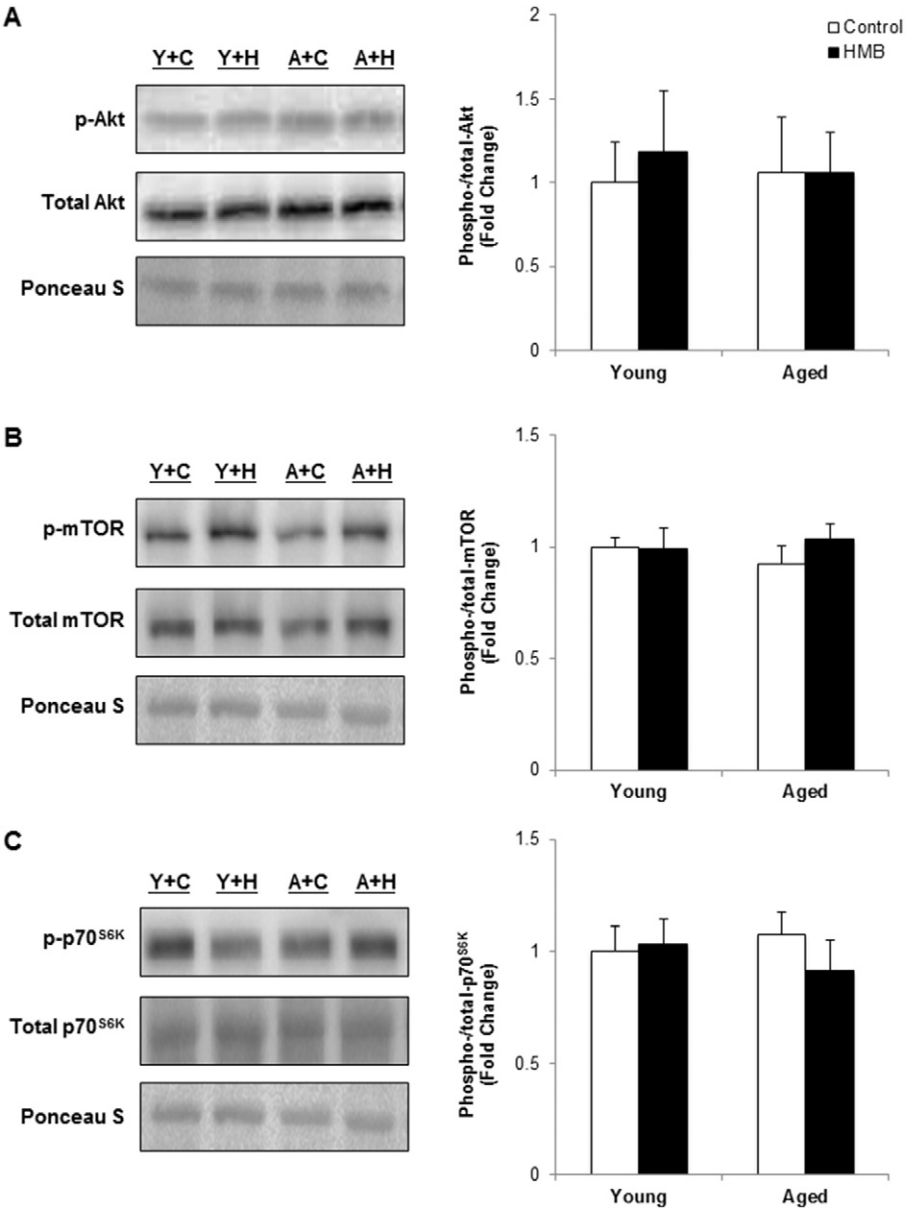

Fig. 2. Hypertrophic signaling in young and aged mice is unaffected by HMB supplementation. Representative immunoblots and summary of data for phospho-/total-Akt (A), mTOR (B), and $p70^{S6K}$ (C). All values are mean ± SEM. All proteins are normalized to Ponceau S and fold change is expressed relative to Y + C group. n = 9–10 per group.

4.1. HMB preserves skeletal muscle strength in aged mice independent of muscle weight or hypertrophic signaling

HMB can positively alter body composition and augment strength in humans when administered in combination with resistance exercise (Gallagher et al., 2000; Wilson et al., 2014). Our findings indicate that in the absence of any external stimulus (*e.g.* exercise, skeletal muscle atrophy), HMB does not significantly influence body or muscle weight in young or aged mice. Similarly, no changes in hypertrophic signaling were detected with age or HMB supplementation. These findings partially conflict with findings from Pimentel et al. (2011), who demonstrated an increase in p70S6K phosphorylation and total mTOR in the extensor digitorum longus muscle of adult male Wistar rats after one month of HMB supplementation. However, no increase in mTOR phosphorylation was reported, nor was there any changes to total or phosphorylated AKT protein. Despite the fact that hypertrophic signaling and muscle weight were not altered, relative peak and mean force were decreased with age, and HMB supplementation was able to prevent this reduction in relative peak force. Thus, short-term HMB supplementation may enhance strength in aged mice independent of changes to skeletal muscle mass. In support of this hypothesis, Vallejo et al. (2016) reported a similar improvement in peak force without any change to muscle mass following 8 weeks of HMB supplementation in aged mice (22 months). Similar improvements in tetanic force were observed in young rats with 4 weeks of supplementation (Pinheiro et al., 2012). Future studies should investigate the impact of HMB on peripheral nerve structure and/or function in order to account for these strength changes.

4.2. HMB fails to improve cognition or enhance neurogenesis in aged mice

Santos-Fandila et al. (2014) verified the presence of HMB in the hippocampus following oral supplementation in Sprague-Dawley rats, but the extent to which HMB can directly or indirectly impact hippocampal neurogenesis and cognition is largely unknown. In the current study, significant reductions in balance and neurogenesis were detected in aged mice compared to young, yet our behavioral assessments failed to

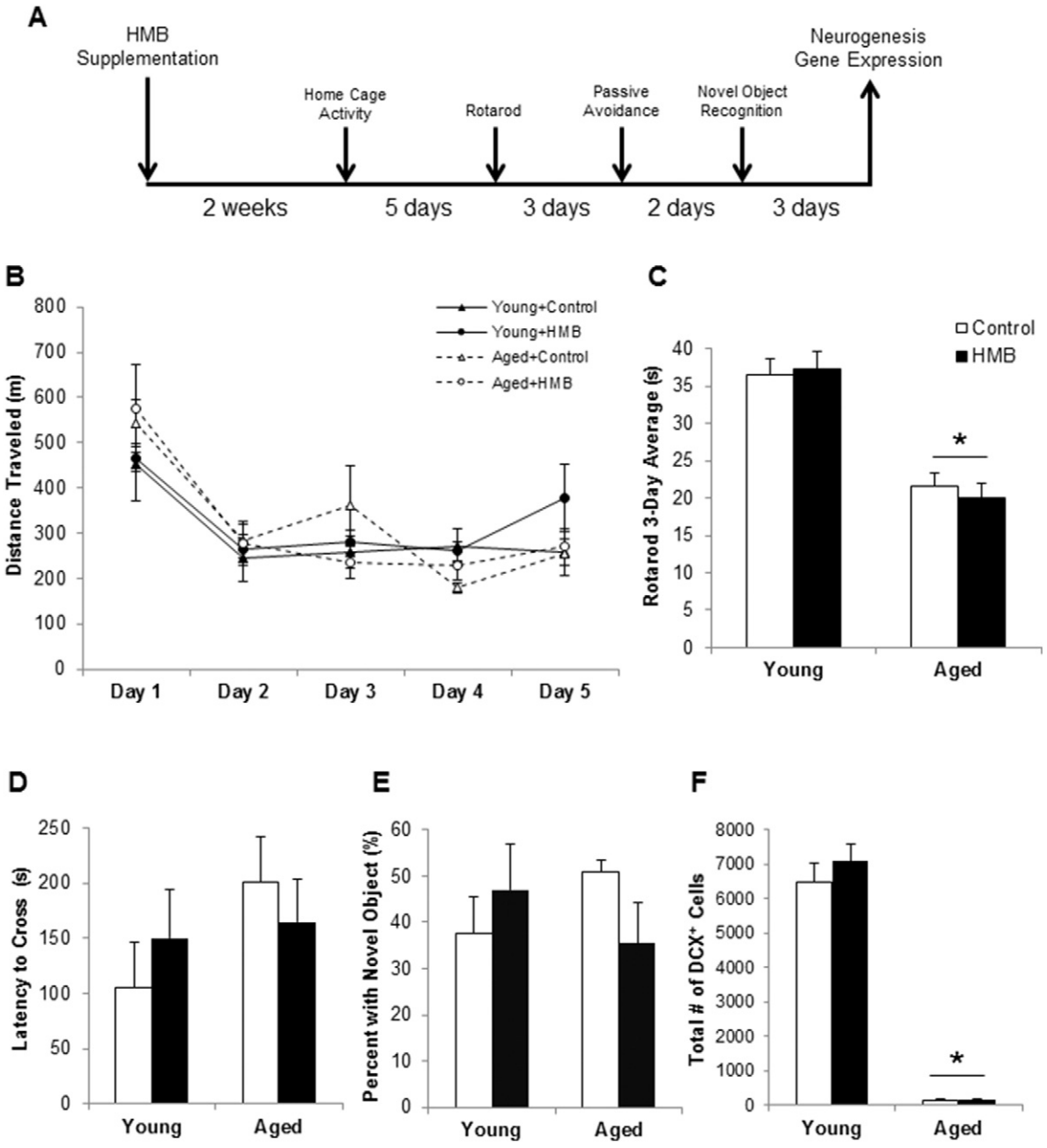

Fig. 3. HMB supplementation does not affect cognitive performance or neurogenesis in young or aged mice. Experimental design for HMB supplementation and behavioral assessments (A). Daily distance traveled was consistent between groups during supplementation as measured by home cage activity monitoring (B). Average time spent on rotarod over 3 days was decreased with age and not recovered with HMB. Neither fear-mediated learning nor was hippocampal-dependent learning different between groups (D and E). Total number of new neurons was significantly reduced in aged mice but not affected by HMB supplementation (F). Values are mean ± SEM. *$p < 0.05$, age main effect. $n = 9–10$ per group.

demonstrate any reduction in performance. In the limited studies that have evaluated the impact of HMB or other nutritional supplements on uncomplicated aging in the rodent model, most do not demonstrate any improvement to cognition when treatment begins at an advanced age (Gibbons et al., 2014; Russ et al., 2015). In contrast, preservation of prefrontal cortex structure and working memory can be observed when treatment begins in middle age and is extended into old age (Hankosky et al., 2016; Kougias et al., 2016). Thus, future work should determine the extent to which long-term HMB supplementation can prevent declines in neuroplasticity and cognitive function across the lifespan.

4.3. HMB selectively restores mMSC function in aged mice

Previous studies have demonstrated the ability of HMB to alter the functional characteristics of different cell types *in vitro*, including myoblasts (Kornasio et al., 2009; Vallejo et al., 2016), myotubes (Aversa et al., 2012) and a neuroblastoma cell line, Neuro2a (Salto et al., 2015). *In vivo*, HMB supplementation can increase satellite cell proliferation in aged rat skeletal muscle during recovery from hindlimb suspension (Alway et al., 2013). These studies indicate some potential for HMB to directly stimulate skeletal muscle stem cell expansion and neuronal elongation and branching. Our study is the first to investigate the effect of age and HMB on mMSC gene expression. With the use of a high throughput screening method, we were able to evaluate growth, neurotrophic, and immunomodulatory factor gene expression in mMSCs from young and aged mice. Interestingly, mMSC gene expression was significantly altered with age, such that age main effects were noted for inflammatory cytokines (TNFα, increased; RPL13a, decreased), several factors that regulate satellite cell activation and muscle repair (HGF, LIF, IGF-1, VEGFα; decreased), and factors that support the growth and survival of developing and mature neurons (EGF, BDNF, NGF, FNDC5; decreased). HMB was able to reverse the impact of age on

Table 1
HMB selectively recovers age-related decrease to mMSC relative mRNA expression. Gene expression of various MSC markers, growth and neurotrophic factors, factors associated with extracellular remodeling, and inflammatory cytokines for each group are presented as mean ± SEM. Age and HMB main effects were considered significant at $p \leq 0.05$ and are in bold. n = 2–10.

Gene	Y + C	Y + H	A + C	A + H	Age effect	HMB effect
Cell surface markers						
Sca-1	1.13 ± 0.18	1.30 ± 0.18	1.23 ± 0.52	0.96 ± 0.34	0.16	0.98
PDGFRα	1.18 ± 0.25	1.88 ± 0.38	1.22 ± 0.37	0.84 ± 0.14	0.07	0.43
CD90	1.13 ± 0.20	1.93 ± 0.36	0.87 ± 0.23	0.84 ± 0.18	**0.01**	0.67
PDGFRβ	1.06 ± 0.14	1.50 ± 0.27	1.39 ± 0.45	0.97 ± 0.24	0.24	0.74
NG2	1.22 ± 0.30	1.26 ± 0.26	0.64 ± 0.28	0.74 ± 0.19	**0.02**	0.56
CD146	1.46 ± 0.38	1.78 ± 0.49	1.79 ± 0.83	1.88 ± 0.81	0.84	0.61
Growth factors						
LIF	1.22 ± 0.33	1.61 ± 0.42	0.40 ± 0.17*	1.09 ± 0.17	**0.02**	**0.03**
HGF	1.51 ± 0.64	1.20 ± 0.23	0.29 ± 0.05*	0.97 ± 0.11	**0.01**	**0.02**
FGF2	1.17 ± 0.21	1.49 ± 0.30	1.42 ± 0.74	1.37 ± 0.34	0.34	0.19
IGF-1	1.09 ± 0.18	1.39 ± 0.27	0.82 ± 0.24	0.54 ± 0.08	**0.01**	0.72
VEGFα	1.16 ± 0.23	1.40 ± 0.22	1.06 ± 0.48	0.35 ± 0.07	<**0.001**	0.41
EGF	1.05 ± 0.14	0.91 ± 0.09	0.67 ± 0.10	0.81 ± 0.11	**0.05**	0.99
Areg	1.34 ± 0.51	2.69 ± 1.47	1.11 ± 0.77	1.13 ± 0.53	0.16	0.47
Neurotrophic factors						
BDNF	1.26 ± 0.46	0.84 ± 0.19	0.41 ± 0.08	0.59 ± 0.09	**0.03**	0.76
NGF	1.28 ± 0.51	1.40 ± 0.40	0.20 ± 0.05	0.80 ± 0.31	**0.03**	0.11
FNDC5	1.45 ± 0.47	1.46 ± 0.37	0.39 ± 0.14	0.36 ± 0.16	**0.01**	0.63
Extracellular matrix remodeling						
MMP2	1.13 ± 0.20	1.50 ± 0.26	1.13 ± 0.45	0.65 ± 0.12	**0.03**	0.80
MMP14	1.13 ± 0.19	1.10 ± 0.18	0.90 ± 0.37	0.66 ± 0.13	**0.02**	0.87
Timp2	1.15 ± 0.24	1.64 ± 0.30	1.19 ± 0.53	0.81 ± 0.14	0.07	0.64
Lama2	1.40 ± 0.27	1.97 ± 0.30	1.51 ± 0.50	0.92 ± 0.18	0.09	0.61
Col1α1	1.10 ± 0.15	1.70 ± 0.29	0.48 ± 0.17	0.36 ± 0.10	<**0.001**	0.77
TGFβ1	1.15 ± 0.22	1.49 ± 0.32	0.89 ± 0.20	0.97 ± 0.18	0.17	0.54
CTGF	1.49 ± 0.51	1.83 ± 0.36	1.25 ± 0.38	1.15 ± 0.14	0.48	0.32
LOX	1.08 ± 0.15	2.42 ± 0.51	1.56 ± 0.35	2.10 ± 0.37	0.62	**0.03**
PAI-1	1.88 ± 0.84	1.72 ± 0.45	0.55 ± 0.13	0.49 ± 0.08	**0.01**	0.77
Eng	1.12 ± 0.20	1.65 ± 0.33	1.45 ± 0.37	1.19 ± 0.18	0.75	0.52
Ang	1.25 ± 0.27	2.63 ± 0.80	1.18 ± 0.41	1.42 ± 0.37	0.23	0.08
FSP-1	1.39 ± 0.43	2.01 ± 0.55	0.56 ± 0.13	1.09 ± 0.23	**0.03**	0.07
Inflammation and oxidative stress						
IL-15	1.18 ± 0.24	1.38 ± 0.39	1.37 ± 0.58	0.61 ± 0.07	0.16	0.71
IL12b	1.11 ± 0.22	0.79 ± 0.34	1.16 ± 0.34	1.19 ± 0.60	0.70	0.25
TNFα	1.28 ± 0.42	0.72 ± 0.07	1.66 ± 0.24	2.11 ± 0.35	<**0.001**	0.76
CCL2	1.24 ± 0.29	2.31 ± 0.70	1.07 ± 0.14	1.80 ± 0.56	0.70	0.24
TLR4	1.50 ± 0.64	1.36 ± 0.25	1.14 ± 0.48	1.15 ± 0.28	0.48	0.85
RPL13a	1.47 ± 0.61	2.80 ± 1.64	0.46 ± 0.30	0.36 ± 0.12	**0.02**	0.56
Mt1	1.19 ± 0.26	4.54 ± 1.62	2.72 ± 1.15	1.23 ± 0.33	0.62	0.66
Mt2	1.92 ± 0.59	2.41 ± 0.80	1.58 ± 0.88	0.93 ± 0.33	0.28	0.91

* Significantly different *vs.* all other groups, $p \leq 0.05$.

HGF and LIF gene expression, and a positive correlation was detected between HGF gene expression and relative peak muscle force, suggesting some improvement in the capacity for muscle repair or function with short-term HMB supplementation (Hunt et al., 2013; Van Mater et al., 2015). Altogether, these findings suggest that mMSC dysfunction in aged mice may contribute to the age-related declines in muscle function, and that HMB possesses the capacity to revitalize mMSC activity.

4.4. mMSC function in vitro is enhanced with HMB treatment

We intentionally chose a relatively short supplementation period to optimize the opportunity to observe changes in mMSC function with HMB treatment. The lack of a potent HMB effect on mMSC gene expression in young and aged mice suggests that there was either a selective effect of HMB on mesenchymal stem/stromal cell function or that the cells were no longer as responsive to supplementation after 5.5 weeks. To address the mMSC response to HMB, an *in vitro* experiment was conducted to evaluate the early gene expression profile of mMSCs following HMB treatment. At 3 hours post-treatment, the cells were highly responsive to HMB. A decline in CCL2 (MCP-1) expression, as well as a trend towards a decrease in GM-CSF expression, suggest that HMB may alter mMSC stromal activity by inhibiting the recruitment of inflammatory cells to skeletal muscle, which would be beneficial in the case of aging when chronic inflammation is persistent. The relative expression of EGF, which can regulate neural stem cell function and increase neurogenesis (Jin et al., 2003; Sütterlin et al., 2013), and the expression of the neurotrophic factor, NTF-3, which can mediate neuronal differentiation and neurite outgrowth (Ammendrup-Johnsen et al., 2015; Oliveira et al., 2013), were both significantly increased in a dose-dependent manner. However, BDNF, similarly characterized as a neurotrophic factor, was not altered *in vitro* (data not shown). Thus, results from *in vivo* and *in vitro* analyses suggest that a combination of mMSC-derived factors, including HGF, LIF, EGF and NTF-3, may contribute to improvements in skeletal muscle function in aged mice with HMB supplementation.

5. Conclusion

Overall, the results from this study suggest that short-term HMB supplementation can preserve strength in aged mice, yet deficits to balance and neurogenesis remain. Future studies are necessary to evaluate the impact of long-term HMB supplementation on the preservation of muscle-resident stromal cell function, strength, neurogenesis, and cognition with age.

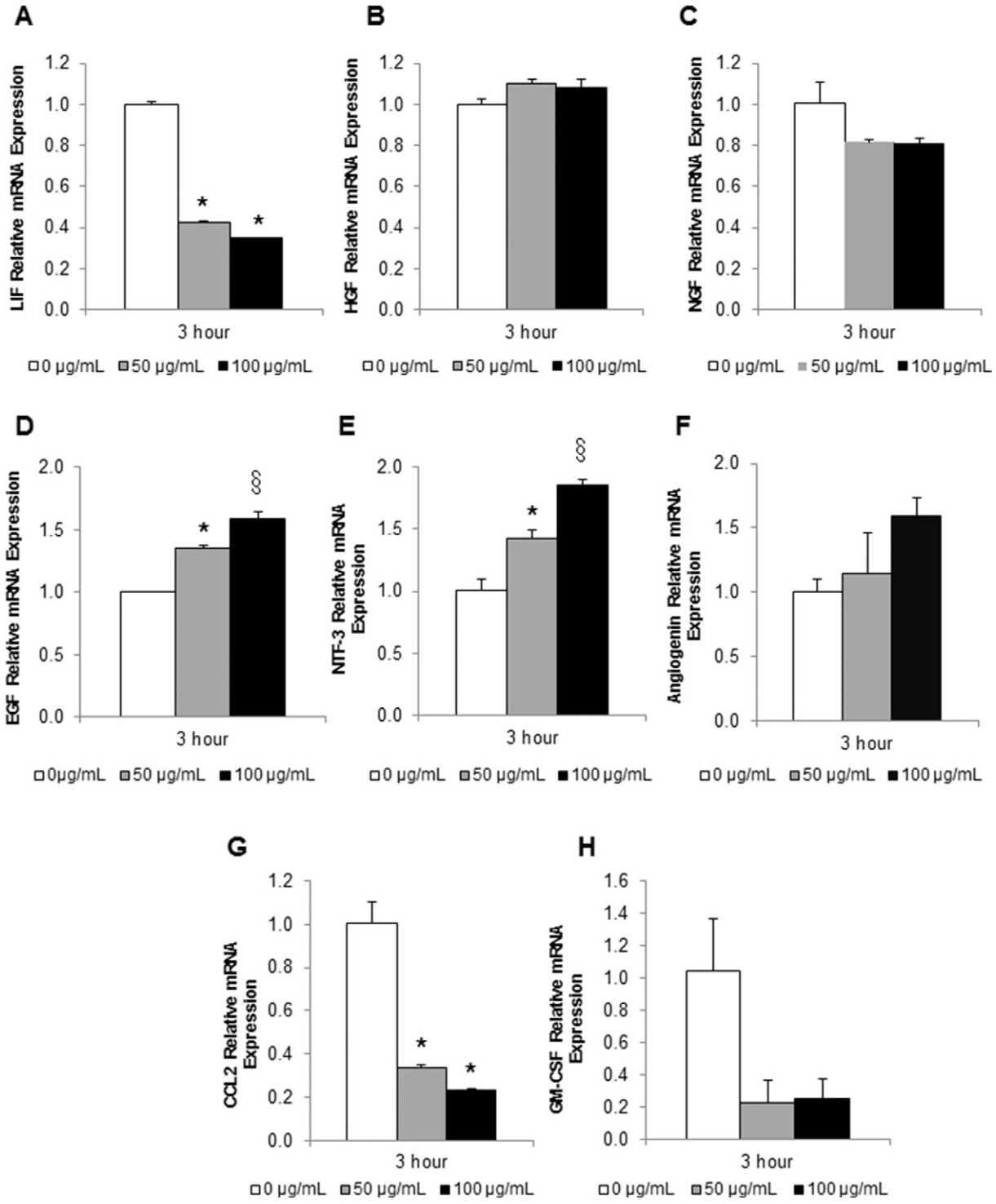

Fig. 4. Isolated mMSCs treated with HMB *in vitro* demonstrate an altered gene expression profile. Relative mRNA expression of factors associated with growth (A–C), neurogenesis (D and E), angiogenesis (F), and inflammation (G and H) are differentially affected by 3 h of treatment with HMB. Values are mean ± SEM. *$p \leq 0.05$ compared to 0 μg/mL; §$p \leq 0.05$ compared to 0 μg/mL and 50 μg/mL. $n = 2$.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.exger.2016.11.010.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors would like to thank Alejandro Barranco and Christopher Moulton (Abbott Industries) for their useful advice and guidance as well as providing Ca-HMB. We would also like to thank Josh Gulley for providing supplies for sipper tubes. This work was supported by a grant from Abbott Industries through the Center for Nutrition, Learning, and Memory at the University of Illinois at Urbana-Champaign (Abbott CNLM ZA68 to MDB).

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