Cognitive function is preserved in aged mice following long-term β-hydroxy β-methylbutyrate supplementation

Michael Munroe[1,2], Ziad S. Mahmassani[1,2], Svyatoslav Dvoretskiy[1,2], Justin J. Reid[3], Benjamin F. Miller [3], Karyn Hamilton [3], Justin S. Rhodes[2,4], Marni D. Boppart [1,2]

[1]Department of Kinesiology & Community Health, University of Illinois at Urbana-Champaign, Urbana, IL, USA, [2]Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign, Urbana, IL, USA, [3]Department of Health and Exercise Science, Colorado State University, Fort Collins, CO, USA, [4]Department of Psychology, University of Illinois at Urbana-Champaign, Urbana, IL, USA

β-hydroxy β-methylbutyrate (HMB) is a nutritional supplement purported to enhance skeletal muscle mass and strength, as well as cognitive function in older adults. The purpose of this study was to determine the potential for long-term HMB supplementation to preserve muscle function and cognition in aged mice, as well as provide evidence of a link between vessel-associated pericyte function and outcomes. Four- (Adult/Ad) and 17 month-old (Aged/Ag) C57BL/6J mice consumed chow containing 600 mg/kg BW/day of either Ca-HMB (Ad, n=16; Ag, n=17) or Ca-Lactate (Ad, n=16; Ag, n=17) for 6 months. HMB did not prevent age-related reductions in muscle mass, strength and coordination (Age main effect, P<0.05). The rate of muscle protein synthesis decreased within the mitochondrial fraction (age main effect, P<0.05), and this decline was not prevented with HMB. Despite no change in muscle mass or function, an age-dependent reduction in active avoidance learning was attenuated with HMB (Age and HMB main effects, P<0.05). Age detrimentally impacted muscle-resident pericyte gene expression with no recovery observed with HMB, whereas no changes in brain-resident pericyte quantity or function were observed with age or HMB. The findings from this study suggest that prolonged HMB supplementation starting in adulthood may preserve cognition with age.

Keywords: HMB, Cognition, Aging, Skeletal muscle, Protein synthesis, Pericyte

Introduction

Aging is accompanied by gradual reductions to skeletal muscle structure and function that ultimately compromises quality of life and increases risk for disability.[1,2] While progressive loss of skeletal muscle mass and strength is commonly observed with aging, severe reductions, clinically diagnosed as sarcopenia, are observed in approximately 11–50% of individuals at or over the age of 80 years old.[3] The mechanistic basis for sarcopenia is not known, yet factors such as inactivity, chronic inflammation, anabolic resistance, impaired vascular function, decreased insulin sensitivity, and altered proteostasis likely contribute.[4] Similarly, impaired executive function and episodic memory are observed in older adults.[5,6] These losses occur concurrent with reductions to cerebral angiogenesis,[7] hippocampal neurogenesis,[8] and microglial quantity.[9] Interestingly, a temporal relationship between loss of muscle mass and dementia has been documented in humans, with progressive and insidious loss of lean muscle mass with age strongly correlating with brain atrophy and declines in short-term memory and learning.[10] The link between muscle and brain health in the aged population suggests a common mechanism for both sarcopenia and dementia that could be potentially addressed with a single therapeutic intervention.

β-Hydroxy β-methylbutyrate (HMB) is a leucine metabolite frequently used as a nutritional supplement to enhance the beneficial physical adaptations associated with exercise training. Increases in lean mass and muscle strength, as well as protection from muscle damage post-exercise, have been observed with HMB supplementation in young individuals beginning a resistance exercise training program.[11–13] Older adults and individuals with disease also benefit from HMB supplementation, as

Correspondence to: Marni D. Boppart, Beckman Institute for Advanced Science and Technology, 405 N. Mathews Ave., MC-251, Urbana, IL 61801, USA. Email: mboppart@illinois.edu

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improvements in body composition and strength have been observed, particularly after long-term use in conjunction with exercise.[14–17] Recent rodent studies have expanded the potential benefits of HMB supplementation to include maintenance of cognitive function with age. Improvements to working memory and visuospatial learning have been observed in aged rats following long-term HMB supplementation.[18,19] These results are promising, yet the specific mechanisms underlying such benefits are not known. In addition, the ability for HMB to concomitantly preserve both muscle mass and cognitive function from middle age to end of life has not been tested.

Pericytes are contractile cells physically embedded within the vascular basement membrane of microvessels, including pre-capillary arterioles, capillaries, and post-capillary venules.[20,21] Pericytes ($CD146^+$ $PDGFR\beta^{+/-}CD31^-CD45^-$) possess potential for mesodermal differentiation and paracrine factor secretion, providing the opportunity to directly and indirectly support tissue repair and remodeling.[22–25] However, loss of pericyte quantity has been observed with aging, diabetic neuropathy, and Alzheimer's disease.[26] Based on these findings, our lab recently assessed perivascular stem cell ($Sca\text{-}1^+CD45^-$; $CD146^{+/-}$) quantity and function in aged mouse muscle. Whereas quantity remained stable, gene expression in isolated cells was markedly altered compared to young controls.[27] Gene expression of several growth, neurotrophic, and angiogenic factors, as well as factors necessary for extracellular matrix (ECM) remodeling, were reduced.[27] Interestingly, 5.5 weeks of HMB supplementation resulted in the partial restoration of perivascular stem cell function in aged mouse muscle, concurrent with a significant improvement in muscle strength. The fact that $PDGFR\beta^+$ pericyte quantity is similarly deficient in the aged mouse hippocampus[28] suggests that pericytes within the microvascular network throughout the body may be vulnerable to aging and possibly represent a common and critical regulator of tissue function across the lifespan.

The purpose of this study was to (1) determine the extent to which long-term HMB supplementation can simultaneously preserve skeletal muscle and cognitive function during the progression of aging in a mouse model, and (2) determine the extent to which tissue-resident (muscle and whole brain) pericyte quantity and/or function is associated with observed outcomes. We hypothesized that HMB supplementation initiated at middle age would help preserve skeletal muscle and cognitive function across the lifespan, and that alterations in pericyte quantity and/or function would accordingly reflect outcome measures of muscle and brain health.

Materials and methods

Animals

All animal use protocols were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign, and all National Institute on Aging (NIA) guidelines for the care and use of animals were followed. Four-month-old (Adult, Ad) and 17-month-old (Aged, Ag) C57BL/6J mice were obtained from Charles River and the NIA, respectively. Same-sex animals were housed together (2–3 mice per cage) in a pathogen-free animal room under controlled conditions (12-hour light/dark cycle, 25°C). Upon arrival, mice were acclimatized to their new environment for seven days and then randomized into four groups based upon body weight (BW): Adult+Control (Ad+C, n=16; 8 males, 8 females), Adult+HMB (Ad+H, n=16; 8 males, 8 females), Aged+Control (Ag+C, n=17; 8 males, 9 females), and Aged+HMB (Ag+H, n=17; 8 males, 9 females). Mice were then fed either an experimental (HMB) or control diet for 24 weeks.

HMB administration and diet

Adult C57BL/6J mice consume approximately 3.5–4.5 g chow/day (for a 25 g mouse). Based upon this average chow consumption, Ca-HMB (Abbott Industries, Champaign, IL, USA) dosage was calculated to achieve a final daily dose of ~600 mg HMB/kg BW/day. An average human dose of HMB is 3 g/day or ~40 mg/kg BW. Using that value as the human equivalent dose (mg/kg BW),[29] the appropriate dose for mice was determined to be approximately 500 mg HMB/kg BW/d, an experimental dosage used previously in rodents.[30,31] However, as this study was over an extended duration (24 weeks), the dosage was increased to 600 mg HMB/kg BW/d in order to account for BW increases that are typically observed with age. Ca-HMB was mixed into an AIN-93G OpenSource Diet (D10012G; Research Diets Inc., New Brunswick, NJ, USA) at a concentration of 3.75 g Ca-HMB/kg chow. To account for the addition of calcium in the Ca-HMB experimental diet, the control diet was supplemented with an equal concentration of Ca-Lactate.

BW and grip strength measures

Each animal was weighed prior to the experiment and weight was recorded weekly thereafter. Four-limb grip strength was measured every two weeks using a Digital Grip Strength Meter (Columbus Instruments, Columbus, OH, USA). 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 grip strength relative to BW (gram force (g)/gram BW (g)). The same tester performed all trials.

Rotarod

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

Active avoidance

Each mouse was placed in a Gemini™ Avoidance System (SD Instruments, San Diego, CA, USA) and allowed to explore both non-illuminated compartments for 120 seconds. Following the acclimatization period, the unoccupied compartment was illuminated for 5 seconds (conditioned stimuli, CS) followed by a 5-second footshock in the non-illuminated/occupied compartment (0.5 mA) (unconditioned stimuli, US). Three possible outcomes were recorded. The mouse could avoid the US by moving to the illuminated compartment during the CS ('Avoidance Response'). The mouse could escape the US by moving to the illuminated compartment during the US, ending the trial ('Escape Response'). If the mouse failed to avoid or escape the US, a 'Non-Response' was recorded. Once the mouse avoided, escaped, or failed to respond, there was a 10-second inter-trial interval before the next trial. Each mouse completed 50 consecutive trials daily for 5 consecutive days for a total of 250 trials.

Heavy water (^2H_2O) incorporation

Heavy water labeling is a safe and reliable method for determining protein synthesis rates for different protein fractions such as myofibrillar, cytoplasmic, and mitochondrial fractions within muscle tissue.[32,33] Heavy water administration began 20 weeks after the start of supplementation. To bring mice up to 5% body water enrichment (based on 60% BW as water), 99% ^2H_2O (with 0.9% w/v NaCl and sterilized via filtration) (Sigma Aldrich) was injected intraperitoneally. Mice were then provided 8% ^2H_2O enriched water for 4 weeks prior to euthanasia.

Tissue collection

After 24 weeks, mice were fully anesthetized using isoflurane and blood was collected via cardiac puncture. Gastrocnemius-soleus muscle complexes were then excised and muscle weight was recorded. One complex was immersed in ice-cold 1× PBS plus penicillin/streptomycin and used for pericyte isolation. The other muscle complex was snap frozen in liquid nitrogen to be used for protein synthesis analysis. The brain was then dissected and immersed in ice-cold MEM with HEPES modification (Sigma-Aldrich, St Louis, MO) and used for pericyte isolation. Collected blood was allowed to clot in a 2.0 mL Eppendorf tube for 30 minutes at room temperature then centrifuged at 1200×*g* for 10 minutes at 4°C. Serum supernatant was transferred to a new 1.5 mL Eppendorf tube and stored at −80°C.

Pericyte isolations and fluorescence-activated cell sorting

For the isolation of $CD146^+CD45^-CD31^-$ pericytes from skeletal muscle, dissected muscle tissue was minced and enzymatically digested for approximately 45 minutes at 37°C. The enzyme solution consisted of 250 U/mL collagenase Type 2 (Worthington-Biochemical Corp., Lakewood, NJ, USA), 2 U/mL neutral protease (Dispase) (Worthington-Biochemical Corp.), 60 U/mL DNase (Sigma Aldrich), 5 mM $CaCl_2$ in 1× Hank's balanced salt solution. Enzymatic reactions were inhibited with a 20% FBS in 1× PBS solution with 1 mM EDTA, and then the cell solution was filtered, first using a 70 μm then a 40 μm filter. The filtered cell suspension was then blocked with anti-mouse CD16/CD32 antibody (eBioscience, San Diego, CA, USA) to prevent Fc-mediated nonspecific binding. After blocking, cells were incubated with a mix of monoclonal anti-mouse antibodies (eBioscience), CD146-phycoerythrin (PE) (1:50), CD45-fluorescein isothiocyanate (FITC) (1:100), and CD31-FITC (1:100), for 1 hour in filtered 2% FBS in 1× PBS at 4°C. Cell suspension was then filtered through a 40 μm filter and kept on ice for fluorescence-activated cell sorting (FACS).

For the isolation of $PDGFR\beta^+CD45^-CD31^-$ pericytes from whole brain tissue, cell isolation procedures were adapted from Boroujerdi et al.[34] Excised brains were minced and the tissue slurry was transferred to a 15 mL tube and centrifuged at 300×*g* for 5 minutes. The tissue slurry was then resuspended in 5 mL of enzyme solution (20 U/mL papain from papaya latex, Worthington-Biochemical Corp.), 1 mM L-cysteine, 0.5 mM EDTA, and 2.5 U/μL DNase I (Worthington-Biochemical Corp.) for approximately 60 minutes at 37°C. The slurry was transferred to a 50 mL tube and titurated ×10 with a 19-gauge needle then ×10 with a 21-gauge needle to disrupt microvessels. An equal volume of 20% BSA inhibition media was added and cells were filtered through a 70 μm and a 40 μm filter. Cells were then

blocked as described above and resuspended in a mix of monoclonal anti-mouse antibodies (eBioscience), PDGFRβ/CD140b-PE (1:50), CD45-FITC (1:100), and CD31-FITC (1:100), for 1 hour in filtered 2% FBS in 1× PBS at 4°C. Cell suspension was then filtered through a 40 μm and kept on ice for FACS.

FACS was performed using a BD FACS Aria II sorter at the University of Illinois at Urbana-Champaign Roy J. Carver Biotechnology Center (CBC, Urbana, IL, USA). Tissue-specific negative and single-stain controls were used to established appropriate gating for FACS, and cells were collected in 2% FBS in 1× PBS solution. Post-sorting, cells were immediately centrifuged at 450×*g* for 5 minutes, lysed in Buffer RLT (Qiagen, Valencia, CA, USA) and snap frozen in liquid nitrogen to be analyzed for relative mRNA expression. Lysed cells were stored at −80°C until analysis.

Protein fractionation

For protein isolation, skeletal muscle was fractionated according to our previously published procedures.[35–39] The muscle complex was homogenized 1:10 in isolation buffer (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM MgCl2, 1 mM EDTA, 1 mM ATP, pH=7.5) with phosphatase and protease inhibitors (HALT, Thermo Scientific, Rockford, IL, USA) using a bead homogenizer (Next Advance Inc., Averill Park, NY, USA). After homogenization, subcellular fractions were isolated via differential centrifugation as previously described.[35–39] Once protein pellets were isolated and purified, 250 μL 1 M NaOH was added and pellets were incubated for 15 minutes at 50°C will slowly mixing. For DNA analysis, DNA was extracted from ~20 mg of tissue or bone marrow suspension using a QiAamp DNA mini kit (Qiagen). Protein was hydrolyzed by incubation for 24 hours at 120°C in 6N HCl. The pentafluorobenzyl-*N*,*N*-di(pentafluorobenzyl) derivative of alanine was analyzed on an Agilent 7890A GC coupled to an Agilent 5975C MS as previously described.[35–39]

To determine body water enrichment, 125 μL of plasma was placed into the inner well of o-ring screw cap and inverted on heating block overnight. Two microliter of 10 M NaOH and 20 μL of acetone were added to all samples and to 20 μL 0–20% D_2O standards and then capped immediately. Samples were vortexed at low speed and left at room temperature overnight. Extraction was performed by the addition of 200 μL hexane. The organic layer was transferred through anhydrous Na_2SO_4 into GC vials and analyzed via EI mode.

The newly synthesized fraction of proteins was calculated from the product enrichment divided by the true precursor enrichment using plasma analyzed for D_2O enrichment and adjusted according to mass isotopomer distribution analysis.[32,33] The fraction new was divided by time and expressed as fractional synthesis rates (FSR: %/day).

Serum 4-HNE analysis

Serum 4-HNE, a marker of lipid peroxidation and oxidative stress, was measured in duplicate using a commercially available ELISA kit (Cell Biolabs OxiSelect™ HNE Adduct ELISA kit, San Diego, CA, USA). The intensity of color was measured at 450 nanometers using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Protein concentrations were quantified using a standard curve (provided by the manufacturer).

RNA isolation, cDNA synthesis, and high throughput microfluidic qPCR using Fluidigm Biomark™ HD

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). Starting RNA concentration of 25 ng was used to perform reverse transcription via the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY, USA) per manufacturer's instructions.

High throughput microfluidic quantitative PCR (qPCR) of synthesized cDNA was ran on a 96×96 Dynamic Array Integrated Fluidic Circuit (IFC, Fluidigm, San Francisco, CA, USA) by the Functional Genomics Unit at the CBC. 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™ (Fluidigm Corporation, San Francisco, CA, USA) HD for qPCR analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene, and relative mRNA expression was expressed relative to the Ad+C group using the ΔΔCt method. Primer information and gene expression assay ID numbers are provided in Supplementary Table 1.

Statistical analysis

All data are presented as mean±SEM. Two-way ANOVA was performed to determine HMB×Age interaction effects and main effects of Age or HMB followed by least significant difference (LSD) *post hoc* analysis when appropriate. BW and grip strength as well as daily rotarod and active avoidance data were analyzed using repeated measures ANOVA followed by LSD *post hoc* analysis. All statistical analyses

were completed using SPSS Ver. 24 (IBM, Chicago, IL, USA). Differences were considered statistically significant at $P \leq 0.05$.

Results

Long-term HMB supplementation does not alter age-related changes to skeletal muscle weight or function

The experimental design for determining the impact of 24 weeks of HMB supplementation on adult and aged skeletal muscle and cognitive function is outlined in Fig. 1A. Ca-HMB and Ca-Lactate were estimated weekly. Chow quantity was weighed at the beginning and end of each week. Based upon the amount of chow consumed during the week, supplement intake was calculated. No differences in average daily supplement consumption were observed between groups over the 24-week period (Ad+C, 380.0±13.3; Ad+H, 391.2±10.8; Ag+C, 388.5±22.0; Ag+H, 367.4±12.2 mg/kg BW/day). After 24 weeks of supplementation, both BW and absolute gastrocnemius-soleus muscle weight were significantly reduced with age (Age main effect, $P=0.01$) (Fig. 1B and C), but not affected by HMB supplementation. Relative muscle weight remained unchanged between groups (Fig. 1D).

Prior to supplementation, relative peak grip strength was lower in the Aged groups compared to the Adult groups (Age main effect, $P=0.01$). After 24 weeks, strength had decreased significantly within each group (Time main effect, $P<0.001$), but no strength differences between groups were noted post-supplementation (Fig. 1E). To assess muscular coordination and balance, mice underwent three days of rotarod testing, with improved performance being measured by an increase in latency to fall (seconds). Mice increased their time on the rotarod each day (Day main effect, $P<0.001$). A significant Day×Age interaction effect ($P<0.001$) was observed attributable to the increased latency in the Adult groups compared to the Aged groups on Days 2 and 3 collapsed across diet (Fig. 1F). The average latency to fall during the three-day testing period was significantly less in the Aged groups compared to Adult groups, but not impacted by HMB supplementation (Age main effect, $P=0.01$) (Fig. 1G).

Skeletal muscle protein synthesis was measured using deuterium oxide (^2H_2O) labeling with separate protein fractions analyzed. Mixed and cytoplasmic fractional synthetic rates (FSR, %/day) trended to increase with age regardless of supplementation (Mixed, $P=0.06$; Cytoplasmic, $P=0.09$) (Fig 1H and I). Aged mice had significantly reduced mitochondrial FSR compared to Adult mice (Age main effect, $P=0.01$) (Fig. 1J). In addition, a trend for an Age×HMB interaction effect ($P=0.06$) was detected as HMB supplementation increased FSR (not significant) in Aged, but not Adult mice. Mitochondrial content and activity within cells contributes to reactive oxygen species production, with 4-hydroxynonenal (4-HNE) being a major byproduct of lipid peroxidation and subsequent oxidative stress.[40] Given the observed alterations to mitochondrial protein synthesis, we assessed serum 4-HNE content using ELISA. While not statistically significant, a trend for an Age x HMB interaction effect ($P=0.07$) was observed post-supplementation (Ad+C, 25.12±1.85; Ad+H, 21.93±0.80; Ag+C, 21.65±1.36; Ag+H, 24.04±1.67 μg/mL).

Age-related reductions in active avoidance learning are attenuated with HMB supplementation

Learning performance on the active avoidance test was measured in response to long-term HMB supplementation. The total number of active avoidances recorded per day is presented in Fig. 2A. A main effect of age was detected, with Adult mice displaying an overall greater number of active avoidances than Aged mice collapsed across days and supplementation (Age main effect, $P<0.001$). A significant main effect of day was also observed (Day main effect, $P<0.001$) suggesting all groups successfully learned the task, as indicated by the increasing number of active avoidances collapsed across groups (Day main effect, $P<0.001$). Additionally, a significant Day×Age interaction effect was observed ($P=0.01$) driven by a progressively greater difference in the number of successful active avoidances between Aged and Adult mice each day. In addition, a trend for an Age×HMB interaction effect was noted ($P=0.08$). Specifically, HMB increased the number of active avoidances achieved in Aged but not Adult mice on Days 4 ($P=0.15$) and 5 ($P=0.04$). On Day 5 alone, both significant Age ($P=0.001$) and HMB ($P=0.04$) main effects were detected with Adult mice successfully avoiding more foot shocks than Aged mice, and HMB supplementation resulting in an increase in the number of avoidances achieved compared to controls (Fig. 2B).

Figure 2C represents the number of escape responses recorded over the five days of active avoidance testing. Both Adult and Aged mice had progressively fewer escape responses over the five days (Day main effect, $P<0.001$) with Adult mice recording fewer escapes overall throughout testing (Age main effect, $P<0.001$). A significant Day×HMB interaction effect ($P=0.001$) was detected driven by significantly more escapes in the Ad+C group on Days 1 and 2 and significantly fewer escapes in the Ag+C group on Days 4 and 5. In addition, a trend for an Age×HMB interaction effect was observed ($P=0.06$)

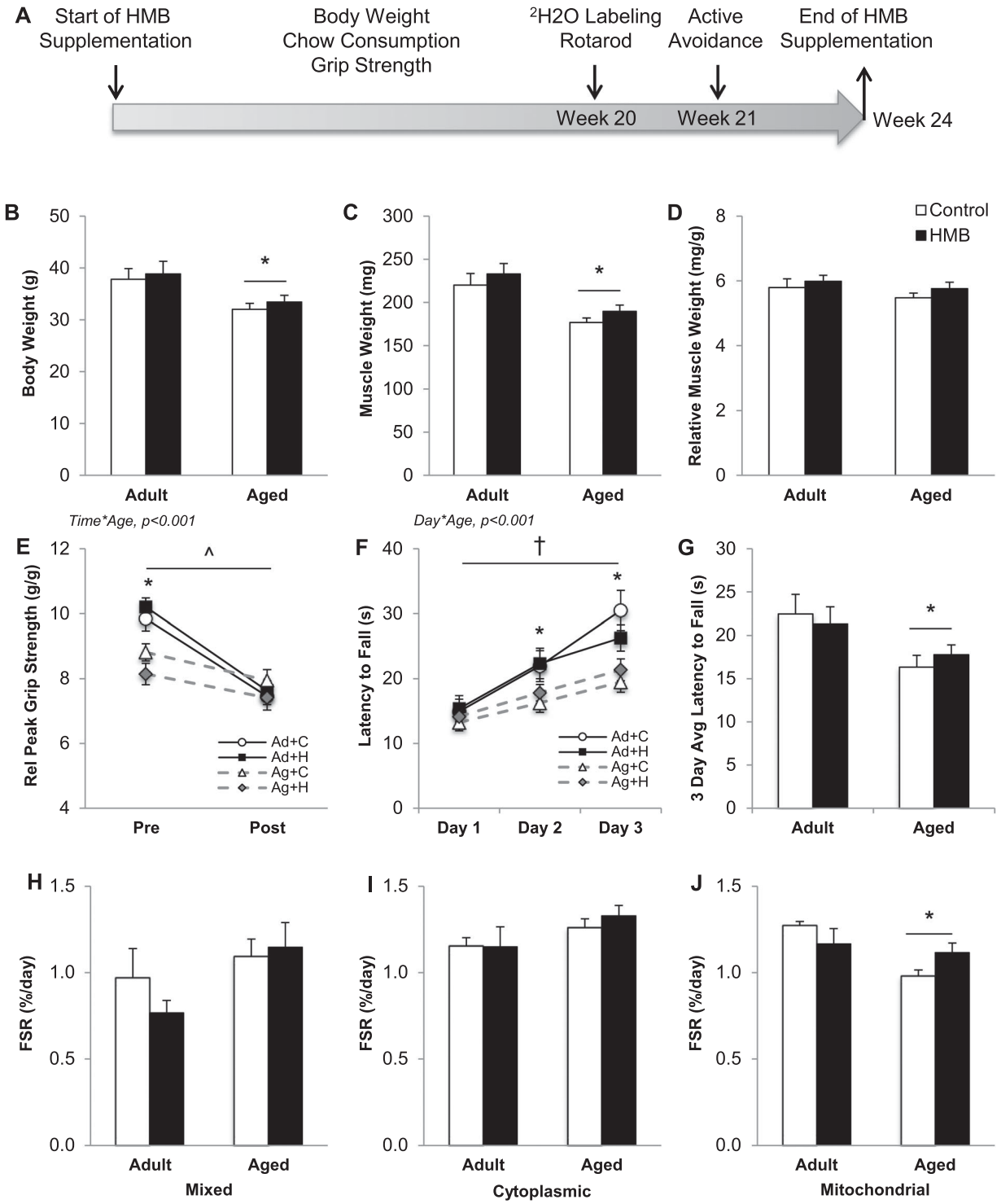

Figure 1 Age-related changes to skeletal muscle mass and function are unaffected by long-term HMB supplementation. Experimental design for supplementation and behavioral testing (A). BW and gastrocnemius-soleus muscle weight were reduced with age after 24 weeks of supplementation (B, C), while relative muscle weight was not different between groups (D). Relative peak grip strength was not different between groups following supplementation (E). Coordination and balance was reduced with age, but not affected by HMB supplementation (F, G). Mixed (H) and cytoplasmic (I) FSR was unaltered by age or HMB, with a significant reduction to mitochondrial FSR (J) observed. Values are mean±SEM. *$P<0.05$, Age main effect;^$P<0.05$, Time main effect; †$P<0.05$, Day main effect. n=16–17 (A–G), n=7–9 (H–J) per group

with HMB supplementation resulting in a significant reduction in the number of escapes in Aged but not Adult mice on Day 5 (P=0.04).

The number of non-responses demonstrated significant Age (P=0.04) and HMB (P=0.04) main effects on Days 1 and 2 (Fig. 2D). In addition, a significant Day main effect ($P<0.001$) was observed over the five days of testing with fewer non-responses recorded daily. A significant Day×Age×HMB interaction effect (P=0.003) was detected driven by the Ad+C group recording significantly more non-responses on Days 1 and 2 compared to the other three groups.

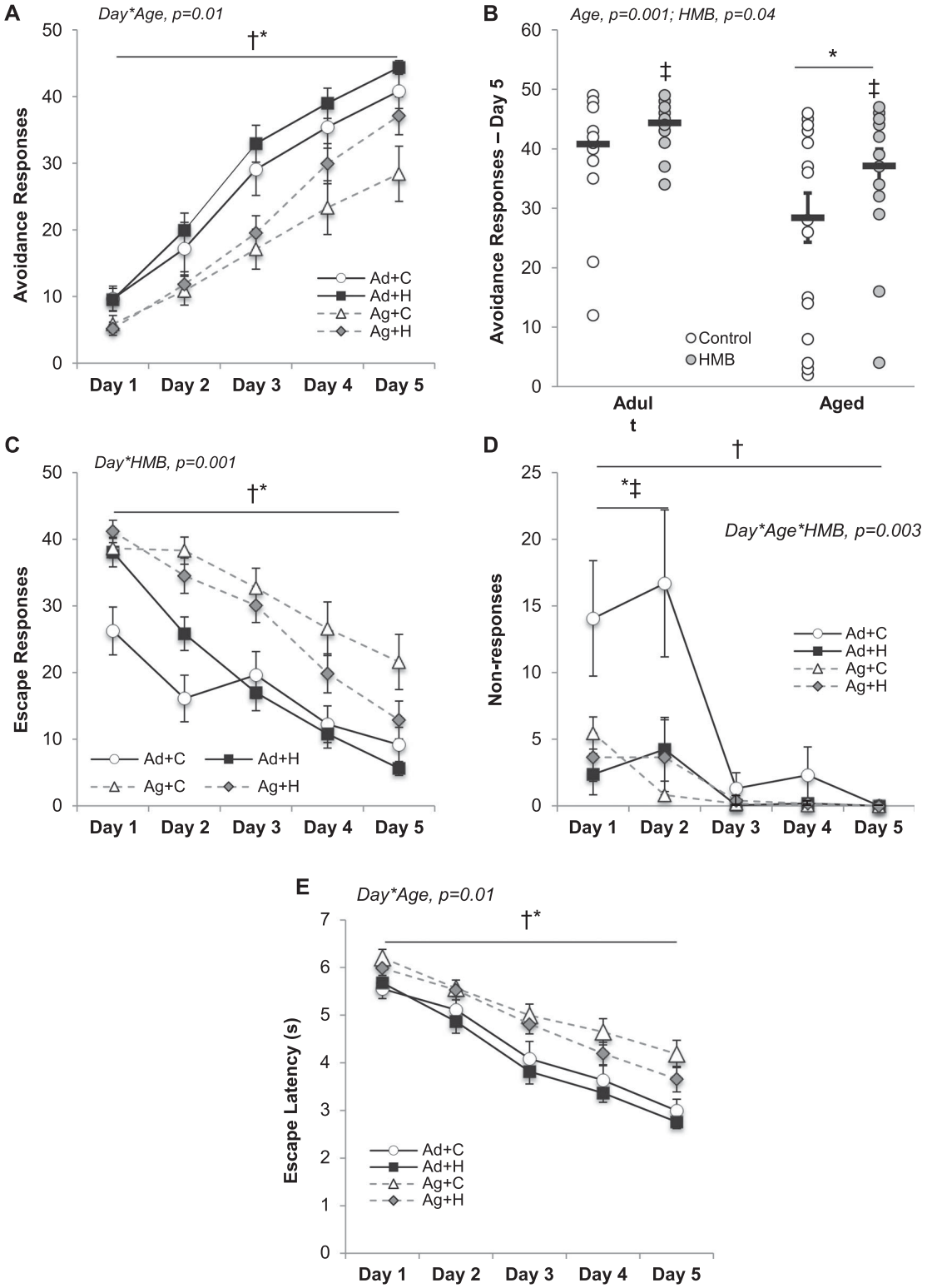

Figure 2 Cognitive function is preserved in aged mice supplemented with HMB. The number of successful avoidances improved daily with greater improvements observed in Adult mice (A). On Day 5, HMB supplementation attenuated age-related reductions to avoidance responses (B). The number of escape responses decreased over the testing period with Aged mice completing more escapes overall (C). The number of non-responses was overall reduced daily, and no non-responses were recorded on Day 5 for any group (D). The escape latency decreased daily with a greater reduction observed in Adult compared to Aged mice (E). Values are mean±SEM. **P*<0.05, Age main effect; ‡*P*<0.05, HMB main effect; †*P*<0.05, Day main effect. *n*=16–17 per group

Importantly, on Day 5, no non-responses were recorded for any group.

Escape latency (seconds) is the time between the onset of the US (foot shock) and a successful escape, which is presented in Fig. 2E. A significant Day main effect ($P<0.001$) was observed indicating all groups improved in their response time to the US. A significant Day×Age interaction effect ($P=0.01$) was

noted due to a shorter response latency in the Adult groups on Days 3–5 compared to Aged counterparts.

Skeletal muscle-derived pericyte myogenic proliferation and differentiation factor gene expression is altered with age and HMB supplementation

Post-supplementation, $CD146^{+}CD31^{-}CD45^{-}$ pericyte quantity was not statistically different between groups (Ad+C, 52.0±11.0%; Ad+H, 53.5±8.7%; Ag+C, 43.3±9.9%; Ag+H, 45.5±9.8%) (P=0.41). Pericyte gene expression, however, demonstrated specific alterations attributable to age or supplementation (Table 1). Specifically, factors related to myogenesis were differentially altered by age and HMB. Paired box 7 (*Pax7*), myogenic factor 5 (*Myf5*), and myogenic differentiation 1 (*Myod*) expression was significantly reduced with age (Age main effect, P<0.05) with *Myf5* expression reduced with HMB supplementation as well (HMB main effect, P=0.03). A significant HMB main effect was observed for relative myogenin (*Myog*) expression (P=0.05), which was significantly elevated in the Ag+C group only. No significant effect of age or HMB was observed for any growth or neurotrophic factors analyzed. Select ECM remodeling and inflammatory/oxidative stress factors were impacted by age, but not HMB supplementation. Matrix metallopeptidase 14 (*Mmp14*), tissue inhibitor of metallopeptidase 1 (*Timp1*), and angiogenin (*Ang*) mRNA expression was significantly reduced with age (Age main effect, P<0.05). Similarly, metallothionein 2 (*Mt2*) and heme oxygenase 1 (*Hmox1*) mRNA expression was reduced in Aged mice (Age main effect, P<0.05). While not statistically significant, tumor necrosis factor alpha (*Tnfa*) expression appeared to increase with age and decrease with HMB supplementation (P=0.06).

Brain-derived pericyte gene expression is largely unaffected by age or HMB after 24 weeks of HMB supplementation

$PDGFR\beta^{+}CD31^{-}CD45^{-}$ pericyte quantity isolated from whole brain tissue was not statistically different between groups (Ad+C, 6.5±3.3%; Ad+H, 8.6±3.4%; Ag+C, 6.4±1.9%; Ag+H, 3.6±1.7%) (P=0.35), and minimal functional changes were observed in response to age or supplementation (Table 2). Leukemia inhibitory factor (*Lif*) mRNA expression was significantly increased with age (Age main effect, P=0.03), while a significant Age×HMB interaction effect was observed for hepatocyte growth factor (*Hgf*) expression (P=0.03). No effect of age or HMB was observed for any neurotrophic factor analyzed. Early B-cell factor 2 (*Ebf2*) has been shown to contribute to the normal development of the cerebellar cortex,[41] and had significantly increased mRNA expression with age (P=0.04). The ECM remodeling proteins, laminin-α2 (*Lama2*) and *Ang*, demonstrated increased relative mRNA expression with age (P<0.05), but neither was affected by HMB supplementation. In contrast, toll-like receptor 3 (*Tlr3*), part of the receptor family of the innate immune system, had significantly reduced expression with HMB (P=0.03). Metabolic regulatory factors, zinc finger protein 423 (*Zfp423*), and peroxisome proliferator-activated receptor gamma co-activator 1 alpha (*Ppargc1*/*Pgc1a*) were differentially altered by age and supplementation. *Zfp423* expression was significantly reduced with HMB supplementation (P=0.03), while *Ppargc1* expression demonstrated a significant Age×HMB interaction effect (P=0.03) attributable to altered expression with supplementation in both Adult and Aged groups

Discussion

The purpose of this study was to evaluate the impact of long-term HMB supplementation on the simultaneous preservation of skeletal muscle and cognitive function from middle age to late life in mice. While HMB supplementation did not effectively maintain absolute muscle weight, strength, or coordination during the progression of aging, supplementation demonstrated strong capacity to preserve active avoidance learning. Our previous work demonstrated that age can markedly reduce muscle-resident perivascular cell function, and that acute HMB supplementation can partially recover selective release of regenerative growth factors.[27] In the current study, a similar decline was noted in muscle-derived pericyte function, yet no recovery was noted with HMB supplementation. Brain-derived pericyte function was largely unaffected by age or HMB. However, brain-derived pericytes were isolated following whole brain dissection, and the changes in gene expression may not accurately reflect pericyte function within regions necessary for active avoidance memory and learning (hippocampus and prefrontal cortex).[42,43] Overall, these data suggest that long-term HMB supplementation preserves certain aspects of cognitive function in aged mice, and future studies are necessary to elucidate the mechanistic basis for this observation.

HMB fails to enhance skeletal muscle mass or function in either adult or aged sedentary mice

The majority of HMB supplementation studies demonstrate beneficial contributions to skeletal muscle mass and strength during resistance training, cachexia, or atrophy,[13,15,44] whereas few studies report the impact of HMB on sedentary skeletal muscle.[14,27,45] When administered in conjunction

Table 1 Impact of age and HMB supplementation on skeletal muscle pericyte gene expression

Gene	Ad+C	Ad+H	Ag+C	Ag+H	Age effect	HMB effect
Growth factors						
Lif	1.02±0.10	1.21±0.21	0.90±0.36	1.31±0.27	0.46	0.23
Hgf	1.02±0.11	1.29±0.21	2.00±1.09	0.77±0.19	0.09	0.25
Fgf2	1.00±0.02	1.21±0.07	0.68±0.25	1.15±0.27	0.17	0.12
Igf1	1.02±0.11	0.89±0.11	0.86±0.30	0.73±0.16	0.23	0.88
Igf2	1.16±0.32	1.13±0.16	1.21±0.30	1.48±0.51	0.91	0.65
Vegfa	1.05±0.18	0.77±0.19	0.55±0.18	1.03±0.26	0.41	0.52
Gdf11	1.01±0.06	2.43±1.04	1.99±0.64	1.43±0.25	0.79	0.49
Myogenic factors						
Pax7	1.02±0.12	0.55±0.09	0.30±0.09	0.17±0.04	**0.001**	0.12
Myf5	1.02±0.11	0.59±0.19	0.27±0.07	0.15±0.02	**<0.001**	**0.03**
Myod	1.10±0.28	1.02±0.34	0.41±0.12	0.33±0.12	**0.01**	0.77
Myog	1.21±0.42	0.85±0.40	3.66±1.14	1.06±0.37	0.09	**0.05**
Neurotrophic factors						
Bdnf	1.11±0.26	1.13±0.10	2.22±0.69	1.18±0.33	0.54	0.67
Ngf	1.00±0.05	0.94±0.03	1.20±0.22	0.80±0.16	0.67	0.35
Fndc5	1.02±0.10	0.91±0.14	3.26±1.71	0.70±0.15	0.79	0.59
Ntf3	1.00±0.04	0.89±0.08	0.83±0.09	0.90±0.19	0.34	0.77
Gdnf	1.13±0.34	0.96±0.37	0.81±0.20	0.69±0.05	0.32	0.87
ECM remodeling						
Mmp2	1.05±0.19	0.68±0.22	0.34±0.12	0.72±0.21	0.40	0.83
Mmp14	1.03±0.13	0.95±0.13	0.46±0.12	0.61±0.18	**0.02**	0.89
Timp1	1.12±0.29	1.14±0.35	0.55±0.12	0.71±0.19	**0.05**	0.61
Timp2	1.75±0.53	1.46±0.43	1.05±0.41	1.21±0.52	0.33	0.85
Lama2	1.05±0.19	0.68±0.22	0.34±0.12	0.72±0.21	0.41	0.98
Col3a1	1.80±0.71	1.23±0.32	0.45±0.20	0.74±0.33	0.18	0.99
Col6a3	1.19±0.38	0.88±0.13	0.40±0.15	0.81±0.34	0.06	0.37
Tgfb1	1.14±0.28	0.87±0.14	1.77±0.61	2.34±1.35	0.18	0.79
Ctgf	1.34±0.33	1.68±0.63	1.40±0.29	1.22±0.40	0.61	0.59
Lox	1.15±0.29	1.36±0.45	0.66±0.19	0.79±0.16	0.20	0.64
PAI1	1.13±0.25	1.07±0.39	0.83±0.32	1.15±0.12	0.58	0.44
Eng	1.70±0.66	1.70±0.63	2.47±0.55	1.89±0.43	0.10	0.89
Ang	1.10±0.26	1.25±0.47	0.40±0.08	0.61±0.07	**0.001**	0.21
Inflammation and oxidative stress						
Tnfa	1.00±0.04	0.56±0.16	2.56±1.00	1.10±0.25	0.06	0.06
Mt1	1.54±0.49	1.44±0.29	0.81±0.19	0.77±0.16	0.09	0.76
Mt2	1.40±0.42	1.00±0.26	0.46±0.25	0.48±0.16	**0.03**	0.59
Hmox1	1.14±0.32	0.53±0.13	0.37±0.08	0.42±0.07	**0.05**	0.55
Hif1a	1.02±0.09	1.23±0.35	1.04±0.13	0.91±0.12	0.69	0.75
Rpl13a	1.05±0.11	1.00±0.17	1.04±0.17	0.83±0.08	0.46	0.43
*Cebpa**	1.45±0.76	0.54±0.12	0.45±0.08	0.64±0.10	0.19	0.58
Cebpb	1.00±0.06	0.77±0.25	0.53±0.14	0.67±0.17	0.28	0.84
Ccl2	1.11±0.25	0.80±0.15	0.61±0.21	0.78±0.05	0.21	0.59
Tlr3	1.51±0.82	2.21±1.35	0.83±0.17	0.71±0.09	0.13	0.72
Tlr4	1.04±0.17	1.22±0.13	0.61±0.24	0.92±0.15	0.07	0.15
Ebf1	1.16±0.32	1.01±0.32	1.01±0.14	1.12±0.28	0.95	0.56
Ebf2	1.01±0.09	0.99±0.08	0.79±0.21	0.90±0.26	0.27	0.72

Notes: Gene expression values are presented as mean±SEM. Age and HMB main effects were considered significant at $P \leq 0.05$ and are in bold. n=3–9 per group.
*Significant Age×HMB interaction effect, P=0.05.

with exercise or disease conditions, HMB can positively impact muscle strength and health,[46,47] however in the absence of stimuli, muscle function appears to be unaffected or equivocal,[14,48–51] Consistent with these reports, HMB supplementation in the current study did not significantly alter BW, muscle weight, or muscle function in sedentary mice when administered during the progression of aging. We previously demonstrated improvement in muscle strength with short-term (5.5 weeks) HMB supplementation in aged mice, yet the degrees of decline and recovery were minimal and did not occur until the last week of administration. We speculate that significant injury, atrophy, and/or inflammation may be necessary for HMB supplementation to exhibit a robust impact on muscle structure and function in the context of aging, conditions that are rare in a mouse model of uncomplicated aging.

Skeletal muscle protein synthesis rates are slightly altered with age but not HMB

Maintenance of muscle mass is achieved through a balance between muscle protein synthesis and breakdown. Muscle protein balance becomes dysfunctional with age, in part due to aged muscle's inability to increase postprandial protein synthesis rates.[52] Only two studies have attempted to determine the impact of HMB on muscle protein balance *in vivo*,

Table 2 Impact of age and HMB supplementation on brain pericyte gene expression

Gene	Ad+C	Ad+H	Ag+C	Ag+H	Age effect	HMB effect
Growth factors						
Lif	1.61±0.83	1.83±0.54	5.24±0.77	2.07±0.69	**0.03**	0.31
*Hgf**	1.29±0.37	2.64±0.79	2.28±0.66	1.06±0.35	0.50	0.71
Fgf2	1.08±0.17	1.29±0.36	2.05±0.81	1.55±0.39	0.36	0.99
Igf1	1.42±0.43	2.12±0.51	3.44±1.36	2.39±0.97	0.62	0.90
Igf2	1.12±0.23	1.10±0.26	1.37±0.21	1.25±0.32	0.44	0.46
Vegfa	1.03±0.09	1.00±0.14	1.42±0.18	0.94±0.09	0.37	0.12
Gdf11	1.25±0.32	1.17±0.19	1.88±0.34	1.28±0.36	0.24	0.34
Neurotrophic factors						
Bdnf	1.06±0.36	0.74±0.13	0.77±0.40	0.74±0.03	0.44	0.59
Ngf	1.41±0.46	1.28±0.35	1.46±0.31	1.28±0.42	0.76	0.66
Fndc5	2.00±0.69	2.88±1.19	4.55±1.59	3.01±1.59	0.59	0.85
Ntf3	1.41±0.51	2.19±0.57	2.28±0.59	2.61±0.41	0.20	0.28
Gdnf	1.24±0.28	2.96±0.91	3.87±1.49	2.50±0.81	0.49	0.67
ECM remodeling						
Mmp2	1.78±0.76	2.85±0.84	4.16±2.14	3.12±1.27	0.96	0.52
Mmp14	1.07±0.15	1.28±0.18	1.39±0.32	1.38±0.23	0.72	0.64
Timp1	1.65±0.51	1.83±0.58	2.54±0.69	1.80±0.46	0.41	0.83
Timp2	1.10±0.20	1.38±0.16	2.17±0.70	1.63±0.25	0.15	0.61
Lama2	1.18±0.26	1.34±0.34	2.02±0.48	1.87±0.43	**0.05**	0.95
Col1a1	1.88±1.02	2.00±0.51	2.85±1.33	1.69±0.39	0.74	0.62
Col3a1	1.49±0.48	1.91±0.38	2.23±0.88	2.00±0.52	0.71	0.36
Col6a3	2.02±0.88	2.40±0.79	8.22±3.35	3.86±1.31	0.14	0.73
Tgfb1	1.03±0.10	0.90±0.20	1.22±0.17	1.05±0.14	0.29	0.26
Ctgf	1.09±0.17	1.05±0.21	1.28±0.24	0.97±0.19	0.98	0.34
Lox	1.24±0.29	1.38±0.40	1.82±0.37	1.34±0.30	0.37	0.60
PAI1	1.13±0.20	0.82±0.16	1.06±0.25	1.08±0.24	0.93	0.62
Eng	1.08±0.21	0.78±0.15	1.08±0.15	0.90±0.24	0.78	0.12
Ang	1.04±0.12	1.16±0.31	2.23±0.26	1.88±0.33	**0.01**	0.27
Inflammation and oxidative stress						
Tnfa	1.69±0.77	2.02±0.59	1.62±0.07	1.46±0.59	0.84	0.56
Mt1	1.10±0.20	1.15±0.21	1.35±0.24	1.49±0.33	0.55	0.89
Mt2	1.25±0.28	1.70±0.39	1.75±0.45	3.25±1.03	0.25	0.32
Hmox1	1.09±0.21	1.24±0.24	1.25±0.32	0.96±0.21	0.41	0.88
Hif1a	1.28±0.30	0.97±0.21	1.69±0.37	1.17±0.33	0.42	0.19
Rpl13a	1.03±0.10	1.15±0.16	1.03±0.15	1.06±0.19	0.58	0.88
Cebpa	1.05±0.13	1.18±0.23	1.65±0.39	1.60±0.09	02.0	0.51
Cebpb	1.16±0.19	0.91±0.19	1.36±0.22	1.17±0.27	0.46	0.23
Ccl2	1.62±0.64	1.77±0.39	2.68±0.82	2.84±0.93	0.08	0.39
Tlr3	1.20±0.30	0.56±0.17	1.25±0.37	0.72±0.20	0.84	**0.03**
Tlr4	1.15±0.22	1.22±0.21	1.41±0.34	1.09±0.25	0.90	0.74
Ebf1	1.12±0.22	0.73±0.18	1.13±0.18	1.04±0.24	0.31	0.11
Ebf2	2.06±0.91	3.01±0.93	7.08±1.94	4.05±1.30	**0.04**	0.79
Zfp423	1.32±0.43	0.63±0.22	1.21±0.26	0.93±0.38	0.51	**0.03**
*Ppargc1a**	1.15±0.30	0.38±0.09	0.99±0.23	1.68±0.90	0.10	0.11

Notes: Gene expression values are presented as mean±SEM. Age and HMB main effects were considered significant at $P \leq 0.05$ and are in bold. n=4–9 per group.
*Significant Age×HMB interaction effect: Hgf, P=0.03; Ppargc1, P=0.03.

and both report increases in protein synthesis immediately after administering a bolus of HMB.[53,54] To our knowledge, this is the first study to investigate fractional protein synthesis rates after long-term HMB supplementation in either adult or aged muscle. There was a trend toward increased myofibrillar (P=0.06) and cytoplasmic (P=0.09) protein synthesis in Aged versus Adult mice, and a significant decrease in mitochondrial protein synthesis in Aged versus Adult mice. These changes are potentially related to the aging process. Specific to mitochondria, Standley et al. (2017) recently observed no impact of HMB supplementation on mitochondrial content following 10-days of bed rest in aged humans.[55] Although content did not change in this study, it is not possible to determine changes in turnover and remodeling from content alone. In the current study, there was a trend for HMB to increase mitochondrial protein synthesis in Aged mice (P=0.06). This potential improvement is notable in that changes in mitochondrial biogenesis may be related to alterations to cognitive function.[56] It is also important to note that our measurements were made in the last 4 weeks of supplementation, so it is possible that there was increased turnover during the initial 20 weeks that remodeled mitochondria to a 'healthier' state prior to our period of measure. Additional studies are necessary to address the interaction between HMB, mitochondrial synthesis and oxidative metabolism.

Decline in active avoidance learning is attenuated with long-term HMB supplementation in aged mice

Active avoidance is a form of operant-based conditioning designed to quantify the rodent's ability to perform the appropriate action (move from one side of the apparatus to the other toward the light cue), in response to presentation of a stimulus (light cue) in order to avoid a negative outcome (shock). Our finding that the aging-related decline active avoidance performance is partially rescued with HMB treatment in aged mice is consistent with a recent study in rats, which found that long-term HMB supplementation maintains cognitive flexibility and dynamic learning on a different operant task.[18] The explanation for the improved performance in aged rodents is not clear. In our study, it could be related to the observation that HMB-treated animals displayed more escapes than no-responses during the early days of training than the control treated. A greater number of escapes might indicate the mice were in the process of learning the rule for avoidance and at least were responding. However, why HMB caused mice to make more escapes, and how it enhanced learning in the aged mice is unclear at this time. Regardless, the current findings indicate that HMB supplementation throughout adulthood and into later life may prevent certain aspects of cognitive decline.

CD146$^+$Lin$^-$ skeletal muscle pericytes exhibit specific changes to myogenic factor gene expression with age and HMB supplementation

We have previously observed the dual effect of age and HMB on perivascular stem cell (Sca-1$^+$CD45$^-$) function in muscle, including HMB's ability to attenuate age-related declines in expression of factors associated with satellite cell activation and myofiber repair (*Hgf*, *Lif*).[27] In the current study, alterations in gene expression were observed with age, yet the changes were less robust and did not include regenerative growth factors. One possible explanation for the discrepancy between our prior and current studies is that we used a new cell marker to isolate muscle-resident pericytes. CD146 is a well-established pericyte marker, and isolation based on this marker, with the elimination of CD45$^+$ hematopoietic and CD31$^+$ endothelial cells during FACS, ensures the specific retrieval of vascular-associated mural cells (designated CD146$^+$Lin$^-$). We have verified that a percentage of CD146$^+$Lin$^-$ pericytes obtained from sorting express Sca-1, yet Sca-1$^+$CD45$^-$ and CD146$^+$Lin$^-$ pericytes are not equivalent and include slightly different cell populations. Despite this limitation, age-related decreases in gene expression were observed in CD146$^+$Lin$^-$ pericytes, including myogenic proliferation/activation factors (*Pax7*, *Myf5*, *Myod*) and factors necessary for ECM remodeling and angiogenesis (*Mmp14, Timp1, Ang*). HMB supplementation reversed *Myf5* and decreased *Myog* gene expression, with no impact on any other factors. We speculate that pericytes isolated from aged muscle might have a reduced capacity for self-renewal, and an increased capacity for terminal differentiation, and HMB can prevent these changes. Finally, it is important to note that *Tnfa* gene expression trended to increase with age (highly significant in our previous study), and a trend toward a decrease was observed with HMB. This would also suggest that pericyte dysfunction may contribute to an elevation in muscle inflammation with aging, and that some of the benefits of long-term HMB may occur via modification of this response.

Brain-derived PDGFRβ$^+$Lin$^-$ pericyte gene expression is minimally affected following long-term HMB supplementation in adult and aged mice

This study is the first to evaluate the impact of age and HMB on whole brain pericyte gene expression. We chose to isolate pericytes using the cell surface marker PDGFRβ to remain consistent with prior studies that have reported a decrease in pericyte quantity in the mouse hippocampus with age.[28] In the current study, PDGFRβ$^+$Lin$^-$ pericyte quantity was highly variable and gene expression was largely unaffected by age or HMB. Gene expression of factors associated with angiogenesis (*Ang*)[57] and cerebellar development (*Ebf2*)[41] was altered with age, while other ECM remodeling and neurotrophic factors remained unaltered. A possible explanation for these inconsistencies could be due to regional differences in pericyte quantity and function, such as the hippocampus or prefrontal cortex. As different sections of the brain help to regulate various physiological processes, it follows that cell types from these regions, including pericytes, might possess different functional capabilities. Future research should focus on evaluating region-specific pericyte function changes with age.

Conclusion

In conclusion, the findings from this study suggest that initiation of HMB supplementation at mid-life can provide benefits to cognitive performance in the years that follow. Pericyte quantity and function as assessed following isolation from whole brain tissue does not appear to provide the basis for this advantage. However, future research is needed to evaluate whether pericytes from specific regions of the brain (e.g. hippocampus and prefrontal cortex) confer positive changes in cognitive performance observed in aged mice supplemented with HMB.

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Disclaimer statements

Contributors M.M, J.R., and M.D.B conceived and designed the research; M.M., Z.S.M., and S.D. performed experiments; M.M., Z.S.M., B.F.M, K.L.H., J.R., and M.D.B analyzed data and interpreted the results of experiments; M.M. and M.D.B prepared figures and drafted the manuscript. All authors revised and approved the final version of the manuscript.

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Ethics approval The research described in the manuscript is in compliance with all NIA guidelines in the treatment of animal subjects and was approved by the Institutional Animal Care and Use Committee at the University of Illinois, Urbana-Champaign.

Supplementary material

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ORCID

Benjamin F. Miller http://orcid.org/0000-0003-3283-0685
Karyn Hamilton http://orcid.org/0000-0002-6320-8824
Marni D. Boppart http://orcid.org/0000-0003-0369-6974

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