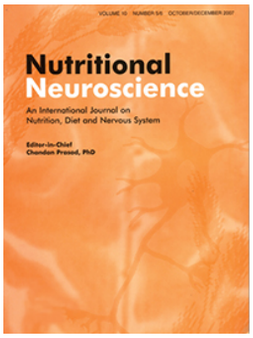

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Effects of β-hydroxy-β-methyl butyrate on working memory and cognitive flexibility in an animal model of aging

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Objectives: Normal aging results in cognitive decline and nutritional interventions have been suggested as potential approaches for mitigating these deficits. Here, we used rats to investigate the effects of short- and long-term dietary supplementation with the leucine metabolite β-hydroxy-β-methyl butyrate (HMB) on working memory and cognitive flexibility.

Methods: Beginning ~12 months of age, male and female Long–Evans rats were given twice daily access to sipper tubes containing calcium HMB (450 mg/kg) or vehicle (285 mg/kg calcium lactate) in a sucrose solution (20% w/v). Supplementation continued for 1 or 7 months (middle- and old-age (OA) groups, respectively) before testing began. Working memory was assessed by requiring rats to respond on a previously sampled lever following various delays. Cognitive flexibility was assessed by training rats to earn food according to a visual strategy and then, once acquired, shifting to an egocentric response strategy.

Results: Treatment with HMB improved working memory performance in middle-age (MA) males and OA rats of both sexes. In the cognitive flexibility task, there was a significant age-dependent deficit in acquisition of the visual strategy that was not apparent in OA males treated with HMB. Furthermore, HMB ameliorated an apparent deficit in visual strategy acquisition in MA females.

Discussion: Together, these findings suggest that daily nutritional supplementation with HMB facilitates learning and improves working memory performance. As such, HMB supplementation may mitigate age-related cognitive deficits and may therefore be an effective tool to combat this undesirable feature of the aging process.

Keywords: Aging, β-Hydroxy-β-methyl butyrate, Working memory, Cognitive flexibility, Prefrontal cortex

Introduction

Normal aging in humans is associated with deficits in several aspects of cognition, including memory, attention, decision-making, and visuospatial skills.[1–3] Cognitive impairment (not associated with dementia) affects more than one in five individuals over 70 years of age and is a risk factor for transitioning to dementia.[4] Furthermore, aging-associated cognitive deficits reduce quality of life, which can manifest as deficits in daily tasks such as preparing healthy meals or taking medications.[5] Recent advances in medical technologies have increased life expectancy such that the population of individuals over 65 is projected to more than double between 2010 and 2050.[6] Due to the growing population of aging individuals, there is a pressing need for solutions to ameliorate aging-associated cognitive decline.

The prefrontal cortex (PFC) appears to be especially sensitive to the detrimental effects of aging. In humans and non-human primates, for example, aging is marked by reductions in synapses and dendritic complexity,[7–9] changes in the integrity of myelination of white matter,[10] and numerous changes in PFC function. The latter effects include reductions in dorsolateral PFC activation and interconnectivity with other brain regions,[11] as well as changes in basal activity in neurons of layers 2/3.[12] Aging-related deficits have also been observed in the rodent PFC. For example, reduced *N*-methyl-D-aspartate receptor-mediated activity,[13] decreased levels of dopamine and norepinephrine,[14] decreased glucose utilization,[15]

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and reductions in spine density and dendritic arborization[16] are observed in aged compared to young mice and rats. These age-related changes have been associated with significant declines in cognitive functioning, including behavioral flexibility,[17–19] working memory,[18,20,21] impulsivity,[22] and decision-making.[23]

Various strategies for mitigating cognitive decline have been proposed, but one that is relatively non-invasive and is often easily implemented is the use of nutritional interventions.[24,25] For example, 8 weeks of cocoa flavanol supplementation improved verbal fluency and visual attention in healthy aged adults[26] and those with mild cognitive impairment.[27] β-Hydroxy-β-methyl butyrate (HMB), which is known to preserve muscle mass in the elderly,[28,29] is another candidate bionutrient that may have pro-cognitive benefits. This leucine metabolite crosses the blood–brain barrier[30] and has the ability to increase protein synthesis and decrease its breakdown through mammalian target of rapamycin (mTOR) and the ubiquitin pathway, respectively.[29,31] In addition, there are indications that HMB promotes neurite outgrowth[32] and upregulates the growth hormone/IGF-1 axis,[33] which also plays a role in maintaining muscle mass and is known to decrease during aging.[34]

We recently demonstrated that in aged rats, daily HMB supplementation mitigated age-related declines in dendritic material and the total number of dendritic spines in the medial PFC.[35] In the current study, we used PFC-sensitive behavioral tasks to investigate the effects of HMB supplementation on working memory and cognitive flexibility in middle- and old-age (OA) male and female rats. To better model the human menopausal condition, female rats were ovariectomized to prevent the modest ovarian hormone release that occurs in rat estropause.[16,36]

Methods and materials

Subjects

Subjects were male ($n = 118$) and female ($n = 107$) Long–Evans rats that were retired breeders (Harlan; Indianapolis, IN). Rats arrived in our colony between 8 and 11 months old and were kept on a 12-hour light/dark cycle (lights on at 08:00) with experiments performed between 10:30 and 18:30. Rats had free access to water and were maintained on *ad libitum* feeding except during behavioral tasks (see below). Experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois, Urbana–Champaign, and were consistent with the *Guide for the Care and Use of Laboratory Animals* [37].

Surgery

At approximately 11 months of age, female and male rats underwent bilateral ovariectomy or sham surgery, respectively, using procedures we described previously.[38] Briefly, rats were anesthetized (2–4% isoflurane) and small bilateral incisions were made through the skin and intramuscular wall of the dorsal torso. For females, the ovaries were removed and the skin and muscle were sutured with nylon thread. Sham surgeries were performed in males the same way except the gonads were left intact. This was done in order to control for the effects of surgery (e.g. isoflurane exposure) that the females received. Rats were given at least 10 days to recover prior to beginning nutritional supplements.

Daily nutritional supplements

Beginning at approximately 12 months of age, sipper tubes containing HMB solution (50 mg/ml HMB calcium + 20% sucrose) or a vehicle solution (32 mg/ml calcium lactate + 20% sucrose) were placed into rats' home cages twice daily (at approximately 08:00 and 20:00 hours) 6 days/week and once on the seventh day in a week (between 18:00 and 20:00 hours). The sipper tubes remained in the rats' cages until the next scheduled dose, though most rats consumed all the solution within 5 minutes of availability. Daily supplementation continued for 1 month in rats from the middle-age (MA) group or 7 months for rats in the OA group before behavioral testing began. Rats continued to receive daily supplements throughout the duration of behavioral testing. The target dose of HMB was 450 mg/kg, which was administered in a volume of 2–6 ml depending on individual body weight. For each rat, the amount consumed was recorded and the actual dose ingested was estimated. At the conclusion of the study, we determined that the mean (±SEM) dose of HMB was 441 ± 1.26 mg/kg for all rats in the HMB-treated groups. The HMB used in this study was provided by Abbott Nutrition (Columbus, OH, USA)

Apparatus

Standard operant chambers (Coulbourn Instruments, Whitehall, PA) that were housed inside sound-attenuating cubicles were used in Experiment 1. The cubicles were equipped with fans that provided ventilation and masked extraneous noise. One wall of each operant chamber was equipped with a centrally located food trough outfitted on either side with two retractable levers that were equidistant (87 mm) from the trough. White cue lights were located above each lever and a white houselight was located near the chamber ceiling on the wall opposite the levers. This wall also contained a recessed nosepoke port located on the center of the wall. Entries into the food trough and nosepoke port were monitored using infrared detectors. A cue light located inside the trough

could be illuminated to signal food pellet delivery. Graphic State (v3.1; Coulbourn Instruments) was used for automated chamber control and data collection. Chambers used for Experiment 2 were identical to those used in Experiment 1, but with two exceptions: nosepoke ports were located on either side of the trough instead of retractable levers and there was no nosepoke port on the back wall. The ports flanking the trough contained a red LED light that could be used as a discriminative stimulus.

Experiment 1: delayed matching to position

MA and OA rats were trained to perform a delayed matching-to-position (DMTP) task as we have described previously.[39] Briefly, rats' body weights were gradually reduced to 80–85% of their free feeding weight over a period of approximately 7–10 days. Rats then received three daily magazine training sessions wherein food pellets were delivered into the recessed food trough on a variable interval (VI) 40-second schedule. Over the next 15 sessions, rats were trained to perform a matching-to-position task using a forward chaining procedure. On this task, an individual trial began with a 5-second intertrial interval (ITI), followed by extension of one of the levers (selected randomly) and the illumination of the cue light above the lever. A response on the lever within 20 seconds (i.e. sample phase) illuminated the nosepoke port on the opposite wall and the lever retracted. If the rat poked its nose into the port on the back wall within 10 seconds, the choice phase began: the port light turned off, both levers extended, and the cue lights above the levers illuminated. If the rat responded on the lever previously presented during the sample phase within 10 seconds, a food pellet was delivered into the trough (illuminated for 3 seconds) and the levers retracted. A response on the non-sample lever was scored as incorrect and a failure to respond on the levers or nosepoke port at any phase was scored as an omission and the next trial began.

During the last phase of training, rats were trained on a DMTP wherein a delay was introduced between the sample and choice phases of each trial. During each session, seven delay intervals were randomly intermixed across trials and each delay was presented for 16 trials (yielding a total of 112 trials/session). The delays ranged from 0 to 24 seconds (delays: 0, 2, 4, 8, 12, 18, and 24 seconds) and rats performed the task for 12 daily sessions.

Data analysis: Experiment 1

Performance on the DMTP task was measured by calculating rats' mean percentage of correct choices across training and delay intervals. For each sex, choice accuracy across sessions and delays was analyzed with three-way repeated measures ANOVA (age × treatment × session) and (age × treatment × delay). Follow-up *post hoc* tests were used to analyze delay-dependent changes in performance during early (sessions 1 and 2) and late (sessions 11 and 12) stages of training.[39] All data are presented as group mean ± SEM.

Experiment 2: operant strategy shifting

Prior to the initiation of training in this version of an operant strategy shifting task that we used previously,[39] rats were food restricted to 80–85% of their free feeding bodyweight over 7 days. Training then began with three daily sessions of magazine training wherein 38 food pellets (45 mg/each; Bio-Serv, F0021) were delivered on a VI 100-second schedule. Subsequently, rats were trained during four 30-minute daily sessions to nosepoke into one of the recessed ports on a fixed ratio 1 (FR1) schedule of reinforcement. For these sessions, access to one of the nosepoke ports was blocked with a metal cover and the port that was accessible (left or right) was alternated each session. The onset of a trial was signaled by illumination of the houselight and red LED within the accessible port. Following a nosepoke into the illuminated port, the LED was extinguished and a food pellet was delivered. Following these four sessions, rats were trained to respond within 10 seconds of trial initiation (i.e. illumination of the houselight and red LED port light) in five sessions where both nosepoke ports were accessible. In these sessions, one of the two ports was illuminated by a red LED light concurrent with houselight illumination; if rats made a nosepoke into the illuminated port within 10 seconds, it was reinforced with a food pellet and a 10-second ITI began. If a rat failed to respond within 10 seconds, the trial was scored as an omission and an additional 10-second time out period (TO) was added to the ITI. The chamber remained darkened during the ITI and TO. During these training sessions, responses into the non-illuminated port had no programmed consequence. In one final training session, each rats' side bias was determined as described by Floresco *et al*.[41]

Strategy shifting sessions commenced the day following assessment of the side bias and consisted of a maximum of 120 trials/day that were each separated by a 10-second ITI. During each trial, one of the two cue lights located above the nosepoke ports (i.e. left- or right-side) was illuminated for 3 seconds. These were presented pseudorandomly across trials, such that no cue light could be illuminated on more than two consecutive trials. Subsequently, the houselight and both of the nosepoke ports were illuminated and rats were required to make a nosepoke response within 10 seconds. Initially, a 'visual strategy' was reinforced: a single food pellet was delivered when

rats made a nosepoke into the port underneath the illuminated cue (i.e. correct choice). A response into the nosepoke port underneath a non-illuminated cue, or no response within 10 seconds of nosepoke port illumination (i.e. omission), resulted in a 10-second TO added to the ITI. Trials with a visual strategy reinforced were continued until rats made eight consecutive correct choices and had completed at least 30 trials. During the session following acquisition of this performance criterion, rats were required to shift to an egocentric 'response strategy'. Here, rats were reinforced for nosepoke responses into the left- or right-side port, regardless of which cue light was illuminated. These trials continued until rats achieved a criterion of eight consecutive correct choices. For each rat, the reinforced response was the port opposite the rat's side bias as assessed on the final day of nosepoke training.

Data analysis: Experiment 2

For the operant strategy shifting task, the number of trials and errors to criterion were recorded. Errors following the shift to the response strategy were classified as perseverative or never reinforced. Perseverative errors were defined as those that would have been correct when the visual strategy was in effect (e.g. responding in the left port when the left cue light was illuminated, but the right port response was the reinforced response). All other errors were scored as never reinforced, as they would not have been correct in a visual strategy and they included responses in the non-reinforced port during the response strategy. Separate two-way ANOVAs (treatment × age) were used to assess performance in male and female rats. Errors were analyzed in separate three-way repeated measures ANOVAs (treatment × age × error type) for males and females. Significant main effects and interactions were investigated with *post hoc* tests. All data are presented as group mean ± SEM. Three control MA females did not acquire the visual strategy within 5 days, and as such, their data were not included in the shift to response strategy.

Results

Experiment 1: DMTP

Working memory performance was assessed using a DMTP task with delays ranging from 0 to 24 seconds (Fig. 1). Three-way repeated measures ANOVA of percent correct choices during the first two sessions of matching-to-position revealed a significant delay × treatment interaction ($F_{6,258} = 2.36$; $P < 0.05$). *Post hoc* analysis indicated that MA and OA males given HMB made significantly more correct choices than their control counterparts at the 24-second delay. Separate three-way repeated measures ANOVAs of the last two sessions revealed a significant main effect of delay ($F_{6,308} = 17.8$; $P < 0.05$) and a significant treatment × age interaction ($F_{1,308} = 4.41$; $P <$

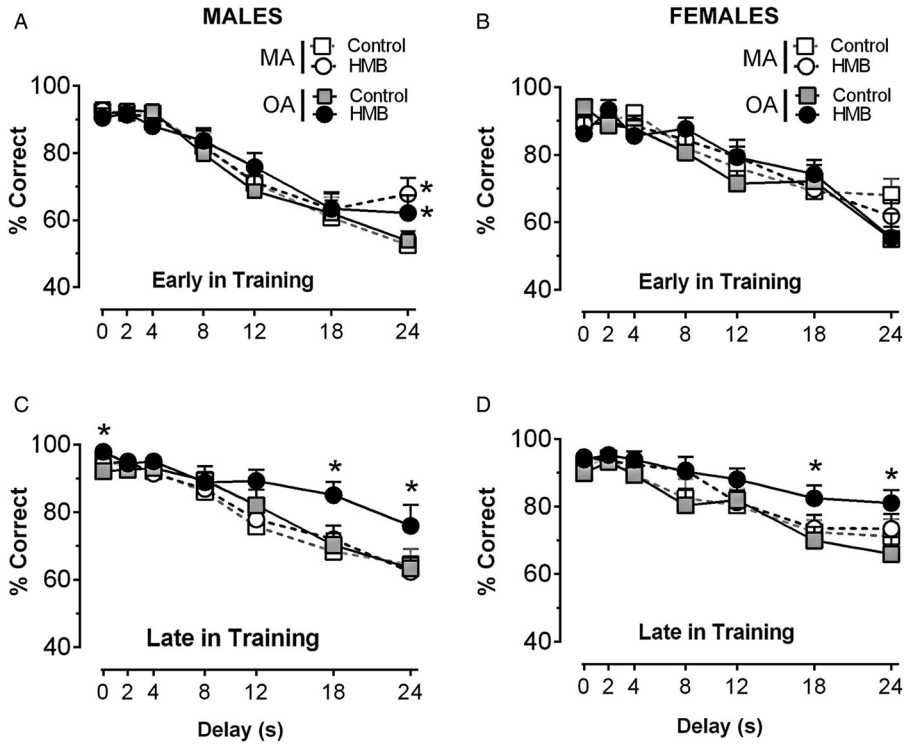

Figure 1 DMTP performance across delay intervals. Mean percent correct across delays during early (sessions 1 and 2) and late (sessions 11 and 12) stages of training. Early in training, HMB-enhanced DMTP performance in males (A) regardless of age, but not females (B). Later in training, OA males (C) and females (D) given HMB showed better accuracy at longer delays than all other groups regardless of age. *$P < 0.05$ vs. controls.

0.05). *Post hoc* analyses indicated that OA HMB-treated males outperformed all other groups at the 18- and 24-second delays.

For females, analysis of percent correct choices during the first two sessions revealed a main effect of delay ($F_{6,228}$= 42.2; $P < 0.05$), but no significant effects of HMB supplementation on performance early in training. A three-way repeated measures ANOVA of the last 2 days revealed significant main effects of delay ($F_{6,228}$= 34.4; $P < 0.05$) and treatment ($F_{1,38}$= 4.45; $P < 0.05$). Follow-up analyses indicated that late in training, OA HMB-treated females outperformed all other groups at the 18- and 24-second delays.

When changes in performance were assessed across the 12 training sessions, we found positive effects of HMB supplementation on working memory during periods of the task when cognitive load was greatest (Fig. 2). Three-way repeated measures ANOVA of percent correct at 18-second delays in males revealed a significant main effect of session ($F_{11,11}$= 5.07; $P <$ 0.05). OA males given HMB tended to perform better than controls across sessions, but the treatment and interaction effects failed to reach statistical significance (P-values >0.05). At 24-second delays, three-way ANOVA revealed significant main effects of treatment ($F_{1,45}$= 4.78; $P < 0.05$) and session ($F_{11,11}$= 3.28; $P < 0.05$), and an age × session interaction ($F_{11,495}$= 2.72; $P < 0.05$). Follow-up analyses of performance at these delays show that OA males given HMB outperformed controls across training. There were no significant effects of treatment on females' performance across training.

Experiment 2: operant strategy shifting

To assess cognitive flexibility, rats were first trained on a visual strategy and were then required to shift to a response strategy. A two-way ANOVA of trials to criterion in males revealed a significant treatment × age interaction ($F_{1,65}$ = 4.8; $P < 0.05$), but no significant main effects (Fig. 3A). *Post hoc* analyses revealed that there was a significant age-associated deficit in acquisition of the visual strategy in control males. OA control males required significantly more trials to reach criterion than their MA control counterparts, however, OA males treated with HMB did not exhibit this age-dependent deficit (Fig. 3A).

In females, HMB enhanced acquisition of the visual strategy. A two-way ANOVA of trials to criterion revealed a significant main effect of treatment only ($F_{1,61}$ = 5.2; $P < 0.05$), such that HMB-treated females outperformed their counterparts. This effect appeared to be largely driven by differences between the MA groups (Fig. 3A). Although OA controls outperformed MA controls, this difference did not reach statistical significance (P = 0.079). There were no significant differences in performance between OA HMB-treated females and their age-matched control group.

During the shift to a response strategy, we found that neither age nor treatment affected performance. Separate two-way ANOVAs of trials to criterion revealed no significant main effects or interactions in males or females. Investigation of the types of errors committed during the shift from visual to response strategy revealed no statistically significant group

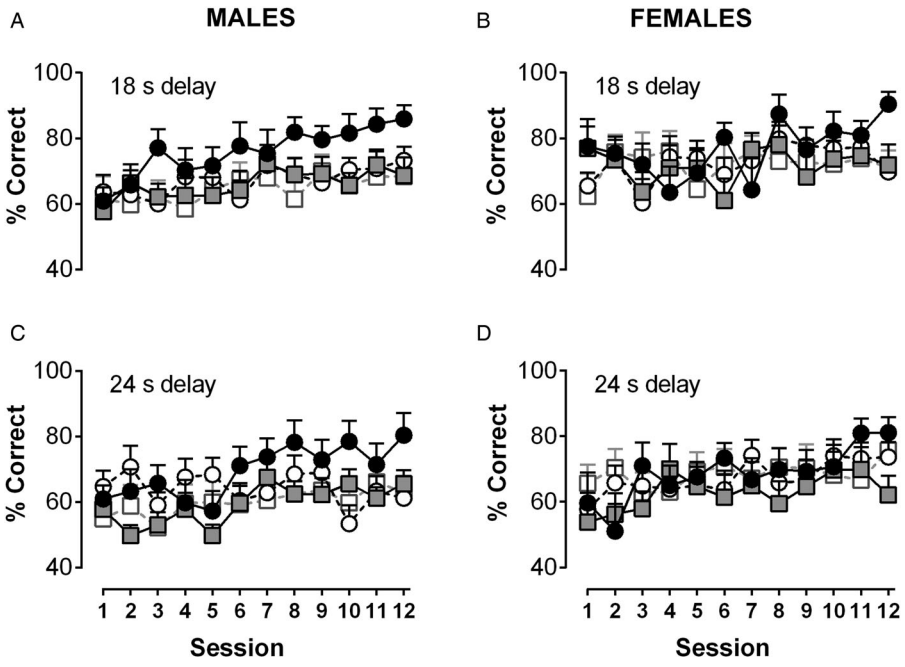

Figure 2 DMTP performance on long delay trials across training. HMB increased choice accuracy in males (A,C), but not females (B,D) across training sessions. OA males given HMB supplementation tended to outperform controls when cognitive load was greatest (18- and 24-second delays).

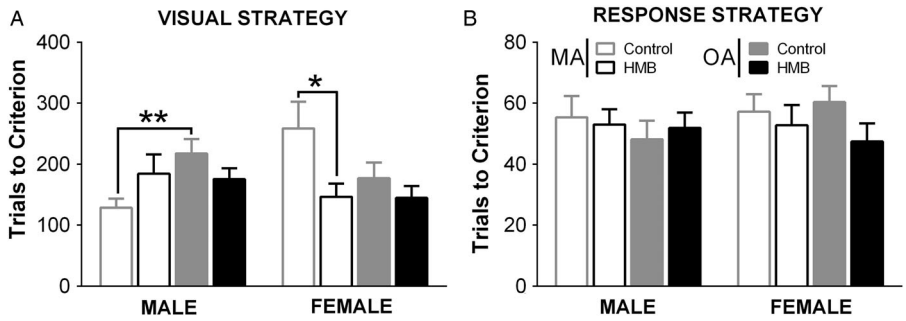

Figure 3 Performance during visual and shift-to-response strategy. Average trials to criterion for visual (A) and response strategies (B). OA males were significantly impaired on acquisition of the visual strategy (A), but not when they were treated with HMB. MA females treated with HMB significantly outperformed their same-age counterparts during acquisition of the visual strategy (A). There were no group differences in the shift to response (B). *$P < 0.05$ vs. MA control females; **$P < 0.05$ vs. MA control males.

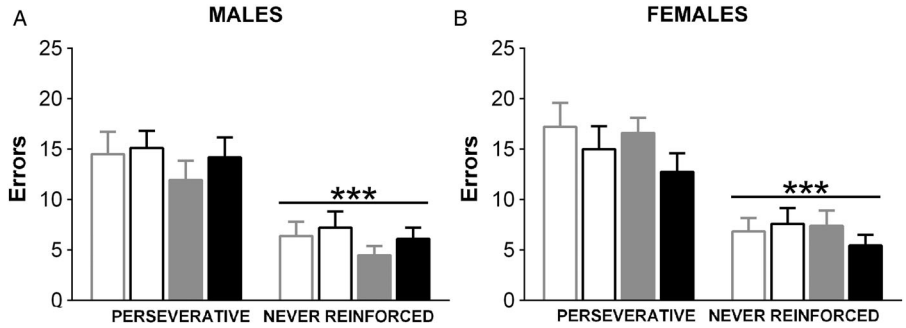

Figure 4 Errors during shift to response strategy. Average perseverative and never reinforced errors committed during the shift from visual to response strategy. Males (A) and females (B) made significantly more perseverative than never reinforced errors. ***$P < 0.001$ vs. perseverative errors.

differences (Fig. 4). However, separate two-way ANOVAs revealed a significant main effect of error type in males ($F_{1,65} = 115.0$; $P < 0.001$) and females ($F_{1,60} = 99.5$; $P < 0.001$), such that all rats committed significantly more perseverative than never reinforced errors.

Discussion

In this study, we used male and female rats to determine if daily HMB supplementation would mitigate age-associated cognitive deficits. We found that HMB-treated rats had enhanced performance in PFC-sensitive tasks assessing working memory and cognitive flexibility. Specifically, HMB enhanced working memory performance in OA males and females. These improvements were most robust later in training and at long delays when cognitive load was greatest. Treatment with HMB also appeared to mitigate an age-related deficit in acquisition of the visual strategy in control OA males. Furthermore, HMB supplementation improved acquisition of the visual strategy in MA females compared to their age-matched controls. Together, our results suggest that HMB supplementation may be an effective nutritional intervention for mitigating age-associated cognitive decline.

Assessment of working memory with a DMTP task revealed beneficial effects of HMB supplementation during aging. OA males outperformed all other groups at the longest delay(s), both early (24 seconds) and late in training (18 and 24 seconds), while OA females outperformed all other groups at the longest delays late in training only. These findings are consistent with a protective effect of HMB supplementation on medial PFC functioning, as working memory has been shown to depend on an intact medial PFC.[42,43] Moreover, the fact that HMB-treated OA males and females outperformed their MA counterparts suggests that HMB supplementation may be most beneficial after prolonged supplementation.

Assessment of cognitive flexibility with an operant strategy shifting task revealed age-associated deficits in acquisition of the initial (visual) strategy in males, but no group differences in the shift to response strategy. These results are consistent with a previous study demonstrating impaired acquisition of an initial, visual strategy in aged monkeys.[44] Importantly, OA males treated with HMB did not exhibit this age-associated deficit, suggesting that HMB may have attenuated age-related impairments in this task. Because

rule learning has been shown to depend on corticostriatal circuits,[45,46] this result suggests that HMB supplementation helped maintain corticostriatal function in OA males. The lack of age-dependent deficit in shift from visual to response strategy is not consistent with previous studies,[18,44] but is likely due to wider age ranges (e.g. 5 and 22 months) used to investigate the effects of aging on cognition in the other studies. In fact, a comparison of the trials to criterion between the current study and the report by Beas et al.[18] reveals that our MA group performed more similarly to their aged group than their young group, suggesting that age-associated deficits in strategy shifting appear prior to 13 months.

In females performing the cognitive flexibility task, we found that HMB improved performance. This effect was driven by a somewhat surprising deficit in acquisition in MA control females. One possible explanation for this is the recent surgery experience in MA females, as post-surgical neuroinflammation has been shown to be associated with deficits in learning and memory[47] for 14 days post-surgery. Heikkinen *et al.*[48] demonstrated that ovariectomy, specifically, resulted in impairments in a working memory task at 4 months, but not 8 or 19 months, post-surgery. These findings suggest that time since ovariectomy impacts performance on cognitive tasks, which is consistent with the transient deficit observed in our control females. MA females were about 2 months out from surgery at the time of testing, while OA females were >7 months post-ovariectomy. As such, the recent loss of circulating ovarian hormones and/or heightened neuroinflammation in MA control females may be responsible for the impaired performance observed during acquisition of the visual strategy.

Interestingly, supplementation with HMB mitigated the deficits in acquisition of the visual strategy that were observed in MA control females. One possible mechanism for this is HMB's downstream actions on the mTOR pathway that may either compensate for the loss of estrogen signaling or aid in the immune response to neuroinflammation. Non-classical actions of estrogen (i.e. binding membrane-bound receptors) can upregulate protein synthesis via the mTOR pathway.[49,50] In fact, inhibition of mTOR prevents estrogen's cognitive enhancing effects.[49] As such, it is possible that the recent loss of circulating estrogen was responsible for the deficits observed in MA control females and that replacement of one of its downstream effectors (e.g. mTOR) in HMB-treated MA females ameliorated these deficits. The lack of these deficits in OA females would suggest that compensatory mechanisms developed during the 7 months since ovariectomy to eliminate this deficit. Another potential mechanism involves mTOR's involvement in adaptive immune responses,[51] which may have mitigated the deleterious effects of post-surgical neuroinflammation on this task. Future studies will be necessary to directly test if these pro-cognitive effects of HMB are mediated by its influence on mTOR.

Notably, we did not observe age-dependent deficits during the working memory task or following the shift from visual to response, both tasks known to rely on an intact medial PFC.[41–43] The lack of age-associated deficits in these tasks is consistent with the assertion that performance on medial PFC-sensitive tasks may become impaired prior to 13 months, a conclusion consistent with other studies.[13,52–54] This suggests that HMB not only mitigated age-associated deficits in medial PFC function, but may have also *reversed* deficits that already existed at the onset of supplementation.

Although the precise mechanism(s) through which HMB confers anti-aging cognitive benefits are unknown, we speculate that HMB may attenuate cognitive decline by maintaining effective synaptic plasticity via its downstream effectors, such as IGF-1 and the mTOR pathway. Reduction of synaptic plasticity in the aged brain is likely caused by changes in cellular and molecular pathways that are required for normal plasticity to occur. For example, IGF-1 has been linked to age-related deficits in synaptic plasticity and cognitive decline,[55] and age-related deficits in mTOR signaling disrupt downstream gene expression that is essential for synaptic plasticity and memory.[56] HMB upregulates IGF-1[33] and HMB's effector, mTOR, is a downstream target of both IGF-1 and BDNF. As such, restoration of these pathways that are required for synaptic plasticity[57–61] may be the mechanism through which HMB exerts its anti-aging cognitive benefits. Regardless of the precise mechanism, our results suggest that HMB ameliorates age-associated cognitive decline and may be an effective nutritional supplement to combat this pervasive problem.

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Disclaimer statements

Contributors E.R.H. assisted with data collection, statistical analysis, interpreting data, and writing the manuscript. L.K.S. assisted with data collection,

statistical analysis, interpreting data, and editing the manuscript. L.A.R., R.M.H., T.K., L.R.H., and D.G.K. assisted with data collection. J.M.J. designed the study, obtained funding for the project, assisted with data interpretation, and helped revise the manuscript. J.M.G. designed the study, obtained funding for the project, assisted with data analysis and interpretation, and helped with writing and revising the manuscript.

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Ethics approval The research described in the manuscript is in compliance with APA ethical standards in the treatment of animal subjects and was approved by the Institutional Animal Care and Use Committee at the University of Illinois, Urbana–Champaign.

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