



Beta-hydroxy-beta-methylbutyrate ameliorates aging effects in the dendritic tree of pyramidal neurons in the medial prefrontal cortex of both male and female rats



Daniel G. Kougias^a, Suzanne O. Nolan^b, Wendy A. Koss^b, Taehyeon Kim^b, Emily R. Hankosky^b, Joshua M. Gulley^{a,b}, Janice M. Juraska^{a,b,*}

^a Neuroscience Program, University of Illinois, Champaign, IL, USA

^b Department of Psychology, University of Illinois, Champaign, IL, USA

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ABSTRACT

Beta-hydroxy-beta-methylbutyrate (HMB), a supplement commonly used to maintain muscle in elderly and clinical populations, has been unexplored in the aging brain. In both healthy aging humans and rat models, there are cognitive deficits associated with age-related dendritic shrinkage within the prefrontal cortex. The present study explores the effects of relatively short- and long-term (7 and 31 weeks) oral HMB supplementation starting at 12 months of age in male and female rats on the dendritic tree of layer 5 pyramidal neurons in the medial prefrontal cortex. Since female rats continue to secrete ovarian hormones after reaching reproductive senescence, middle-aged female rats were ovariectomized to model humans. As expected, there were fewer spines and a retraction of dendritic material in the apical and basilar trees in old age controls of both sexes compared with their middle-aged counterparts. However, these losses did not occur in the HMB-treated rats in either dendrites or the total number of dendritic spines. Thus, HMB forestalled the effects of aging on the dendritic tree of this population of neurons.

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1. Introduction

With medical advances and increases in life expectancy, there is a large, rapidly growing population of people 65 years and older. As a result, there is a need to explore preventative measures directed at the cognitive and neural declines that accompany normal aging. Normal aging humans show deficits in several modalities of executive functioning such as memory, attention, decision making, and visuospatial abilities (reviewed in Erickson and Barnes, 2003; Gallagher and Rapp, 1997). This cognitive decline in normal healthy aging is unsurprisingly accompanied by age-related changes in neurons within the prefrontal cortex (PFC) and medial temporal lobe, which are brain areas associated with the aforementioned functions (Burke and Barnes, 2006). Notably, in the PFC there is a significant decrease in neuropil, specifically in dendritic arborization, spine density, and spine number (de Brabander et al., 1998; Jacobs et al., 1997), which contributes to the age-related decreases in brain and gray matter volume (Coffey et al., 1992; Tisserand et al., 2002).

Similar to humans, aging animals exhibit a decline in performance on tasks involving working memory, reversals, and spatial learning (reviewed in Erickson and Barnes, 2003; Gallagher and Rapp, 1997). In addition, this decline in performance is associated with age-related neural losses in the PFC. In particular, a loss of both dendrites and spines has been well-characterized in aging rhesus monkeys (Cupp and Uemura, 1980; Duan et al., 2003; Kabaso et al., 2009; Uemura, 1980). Likewise, there is a reduction in dendritic arborization with fewer dendritic spines in the medial PFC (mPFC) of aged rats (Allard et al., 2012; Markham and Juraska, 2002).

In an effort to combat age-related cognitive decline and the associated neuroanatomic changes, dietary nutrients are being investigated, and some appear to correlate with significant effects in both cognitive function and brain volume in normal aging humans (Bowman et al., 2012). One such nutrient, beta-hydroxy-beta-methylbutyrate (HMB), known to be beneficial for aging muscles, may have potential for ameliorating cognitive decline in the normal healthy aging population. Although HMB has been extensively studied in peripheral tissue (i.e., muscle), considerably less is known about its effects in the brain. This is a gap in knowledge that needs to be addressed as many peripheral mechanisms by which HMB exerts its positive effects parallel mechanisms that may

* Corresponding author at: Department of Psychology, University of Illinois, 603 E. Daniel, Champaign, IL, 61820, USA. Tel.: +1 217 333 8546; fax: +1 217 244 5876. E-mail address: jjuraska@illinois.edu (J.M. Juraska).

be beneficial for the aging brain. These peripheral effects of HMB include saturating the rate-limiting enzyme in cholesterol synthesis (reviewed in [Nissen and Abumrad, 1997](#)), increasing protein synthesis via the mechanistic target of rapamycin (mTOR) pathway ([Eley et al., 2007](#); [Hoeffler and Klann, 2010](#)), and upregulating the growth hormone and/or insulin-like growth factor-1 (GH/IGF-1) axis ([Gerlinger-Romero et al., 2011](#)). These are mechanisms that may be vulnerable to aging and contribute to deficits in the aging brain ([Thornton et al., 2000](#); [Yang et al., 2014](#)).

Although most literature on the aging rat model focuses solely on males, it is pertinent to use both male and female rats to reflect the aging human population, which has proportionately more females. As human females age, they experience reproductive senescence (menopause), which is the cessation of gonadal hormone cyclicality accompanied by a dramatic loss of ovarian hormone production ([Perheentupa and Huhtaniemi, 2009](#)). In contrast, this cessation in aging female rats does not include such a dramatic decline of ovarian hormones ([Huang et al., 1978](#); [Wise and Ratner, 1980](#)). Rather, female rats' ovaries continue to secrete low to moderate levels of estrogen and progesterone after their cycles have stopped, and the relative amounts depend on their estrous status. Since there is evidence that circulating estrogens are neuroprotective in aged female rats ([Chisholm and Juraska, 2012](#); [Chisholm et al., 2012](#); [Garcia-Segura et al., 2001](#)), female rats in the present study were ovariectomized (OVX) in middle age to model normal human female aging following menopause, whereas males underwent sham surgery.

In this study, middle-aged (12-month old) rats were supplemented with an HMB solution or vehicle for either a relatively short (7 weeks) or long (31 weeks) period of time. We use a middle-age time point to assess whether HMB has any direct effects on neuronal morphology and an aged time point to determine whether HMB supplementation mitigates the normal neuroanatomic changes that are associated with aging. We focused our investigation within the mPFC on neuronal morphology to dendritic arborization, spine density, and spine number measurements of layer 5 pyramidal neurons as this layer appears to be vulnerable to age-related changes in both humans and rats ([de Brabander et al., 1998](#); [Markham and Juraska, 2002](#)).

2. Materials and methods

2.1. Subjects

Subjects were male ($n = 37$) and female ($n = 32$) Long Evans hooded rats obtained from Harlan Laboratories (Indianapolis, IN, USA) at approximately 10 months of age. The subjects were housed individually in standard clear Plexiglas laboratory cages, fed and hydrated ad libitum, and weighed weekly. The colony was maintained on a 12-hour light/dark cycle, with lights on at 0800 hours. At 11 months, all subjects were anesthetized with isoflurane vapors and underwent surgery. In accordance with animal care policy, rats were administered the analgesic carprofen (5 mg/kg delivered subcutaneously) immediately after anesthetization and again 6- to 12-hour later. Female rats were OVX via bilateral dorsal incisions, whereas male rats underwent a sham surgery and retained their gonads.

2.2. Dosing regimen

HMB levels peak in the circulation by 1–2 hours after ingestion and reach baseline levels by 9 hours ([Vukovich et al., 2001](#)). Thus, HMB was administered twice daily with a target dose of 450 mg calcium-HMB (Ca-HMB)/kg body weight (BW), a dose shown to aid aging muscle ([Wilson et al., 2012](#)). Sipper tubes containing the

vehicle (32 mg/mL calcium lactate + 20% sucrose) or HMB solution (50 mg/mL Ca-HMB + 20% sucrose) were placed into rats' home cages twice daily (~727 mg HMB/kg BW/day) Monday–Saturday (at approximately 0800 and 1800 hours) and once (~364 mg HMB/kg BW/Sunday) on Sunday (between 1800 and 2000 hours). Most rats began consuming their solution immediately and finished within 60 minutes of it being placed in their cage. However, tubes remained in cages until the next dose to maximize consumption. For each rat, the amount consumed was recorded, and the actual dose ingested was estimated.

Dosing with either vehicle or HMB solution began at 12 months and continued until day of sacrifice. The short-term treatment (middle-age) group was dosed for a month before the start of behavioral testing in the water maze (not reported here), whereas the long-term treatment (aged) group was dosed for 7 months before behavioral testing. Dosing continued through the week of behavioral testing in a water maze (a day of pretraining, a day of rest, and then 4 consecutive days of testing) and for the 2 weeks after training until sacrifice. Rats were injected with sodium pentobarbital (100 mg/kg; Sigma, St. Louis, MO, USA), and their brains were harvested for Golgi Cox staining and processing. Within the short-term treatment (middle-age) group, 7 males and 9 females were administered vehicle, and 10 males and 9 females were administered HMB. Within the long-term treatment (aged) group, 11 males and 8 females were administered vehicle, and 9 males and 6 females were administered HMB. Animal care and experimental procedures were in accordance and approved by the Institutional Animal Care and Use Committee at the University of Illinois Urbana-Champaign.

2.3. Neuroanatomy

2.3.1. Histology

All brains were Golgi impregnated by methods similar to those described previously ([Glaser and Van der Loos, 1981](#)) and in work from our laboratory ([Koss et al., 2014](#); [Markham and Juraska, 2002](#)). Golgi-Cox solution was prepared from 5% solutions of potassium dichromate, mercuric chloride, and potassium chromate in a volume ratio of 5:5:4. At sacrifice, the brain was placed into Golgi-Cox solution for approximately 20 days and coded to keep the experimenter blind to the animal's identity. Coronal test-slices were cut periodically near the region of interest to ensure adequate staining. Once neurons were entirely filled, the brains were dehydrated, embedded in 12% celloidin solution, hardened in a desiccator, and stored in butanol until slicing. Brains were coronally sliced on a microtome at 150 μm to capture the entire extent of the dendritic tree of neurons. Tissue slices were developed in a series of solutions, mounted on slides with permount, and coverslipped.

2.3.2. Identification of layer 5 pyramidal neurons in the mPFC

Neurons were sampled in the mPFC from the initial appearance of cortical white matter (i.e., the forceps minor) to the first appearance of the genu of the corpus callosum. The ventral and dorsal boundaries of the prelimbic and infralimbic areas of the mPFC were conservatively identified based on previous work from our laboratory ([Koss et al., 2014](#); [Markham and Juraska, 2002](#)), while referencing Nissl-stained brain tissue. Neurons from layer 5 were selected based on a pyramidal morphology within approximately 500–750 μm from the top of layer 1 that had a thick apical dendrite extending at least 250 μm toward the pial surface ([Fig. 1](#)).

2.3.3. Quantification of dendritic arbor

Three dimensional tracing of neurons was carried out using a 63X objective on a Zeiss Axioimager A1 light microscope with NeuroLucida 9.0 software, whereas subsequent analyses of

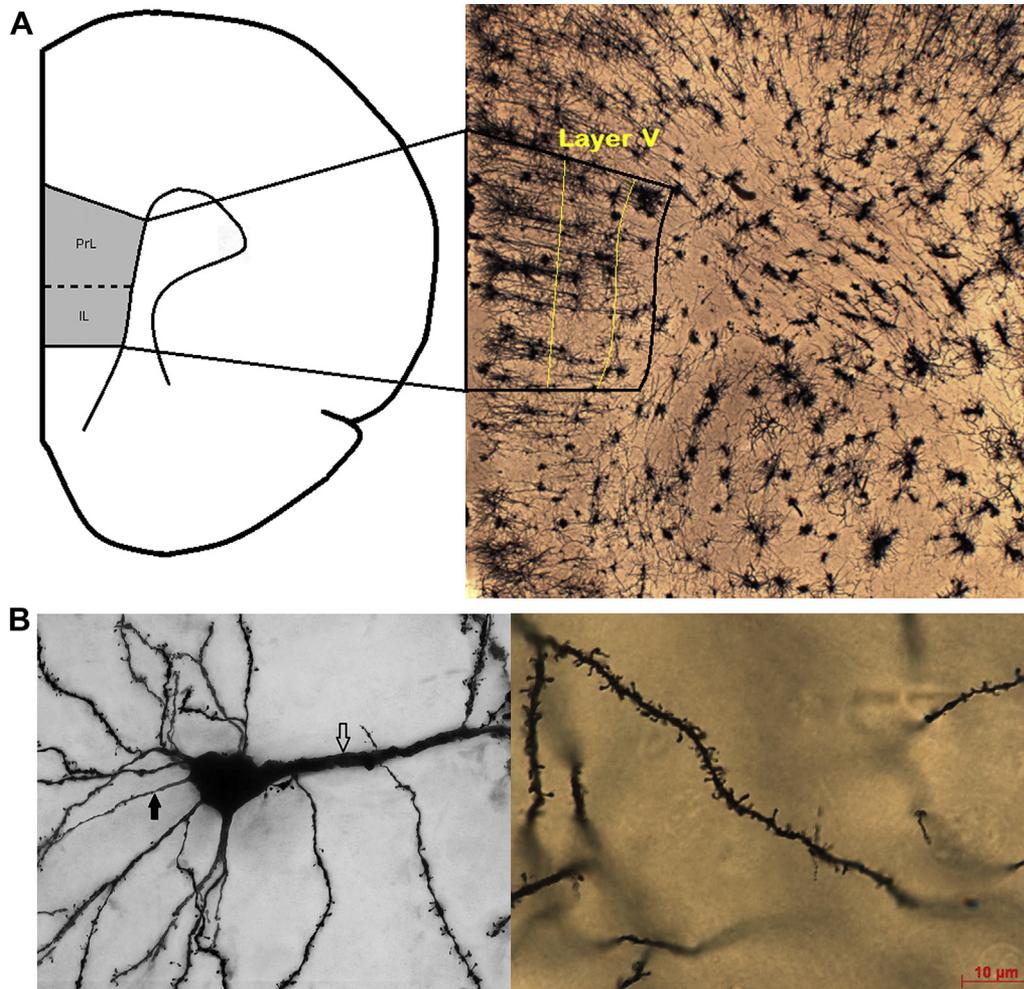


Fig. 1. (A) Prelimbic (PrL) and infralimbic (IL) areas of the mPFC (left) parcellated on Golgi Cox impregnated tissue with layer 5 (V) indicated (right). (B) A Golgi Cox impregnated layer 5 pyramidal neuron in the mPFC (left) and dendritic spines on a dendrite (right). Filled arrow: axon. Unfilled arrow: apical dendritic shaft. Abbreviations: mPFC, medial prefrontal cortex.

dendritic extent were performed with Neurolucida Explorer 4.7 (Microbrightfield). Ten neurons were sampled per brain and entirely traced except for the apical tufts in layer 1 to avoid inconsistencies as some neurons possess tufts and others do not. The total length, length of higher order ($3^{\circ}+$) branches (with branches from the soma as 1°), and branch points of the basilar tree were measured. Because the Neurolucida Program did not separate the apical shaft (which is standard for pyramidal neurons) from the oblique dendrites that emanate from the shaft, a Sholl analysis, rather than branch analysis, of the apical tree was performed. The number of intersections between dendrites and concentric spheres every $20\ \mu\text{m}$ from the cell body was calculated (Sholl, 1956). The Sholl analysis of the apical tree was only performed up to $240\ \mu\text{m}$ away from the cell body to include all neurons since some apical shafts end or are obscured and/or truncated before reaching the top of the cortex. The branch points of the apical tree were also measured within $240\ \mu\text{m}$ from the cell body. All measurements were averaged within each animal, such that animal was the unit of analysis.

2.3.4. Quantification of dendritic spines

Dendritic spines were assessed on 10 segments of the basilar tree of pyramidal neurons per animal using a 100X objective on a Zeiss light microscope with an attached camera lucida. All segments

within an animal were sampled from different neurons. Counts were made on segments of at least $10\ \mu\text{m}$ (average $\sim 15\ \mu\text{m}$) of higher order ($3^{\circ}+$) branches that were in a single z-plane of focus to avoid errors in calculating segment length. This method does not include spines hidden by the dendrite itself but does result in counts that are proportional between groups (Mancuso et al., 2013). Spine density was calculated as the average number of spines per $10\ \mu\text{m}$ of dendritic length per animal. Because spine density measurements were made on higher order ($3^{\circ}+$) branches, the total number of spines was calculated from the product of the spine density and total length of higher order ($3^{\circ}+$) branches of the basilar tree for each animal.

2.4. Statistical analyses

All analyses were performed using Systat 12.0. The neuroanatomic measurements were analyzed using 3-way Analysis of Variances (ANOVAs; age, treatment, and sex). Post-hoc comparisons were done on those with significant interactions. In addition, the $20\text{-}\mu\text{m}$ concentric spheres were a repeated measure for the Sholl analyses, which was analyzed using a 3-way ANOVA (age, treatment, and sex). Post-hoc Bonferroni comparisons were performed on individual spheres for factors that significantly interacted with sphere.

3. Results

3.1. Dose

The mean dose of HMB consumed by male and female rats in either age group was not significantly different. The mean (\pm standard error of mean [SEM]) dose of Ca-HMB consumed by male and female rats in the middle-age groups was 423 ± 5.46 mg/kg ($\sim 635 \pm 8.19$ mg HMB/kg BW/day). Likewise, the mean (\pm SEM) dose consumed by male and female rats for those in the aged groups was 415 ± 8.93 mg/kg ($\sim 623 \pm 13.40$ mg HMB/kg BW/day).

3.2. Body weight

The average BWs across all experimental groups did not significantly differ from each other before dietary supplementation, except for the obvious sex difference ($p < 0.001$). However, BWs on sacrifice exhibited main effects of age [$F(1, 61) = 29.099$, $p < 0.001$] and sex [$F(1, 61) = 312.241$, $p < 0.001$], such that middle-aged animals had lower BWs than aged animals and females had lower BWs than males (Fig. 2). There were also significant sex by treatment [$F(1, 61) = 4.893$, $p = 0.031$] and sex by treatment by age [$F(1, 61) = 5.208$, $p = 0.026$] interactions, which revealed that old-age females treated with HMB had lower weights than old-age females treated with vehicle [$t = 2.254$, $p = 0.046$], and that there was a trend for old-age males treated with HMB having greater BWs than old-age males treated with vehicle ($t = -1.935$, $p = 0.07$). Furthermore, it is worth noting that there were no correlations between BWs and any of the subsequent neuroanatomic measurements.

3.3. Dendritic tree

3.3.1. Total length of the basilar tree

There were main effects of age [$F(1, 61) = 9.8$, $p = 0.003$] and treatment [$F(1, 61) = 7.9$, $p = 0.007$], such that middle-aged animals had more extensive basilar trees than aged animals and HMB-

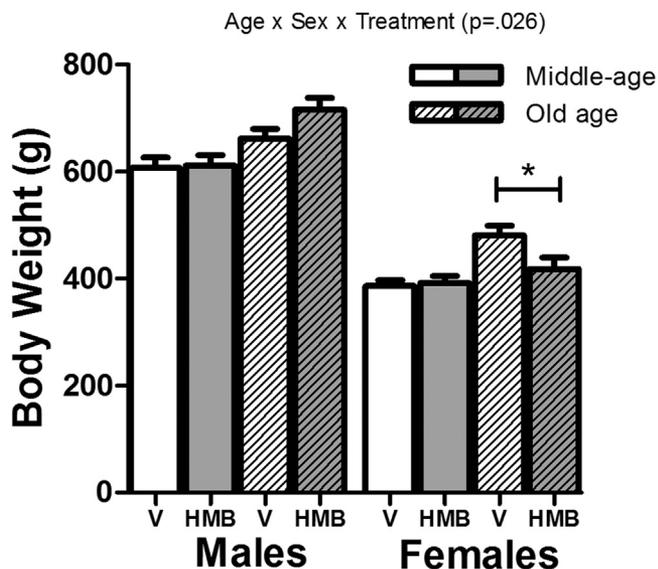


Fig. 2. The average BW (\pm SEM) at sacrifice. Middle-aged animals had lower BWs than aged animals ($p < 0.001$) and females had lower BWs than males ($p < 0.001$). Significant sex by treatment ($p = 0.031$) and sex by treatment by age ($p = 0.026$) interactions revealed that old HMB-treated females had lower weights than old vehicle-treated females ($p = 0.046$). Abbreviations: BW, body weight; HMB, beta-hydroxy-beta-methylbutyrate; SEM, standard error of mean; V, vehicle-treated.

treated animals had more extensive basilar trees than vehicle-treated animals (Fig. 3A). There was also a nonsignificant weak treatment by age interaction [$F(1, 61) = 2.9$, $p = 0.096$]. There were no significant effects of sex or any interaction between sex and the other factors.

3.3.2. Length of higher order ($3^\circ+$) branches of the basilar tree

Again, there was a significant main effect of age [$F(1, 61) = 8.6$, $p = 0.005$] and of treatment [$F(1, 61) = 6.0$, $p = 0.017$; Fig. 3B]. Middle-aged animals had a greater total length of dendritic branches that were 3° and above than did old-age animals, and HMB-treated animals had a greater total length of higher order branches compared with vehicle-treated animals. A weak age by treatment interaction [$F(1, 61) = 2.9$, $p = 0.095$] occurred. Moreover, there were no sex differences or any interactions with sex.

3.3.3. Branch points of the basilar tree

Similarly, there were main effects of age [$F(1, 61) = 7.4$, $p = 0.009$] and of treatment [$F(1, 61) = 7.0$, $p = 0.010$; Fig. 3C]. Middle-aged animals had more branch points than did old-age animals, and HMB-treated animals had more branch points compared with vehicle-treated animals. A weak age by treatment interaction [$F(1, 61) = 3.0$, $p = 0.087$] occurred. Moreover, there were no sex differences or any interactions with sex.

3.3.4. Sholl analysis of the apical tree

The ANOVA revealed significant interactions between treatment and sphere [$F(11, 671) = 1.8$, $p = 0.047$] and age and sphere [$F(11, 671) = 3.6$, $p < 0.001$; Fig. 4A]. Further analysis with post hoc Bonferroni comparisons revealed that at $160 \mu\text{m}$ away from the cell body both HMB-treated animals showed a trend toward having more intersections than vehicle-treated animals ($p = 0.068$) and middle-aged animals also showed a trend toward having more intersections than aged animals ($p = 0.067$). The ANOVA also revealed a nonsignificant treatment trend [$F(1, 61) = 3.5$, $p = 0.065$] and a weak trend for a sphere by sex interaction [$F(11, 671) = 1.6$, $p = 0.084$]. Furthermore, when blocking the first 6 inside spheres and the latter 6 outside spheres (Fig. 4B), the Bonferroni-adjusted test indicated that although in the inner spheres there were no significant effects, in the outer spheres both HMB-treated animals had more intersections than vehicle-treated animals ($p = 0.010$) and middle-aged animals had more intersections than aged animals ($p = 0.007$).

3.3.5. Branch points of the apical tree

The ANOVA revealed no significant effects; however, there was a trend for age [$F(1, 61) = 3.688$, $p = 0.059$] such that middle-aged animals had a tendency to have more apical branch points than old-age animals (8.96 ± 0.33 middle aged; 8.09 ± 0.23 old age).

3.4. Dendritic spines

3.4.1. Spine density

Aged animals had a greater spine density than did middle-aged animals [$F(1, 61) = 16.6$, $p < 0.001$; Fig. 5A]. An age by treatment interaction [$F(1, 61) = 4.3$, $p = 0.043$] showed that middle-aged vehicle-treated animals had a greater spine density than middle-aged HMB-treated animals ($t = 2.901$, $p = 0.007$). It also revealed that aged HMB-treated animals had a greater spine density than middle-aged HMB-treated animals ($t = -4.75$, $p < 0.001$). Again, there were no significant effects of sex or significant interactions between sex and other factors.

3.4.2. Total spine number

The number of spines was calculated by multiplying spine density (number and/or length) by the length of the dendrites on

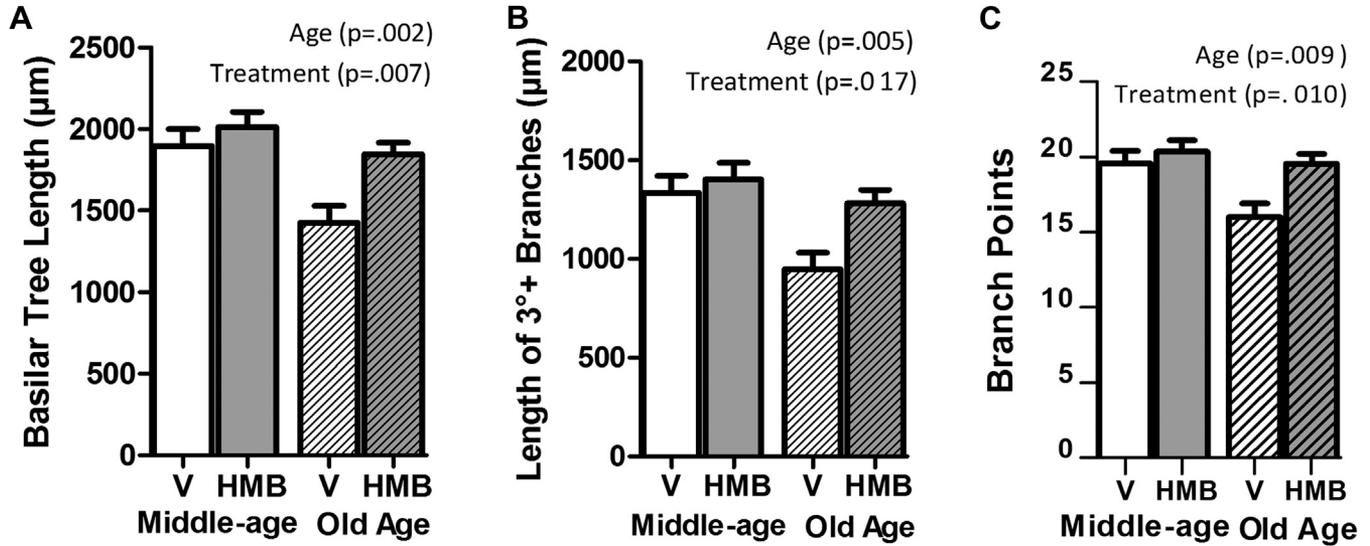


Fig. 3. The basilar tree for both sexes combined (no sex differences). (A) The average length ($\mu\text{m} \pm \text{SEM}$) of the basilar tree per neuron. Middle-aged animals had a greater length than old-age animals ($p = 0.002$), and HMB-treated animals had a greater length compared with vehicle-treated (V) animals ($p = 0.007$). (B) The average length ($\mu\text{m} \pm \text{SEM}$) of higher order ($3^{\circ+}$) branches of the basilar tree per neuron. Middle-aged animals had a greater length than did old-age animals ($p = 0.005$), and HMB-treated animals had a greater length compared with vehicle-treated (V) animals ($p = 0.017$). (C) The average number of branch points ($\pm \text{SEM}$) in the basilar tree per neuron. Middle-aged animals had more branch points than did old-age animals ($p = 0.009$), and HMB-treated animals had more compared with vehicle-treated (V) animals ($p = 0.010$). Abbreviations: HMB, beta-hydroxy-beta-methylbutyrate; SEM, standard error of mean; V, vehicle-treated.

which the spines were counted ($3^{\circ+}$) for each animal. An ANOVA of spine number resulted in an age by treatment interaction [$F(1, 61) = 5.4, p = 0.024$] which showed that aged vehicle-treated animals had significantly fewer spines than middle-aged vehicle-treated animals ($t = 2.385, p = 0.023$; Fig. 5B). Furthermore, there was a lack of a significant difference between middle-aged HMB-treated animals and aged HMB-treated animals ($t = -0.8, p = 0.43$). There was a weak trend [$F(1, 61) = 2.9, p = 0.093$] for the main effect of treatment. In addition, there were no sex differences or any interactions with sex.

4. Discussion

The present study indicated that daily ingestion of HMB increases the size of the basilar dendritic tree in the rat mPFC of both sexes. This increase, which appeared in both middle and old-aged animals, is especially important for the aged where there is regression of dendrites. It did not appear to directly affect spines in that density was not increased in the HMB-fed groups, but spine numbers did not regress during aging because HMB was increasing the dendritic tree and the spine density did not go down. These

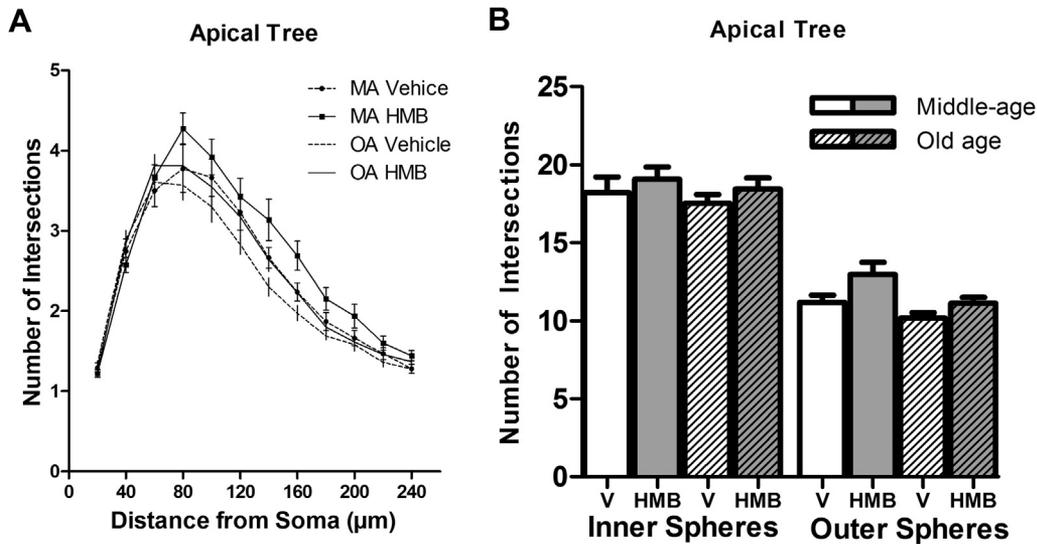


Fig. 4. Sholl analyses of the apical tree for both sexes combined (no sex differences). An intersection denotes the intersection of apical dendrites with concentric spheres superimposed every 20 μm from the cell body. (A) The average number of intersections ($\pm \text{SEM}$) per neuron in the apical tree at concentric spheres every 20 μm from the soma. There was a treatment by sphere interaction ($p = 0.047$), and a significant age by sphere interaction ($p < 0.001$). (B) The total number of intersections ($\pm \text{SEM}$) per neuron in the apical tree at concentric spheres every 20 μm from the soma summed into 2 blocks: inner spheres (20–120 μm) and outer spheres (140–240 μm). In the outer spheres, HMB-treated animals had more intersections than vehicle-treated animals ($p = 0.010$) and middle-aged animals had more intersections than aged animals ($p = 0.007$). There were no significant differences in the inner spheres. Abbreviations: HMB, beta-hydroxy-beta-methylbutyrate; MA, middle-aged; OA, old age; SEM, standard error of mean; V, vehicle-treated.

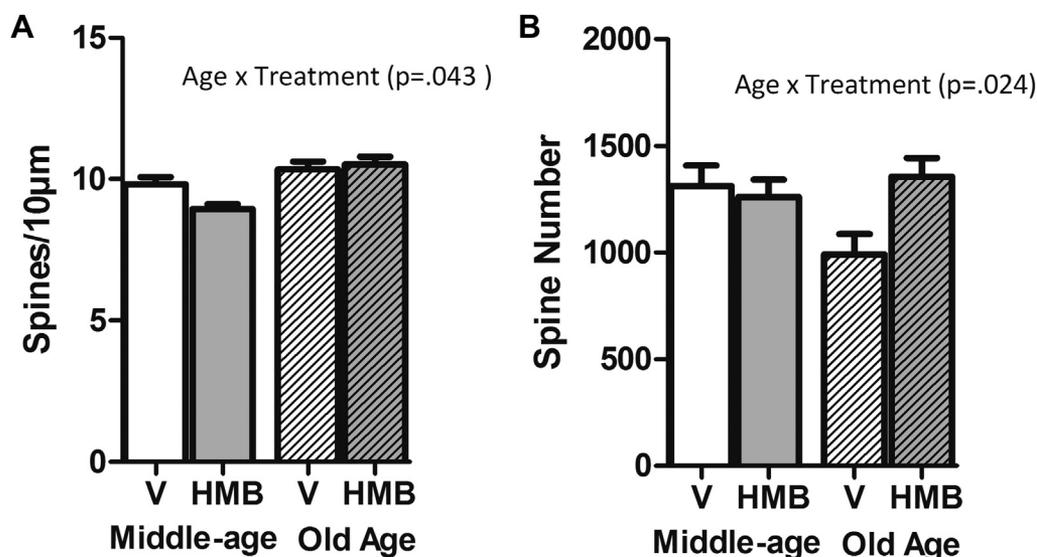


Fig. 5. Spines on higher order ($3^{\circ}+$) branches of the basilar tree from both sexes combined (no sex differences). (A) The average spine density (spines/ $10\mu\text{m} \pm \text{SEM}$) on higher order ($3^{\circ}+$) branches of the basilar tree per neuron. Aged animals had a greater spine density than did middle-aged animals ($p < 0.001$). An age by treatment interaction ($p = 0.043$) showed that middle-aged vehicle-treated (V) animals had a greater spine density than middle-aged HMB-treated animals ($p = 0.007$). It also revealed that HMB-treated animals had a greater spine density than middle-aged HMB-treated animals ($p < 0.001$). (B) The average total number of spines ($\pm \text{SEM}$) on higher order ($3^{\circ}+$) branches of the basilar tree per neuron. An age by treatment interaction ($p = 0.024$) showed that aged vehicle-treated (V) animals had a significantly smaller number of spines than middle-aged vehicle-treated animals ($p = 0.023$). Abbreviations: HMB, beta-hydroxy-beta-methylbutyrate; SEM, standard error of mean; V, vehicle-treated.

positive effects of HMB on dendrites appear similar to its effects on muscle atrophy. It remains to be tested whether other situations with neural regression such as degenerative diseases might be ameliorated by HMB.

HMB's mechanisms of action in the periphery may be relevant for the brain as many of the molecular processes in the periphery and brain are similar. HMB is postulated to increase sarcolemmal (i.e., muscle cell wall) integrity by upregulating cholesterol synthesis to permit maintenance of the plasma membrane while providing maximal growth and cell function (reviewed in Routhier and Stacy, 2007). Given that HMB crosses the blood brain barrier (Santos-Fandila et al., 2014), it may be similarly metabolized into cholesterol and provide the raw material necessary for the maintenance of dendrites and spines. Cholesterol is speculated to enhance neuronal synaptogenesis, specifically dendrite and synaptic terminal differentiation (Clarke and Barres, 2013; Mauch et al., 2001). Moreover, the addition of cholesterol significantly increases the number of excitatory synapses and presynaptic vesicle release (Goritz et al., 2005; Mauch et al., 2001).

In addition to its putative role in saturating the cholesterol synthesis pathway, HMB also enhances muscle protein synthesis via upregulation of the mTOR/p70^{S6k} pathway (Eley et al., 2007), a pathway known to decrease in activity within the aging brain (Yang et al., 2014). This may be another mechanism of HMB within the brain since HMB has been demonstrated in neural cells to regulate mTOR (Lopez-Pedrosa et al., 2011) via upstream signaling pathways that consequently promote neurite outgrowth through downstream transcriptional activity (Salto et al., 2015). Furthermore, mTOR signaling has been implicated with memory facilitation, synaptic plasticity (Hoeffler and Klann, 2010), dendritic growth (Urbanska et al., 2012), and synaptogenesis through the regulation of localized protein synthesis within dendrites (Glanzer, 2003). Notably, not only the maintenance but also the maturation of dendritic spines is contingent on mTOR/p70^{S6k} activation (Lai et al., 2013).

HMB also upregulates the growth hormone (GH) and/or insulin-like growth factor-1 (IGF-1) axis by increasing pituitary output of GH and as a result hepatic output of IGF-1 (Gerlinger-Romero et al.,

2011). There is a decrease in IGF-1 and IGF-1 receptors in the aging brain (Sonntag et al., 1999), which is associated with cognitive decline (Aleman and Torres-Alemán, 2009). In addition, administration of the upstream GH-releasing hormone restores GH and IGF-1 levels and enhances Morris water maze performance in rats with spatial learning and reference memory deficits associated with the hormonal decline (Thornton et al., 2000). More pertinent to our findings, it is known that IGF-1 is crucial for dendritic growth (Cheng et al., 2003). Furthermore, considering GH permeates across the blood brain barrier (Pan et al., 2005), GH may be contributing to HMB's effects since there is evidence that GH increases spine density in the amygdala (Gisabella et al., 2014).

The effects of HMB ingestion on the apical dendrites were minimal. This may be the result of the apical dendrites not being quantified beyond $240\mu\text{m}$ of length from the cell body because of the long apical often being obscured and/or cutoff beyond this point. More pronounced aging and HMB effects might have been found if the whole apical tree was available for quantification. However, it is also possible that HMB does not affect the dendritic tree uniformly and the effects on dendritic branching and spine numbers found in the basilar tree are an overestimate of the magnitude of effects on the whole neuron or on the other neurons within the mPFC. This is a limitation of the present study that awaits further investigation.

Spine density (number of spines/unit length) is just a first step in quantifying the number of spines, and it can be misleading when dendritic branches are changing, as is seen here. However, both spine density and the size of the dendritic tree generally regress with age, including in the mPFC (Bloss et al., 2013; Markham and Juraska, 2002). In contrast, the present study showed spine density to increase between the middle- and old-age controls, even as spine number per neuron decreased. We do not know why this happened but one speculation is that the sucrose solution that both the control and HMB groups received twice a day may have slowed spine regression during aging. There is a literature indicating that glucose is beneficial for performance in cognitive tasks in aging rats (reviewed in Gold, 2005) and may be mTOR-dependent (Dash et al., 2006). In addition, the increased activity in the colony with HMB

administration twice a day over the long months of aging, and the behavioral testing, may have contributed enough “enrichment” to slow spine loss in the most aged. Since the effects of HMB on the aged were compared with the vehicle group that experienced the same activity and glucose intake, the within age effects are not confounded.

Aside from the neuroanatomic effects, HMB also had effects on BW in aged animals that varied with sex; however, this gross measurement did not correlate with any neuroanatomic measurements. In aging male and female rats, there typically is an increase in BW (Mazzeo and Horvath, 1986; Thomas et al., 2002) and changes in body composition. Generally, these changes are marked by a progressive decline in lean body and skeletal muscle mass and a counterbalancing increase in body fat (French et al., 2008; Garthwaite et al., 1986). In the present study, both male and female rats had increased BWs with age; however, old HMB-treated females weighed less than old vehicle-treated females, whereas old HMB-treated males, though not significant, had a tendency to weigh more than old vehicle-treated males. Considering the lack of body composition data and female rat literature, it is difficult to speculate how HMB is differentially affecting BW in both sexes although HMB has been previously shown to maintain, and occasionally increase, muscle and lean body mass while also decreasing fat mass in aging male rats (Wilson et al., 2012).

The present study is unique in examining the dendritic structure between middle- and old-age rats of both sexes using OVX females to model human postmenopausal aging. Here, there was an absence of sex differences in both middle- and old-age groups and in the changes between the ages, such that OVX females aged similar to males. This contrasts with previous work from our laboratory on the same neuronal population where young intact adult (3–5 months) females had smaller dendritic trees and lower spine density than young males of the same age. Some of these sex differences persisted into old age (20–24 months), whereas others faded indicating that females had less pronounced age-related decreases (Markham and Juraska, 2002). This was likely because of the circulating ovarian steroids in both intact young adult and old females, and these hormones were eliminated in the present study. In support of this, our laboratory has previously shown that aged (19–20 months) female rats that were OVX at middle-age (12–13 months) and replaced with 17 β -estradiol and medroxyprogesterone acetate had more synapses (Chisholm and Juraska, 2012) and dopaminergic fibers (Chisholm et al., 2012) compared with OVX females with no replacement as assessed by synaptophysin-labeled boutons and tyrosine hydroxylase fiber density, respectively.

This study establishes beneficial effects of HMB on the aging brain, and it provides the foundation for further investigation of the potential nootropic effects of HMB in the cognitive and neural decline of normal healthy aging. Studies examining the effects of HMB on cognitive performance in middle age and aging rats are in progress and indicate that HMB is often beneficial (Gulley et al., 2014; Juraska et al., 2014; Kougias et al., 2013).

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