A blueberry-enriched diet provides cellular protection against oxidative stress and reduces a kainate-induced learning impairment in rats

Kara B. Duffya, Edward L. Spanglera, Bryan D. Devana, Zhihong Guob, Jonna L. Bowker a, Anne M. Janas a, Adrienne Hagepanos b, Robin K. Minor a, Rafael DeCabo a, Peter R. Moutona, Barbara Shukitt-Hale c, James A. Joseph c, Donald K. Ingrama,d,∗

a Laboratory of Experimental Gerontology, Intramural Research Program, National Institute on Aging, National Institutes of Health, 5600 Nathan Shock Drive, Baltimore, MD 21224, USA
b Laboratory of Neurosciences, Intramural Research Program, National Institute on Aging, National Institutes of Health, 5600 Nathan Shock Drive, Baltimore, MD 21224, USA
c USDA-ARS, Human Nutrition Research Center on Aging, Tufts University, Boston, MA 02111, USA
d Nutritional Neuroscience and Aging Laboratory, Pennington Biomedical Research Center, Louisiana State University System, 6400 Perkins Road, Baton Rouge, LA 70808, USA

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Abstract
Young male Fischer-344 rats were fed a diet containing 2% blueberry (BB) extract or control diet for at least 8 weeks and then received bilateral hippocampal injections of kainic acid (KA 200 ng/0.5 μl) or phosphate buffered saline (PBS). One week later rats were trained in one-way active footshock avoidance in a straight runway followed the next day by training in a footshock motivated 14-unit T-maze with documented sensitivity to hippocampal glutamatergic manipulations. Based on analyses of several performance variables, KA-treated rats exhibited clearly impaired learning performance; however, the BB diet significantly reduced this impairment. Supporting the behavioral findings, stereological assessment of CA1 pyramidal neurons documented greater neuronal loss in KA-treated controls compared to KA-treated rats on the BB diet. In an in vitro experiment, FaO cells grown in medium supplemented with serum from BB-fed rats had enhanced viability after exposure to hydrogen peroxide. These findings suggest that BB supplementation may protect against neurodegeneration and cognitive impairment mediated by excitotoxicity and oxidative stress.

Keywords: Flavanoid; Neurotoxicity; Nutrition; Memory; Glutamate; Hippocampus; Alzheimer’s disease

1. Introduction
Diets rich in fruits and vegetables appear to be associated with lowered risk for several categories of age-related chronic illnesses, including heart disease and cancer (Duthie et al., 2006; Giugliano and Esposito, 2005; Havsteen, 2002). Cognitive and motor abilities decline during normal aging, and these declines are exaggerated in neurodegenerative disease, which can emerge through different mechanisms. Epidemiological evidence indicates that antioxidant supplementation may provide neuroprotection against age-related neurodegenerative disorders, including Parkinson’s disease (de Rijk et al., 1997), amyotrophic lateral sclerosis (Ascherio et al., 2005; Di Matteo and Esposito, 2003) and Alzheimer’s
disease (Dai et al., 2006; Maxwell et al., 2005). Studies using animal models have reported experimental evidence linking consumption of specific fruits and vegetables to reduced age- and disease-related neurodegeneration (Bickford et al., 2000; Galli et al., 2002; Joseph et al., 1999; Sweeney et al., 2002; Wang et al., 2005b; Youdim et al., 2000). To date, however, the mechanism underlying the apparent beneficial effects of fruits and vegetables remains unclear.

Among the possible neuroprotective agents emerging from these studies are dietary components that contain relatively large amounts of polyphenolic compounds, suggesting higher levels of antioxidant activity may be involved in the putative protective effects (Cao et al., 1996, 1995; Prior et al., 1998, 2003; Wang et al., 1996; Wu et al., 2004). Blueberries (BBs) contain high levels of polyphenolic flavonoids, ranking them among the foods highest in antioxidant activity (Prior et al., 1998; Wu et al., 2004). Rodents fed a BB-supplemented diet prior to introduction of brain trauma have reduced neuronal damage following both ischemia (Sweeney et al., 2002; Wang et al., 2005b) and 6-hydroxydopamine neurotoxicity (Stromberg et al., 2005). A study with BB-supplemented diet in a double transgenic murine model of Alzheimer’s disease (AD) demonstrated increased performance compared to mice on a control diet in a Y-maze, in the absence of significant effects on amyloid-β deposition (Joseph et al., 2003).

In the present study, we tested the hypotheses that dietary pretreatment with BB would protect against neurotoxicity caused by central injections of kainic acid (KA) in rats and that plasma collected from BB-fed rats would protect cells from H2O2 mediated stress. This approach addressed the excitotoxicity hypothesis of Alzheimer’s disease, which indicates that overstimulation of glutamate receptors can lead to neurodegeneration through oxidative stress (Coyle, 1983; Coyle and Puttfarcken, 1993; Hynd et al., 2004; Wang et al., 2005a). For the in vivo study, we utilized a well-established complex maze task, the Stone 14-unit T-maze, to assess the neuroprotective effects of BB supplementation on learning performance in rats. Previous rat studies have demonstrated that performance in this maze task declines with advancing age and is sensitive to manipulation of glutamate receptors (Ingram et al., 1992, 1994b) as well as to damage to various hippocampal circuitry, including the CA1 region (Ingram et al., 1994a). For the in vitro study, we examined FaO cell viability using a model system shown to increase cell viability when cultured in serum from animals on calorie restriction (Cohen et al., 2004; de Cabo et al., 2003). Rats received a diet containing a 2% BB extract for at least 8 weeks and were then subjected to KA infusion into the CA1 region of the hippocampus. Subsequent behavioral and stereological studies indicated that rats on the BB diet experienced less KA-induced neuronal loss and superior learning performance compared to rats on control diet treated with KA. When FaO cells were grown in serum obtained from rats on the BB diet, we observed improved viability following treatment with hydrogen peroxide as an oxidative stressor.

2. Materials and methods

2.1. Subjects

The study involved two separate experiments using nearly identical protocols. For Experiment 1, 40 male Fischer-344 rats approximately 5 months old were shipped to the Gerontology Research Center (GRC: Baltimore, MD) from the contract colony maintained by the National Institute on Aging at Harlan Sprague–Dawley (Indianapolis, IN). For Experiment 2, 75 male Fischer 344 rats 3–4 months old were shipped to the GRC from the same source. The rats were pair-housed in large, suspended plastic cages located in a movable metal rack in a vivarium maintained at 21 °C with a 12-h light:12-h dark photocycle. All animals were allowed ad libitum access to food and water. The rats were acclimated in this setting for at least 1 week prior to initiating the feeding protocols. All rats were maintained on their respective diets for at least 8 weeks before beginning surgical procedures.

All experimental procedures included in the study complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No. 85-23, revised 1996) and were approved by the GRC Institutional Animal Care and Use Committee.

2.2. Diet and diet groups

The diets were prepared as previously described (Youdim et al., 2000). Briefly, freeze dried extracts were shipped to Harlan Teklad (Madison, WI, USA), where they were combined with the control diet (20 g/kg diet), a modified NIH-31 diet (Youdim et al., 2000). The amount of BB extract added, 2%, was similar to that of earlier studies (Joseph et al., 1999) in which beneficial effects were first observed. The control diet was supplemented with 2% dried corn to approximate the carbohydrate content of the BB diet (Goyarzu et al., 2004). The two diets were isocaloric within the margin of error attributable to routine variations in the nutritional value of the natural ingredients of the NIH-31 diet. Rats were assigned randomly to a control (CN) diet group or to the 2% blueberry (BB) diet group that were fed the diet for at least 8 weeks before surgery and behavioral testing.

2.3. Animal monitoring and surgical procedures

Body weight and food consumption were monitored and measured weekly throughout the course of each experiment. After surgical procedures were performed, rats were monitored daily.

In Experiment 1, rats from each diet group were assigned randomly to surgical groups so that equivalent numbers received stereotaxic surgery on weeks 14 and 15. Surgical procedures for Experiment 2 were started during weeks 9–10 following initiation of the diets, with equivalent numbers of rats from the BB and CN groups undergoing surgery each week. Under isoflurane anesthesia, all rats received
bilateral intracranial injections (from bregma: A/P −3.6 mm, M/L ± 2.4 mm, D/V −2.4 mm) of either 0.50 μl phosphate buffered saline (PBS) or 200 ng kainic acid (KA: Sigma, St. Louis, MO) in 0.50 μl PBS (0.25 μl to each side) to constitute the following groups: CN PBS; BB PBS; CN KA; BB KA.

2.4. 14-unit T-maze

One week following surgery, rats were transported to the testing room and allowed to acclimate for 30 min prior to training in one-way active avoidance. As described in detail previously (Spangler et al., 1986), all rats were trained to a criterion of 13 out of 15 correct foot shock avoidances (0.8 mA; maximum 30 trials) in a straight runway (2 m long). For each trial the rat was gently pushed from a start box and was required to move down the runway to the goal box within 10 s to successfully avoid footshock. After 10 s a timer initiated scrambled footshock that remained activated until the rat escaped to the goal box. If a rat failed to escape the shock within 120 s, it was removed from the straight runway. Following pretraining each rat was returned to its home cage.

On the following day each rat that passed pre-training was tested in a 14-unit T-maze (Fig. 1) requiring the rat to make 14 successive position discriminations (arrows denote correct pathway) as it traversed the maze from start (S) to goal (G). The maze is divided into five sections and requires the rat to locomote through each section within 10 s to avoid footshock or escape footshock once initiated, as in straight runway training (Spangler et al., 1986). Each rat received 20 massed practice trials with a 2-min inter-trial interval. Data for error (deviations from the correct pathway) and runtime performance were collected via an automated system that used infrared sensors located within the maze wired to a microprocessor connected to a personal computer. In brief, once the rat left the start area and broke a sensor beam, a clock in the microprocessor was initiated, and the path ambulated by the rat could be recorded as the infrared beams were broken as the rat negotiated the maze. Entry to the goal area broke the final sensor beam that stopped the clock. Data were transferred from the microprocessor to a personal computer after each trial and later analyzed with a program designed to score errors and runtime performance for each rat. The shock duration and frequency data were collected by hand and entered into a computer for analysis.

2.5. Histology

Shortly after completing maze testing, rats were anesthetized deeply by injection with a dose of sodium pentobarbital (50 mg/ml, i.p.) and perfused transcardially with 1 × PBS followed by 4% paraformaldehyde (PFA). After removal, brains were placed into glass vials and post-fixed for 48 h in 4% PFA. Afterwards they were dehydrated in 30% sucrose at 5 °C until brains sank to the bottom of the vial and then frozen in isopentane for storage in a −80 °C freezer.

Brains from Experiment 1 were not analyzed histologically due to errors in tissue processing. Brains from Experiment 2 were sliced on a freezing microtome at 50 μm, and sections, including hippocampus, were placed into wells containing cryopreservation buffer. All sections were stored at −20 °C until initiation of cresyl violet (CV) staining. Every 6th section through the hippocampus was mounted onto gelatin-subbed slides and allowed to dry for at least 3 days. After drying, the slides were stained following a standard CV protocol and cover-slipped using permount. Slides were dried for at least 1 week prior to morphometric analysis using computer-assisted stereology (Stereologer, Systems Planning and Analysis, Inc., Alexandria, VA).

2.6. Stereology

Using standard methods of design-based stereology, systematic-random sampling in combination with the optical fractionator was used to quantify the total number of pyramidal neurons in the CA1 region of the hippocampus (West et al., 1991). Sampling every 6th section with a random start generated a total of 14–18 sections through the CA1 region of each brain. Post-processing section thickness was measured at each counting location, yielding an average section thickness of 18.83 ± 0.08 μm. The counting item within the CA1 region was CV-positive neurons with clear evidence of nucleoli, nuclear membrane, cytoplasm and cell membrane to identify cells with neuronal morphology in the CA1 region. Clear anatomical definitions of the CA1 region were based on Paxinos and Watson (1998) rat atlas and previous studies in hippocampus by West et al. (1991). The sampling was optimized for maximal efficiency, with a final mean coefficient of error (CE) per group less than 10%, as detailed elsewhere (Mouton, 2002).
2.7. In vitro assay

Cell culture assays were completed to assess survivability following oxidative stress. FaO rat hepatoma cells were cultured in Ham’s F12 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics (Gibco). Prior to oxidative stress exposure, cells were pretreated with medium supplemented with 10% serum obtained from tail bleeds of rats fed the BB diet or control diet for 8 weeks as previously described (de Cabo et al., 2003). Following 24 h of serum incubation, cells were exposed to freshly prepared hydrogen peroxide (H$_2$O$_2$, 0–0.5 mM) for 2 h after which the H$_2$O$_2$-medium was removed, and serum-free medium was added to the cells. The FaO cells were then plated and incubated for 2 h, and cell viability was determined by the addition of Thiazolyl blue tetrazolium bromide (MTT, Sigma) as previously reported (Denizot and Lang, 1986). Cell viability is expressed as the ratio of MTT production in the BB serum-treated cells to control serum-treated cells with the percent increase in BB serum-treated survivorship representing means ± S.E.M. from assays run in triplicate from independent experiments.

2.8. Statistical analysis

Body weight and food consumption data were analyzed by repeated measures analysis of variance (ANOVA). All maze data were analyzed by one-way ANOVAs to determine treatment group effects for mean scores per trial and blocks of five trials for each of the dependent variables: errors, runtime, shock duration and frequency. Dunnett post hoc comparisons using the CN PBS group as the control group were utilized to determine the locus of group treatment effects for behavioral data. Stereological assessment in the CA1 region of the hippocampus was analyzed by one-way ANOVA and orthogonal contrasts were used to determine significant differences in neuronal loss. The specific a priori hypotheses tested in this experiment were the following: (h1) animals injected with KA would have significantly more neuronal loss than animals injected with PBS; (h2) animals on the CN diet would have significantly more neuronal loss compared to animals on the BB diet after treatment with KA; (h3) animals on the CN diet and animals on the BB diet would not differ significantly after treatment with PBS. The number of planned comparisons for each dependent measure was restricted ($k−1$) to test the three above hypotheses in order to minimize family wise type I error. Cell culture data were analyzed by t-test to compare CN and BB survivability after treatment with H$_2$O$_2$. Statistical significance was accepted as $p<0.05$.

3. Results

3.1. Body weight and food consumption

All rats gained weight throughout the study and consumed approximately 19–21 g of diet daily. As observed in Fig. 2, there were no significant differences among groups in either body weight ($n$’s: BB1 = 20, CN1 = 20, BB2 = 38, CN2 = 37) or food intake ($n$’s: BB1 = 10, CN1 = 10, BB2 = 19, CN2 = 19). Repeated measures ANOVA tests for body weight and food consumption recorded in Experiment 1 yielded omnibus values of $F_{(1,38)} = 0.032$ ($p=0.858$) and $F_{(1,19)} = 0.001$ ($p=0.974$), respectively. Repeated measures ANOVA results for Experiment 2 data were $F_{(1,73)} = 0.90$ ($p=0.765$) and $F_{(1,36)} = 0.867$ ($p=0.384$) for body weight and food consumption, respectively.

3.2. 14-unit T-maze

As observed in Fig. 3, rats receiving KA injections showed impaired maze performance; however, rats on the BB diet exhibited a diminished learning impairment. In Experiment 1, results of a one-way ANOVA of errors per trial revealed a significant effect of group, $F_{(3,24)} = 4.28$, $p=0.015$. The CN KA ($n=6$) group made the most errors per trial, with the BB KA ($n=6$) group making about half the number of errors of this group. Using the CN PBS ($n=8$) group as the control group for post hoc Dunnett comparisons, only the CN KA ($n=6$) group making about half the number of errors of this group. The behavioral results observed in Experiment 1 were replicated in Experiment 2. Results of a one-way ANOVA of maze errors revealed a significant group effect, $F_{(3,31)} = 10.07$, $p<0.01$. The CN KA ($n=8$) group made
Fig. 3. Effects of BB diet after hippocampal injections of kainic acid on mean performance (±S.E.M.) during acquisition training in the 14-unit T-maze. Symbol-line graph shows mean error (top) and runtime (bottom) data plotted in blocks of 5 trials for Experiment 1 (left) and 2 (right). See text for description of statistical results. Abbreviations: BB, blueberry; PBS, phosphate buffered saline; CN, control; KA, kainic acid.

the most errors per trial, with the BB KA (n = 8) group making about half the number of errors. Dunnett comparisons revealed that only the CN KA differed significantly from CN PBS (n = 9) group (p < 0.05; Fig. 4). Similar results were found for diet effects on runtime (Fig. 4), shock duration and shock frequency (Fig. 5), that is, only the CN KA group differed significantly from controls, the CN PBS group (p’s < 0.05). In addition, for the shock frequency measure, the BB KA group also differed significantly from the CN PBS group (p < 0.05).

Fig. 4. Effects of BB diet after hippocampal injections of kainic acid on overall mean performance (±S.E.M.) during acquisition training in the 14-unit T-maze. Mean error (top) and runtime (bottom) data plotted for Experiment 1 (left) and 2 (right). Symbols: *, significantly different from CN PBS group. See text for description of statistical results. Abbreviations: BB, blueberry; PBS, phosphate buffered saline; CN, control; KA, kainic acid.

Taken together, the results for various maze learning parameters collected in two independent experiments indicated that the BB KA group had enhanced performance compared to the CN KA counterparts on three of the four measures of learning.

3.3. Stereological assessment

The cannula insertion sites could be identified on tissue sections and were noted to be similar across groups. All rats
receiving KA acid injections exhibited loss of CA1 neurons; however, this loss was reduced in rats on the BB diet. Neuronal loss is visualized in Fig. 6, which shows representative images of the CA1 region within the hippocampus from each treatment group. All images were taken between approximate locations of A/P \(-4.8\) to \(-5.2\) mm from bregma.

Fig. 7 presents the estimated neuron number in the CA1 region of the hippocampus by diet group (n’s: BB PBS = 6, CN PBS = 7, BB KA = 7, CN KA = 7). A one-way ANOVA for an effect of group, $F_{(3,23)} = 10.13, p < 0.01$, was significant for neuron number. Planned comparisons revealed significantly less neurons after treatment with KA compared to animals injected with PBS ($p < 0.05$). Rats on the CN diet had significantly less neurons than those on the BB diet after treatment with KA ($p < 0.05$). Rats on the CN and BB diets injected with PBS did not differ significantly ($p > 0.05$).

3.4. In vitro assay

All cells treated with $\text{H}_2\text{O}_2$ had decreased survivability; however, at the concentrations of 0.25 mM and 0.50 mM, viability of BB-treated FaO cells was significantly higher.
KA injections into the hippocampus or lateral ventricles can induce selective neuronal loss to hippocampal neurons containing ionotropic kainate glutamatergic receptors (Gobbo and O’Mara, 2005). The hippocampus contains a high concentration of glutamate receptors, particularly the CA1 region, and manipulation of receptor activity has been demonstrated to affect performance in a wide variety of learning and memory tasks in a variety of animal models. In AD, over-activity of glutamate receptors has been hypothesized to be a mechanism of neuronal loss (Coyle, 1983; Coyle and Puttfarcken, 1993; Hynd et al., 2004; Wang et al., 2005a). Hyperstimulation of these receptors is thought to lead to increasing generation of reactive oxygen species (ROS) and significantly higher levels of oxidative stress via an excitotoxic cascade (Atlante et al., 2001). In support of NMDA receptor mediated dysfunction, the NMDA glutamate receptor antagonist, memantine, has been approved for therapeutic management of late stages of AD. Although it has been demonstrated to impair memory in normal adult rats (Creeley et al., 2006), memantine shows clinical efficacy in slowing the progression of AD in advanced stages (Koch et al., 2004; Lipton, 2005; Rogawski and Wenk, 2003; Rossom et al., 2004).

The present findings indicate that rats exhibited impaired performance in maze learning following intrahippocampal injection of KA and that a BB enriched diet provided significant protection against these decrements in performance. Additionally, stereological analysis was utilized to estimate the number of neurons in the CA1 region and documented clear evidence that the BB-enriched diet reduced neuronal loss resulting from the excitotoxic effects of KA. Results of the in vitro experiment further confirmed that a BB supplemented diet could provide protection against an oxidative stress. Specifically, FaO cells that were reared in serum obtained from rats on the BB diet were more protected against death induced by H2O2 compared to cells reared in control serum. Thus, the BB diet induces factors in circulation that provide cellular protection against stressful stimuli. These results add to the literature demonstrating that a BB enriched diet can attenuate brain aging and age-related decline in behavioral performance of rodents (Goyarzu et al., 2004; Lau et al., 2005) as well as provide protection against neurotrauma, such as stroke (Sweeney et al., 2002; Wang et al., 2005b).

The mechanisms involved in this protection remain to be elucidated. Anthocyanins are a subset of a larger class of polyphenols known as flavonoids. Over 4000 flavonoids have been identified in plants. They are also abundant in seeds, fruits and plant-derived oils, such as olive oils, as well as tea and red wine. Thus, they are part of the human diet. Plants and spices containing flavonoids have been used for many years in Eastern medicine. Flavonoids have been reported to inhibit lipid peroxidation in several biological systems including mitochondria and microsomes (Bindoli et al., 1977; Cavallini et al., 1978) as well as erythrocytes (Maridonneau-Parini et al., 1986; Sorata et al., 1984) and liver (Kimura et al.,

4. Discussion

Rodent models provide valuable tools for assessing pathogenesis associated with amyloid-β deposition, impairments in cognitive function and development of novel strategies for the therapeutic management of AD. While transgenic mouse models demonstrate expression of mutant proteins associated with familial AD, these mice show little or no evidence of neuronal loss (German and Eisch, 2004) in brain regions that exhibit AD pathology, including the hippocampal formation (Zarow et al., 2005). In contrast, the KA model in rats provides a useful tool for analyzing neuronal vulnerability to excitotoxicity relevant to AD (Chandrasekaran et al., 2004; Sharma and Kaur, 2005).
They are inhibitors of both NADPH and CCl₄-induced lipid peroxidation (Afanas’ev et al., 1989) and to some extent they appear to have iron chelating ability (HOult et al., 1994) as well as the ability to upregulate endogenous antioxidants, such as glutathione (Schroeter et al., 2002; Zipper and Kaur, 2000).

While the original rationale for examining effects of BB supplemented diets was to assess antioxidant protection, more recent research has emphasized other biological effects beyond antioxidant activities. For instance, there is evidence to suggest that flavonoids have possible MAP-kinase (MAPK) altering activity. Because MAPKs are involved in numerous biological activities, findings that flavonoids may influence such signaling suggest that their potential benefits may involve properties other than those mediating antioxidant or anti-inflammatory effects. Delphinidin, a primary plant pigment, inhibits endothelial cell proliferation and cell cycle progression by ERK 1/2 activation (Martin et al., 2003), while grape seed proanthocyanidin can reduce ischemia reperfusion-induced activation of JNK-1 and c-Jun and reduce cardiomyocyte apoptosis (Sato et al., 2001). Additional research indicates that phytochemicals can regulate MAP kinase and other signaling pathways at the level of transcription (Frigo et al., 2002). These findings, coupled with numerous studies demonstrating the involvement of ERK in diverse pathways, such as contextual fear conditioning (English and Sweatt, 1996), long-term potentiation (English and Sweatt, 1997), striatal-dependent learning and memory (Mazzucchelli and Brambilla, 2000), hippocampal-dependent spatial memory (Selcher et al., 1999) and inhibitory avoidance (Schafe et al., 2000, 1999; Wilensky et al., 2000) suggest that interventions which influence MAPK signaling may have beneficial effects on cognition. Thus, it may be that alterations in the putative signal modifying properties of flavonoids may prove to be invaluable in altering the neuronal and behavioral effects of neurotoxins, such as KA. Previous research has shown that in COS-7 cells transfected with M1 muscarinic receptors, there is increased vulnerability to toxicity induced by dopamine and Aβ 1-42. However, if the cells are pretreated with one of several berryfruits (e.g., blueberries), deficits in calcium buffering in these cells induced via the oxidative stressors (e.g., dopamine) could be prevented (Joseph et al., 2004). A subsequent study indicated that increases in MAPK were associated with this protection (Joseph et al., 2006). Moreover, in a study using APP/PS1 transgenic mice given a control or BB-supplemented diet, findings indicated that mice supplemented with BB exhibited Y-maze performance similar to those seen in non-transgenic mice and significantly greater than that observed in non-supplemented transgenic animals (Joseph et al., 2003). Interestingly, there was a dichotomy between the plaque burden and behavior in the BB supplemented transgenic mice. No differences between the supplemented and non-supplemented APP/PS1 mice in the number of plaques were observed, even though behavioral declines were prevented in the BB supplemented animals. However, there were significant increases of ERK in the BB-supplemented APP/PS1 mice that correlated with their performance in the Y-maze. These findings, combined with additional research showing that BB supplementation in addition to altering ERK activity may also increase hippocampal neurogenesis (Casadesus et al., 2004), suggests that at least part of the efficacy of the BB supplementation may be to enhance neuronal signaling in areas of the brain affected by KA. This would allow more effective intra-and inter-area communication and ultimately facilitate both cognitive and motor function.

In summary, there is emerging evidence that dietary interventions might provide an effective strategy for preventing or treating AD, and possibly other neurodegenerative disorders. Based on data from a variety of rodent models, multiple mechanisms appear to be involved in the neuroprotection provided by a BB diet. The current findings would suggest that a diet enriched in BB might attenuate degenerative processes due to oxidative or inflammatory stressors similar to the effectiveness of pharmacological strategies related to this hypothesis of AD.

Conflict of interest

The authors certify that they have no actual or potential conflicts of interest regarding the research reported in this paper.

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