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Anthocyanin-enriched bilberry and blackcurrant extracts modulate amyloid precursor protein processing and alleviate behavioral abnormalities in the APP/PS1 mouse model of Alzheimer's disease☆

Saila Vepsäläinen[a], Henna Koivisto[b], Elina Pekkarinen[b], Petra Mäkinen[a], Gary Dobson[c], Gordon J. McDougall[c], Derek Stewart[c,d], Annakaisa Haapasalo[a], Reijo O. Karjalainen[e], Heikki Tanila[b,f], Mikko Hiltunen[a,f,*]

[a]Institute of Clinical Medicine–Neurology, University of Eastern Finland, Kuopio, Finland
[b]A.I. Virtanen Institute, University of Eastern Finland, Kuopio, Finland
[c]The James Hutton Institute, Enhancing Crop Productivity and Utilization Theme, Invergowrie, Dundee, UK
[d]School of Life Sciences, Heriot-Watt University, Riccarton, Edinburgh, UK
[e]Department of Biosciences, University of Eastern Finland, Kuopio, Finland
[f]Department of Neurology, Kuopio University Hospital, Kuopio, Finland

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Abstract

A growing body of epidemiological evidence suggests that fruit and vegetable juices containing various phenolic compounds can reduce the risk of Alzheimer's disease (AD). As the altered amyloid precursor protein (APP) processing leading to increased β-amyloid (Aβ) production is a key pathogenic feature of AD, we elucidated the effects of different polyphenols on neuroprotection and APP processing under different *in vitro* stress conditions. The effects of these compounds were also investigated in transgenic AD mice (APdE9). Free radical toxicity and apoptosis were induced in human SH-SY5Y neuroblastoma cells overexpressing APP751. Menadione-induced production of reactive oxygen species was significantly decreased upon treatment with myricetin, quercetin or anthocyanin-rich extracts in a dose-dependent manner. However, these extracts did not affect caspase-3 activation, APP processing or Aβ levels upon staurosporine-induced apoptosis. APdE9 mice fed with anthocyanin-rich bilberry or blackcurrant extracts showed decreased APP C-terminal fragment levels in the cerebral cortex as compared to APdE9 mice on the control diet. Soluble Aβ40 and Aβ42 levels were significantly decreased in bilberry-fed mice as compared to blackcurrant-fed mice. Conversely, the ratio of insoluble Aβ42/40 was significantly decreased in blackcurrant-fed mice relative to bilberry-fed mice. Both berry diets alleviated the spatial working memory deficit of aged APdE9 mice as compared to mice on the control diet. There were no changes in the expression or phosphorylation status of tau in APdE9 mice with respect to diet. These data suggest that anthocyanin-rich bilberry and blackcurrant diets favorably modulate APP processing and alleviate behavioral abnormalities in a mouse model of AD.

Keywords: Apoptosis; Amyloid precursor protein; Anthocyanin; Bilberry; Blackcurrant; Cell viability; APdE9 mice; Cognitive impairment; Flavonols

1. Introduction

Recent epidemiological and experimental data suggest that fruit and vegetable juices containing various phenolic compounds can reduce the risk of Alzheimer's disease (AD) [1]. Individuals drinking three or more glasses of fruit or vegetable juice per week were shown to have over 50% lower risk of AD compared to those who had less than one serving per week. Polyphenols, which are abundant in red wine, bilberry and blackcurrant, have been shown to inhibit the formation and extension of β-amyloid (Aβ) fibrils in a dose-dependent manner and to destabilize the preformed Aβ fibrils *in vitro* [2–4]. These are important observations considering that Aβ, which is released after sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases, is a key determinant in AD pathogenesis [5]. Aβ-induced formation of reactive oxygen species (ROS) is also inhibited by flavonols from many plants (e.g., grapes, blackcurrant and green tea) [6]. Moreover, transgenic AD model mice (APPswe) that received pomegranate juice had about 50% less accumulation of soluble Aβ in the hippocampus than sugar water-treated control mice [7]. These mice were also able to learn more quickly and swim faster than the controls in the Morris water maze test. Bilberry supplementation did not affect Aβ burden in the brain tissue of transgenic APP/PS1 mice but, in turn, prevented behavioral deficits in this AD mouse model [8]. Collectively, these encouraging observations indicate that polyphenols may be promising dietary means to delay the

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* Corresponding author. Institute of Clinical Medicine–Neurology, University of Eastern Finland, P.O. Box 1627, 70211 Kuopio, Finland. Tel.: +358 40 3552014; fax: +358 17 162048.
E-mail address: mikko.hiltunen@uef.fi (M. Hiltunen).

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onset of AD. However, before novel polyphenol-based dietary applications are considered for usage in practice to reduce the AD risk, more information is needed about the specific mechanisms by which fruit polyphenols exert their potential neuroprotective actions.

Blackcurrant is a strong candidate fruit to provide neuroprotection in AD. Among the 143 vegetable foods analyzed, blackcurrant was included in the top list in terms of polyphenol concentration [9]. Anthocyanins are the major group of polyphenols in blackcurrant, accounting for about 80% of the total amount of quantified compounds [10,11]. Four major anthocyanins (delphinidin 3-*O*-glucoside, delphinidin 3-*O*-rutinoside, cyanidin 3-*O*-glucoside and 3-*O*-cyanidin rutinoside) have been reported in blackcurrant [10]. Berry-derived anthocyanins possess high antioxidant activity [12] and neuroprotective activity in aging mice [13]. Blackcurrant also contains a wide range of flavonols, including high levels of myricetin and a relatively high amount of quercetin derivates, which possess strong neuroprotective activity [14]. As compared with other fruits, blackcurrant has also a high antioxidant activity.

In this study, we set our goal to determine whether flavonols and anthocyanin-rich extracts from blackcurrant exert neuroprotective activity and affect APP processing under different *in vitro* stress conditions, such as free radical-mediated toxicity and apoptosis. We induced these stress conditions in human SH-SY5Y neuroblastoma cells stably overexpressing APP751 isoform (SH-SY5Y-APP751) and subsequently investigated the effects of myricetin, quercetin and anthocyanin-rich extracts on cell viability, production of ROS, caspase-3 activity and APP processing. Furthermore, we compared the effects of bilberry supplementation to blackcurrant supplementation in terms of APP processing, Aβ degradation status and tau pathology as well as assessed behavioral outcome measures in a common AD model mouse (APdE9) overexpressing mutated human APP (APPswe) and presenilin-1 (PS1dE9) proteins [15]. We started the dietary intervention at the age of 2.5 months before any Aβ plaque deposition is detectable in the brain of these mice (from 4 months of age onward) [16] and continued the study until the age of 13 months, when the mice show clear memory deficits and massive Aβ deposition in the brain [17].

2. Methods

2.1. Myricetin, quercetin and anthocyanin-rich blackcurrant extracts used in vitro studies

Myricetin (70050) and quercetin (Q4951) were obtained from Sigma-Aldrich. Purified anthocyanin fractions were prepared from commercially available blackcurrant powders (BerryPharma, Appenzell, Switzerland; Products V0140001 and V0140006). In short, polyphenol-rich fractions devoid of sugars, organic acids and minerals were prepared using solid-phase extraction (SPE) on C18 units as previously [18]. SPE of one of the original powders yielded an anthocyanin content of 46% of the total phenolic content. A highly enriched anthocyanin sample was obtained by further fractionation on polyamide SPE cartridges using elution with 40% aqueous methanol, based on previous method [19]. Liquid chromatography–mass spectrometry (LC–MS) showed that the fractions contained all four anthocyanins in similar proportions to the original powder. However, the enriched fraction was almost completely devoid of flavonols, and the anthocyanins accounted for about 86% of the total phenolics. The anthocyanin-rich BC fractions were aliquoted in 250 μg total phenol amounts and lyophilized. The LC–MS profile and total anthocyanin and phenol contents of the dried aliquots of each of the samples were subsequently rechecked. Dried 46% and 86% anthocyanin extracts were dissolved to Dulbecco's modified Eagle's medium (DMEM) culture medium to be used in cell culture experiments.

2.2. Cell cultures

Human SH-SY5Y neuroblastoma cells stably overexpressing APP751 (SH-SY5Y-APP751) were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 200 μg/ml of G418, as previously described [20].

2.3. Cell viability assay

Cell viability was measured by 3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) and/or propidium iodide (PI)/digitonin (DIG) assays as previously described [20–22].

2.4. ROS measurements under menadione-induced cellular stress

ROS were assayed in living cells as previously described [21]. Briefly, intracellular ROS production was assayed from living SH-SY5Y-APP751 cells by treating them with fluorescent probe 2′,7′-dichlorofluoresceindiacetate (DCFH-DA) for 20 min. After washings, cells were treated for 60 min with 50 μM menadione (Sigma-Aldrich) and with or without myricetin, quercetin or anthocyanin-rich extracts. As a nonpolar compound, DCFH-DA crosses cell membranes, and due to cellular esterases, diacetate groups are cleaved from DCFH-DA to form nonfluorescent 2′,7′-dichlorofluorescein (DCFH). In the presence of intracellular ROS, DCFH is oxidized very quickly to highly fluorescent DCFH. ROS were measured at time points of 0, 5, 20, 40 and 60 min after the treatments using a fluorometer [excitation 485 nm/emission 535 nm; PerkinElmer HTS 7000 Plus Bio Assay Reader (Norwalk, CT, USA)].

2.5. Animals

The animals were male APdE9 mice (n=51) and age-matched wild-type littermates (n=16). Their age at the time of behavioral testing was 12 months, and they were euthanized at the age of 13 months. The APdE9 colony founders were obtained from D. Borchelt and J. Jankowsky (Johns Hopkins University, Baltimore, MD, USA), while the mice were raised locally at the Laboratory Animal Center in Kuopio, Finland. The APdE9 mice were created by coinjection of chimeric mouse/human APPswe and human PS1dE9 (deletion of exon 9) vectors controlled by independent mouse prion protein promoter elements. The two transgenes cointegrated and cosegregate as a single locus [15]. The line was originally maintained in a C3HeJxC57BL/6J hybrid background. By the time of the present work, the mice were backcrossed to C57BL/6J for 14 generations. The mice were housed in individual cages in a controlled environment (constant temperature, 22°C ± 1°C, humidity 50%–60%, lights on 0700–1900 h). A plastic "igloo" (diameter 10 cm, Mouse Igloo; Bio-Serve, Frenchtown, NJ, USA) with three openings was included as an environmental enrichment in all cages. Animals had food and water available *ad libitum*. All animal procedures were carried out in accordance with the guidelines of the European Community Council Directives 86/609/EEC and approved by the Animal Experiment Board of Finland.

2.6. Dietary intervention

All experimental diets were prepared by Research Diets Inc (New Brunswick, NJ, USA). The control diet was the standard Teklad 2016 diet (Teklad Diets, Madison, WI, USA) consisting 16.4% of crude protein, 4% fat and 3.3% of crude fiber. Energy density was 3.0 kcal/g (12.6 kJ/g), and originated from different dietary components as follows: protein 22%, fat 12% and carbohydrates 66%. Anthocyanin-rich diet was based on purified bilberry and blackcurrant anthochyanin powders (from BerryPharma Inc, Appenzell, Switzerland). The total amount of anthocyanins in bilberry and blackcurrant-enriched chow was 1.53 and 1.43 mg/g, respectively. No changes in the levels of anthocyanins were observed during the 6 months of storage. The quantification of anthocyanins from bilberries and blackcurrants was based on HPLC–MS methods described previously [23,24].

At the age of 2.5 months, APdE9 mice were assigned to three diet groups. One group (n=17) continued to receive control chow that also was the base for the two polyphenol-enriched chows. The second group (n=16) received chow supplemented with anthocyanin-enriched bilberry (BB) extract and the third group (n=18) chow supplemented with anthocyanin-enriched blackcurrant (BC) extract. The wild-type control mice (n=16) received control chow. All mice were fed with respective chows until euthanization at the age of 13 months. The body weight was measured first every 2 weeks and then once a month to ensure adequate intake of the experimental chow.

2.7. Behavioral tests

At the age of 12 months, the mice underwent a 3-week battery of neurological tests with the following tests in this order. Spontaneous explorative activity was tested using an automated activity monitor (TruScan; Coulbourn Instruments, Allentown, PA, USA) based on infrared photodetection. The system consisted of a transparent observation cage (26 × 26 × 39 cm) and two rings of photodetectors enabling separate monitoring of horizontal (XY-move time) and vertical activity (rearing). The test cage was cleaned with 70% ethanol between each mouse to avoid odor traces. The test session took 10 min and was replicated after 48 h to assess the extent of habituation to the test cage.

The marble burying task for object neophobia was run in the home cage (27 × 45 × 14.5 cm). In the afternoon, 1 L of extra bedding was added to cage bottom and nine glass marbles (1 cm in diameter) were left on the top of the new bedding in a 3 × 3 array. Next morning (16–18 h later), the number of uncovered marbles was counted.

The delayed alternation test for spatial working memory was conducted during the second test week. To ensure motivation, the mice were food deprived from the previous afternoon until the first test session and were given only 1.1–1.4 g of their chow between the two test days. The test was run in the home cage. First, the plastic igloo (diameter 10 cm) was removed and an opaque partition was placed in the middle of the cage. It had a mouse-size opening that could be closed with a slide door. Two modified plastic igloos with only one opening were placed in the half opposite to the mouse. During the first trial of the session, both igloos were baited with a piece of rice cereal (Rice Crispies). On the following trials, only the igloo not visited during the previous trial was baited. No

correction procedure was used, that is, the food reward remained in its location until the mouse made a correct visit and ate it. Once the mouse had returned to its side of the cage, a 15-s delay ensued. Ten trials were run in a session, and two test days both included two sessions, one in the morning and the second 3 h later.

The Morris swim navigation task (water maze) was used to test spatial learning and memory. The apparatus was a white plastic pool with a diameter of 120 cm. A transparent escape platform (14 × 14 cm) was hidden 1.0 cm below the water surface. The temperature of the water was kept at 20°C ± 0.5°C throughout the experiment, and a 5-to 10-min recovery period was allowed between the training trials. First, the mice were pretrained (2 days) to find and climb onto the submerged platform, aided by a guiding alley (1 m × 14 cm × 25 cm) leading to the platform. In the testing phase (Days 1–4), five 60-s trials/day were conducted with a hidden platform. The platform location was kept constant and the starting position varied between four constant locations at the pool edge, with all mice starting from the same position in any single trial. Each mouse was placed in the water with its nose pointing toward the wall. If the mouse failed to find the escape platform within 60 s, it was placed on the platform for 10 s by the experimenter. The first and last trials on Day 5 were run without the platform to test the search bias. The experimenter marked the start of the trial using a remote controller, while the end of the trial was automatically detected by the video-monitoring program. A computer connected to an image analyzer (Ethovision; Noldus, Wageningen, the Netherlands) calculated the escape latency (time between the start and the end), swim path length, swimming speed, time in the 10-cm-wide wall zone, number of clockwise or counterclockwise rotations (summed up into a single parameter), average distance to the platform and during the probe trial time in the platform zone (30-cm in diameter) and number of platform crossings.

2.8. Brain tissue samples

At the end of the experiment, the mice were deeply anesthetized with pentobarbiturate–chloralhydrate cocktail (60 mg/kg each) and perfused transcardially with 50 ml heparinized ice-cold 0.9% saline (10 ml/min). Brains were then removed, and one hemibrain was dissected on ice into frontal, parietal and temporal posterior cortex and hippocampus. The brain blocks were snap frozen in liquid nitrogen and stored at −70°C for biochemical assays.

2.9. Western blotting

For Western blotting, total protein lysates were extracted from SH-SY5Y-APP751 cells or brain tissue (ventral cortex) using tissue protein extraction reagent (TPER) extraction buffer (Pierce) containing EDTA-free protease inhibitor mixture (Thermo Scientific). After protein quantification using the BCA protein assay kit (Pierce), 30 μg of total protein lysates were subjected to 4%–12% BisTris–PAGE (Invitrogen) and subsequently blotted onto Immun-Blot polyvinylidene fluoride membranes (Bio-Rad). Primary antibodies against APP C-terminus (A8717; Sigma), caspase-3 (detecting the activated caspase-3 fragments and procaspase-3; Cell Signaling), IDE (Abcam), NEP (AF1126; R&D Systems), PHF-Tau (AT8; Thermo Scientific), total tau (ab47579; Abcam) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Ab8245; Abcam) were used for immunoblotting. After incubation with appropriate horseradish peroxidase-conjugated secondary antibodies (GE Healthcare), enhanced chemiluminescence substrate (Amersham Biosciences) was applied to membranes. Detection of protein bands was performed with ImageQuant RT ECL Imager (GE Healthcare). Western blot images were quantified using Quantity One software (Bio-Rad).

2.10. Aβ and sAPP measurements from cell culture medium and brain tissue

Aβ40 and Aβ42 levels were determined from conditioned SH-SY5Y-APP751 cell culture media using monoclonal and horseradish peroxidase-conjugated antibody-based Human/Rat β Amyloid 40 (294-62501) and β Amyloid 42 (high-sensitive; 290-62601) ELISA Kit (Wako). Soluble APP (sAPP) levels were measured from the cell culture medium using Western blotting with antibodies specific for sAPPα (6E10; Biosite) and sAPPtotal (22C11; Millipore). Brain tissue samples from the dorsal cortex were homogenized in phosphate-buffered saline with phosphatase and protease inhibitor cocktail. After centrifugation in 100,000×*g* for 1 h at +4°C, supernatants were collected (soluble fraction), while the membrane pellets were homogenized into 5 M guanidine, pH 8.0 with EDTA-free phosphatase and protease inhibitor cocktail (insoluble fraction). The levels of soluble and insoluble human Aβ1-40 and Aβ1-42 were quantified using the Signal Select TM Beta Amyloid ELISA Kits (BioSource International Inc., Camarillo, CA, USA). sAPPα and sAPPtot (=sAPPα+sAPPβ) levels from the brain samples were determined from the soluble protein fraction after centrifugation in 100,000×*g* for 1 h at +4°C using Western blotting with 6E10 (Biosite) and 22C11 (Millipore) antibodies, respectively. Aβ40, Aβ42 and sAPP levels were normalized to the total protein levels determined from the respective cell culture medium or brain lysates.

2.11. γ-Secretase activity assay from brain tissue

γ-Secretase activity measurements were performed from CHAPSO-solubilized ventral cortical membranes as previously described [25]. Briefly, membrane fractions from ventral cortex were homogenizing in buffer B [20 mM HEPES pH 7.5, 150 mM KCl, 2 mM EGTA+protease inhibitor mixture (Thermo Scientific)] followed by centrifugation in 100,000×*g* for 1 h at +4°C. Membrane pellets were washed with buffer B, centrifuged at 100,000×*g* for 30 min, resuspended in buffer B+1% CHAPSO, and finally centrifuged at 100,000×*g* for 30 min. Equal amounts of CHAPSO-solubilized membrane lysates (100 μg) were incubated overnight at 37°C in 150 μl of substrate buffer (50 mM Tris–HCl pH 6.8, 2 mM EDTA, 0.25% CHAPSO) with 8 μM fluorogenic γ-secretase substrate peptide NMA-GGVVIATVK(DNP)-*DRDRD*R-NH2 (Cat# 565764; Calbiochem). Samples treated with 100 μM γ-secretase inhibitor L685,458 or dimethyl sulfoxide were used to validate the specificity of the γ-secretase activity assay. Samples were briefly centrifuged, and supernatants were transferred to a 96-well plate. Fluorescence was measured using Wallac 1420 Victor Multilabel Counter (PerkinElmer) plate reader with an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

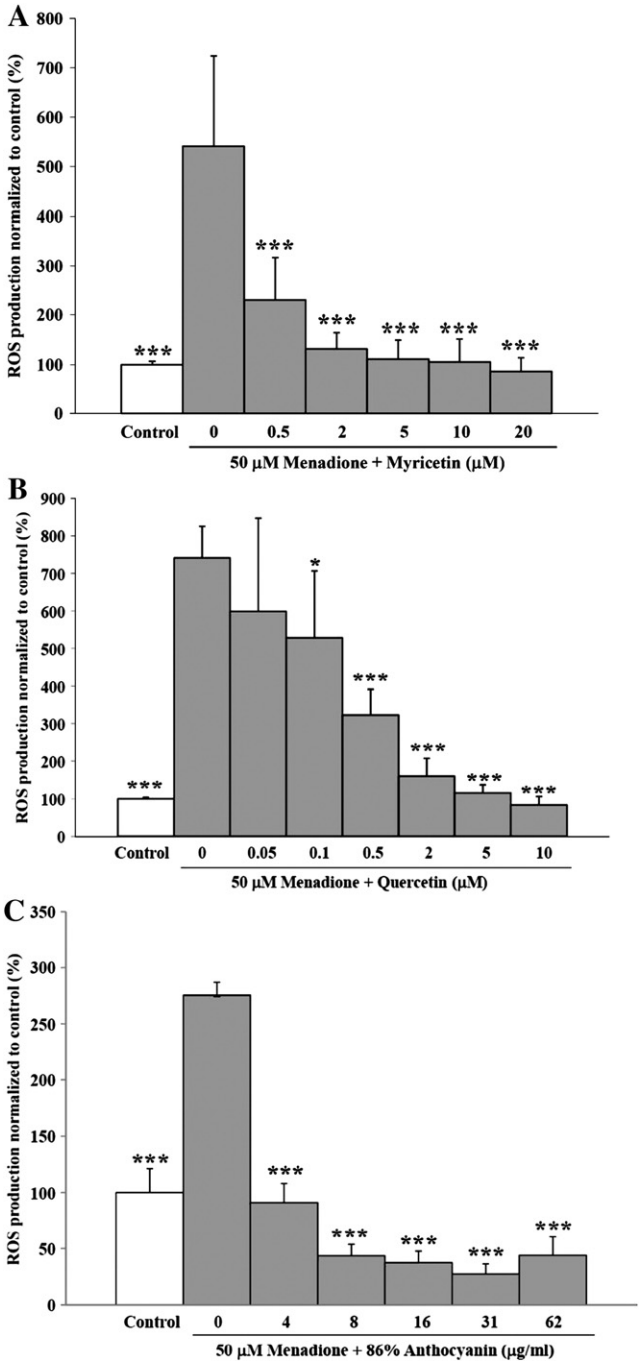

Fig. 1. Myricetin, quercetin and anthocyanin-rich blackcurrant extracts alleviate menadione-induced ROS production in SH-SY5Y-APP751 cells. The cells were treated with 50 μM menadione together with different concentrations of myricetin (A), quercetin (B), or 86% anthocyanin extracts for 60 min (C), after which ROS production was measured. Groups are compared to menadione-treated samples. ***$P<.001$, *$P<.05$; one-way ANOVA with Bonferroni correction, $n=4–6$, S.D.

2.12. Statistical analyses

All statistical analyses were done with SPSS 14.0 Software. One-way analysis of variance (ANOVA) test, followed by Bonferroni post hoc comparisons or independent samples *t* test, was used to determine statistical significance in the *in vitro* part. In the *in vivo* part, all tests involving multiple test sessions (spontaneous exploratory activity, delayed alternation, Morris swim test) were analyzed using ANOVA for repeated measures with test group as between-subject factor. Tests with only one time point were analyzed with one-way ANOVA. Tukey's honestly significant difference (HSD) was used as a post hoc test. Values are expressed as mean ± standard deviation (S.D.) or standard error of mean (S.E.M.). Statistical significance was set to $P<.05$.

3. Results

3.1. Myricetin, quercetin and anthocyanin-rich blackcurrant extracts alleviate the menadione-induced ROS production in SH-SY5Y-APP751 cells

Before elucidating the effects of flavonols and anthocyanin-rich extracts upon various stress conditions, MTT or PI/DIG assays were used to assess whether different concentrations of myricetin, quercetin or anthocyanin-rich extracts (46% and 86%) affect the viability of the SH-SY5Y-APP751 cells in normal growth conditions (Supplementary Figure 1). Although 24-h treatment with myricetin or quercetin did not affect cell viability at lower concentrations, quercetin at concentrations ≥20 μM significantly reduced viability of the SH-SY5Y-APP751 cells (Supplementary Figure 1B). Interestingly, 46%, but not 86%, anthocyanin-rich extract moderately increased the cell viability at concentrations ranging from 0.25 to 31 μg/ml (Supplementary Figure 1C, data not shown). Next, the impact of myricetin, quercetin and anthocyanin-rich extracts on menadione-induced ROS production [26] was tested in SH-SY5Y-APP751 cells. After a 60-min menadione treatment, myricetin, quercetin or anthocyanin-rich extracts (46% and 86%) significantly decreased ROS production in a dose-dependent manner (Fig. 1, data not shown). These data suggest that myricetin, quercetin and anthocyanin-rich extracts exert protective effects in SH-SY5Y-APP751 cells by alleviating menadione-induced ROS production.

3.2. High quercetin concentration decreases APP maturation in SH-SY5Y-APP751 cells under normal growth conditions

To assess whether myricetin, quercetin and anthocyanin-rich extracts alter APP processing under normal growth conditions, SH-SY5Y-APP751 cells were treated with these compounds for 24 h. Interestingly, 10 μM quercetin significantly increased the levels of total APP (APPtot) and immature APP (APPim), but not mature APP (APPm), resulting in a decreased ratio of APPm/APPim (Fig. 2A). Levels of APP C-terminal fragments (CTFs) (C83 and C99) were unaltered. Assessment of the total protein-normalized levels of soluble APPα (sAPPα) and sAPPtotal (sAPPtot=sAPPα+sAPPβ) from SH-SY5Y-APP751 cell culture medium did not reveal significant changes (Fig. 2B). Based on the cell survival analysis (Supplementary Figure 1C), 8 and 31 μg/ml anthocyanin-rich extract (86%) concentrations were used to assess

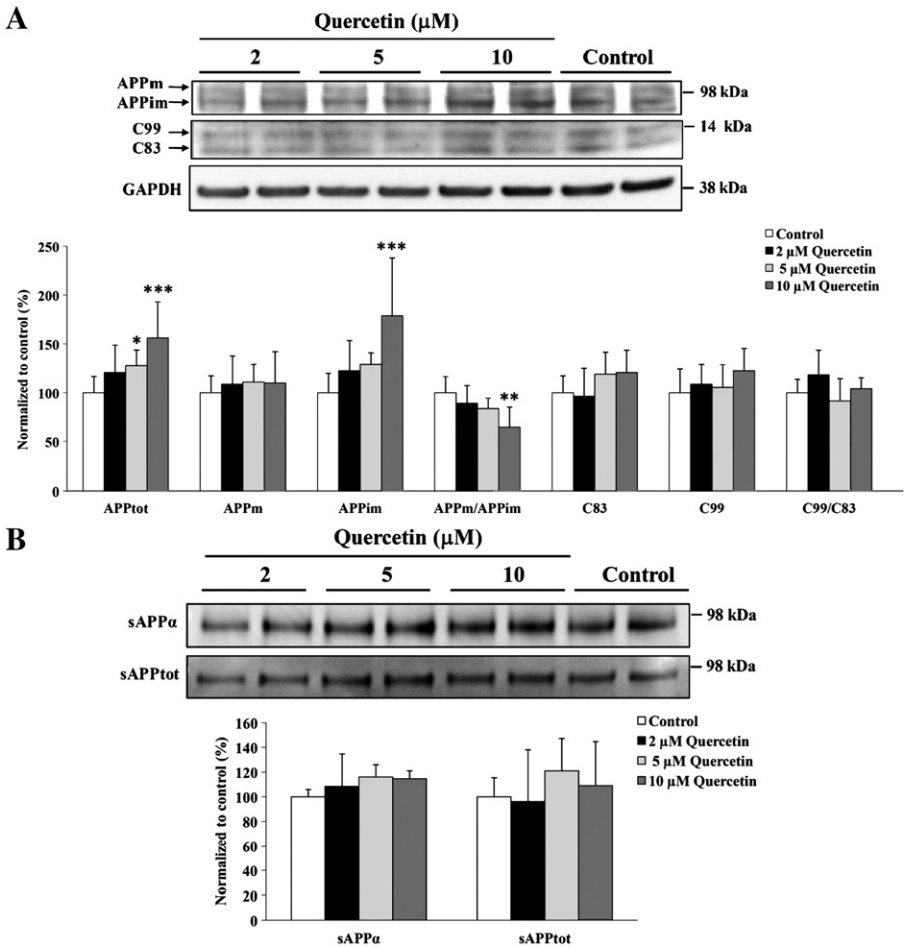

Fig. 2. Quercetin affects APP maturation in SH-SY5Y-APP751 cells under normal growth conditions. The cells were treated with different concentrations of quercetin for 24 h. (A) The effects on GAPDH-normalized APPtot, APPmature (APPm), APPimmature (APPim) and APP C-terminal fragment (C83 and C99) levels were determined from the total protein lysates. (B) Total protein-normalized sAPPα and sAPPtot levels were determined from SH-SY5Y-APP751 cell culture medium. ***$P<.001$, **$P<.01$, *$P<.05$; one-way ANOVA with Bonferroni correction, $n\geq6$, S.D.

alterations in APP processing. No changes were observed in APP processing or total protein-normalized sAPP levels in the cell culture medium of SH-SY5Y-APP751 cells after 24 h of treatment (data not shown). Also, treatment of SH-SY5Y-APP751 cells with 2 and 20 μM myrcitetin did not affect APP processing or sAPP secretion (data not shown). Collectively, these data indicate that under normal growth conditions, the highest quercetin concentration tested (10 μM) decreased the ratio of APPm/APPim, suggesting that APP maturation is reduced in SH-SY5Y-APP751 cells due to quercetin-related effects.

3.3. Anthocyanin-rich extracts do not affect caspase-3 activation or APP processing in SH-SY5Y-APP751 cells under apoptotic conditions

Next, the effects of anthocyanin-rich extracts on APP processing were studied under staurosporine-induced apoptosis. Treatment of SH-SY5Y-APP751 cells for 24 h with 0.15 μM staurosporine significantly increased caspase-3 activation. This was assessed by determining the ratio of the levels of active caspase-3 fragments (17 and 19 kDa) vs. those of procaspase-3 (Fig. 3A). Coinciding with caspase-3 activation, APP full-length (APPtot) and CTF (APP–CTF; C83 and C99) levels significantly increased in staurosporine-treated samples. Consistent with this, total protein-normalized sAPP and Aβ levels were significantly elevated in the SH-SY5Y-APP751 cell culture medium (Fig. 3B). Simultaneous treatment of SH-SY5Y-APP751 cells with staurosporine (0.15 μM) and 86% anthocyanin extracts (8 and 31 μg/ml) for 24 h did not affect APP processing or Aβ levels (Fig. 3). Similarly, staurosporine treatment together with different concentrations of myricetin (2 and 20 μM) for 24 h did not affect caspase-3 activation, APP processing or Aβ levels (data not shown) when compared to samples treated only with staurosporine. At the same time, however, different myricetin concentrations ranging

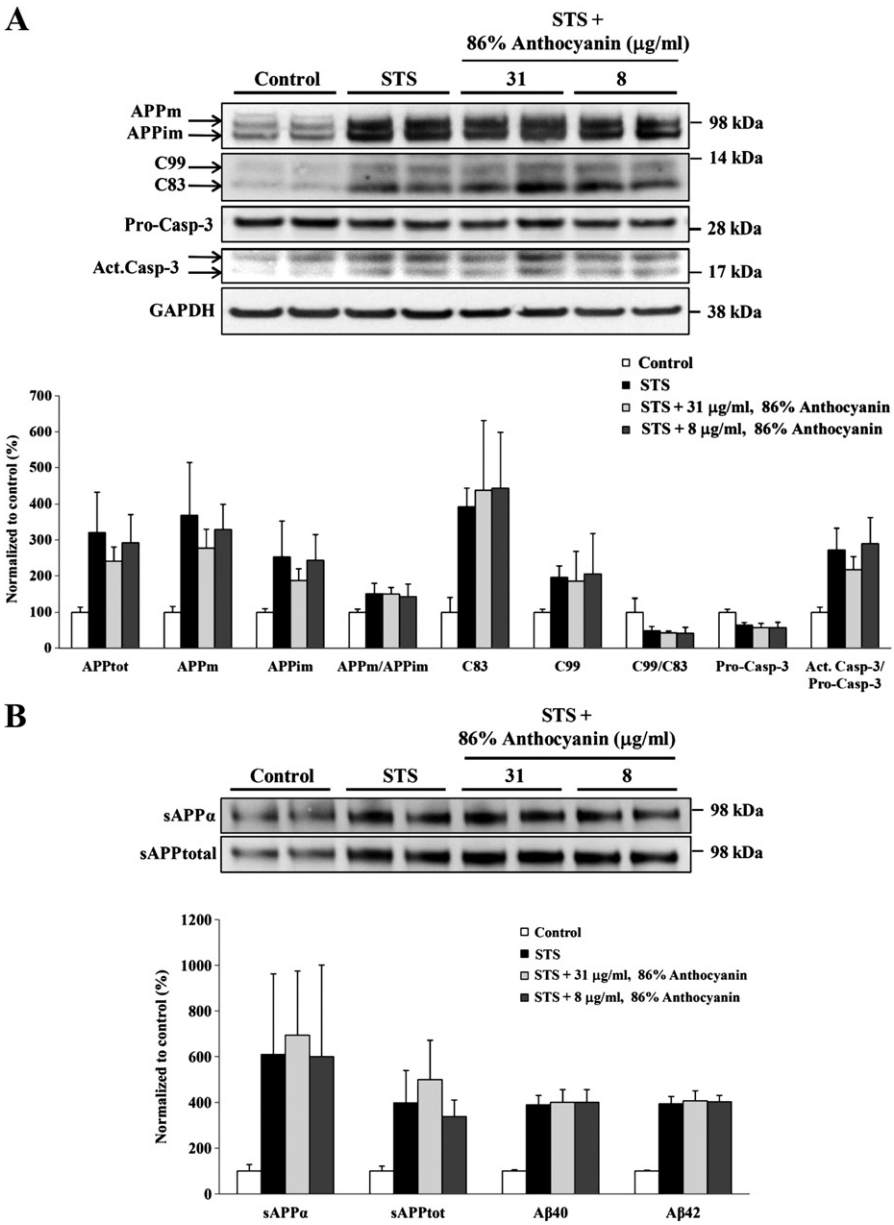

Fig. 3. Anthocyanin-rich blackcurrant extracts do not affect caspase-3 activation or APP processing in SH-SY5Y-APP751 cells under apoptotic conditions. The cells were treated with 0.15 μM staurosporine (STS) together with different concentrations of 86% anthocyanin extract for 24 h, after which effects on caspase-3 activity and APP processing were assessed in total protein lysates (A) and cell culture medium (B). Anthocyanin and staurosporine-treated samples are compared to the samples prepared from the cells treated with staurosporine only. One-way ANOVA with Bonferroni correction, n=5–6, S.D.

from 2 to 20 μM moderately increased cell viability when compared to samples treated only with staurosporine (Supplementary Figure 2). These data suggest that anthocyanin-rich extracts and myricetin at the used concentrations do not affect caspase-3 activation or APP processing in SH-SY5Y-APP751 cells under apoptotic conditions and therefore do not effectively protect the cells from staurosporine-induced apoptosis.

3.4. Anthocyanin-enriched bilberry and blackcurrant extracts alleviate behavioral abnormalities in transgenic APdE9 mice

The behavioral effects of anthocyanin-enriched bilberry (BB) and blackcurrant (BC) supplementation diets were assessed in the APdE9 mice, a well known mouse model of AD [15]. The mice started BB, BC or control diet at the age of 2.5 months and were monitored for their body weight throughout the BB and BC supplementation period (~10 months). At the age of 12 months, APdE9 mice underwent a comprehensive behavioral test battery and were killed for biochemical analyses at the age of 13 months.

3.4.1. Body weight

The weight gain of all mice was within normal limits, although mice receiving BB or BC extract containing chow showed a less steep weight increase (age×test group: $F_{21,177}=2.3$, $P=.002$; Supplement Figure 3). This suggests that the maximum tolerated (taste-wise) concentration of berry extracts was reached.

3.4.2. Spontaneous activity and neophobia

The test groups differed significantly in ambulatory distance (gross horizontal locomotion) in a new test cage ($F_{3,63}=4.2$, $P=.009$). As illustrated in Fig. 4A and confirmed by post hoc testing, ApdE9 mice on control chow were clearly hyperactive compared to wild-type littermates ($P=.008$). The BB group also significantly differed from the wild-types ($P<.05$), but the BC group did not ($P=.07$). In contrast, the test groups did not differ in rearing time ($F_{3,63}=0.7$, $P=.53$). APdE9 mice tended to leave more marbles uncovered than their wild-type littermates. This tendency was reduced to the level of wild-type mice by the berry extract diets (BB or BC) (Fig. 4B). However, the group differences were nonsignificant ($P=.58$).

3.4.3. Spatial working memory

APdE9 mice on control chow made more errors in the delayed alternation task than their wild-type littermates and did not show any improvement across the testing days (Fig. 4C). In contrast, APdE9 receiving berry extract-enriched (BB or BC) chow did improve. The overall group difference was marginally significant ($F_{3,59}=2.7$, $P=.056$). In the post hoc test, APdE9 mice on control chow significantly differed from the wild-type mice ($P=.03$), while the BB ($P=.34$) and BC ($P=.40$) groups did not. On the other hand, the difference between APdE9/control vs. APdE9/BB ($P=.34$) and APdE9/control vs. APdE9/BC ($P=.41$) mice did not reach significance. This finding indicates that the berry extract diets alleviated the spatial working memory deficit of aged APdE9 mice, although the effect size was small.

3.4.4. Morris swim task

As found in numerous experiments before, aged APdE9 male mice showed a dramatic impairment in the acquisition of the Morris swim task in terms of escape latency. The overall difference between the test groups was highly significant ($F_{3,63}=8.5$, $P<.001$). As illustrated in Fig. 5A, this learning impairment was alleviated by the BC chow. This improvement was, at least partially, due to diet effects on swimming speed. Namely, the overall group difference in swimming speed was significant ($P=.03$), and in the post hoc test, only the BC group swam significantly faster than the wild-type control mice ($P=.02$). A characteristic feature of the APdE9 mice is to stick to an inefficient strategy to search for an escape in the pool wall

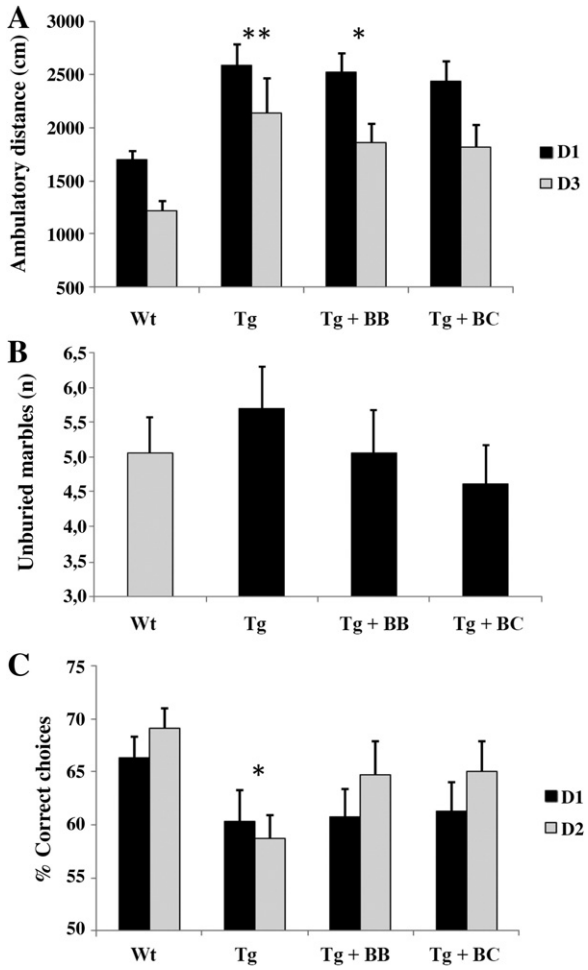

Fig. 4. Anthocyanin-enriched bilberry and blackcurrant extracts alleviate hyperactivity and spatial working memory deficit in transgenic APdE9 mice. (A) Ambulatory distance in a new test cage at two 10-min sessions on Day 1 (D1) and Day 3 (D3). Note the hyperactivity of APdE9 (tg) mice compared to their wild-type (Wt) littermates. BB=bilberry, BC=blackcurrant. Group means+S.E.M.s are shown. Different from wild-type controls: $^{*}P<.05$, $^{**}P<.01$ (Tukey HSD test). (B) Mean number of uncovered marbles overnight. Conventions as in panel A. (C) Correct choices (as %) in the delayed alternation task on D1 and D2. Conventions as in panel A; $n=6$ mice/group.

(thigmotaxis). This behavior accounts for the overall group difference in the time spent in the wall zone ($P=.04$). Interestingly, the berry extract groups abandoned this strategy earlier than APdE9 mice on the control chow (Fig. 5B). This was confirmed by post hoc analysis showing that while APdE9 mice on control chow differed from wild-type mice ($P=.02$), neither of the berry extract groups did ($P=.30$ and .39). Another characteristic behavioral pattern of APdE9 mice in this task is to do small rotations while swimming. This appears to be an adaptive behavior to compensate for the impaired memory of the exact platform location. The overall group difference in these search rotations was significant ($P=.03$). The APdE9 mice in the berry extract groups used this strategy less than the APdE9 mice on the control chow (Fig. 5C). Only the APdE9 mice on the control chow differed from the wild-type in the post hoc test (control: $P=.02$, BB: $P=.07$, BC: $P=.26$). To assess the memory for the platform location, a probe trial was run at the end of the 5-day task acquisition without the platform to determine the search bias. The overall group difference was significant ($F_{3,63}=4.8$, $P=.004$), reflecting impaired memory of the APdE9 mice compared to their wild-type littermates (Fig. 5D). In the post hoc test, APdE9 mice on control ($P=.03$) and BC chow ($P=.003$) were clearly impaired

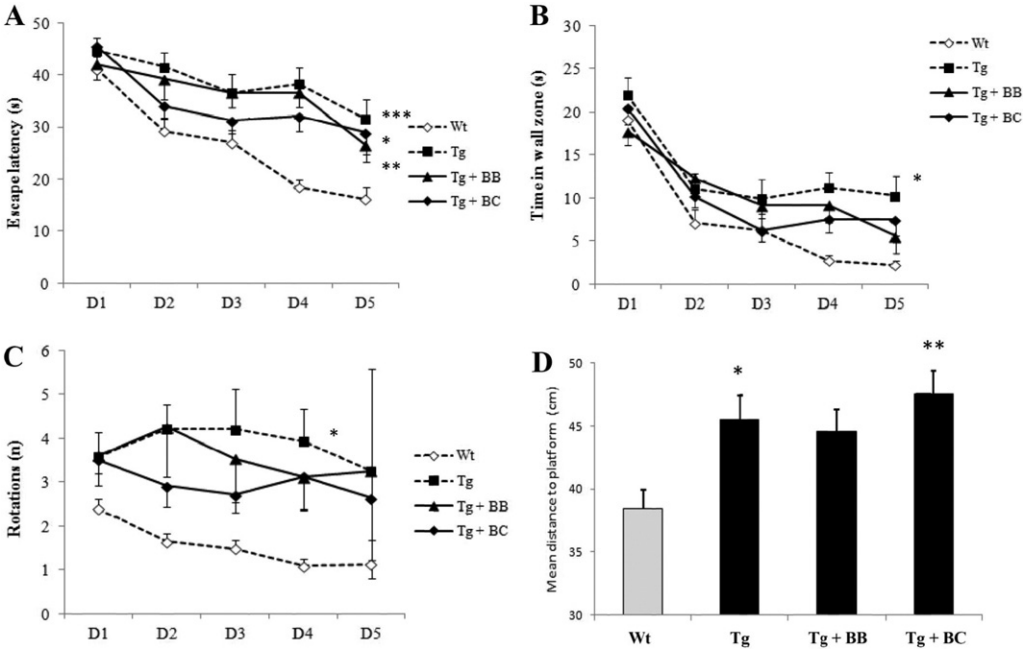

Fig. 5. Anthocyanin-enriched bilberry and blackcurrant extracts improve spatial learning in the Morris swim navigation task in transgenic APdE9 mice. Escape latency (A), time in the 10-cm-wide wall zone (B), number of search rotations (C) and mean distance to the former platform center during the probe test on the fifth trial day (D). Group means and S.E.M.s are shown. Different from wild-type controls: *P<.05, **P<.01, ***P<.001 (Tukey HSD test); n=6 mice/group.

compared to the wild-type mice, while APdE9 mice on BB chow were only marginally impaired (P=.09).

3.5. Anthocyanin-enriched bilberry and blackcurrant extracts affect APP processing, but not the expression or phoshorylation status of tau in the cerebral cortex of transgenic APdE9 mice

Finally, the effects of BB and BC supplementation diets on cellular processes relevant for AD pathogenesis were assessed in the cerebral cortex of APdE9 mice. Both BB and BC supplemented APdE9 mice showed decreased APP–CTF levels normalized to APPtot levels in the ventral posterior (temporo-occipital) cortex as compared to APdE9 mice on the control diet (Fig. 6A). Furthermore, BC supplementation in APdE9 mice revealed a trend to an increase in sAPPα levels as compared to BB or control mice (Fig. 6B). To address possible changes in the Aβ levels due to the BB and BC supplementation diet, soluble and insoluble Aβ40 and Aβ42 levels were measured from dorsal posterior (parietal–occipital) cortex using Aβ-ELISA (Fig. 7). BB-fed APdE9 mice revealed ~30% reduction in both soluble Aβ40 and Aβ42 levels, while the insoluble Aβ40 and Aβ42 levels were not altered as compared to APdE9 mice on the control diet (Fig. 7A, B). Interestingly, BB-fed APdE9 animals had significantly lower levels of both soluble Aβ40 (P<.05) and Aβ42 (P<.05) levels as compared to BC-fed APdE9 mice (Fig. 7A). Conversely, the ratio of insoluble Aβ42/40 was significantly decreased (P<.05) in BC-fed APdE9 mice as compared to BB-fed APdE9 mice (Fig. 7C). Although the γ-secretase activity assay did not reveal significant alterations between different groups (Fig. 6A), there was a statistically significant negative correlation between insoluble Aβ40 levels and γ-secretase activity (r=−0.50, P<.05). Assessment of IDE and NEP levels from the ventral cortex did not reveal significant alterations between groups (Fig. 8A), suggesting that the Aβ degradation status was not affected due to the BB or BC supplementation diet. Also, total tau levels and the Ser202/Thr205 phosphorylation status of tau were unaffected by the BB or BC supplementation diet (Fig. 8B). Collectively, these results suggest that BB and BC supplementation diets affect APP processing, but not tau isoform expression or phoshorylation status in the cerebral cortex of APdE9 transgenic mice.

4. Discussion

Here we have investigated the effects of pure flavonols and anthocyanin-rich blackcurrant extracts on neuroprotection and APP processing under AD-related stress conditions in human SH-SY5Y neuroblastoma cells overexpressing APP751 isoform. Furthermore, we wanted to assess whether bilberry or blackcurrant supplementation diets influence the Aβ and tau pathology as well as behavioral deficits in the APdE9 mouse model of AD [15]. Abnormalities in APP processing that lead to increased production of Aβ peptide are known to play a key role in AD pathogenesis [5]. APP undergoes sequential cleavage by α- or β- and γ-secretases resulting in the generation of p3 fragment and sAPPα (nonamyloidogenic pathway) or a self-aggregating Aβ peptide consisting of 37 to 42 amino acid residues and sAPPβ (amyloidogenic pathway), respectively. Maintenance of the balance between the amyloidogenic and the nonamyloidogenic pathway is crucial, since only small changes in this fine equilibrium may initiate pathogenic cascades, ultimately leading to the onset of AD. Polyphenols, which are abundant in bilberry and blackcurrant, have been shown to inhibit the formation and extension of Aβ fibrils in a dose-dependent manner and to destabilize preformed Aβ fibrils *in vitro* [2–4]. These experimental findings along with the epidemiological data, which indicate that fruit and vegetable juices containing various phenolic compounds can reduce the risk of AD [1], hold great promise that active dietary choices can modulate the development and progression of AD.

We first assessed whether flavonols and anthocyanin-rich blackcurrant extracts affect APP processing under normal growth conditions. Interestingly, high quercetin concentration (10 μM), but not myricetin, consistently increased the levels of APPim, but did not affect APPm levels. This led to a decreased ratio of APPm vs. APPim (APPm/APPim). A similar trend was observed at lower quercetin concentration (5 μM). A decreased APPm/APPim ratio suggests that APP maturation is reduced in response to quercetin treatment. Since APP maturation (*N*- and *O*-linked glycosidation) is tightly linked to its trafficking through the secretory pathway [27], it is anticipated that quercetin treatment at high concentrations would results in less APP reaching the plasma membrane. Consequently, there would be less APP available on the

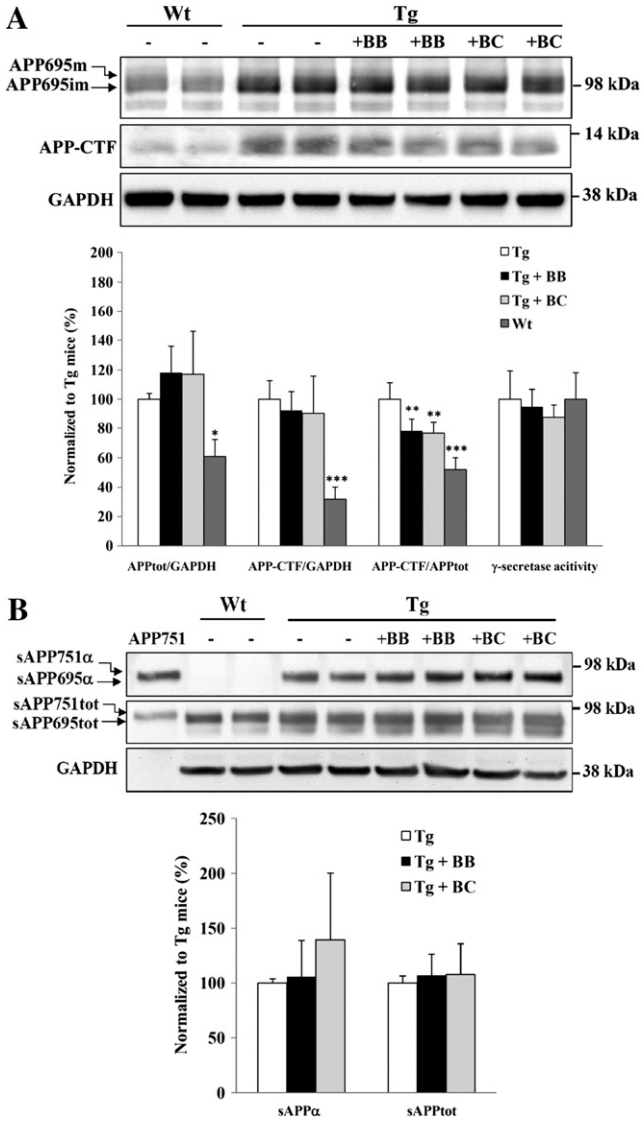

Fig. 6. Anthocyanin-enriched bilberry and blackcurrant extracts affect APP processing in the cerebral cortex of transgenic APdE9 mice. Transgenic APdE9 mice were fed with bilberry (Tg+BB), blackcurrant (Tg+BC) or control diet for ~10 months. Effects of BB and BC supplementation on APP processing were assessed from total protein lysates (A) or soluble protein fractions (B) extracted from the ventral posterior cortex of transgenic APdE9 mice (Tg). APPtot-normalized APP–CTF levels were significantly decreased in BB- and BC-fed APdE9 mice. There were no statistically significant changes in secreted APP levels (sAPPα or sAPPtot). Cell culture medium from SH-SY5Y-APP751 cells (APP751) and soluble fraction from wild-type mice (Wt) were used as controls for assessment of APP-related changes. 6E10 antibody detected only the human-specific sAPPα in the APdE9 mice, but not the mouse-specific sAPPα in the Wt mice. ***P<.001, **P<.01, *P<.05 when normalized to APdE9 mice on the control diet; one-way ANOVA with Bonferroni correction, n=6 mice/group, S.D.

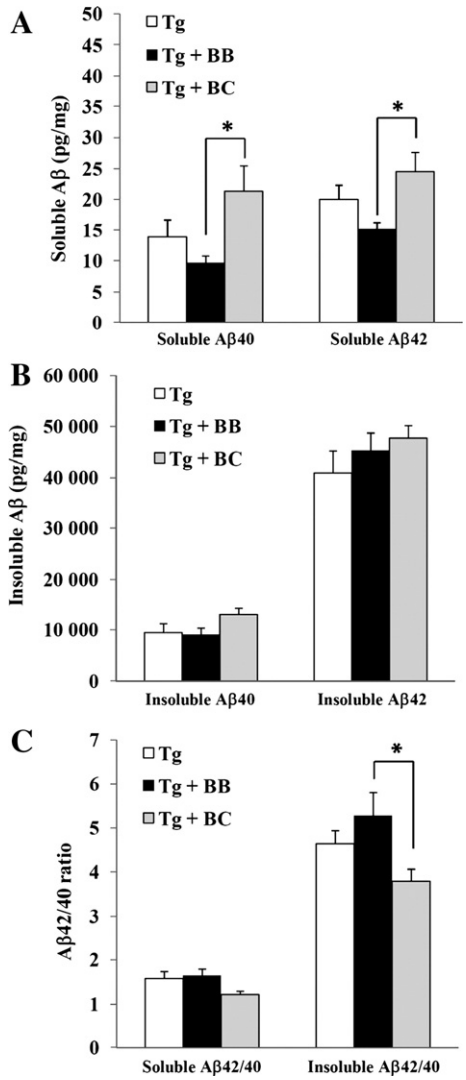

Fig. 7. Aβ40 and Aβ42 levels as well as ratio of Aβ42/40 are differentially affected by anthocyanin-enriched bilberry and blackcurrant extracts in the cerebral cortex of transgenic APdE9 mice. (A) Although the soluble Aβ40 and Aβ42 levels were not significantly changed between bilberry (Tg+BB) and control diet (Tg) or blackcurrant (Tg+BC) and control diet (Tg) mice groups, both soluble Aβ40 (P<.05) and Aβ42 (P<.05) levels were significantly decreased in BB-fed mice as compared to BC-mice in the dorsal posterior cortex. (B) There were no statistically significant changes in insoluble Aβ40 and Aβ42 levels between mice groups. (C) The ratio of insoluble Aβ42/40 was significantly decreased (P<.05) in BC-fed mice as compared to BB-fed mice. One-way ANOVA with Bonferroni correction, n=6 mice/group, *P<.05, S.E.M.

plasma membrane for the α-secretase-mediated cleavage to release the potentially neuroprotective sAPPα. Thus, it is likely that the observed reduction in APP maturation due to a high concentration of quercetin is not beneficial to the cells. This idea is corroborated by the finding that high concentrations of quercetin (≥20 μM) significantly decreased the viability of SH-SY5Y-APP751 cells. However, since we found not major changes in APP cleavage products like sAPPα, it is difficult to determine the relevance of the effects of high doses of quercetin in the long-term, indicating that further studies in a relevant *in vivo* model of AD are needed. Nevertheless, our data suggest that under normal growth conditions, flavonols such as quercetin may exert variable, concentration-dependent effects on APP processing.

We also elucidated the effects of these compounds under different stress conditions, such as free radical-mediated toxicity. Acute ROS production in SH-SY5Y-APP751 cells was induced using menadione, which generates ROS via redox cycling and ultimately leads to apoptosis [26]. At the same time, the cells were treated with different concentrations of flavonols or anthocyanin-rich blackcurrant extracts. Subsequent studies revealed that myricetin, quercetin and anthocyanin-rich extracts significantly reduced ROS production in a dose-dependent manner. These results indicate that both flavonols and anthocyanin-rich blackcurrant extracts have antioxidant-like properties, which is consistent with a recent observation that flavonols inhibit the Aβ-induced ROS production in various neuronal cells [28,29]. Furthermore, these data confirmed that the anthocyanin-rich extracts processed from blackcurrant powder were biologically active. Prompted by these findings, we subsequently assessed the effects of flavonols and anthocyanin-rich extracts on neuroprotection

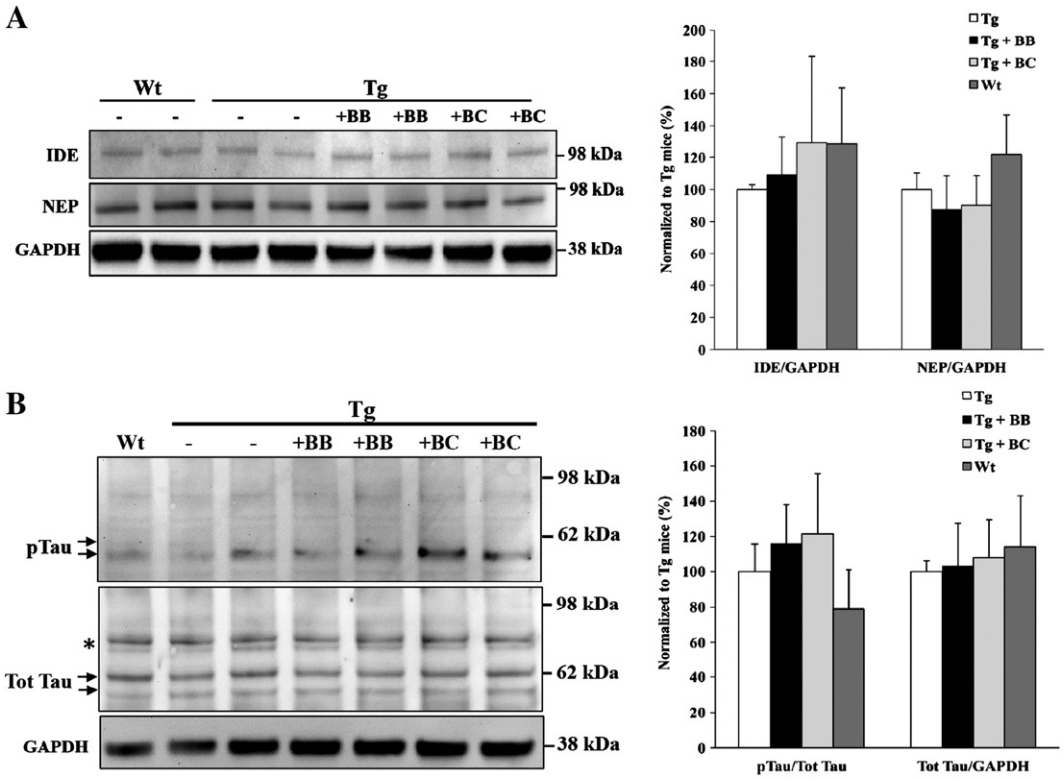

Fig. 8. Anthocyanin-enriched bilberry and blackcurrant extracts do not affect Aβ degradation status or tau isoform expression and phoshorylation in the cerebral cortex of transgenic APdE9 mice. (A) Bilberry (BB) or blackcurrant (BC) supplementation did not significantly affect GAPDH-normalized IDE or NEP levels in ventral posterior cortex of APdE9 mice. (B) Tau isoform expression or total tau (Tot Tau)-normalized phosphorylated tau (pTau, Ser202/Thr205 epitopes) levels were not significantly changed between different groups. Wild-type mice (Wt) were used as controls. An ~80-kDa protein not matching the expected size of tau isoforms was recognized by the total tau antibody in Western blot gels (indicated as *). One-way ANOVA with Bonferroni correction, n=6 mice/group, S.D.

and APP processing in more sustained stress conditions by inducing apoptosis using a low concentration of staurosporine in SH-SY5Y-APP751 cells. As shown previously in SH-SY5Y cells [20], a 24-h staurosporine treatment markedly induced caspase-3 activation and robustly increased (~4–6-fold) sAPP and Aβ levels in the cell culture medium. However, treatment with myricetin or anthocyanin-rich extracts together with staurosporine did not affect APP processing or Aβ production as compared to the samples treated only with staurosporine, suggesting that these compounds were not able to efficiently prevent induction of apoptosis. Although we did not observe changes in APP processing after the induction of apoptosis in response to myricetin treatment, we detected a moderate but dose-dependent increase in the viability of SH-SY5Y-APP751 cells, suggesting that myricetin may provide cytoprotection. The finding that Aβ levels remained unchanged after myricetin treatment contradicts the recent observations in primary cortical neurons, in which natural flavonoids were able to reduce secreted Aβ levels due to β-secretase inhibition [30]. Despite this, our present findings emphasize that myricetin possesses cytoprotective activity upon sustained apoptotic stress in SH-SY5Y-APP751 cells.

In addition to *in vitro* analyses, we also investigated the effects of diets supplemented with bilberry or blackcurrant on APP processing, Aβ levels, Aβ degradation status and the expression and phoshorylation status of tau in the cerebral cortex of APdE9 mice. Consistent with the previous effects of polyphenol diet in different AD mouse models [4,7,8,31,32], both bilberry- and blackcurrant-fed APdE9 mice showed altered APP processing and Aβ levels in the cerebral cortex. More specifically, both berry extract-fed APdE9 mouse groups showed similar reduction in APPtot-normalized APP–CTF levels, while the diet effects on soluble Aβ40 and Aβ42 levels and the ratio of Aβ42/40 in the dorsal cortex were different. Interestingly, bilberry supplementation reduced both soluble Aβ40 and Aβ42 levels as compared to blackcurrant-fed mice. In contrast, a reduced ratio of insoluble Aβ42/40 and moderately increased sAPPα levels were observed in blackcurrant-fed mice, but not in bilberry-fed mice. These are important findings considering that the increased ratio of Aβ42/40 is a key pathogenic feature observed in the familial AD patients with autosomal dominant mutations [5] and that sAPPα is known to exert neuroprotective effects. A recent study showed that tannic acid acted as a β-secretase inhibitor and prevented cognitive decline and mitigated the AD-like pathology in a mouse model of AD [31]. Consistent with this, we observed that APP–CTF levels were reduced in both bilberry- and blackcurrant-fed APdE9 mice, supporting the idea that the berry supplements may have an inhibitory effect on β-secretase. On the other hand, the decreased ratio of insoluble Aβ42/40 in blackcurrant-fed mice may rather be attributable to the modulation of γ-secretase function than β-secretase inhibition. However, as the γ-secretase activity measurements did not reveal significant activity changes, it appears unlikely that the cellular mechanisms induced by bilberry and blackcurrant supplementation diets would underlie alterations in the function of γ-secretase.

Changes in Aβ degradation or clearance might also have contributed to the observed Aβ changes. IDE and NEP are the key proteases involved in Aβ degradation in the brain [33]. Thus, we also assessed the expression status of IDE and NEP in the cerebral cortex of APdE9 mice fed with bilberry or blackcurrant, but no alterations in the levels of these proteases were detected. However, we cannot rule out the possibility that some of the other proteases/enzymes involved in Aβ degradation play a role upon bilberry and blackcurrant supplementations. Apart from APP- and Aβ-related changes, phosphorylation of tau protein at certain epitopes, such as Ser202/Thr205, is increased in AD. This leads to tau aggregation and formation of

neurofibrillary tangles. We have recently shown that phosphorylation of tau at the Ser202/Thr205 epitopes was robustly increased in APdE9 mice fed with high-fat diet [34]. This finding indicates that diet-induced changes in tau phosphorylation at these particular epitopes are relevant in terms of tau pathology. Interestingly, a grape-derived polyphenol extract was shown to attenuate tau pathology in an AD mouse model carrying mutated human tau transgene via mechanisms coinciding with signaling changes of extracellular-receptor kinase 1/2 [35]. In the present study, we did not find any significant alterations in tau isoform expression or Ser202/Thr205 phosphorylation status between the diet groups. However, there are several other AD-related tau phosphoepitopes, which might also be affected by dietary factors and contribute to tau pathology.

Finally, bilberry and blackcurrant supplementation diets attenuated specific behavioral abnormalities in APdE9 mice. Hyperactivity in a new environment is a consistent finding in APdE9 mice [31]. This was alleviated to some extent by both bilberry and blackcurrant diets, but significantly only in the blackcurrant group. Interestingly, under a stressful swimming condition, blackcurrant diet increased swimming speed, ruling out the possibility that this derives from some kind of motor impairment. The observed reduction of APdE9 transgene-related hyperactivity by polyphenol-supplemented diet is consistent with a recent study showing amelioration of hyperactivity in the same APdE9 mice by the flavonoid tannic acid [31]. The most striking effect of berry extracts was observed in the food-motivated spatial working memory task, in which both bilberry and blackcurrant attenuated the APdE9 genotype-linked impairment. A moderate beneficial effect of the berry extracts was also observed in the strategy of solving the Morris swim task: both the time spend near the pool wall and search rotations while swimming were decreased in the bilberry and blackcurrant groups compared to the mice receiving regular chow. In contrast, the blackcurrant diet in particular did not have a beneficial effect on the most sensitive measure of spatial long-term memory, the search bias in the probe test. This pattern of effects suggests that polyphenol supplementation augmented executive functions that draw on the medial frontal cortex but did not affect hippocampus-dependent encoding into long-term memory [36,37]. The finding of improved spatial working memory by bilberry and blackcurrant diets is fully consistent with an earlier report showing a beneficial effect of blueberry extract on spontaneous alternation in the Y-maze in another APP/PS1 mouse line [8]. Improved learning in the Morris swim task has also been reported for tannic acid, although in that study the effect could also be seen in the probe trial testing memory retention [31]. In addition, amelioration of hyperactivity and improved spatial learning in the Morris swim task have been reported in APPswe transgenic mice given the LMN diet, which is rich in polyphenols, but also in polyunsaturated fatty acids [32]. Collectively these findings in three different mouse lines overexpressing the APPswe transgene suggest that polyphenols alleviate both hyperactivity and age-associated cognitive impairment common to all APP transgenic mouse lines.

Taken together, our findings suggest that the flavonols and anthocyanin-rich blackcurrant extracts studied here exert protective effects in different *in vitro* stress conditions, particularly in menadione-induced ROS production. Interestingly, myricetin consistently encompassed a protective role in all the studied stress conditions, indicating that this flavonol is a potent bioactive compound with a strong antioxidant-like activity. However, the fact that we only observed moderate alterations in APP processing emphasized the importance of pursuing these studies also in an *in vivo* model of AD. Long-lasting supplementation of APdE9 mice with bilberry or blackcurrant revealed beneficial effects on APP and Aβ metabolism, but not tau-related changes. In addition, these supplementations alleviated behavioral abnormalities in a well-characterized AD mouse model. Based on the present study, it is anticipated that bilberry- and blackcurrant-derived bioactive compounds display beneficial effects on neuroprotection, behavioral outcome and APP processing and Aβ accumulation. Thus, the present study emphasizes the importance of assessing both *in vitro* and *in vivo* effects of dietary factors on pathways relevant for AD, such as APP processing and Aβ production. The fact that we observed Aβ-related changes in APdE9 mice but not in neuroblastoma cells suggests that long-term supplementation with bioactive compounds in *in vivo* settings is needed to reveal the beneficial outcome measures related to Aβ pathology and subsequent behavioral outcome. Conversely, *in vitro* screening of bioactive compounds may function as an efficient tool to assess short-term effects of these compounds on cell survival and ROS production. Finally, our findings emphasize that the individual bioactive compounds used and their concentrations in the dietary supplements need to be carefully characterized when applied for intervention strategies against AD.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2012.07.006

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