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Cognitive-Enhancing Effects of a Polyphenols-Rich Extract from Fruits without Changes in Neuropathology in an Animal Model of Alzheimer's Disease

Alexandre Dal-Pan[a,b,d,e], Stéphanie Dudonné[b,e], Philippe Bourassa[a,b,c,d], Morgane Bourdoulous[a], Cyntia Tremblay[a], Yves Desjardins[b,e] and Frédéric Calon[a,b,c,d,e,*] on behalf of the Neurophenols consortium[e]

[a]*CHU de Québec Research Center, Quebec, QC, Canada*
[b]*Institute of Nutrition and Functional Foods, Laval University, Quebec, QC, Canada*
[c]*Faculty of Pharmacy, Laval University, Quebec, QC, Canada*
[d]*OptiNutriBrain International Associated Laboratory (NutriNeuro France-INAF Canada)*
[e]*http://www.neurophenols.org*

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Abstract. No effective preventive treatment is available for age-related cognitive decline and Alzheimer's disease (AD). Epidemiological studies indicate that a diet rich in fruit is associated with cognitive improvement. It was thus proposed that high polyphenol concentrations found in berries can prevent cognitive impairment associated with aging and AD. Therefore, the Neurophenols project aimed at investigating the effects of a polyphenolic extract from blueberries and grapes (PEBG) in the triple-transgenic (3xTg-AD) mouse model of AD, which develops AD neuropathological markers, including amyloid-β plaques and neurofibrillary tangles, leading to memory deficits. In this study, 12-month-old 3xTg-AD and NonTg mice were fed a diet supplemented with standardized PEBG (500 or 2500 mg/kg) for 4 months ($n = 15$–20/group). A cognitive evaluation with the novel object recognition test was performed at 15 months of age and mice were sacrificed at 16 months of age. We observed that PEBG supplementation with doses of 500 or 2500 mg/kg prevented the decrease in novel object recognition observed in both 15-month-old 3xTg-AD mice and NonTg mice fed a control diet. Although PEBG treatment did not reduce Aβ and tau pathologies, it prevented the decrease in mature BDNF observed in 16-month-old 3xTg-AD mice. Finally, plasma concentrations of phenolic metabolites, such as dihydroxyphenyl valerolactone, a microbial metabolite of epicatechin, positively correlated with memory performances in supplemented mice. The improvement in object recognition observed in 3xTg-AD mice after PEBG administration supports the consumption of polyphenols-rich extracts to prevent memory impairment associated with age-related disease, without significant effects on classical AD neuropathology.

Keywords: 3xTg-AD, age-related cognitive decline, Alzheimer's disease, polyphenols

*Correspondence to: Frédéric Calon, PhD, Centre de Recherche en Endocrinologie Moléculaire et Oncologique, Centre de Recherche du CHUL (CHUQ), 2705, Boulevard Laurier, Room T2-05, Québec, QC, G1V 4G2, Canada. Tel.: +1 418 656 4141/Ext. 48697; Fax: +1 418 654 2761; E-mail: Frederic.Calon@crchul.ulaval.ca.

INTRODUCTION

Life expectancy is reaching its highest level in human history. Consequently, the demand for interventions to prevent cognitive decline associated with age and neurodegenerative diseases has never been more urgent. Among age-associated diseases, Alzheimer's disease (AD) is the most common form of dementia [1, 2] and one of the most costly to society [3]. AD is clinically characterized by a decline in episodic memory function often coupled with impairments in verbal fluency, visuospatial abilities, and activities of daily living [4]. From a neuropathological standpoint, AD is a progressive proteinopathy characterized by the accumulation of oligomerization-prone amyloid-β (Aβ) fragments and phosphorylated tau aggregated into neurofibrillary tangles in multiple regions of the brain [5–7]. Massive neuronal and synaptic losses are also observed in the cerebral cortex and subcortical regions, inducing an important atrophy of the affected regions [8]. Although the accumulation of plaques and tangles correlate to some extent with symptoms [5, 6], the exact neuropathological substrate of cognitive impairment in AD remains poorly defined.

Unfortunately, in the last decades, most randomized controlled trials of potential AD therapeutics have failed to show cognitive benefits [9–11]. The absence of well-defined drug targets probably explains in part the disappointing results of these randomized controlled trials [12] but also the fact that AD is a heterogeneous disease. Specific targeting of a single mechanism of action might hence only benefit a limited subpopulation of individuals among those classified as AD patients [13, 14]. There is thus a strong need for developing cognitive enhancers effective in a large range of individuals suffering from various subtypes of age-related cognitive impairment and with a favorable safety profile. Therefore, it is understandable that nutraceutical or dietary approaches based on natural food compounds have recently received considerable interest to reverse cognitive decline induced by AD or other age-related diseases [13, 15].

This is particularly the case with polyphenols or food with high polyphenol concentrations, such as berries and grapes. Indeed, results from preclinical and clinical studies suggest that berries deserve further studies as nutraceutical tools to prevent cognitive impairment associated with aging and AD, either *per se* or through their metabolites [16, 17]. Although the cerebral bioavailability of polyphenols is variable and limited, three major phenolic compounds, namely gallic acid, catechin, and epicatechin, can be found in the brain of rats fed a naturally derived grape seed phenolic extract (GSPE) preparation [18]. In randomized clinical trials, mild cognitive impairment patients fed a mix of flavonoid compounds displayed improved verbal cognitive tasks [19–21]. Moreover, elderly people with age-related cognitive decline showed an improvement of their cognitive abilities and memory after consuming food concentrated in polyphenols, such as juices from blueberries [20] or grapes [19, 22, 23]. Correlative epidemiological studies also pinpoint that a high intake of flavonoids is associated with a slower progression of cognitive decline and a lower risk of dementia in older adults [24–26]. Among the mechanisms of action proposed, activation of signaling pathways involving brain-derived neurotrophic factor (BDNF) [27] and induction of vascular changes leading to enhanced blood flow have received most attention [28, 29]. However, it has been suggested that the mechanisms of action of polyphenols from each family of fruits are different and may therefore have a synergistic effect [30].

Benefits of blueberries and grapes have been shown separately in AD mouse models. For instance, when orally administered to Tg2576 mice, a GSPE preparation was shown to significantly limit AD-like cognitive deterioration coincidentally with reduced high-molecular-weight soluble oligomeric Aβ in the brain [31]. The same grape extract was shown to significantly attenuate the development of AD-like tau neuropathology in the brain of TMHT mouse model of AD [32]. Better performance in Y-maze test was observed in APP-PS1 transgenic mice fed a blueberry extract for 8 months with no alterations in Aβ burden, compared to age-matched APP-PS1 mice without any treatment [33]. Yet, data about the potential synergistic effect of each family of fruits still remain limited.

In this context, the *Neurophenols* project was developed to characterize and formulate extracts from both blueberries and grapes for clinical trial against age-related cognitive decline. As part of this project, we investigated the effects of a 4-month dietary supplementation with a standardized polyphenolic extract from blueberries and grapes (PEBG) in the triple-transgenic (3xTg-AD) mouse model of AD, which is known to develop memory deficit and neuropathological markers of the disease [34–36]. The main objective of this study was to determine the effect of a PEBG-supplemented diet on cognitive performance and brain neuropathological markers in the 3xTg-AD mouse.

MATERIAL AND METHODS

The Neurophenols consortium is a Europe-North America collaboration dedicated to the research and development of natural ingredients and products to prevent age-related cognitive decline in humans and pets. The consortium brings together scientists in the fields of phytochemistry, neuroscience, psychology, and nutrition with companies specialized in the development of active ingredients and food supplements. The specific aims of the program are to characterize and formulate extracts from each family of fruits and to evaluate their safety and efficacy in pre-clinical and clinical trials.

Animals

The homozygous 3xTg-AD mouse model used here has already been described elsewhere [34, 35, 37–39]. Briefly, this mouse model of AD harbors mutant presenilin-1 ($PS1_{M146V}$), APP_{swe}, and tau (P301L) transgenes. The human APP and tau-independent transgene constructs have been co-injected in embryos harvested from mutant homozygous $PS1_{M146V}$ knock-in mice. Both human APP and tau transgenes are under the control of the Thy1.2 regulatory elements, co-integrated in the same locus and therefore inherited together [34]. The model progressively develops the two main pathological hallmarks of AD, namely Aβ deposits and neurofibrillary tangles (tau pathology), but also shows significant synaptic and cognitive deficits [35, 36]. It is thus an appropriate model for preclinical studies on potential drug therapies in early AD. Non-transgenic (NonTg) mice were derived from the original mouse line and were from the same genetic background. One hundred and twenty (120) non-transgenic (NonTg) and triple-transgenic (3xTg-AD) 12-month-old mice (60 females and 60 males) were assigned to this study. Seven mice (evenly distributed between groups) were found dead and were excluded from our analyses. Their death was unrelated to experimental treatment according to histopathological analyses performed by the staff of the animal facility. Moreover, an additional group of 4-month-old C57BL/6 mice ($n = 10$) were used as a young control group.

Drugs

The Neurophenols consortium extract is made of blueberries (*Vaccinium angustifolium*) and grapes (*Vitis vinifera* L.) extracts containing specific low-molecular-weight polyphenols (monomers, oligomers, flavonols, anthocyanins, phenolic acids, and resveratrol), formulated in a unique patented ratio of molecules. The Neurophenols consortium extract was introduced in mouse pellets in order to achieve constant plasma levels in mice. Mice were fed a control diet (named "Tpolyph") or 500 mg of extract/kg(bw) per day (named "Polyph1") or 2500 mg of extract/kg(bw) per day (named "Polyph2"). These doses correspond to a 2–12 g/day intake of the Neurophenols consortium extract according to U.S. Food and Drug Administration's table for conversion between species for a human weighing 60 kg and assuming body surface area equivalence. Proportions for each polyphenol class were adjusted for both PEBG doses, which mainly contain flavan-3-ol monomers from French grape extract at respectively 129 mg/kg(bw) per day and 644 mg/kg(bw) per day, and flavonols from blueberry at respectively 4.6 mg/kg(bw) per day and 22.3 mg/kg(bw) per day. Proportions of other components of the Neurophenol consortium extract remain confidential. The dose of 500 mg/kg(bw) per day was determined based on a review of the literature from cognitive studies using grape seed or blueberry extracts in rodents and on the fact that adjustments had to be done to obtain the specific proportions for each polyphenol class mentioned above. We added a higher dose, 2500 mg/kg body weight, to evidence a dose-response effect and to allow us to perform correlative analysis between phenolic content in plasma and other endpoints.

Experimental schedule

The timeline of the study is shown in Fig. 1. For colony maintenance and to avoid interfering with developmental processes, all mice were kept on the mouse breeder chow (Teklad 2018, Harlan Laboratories, Canada) at our animal facility for the first 12 months. High dietary fat intake is widespread in our modern society and has been identified as a risk factor underlying the constant increase of AD prevalence over the last few years [40, 41]. The deleterious effect of high-fat diet on AD-like neuropathology and cognitive performance has been consistently replicated in animal models [37, 39, 42, 43]. Therefore, to mimic the current diet composition of what most people actually eat in industrialized nations, the animals in this study were fed a "Western diet" with 20% (w/w) of total fat. Table 1 summarizes diet composition,

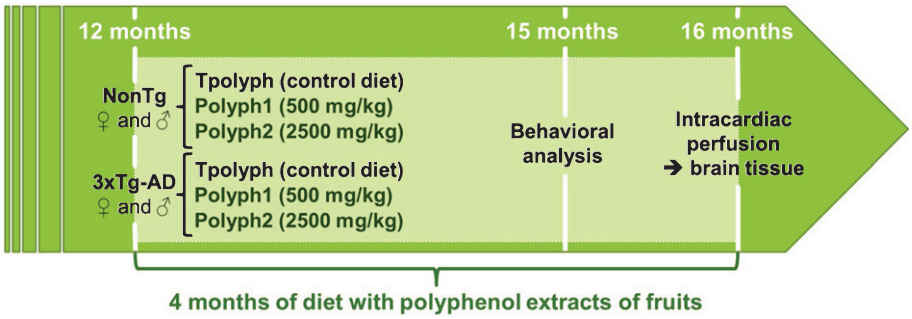

Fig. 1. Experimental schedule of the study. Non-transgenic (NonTg) and triple-transgenic (3xTg-AD) mice were fed from the age of 12 months a "Western diet", more representative of what most people eat in developed countries, supplemented or not with the Neurophenols consortium polyphenolic extract from grapes and blueberries (PEBG) for 4 months. Two different doses, 500 and 2500 mg/kg body weight, were used to study potential dose-response effects. Behavioral and cognitive performance were investigated at the age of 15 months (3 months after the beginning of supplementation). Animals were sacrificed at the age of 16 months and brain tissues were collected to detect changes in neuropathological markers of AD. An additional group of 4-month-old NonTg mice (NonTg_CTL) without any treatment was added to verify potential age effect.

with fatty acid content confirmed by gas chromatography. The purified diet formula was produced by Research Diet Inc. (New Brunswick, USA) and precisely determined to avoid batch-to-batch variations commonly found in laboratory chow.

Experimental PEBG treatments were started at 12 months and lasted 4 months, as described in Fig. 1. A cognitive evaluation was performed at 15 months of age and all mice were sacrificed at 16 months of age. Thus, animals used in this study were divided into six groups: *NonTg_T* for non-transgenic mice fed control diet, *NonTg_P1* for non-transgenic mice fed "Polyph1" diet, *NonTg_P2* for non-transgenic mice fed "Polyph2" diet, *3xTg-AD_T* for triple-transgenic mice fed control diet, *3xTg-AD_P1* for triple-transgenic mice fed "Polyph1" diet and *3xTg-AD_P2* for triple-transgenic mice fed "Polyph2" diet. An additional group of 4-month-old NonTg mice (*NonTg_CTL*) without any treatment was added as a control to evidence age-related cognitive impairment in older animals. All procedures were approved by the Laval University animal ethics committee and were performed in accordance with the Canadian Council on Animal Care guidelines.

Behavioral analysis

Motor performance

Motor coordination and postural stability were estimated with the rotarod device (San Diego Instruments, San Diego, CA, USA). Facing away from the experimenter's view, the mice moved on top of a rotating rod (diameter: 3 cm, width: 9.5 cm, height: 45 cm) increasing progressively in smooth, graduated steps up to 40 rpm in 3 min. In this test, the integrity of motor coordination was assessed on the basis of endurance time of the animals on the rotating rod. After one habituation trial, latencies before falling were measured in a single 3-trial session and the mean score was retained [44].

Exploratory behavior

Locomotor activity was assessed using an open field apparatus consisting of ten Plexiglas cages with translucent walls (40 cm × 40 cm) homogenously illuminated (around 400 lux) and divided into peripheral and central regions. Movements were tracked by the automated recording of photobeam breaks (San Diego Instruments) to measure horizontal (for example, distance traveled) and vertical activity (for example, rearing). Mice were placed individually in the center of the open field and movements were recorded for 1 h with the experimenter out of their view [39, 45].

Anxiety-like behavior

Anxiety was assessed with the dark-light emergence test as previously described [39, 46, 47]. Animals were positioned in the dark compartment and the time spent in the illuminated compartment was measured for 5 min. A reduction in the time spent in the illuminated compartment was interpreted as increased anxiety. However, in our study, a large number of mice (41%, evenly distributed between groups) did not come out of the dark compartment before the end of the test, limiting the statistical power of our analysis of the time spent in the white compartment. Thus, in addition to this parameter chosen to highlight

Table 1
Diet composition

	Western diet
General information (kcal%)	
Protein	18.8
Fat	40.9
Carbohydrate	40.3
Total	100
(kcal/g)	4.5
Ingredients (% w/w)	
Casein	200
DL-Methionine	3
Corn starch	75
Maltodextrine 10	75
Sucrose	223
Cellulose, BW200	50
Safflower oil	40
Butter, anhydrous	92
Lard	41
Ethoxyquin	0.001
Mineral mix S19101	35
Sodium selenite (45,7% Se)	0.00028
Vitamin mix V15908	10
Choline bitartrate	2
Cholesterol, USP	1.5
Fatty acid content (g/kg)†	
C16:0	37.0
C18:0	12.2
C20:0	0
C22:0	0
SFA	61.6
MUFA	37.0
C18:1 n-9 (OA)	30.1
C18:2 n-6 (LA)	25.4
C20:4 n-6 (AA)	0
C22:4 n-6 (DTA)	0
C22:5 n-3 (DPA)	0
C18:3 n-3 (LNA)	0.8
C20:5 n-3 (EPA)	0
C22:6 n-3 (DHA)	0
n-6 PUFA	27.3
n-3 PUFA	1.5
total PUFA	28.8
n-6:n-3 ratio	18
Cholesterol	1.8

SFA, saturated fatty acid; MUFA, monounsatured fatty acid; OA, oleic acid; LA, linoleic acid; AA, arachidonic acid; DTA, docosatetraenoic acid; DPA, docosapentaenoic acid; LNA, linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid. †Measured by gas chromatography.

anxiety level in mice, the latency to nose entry in the white compartment was also analyzed (while leaving at least 2 feet out of 4 inside). A longer latency was interpreted as increased anxiety.

Memory performance

Recognition memory was assessed as previously described with the novel object recognition (NOR) task, which is based on the spontaneous tendency of rodents to explore a novel object longer than a familiar one [39, 45, 46]. The NOR test was used to assess memory performance in this study because, compared to other tests like the Morris water maze or the Barnes maze, it is easier to perform and allows for a better distinction between cognitive and motivational domains, without depending on sensorimotor function and anxiety, which have been shown to be impaired in the 3xTg-AD mouse [39, 45, 46, 48]. Moreover, this test has been successfully used with several other AD mouse models [49]. The objects used here were comparable in size, texture, and shape complexity. During the conditioning phase, animals were placed individually in the cage (29.2 cm × 19 cm × 12.7 cm) containing two identical cylindrical objects (4 cm diameter × 6 cm high) for a period of 5 min. The test was repeated 1 h later with a familiar object and a new one, which was rectangular (4 cm × 4 cm × 6 cm). The time spent exploring and sniffing each object was recorded. After each trial, objects were cleaned and dried to minimize olfactory cues. The NOR index was determined as the time spent interacting with the novel object divided by the total time of exploration during the testing phase. Animals whose exploration was considered insufficient to allow recognition in the conditioning phase (<5 s per item) or whose NOR index was below 30% were excluded from analysis. Statistical analyses were performed by comparing the average of each group with the threshold of 50%, which corresponds to a mouse randomly visiting each object.

Samples preparation

Animals were placed under deep anesthesia with ketamine/xylazine and then, intracardiac blood samples were drawn. Mice were perfused via intracardiac perfusion with phosphate saline buffer (PBS 1X, 1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing a cocktail of protease inhibitors (SIGMA*FAST*^{TM} Protease Inhibitor Tablets, Sigma-Aldrich, St. Louis, MO) along with phosphatase inhibitors (1 mM sodium pyrophosphate and 50 mM sodium fluoride). Extracts of the parietotemporal cortex were dissected, snap frozen on dry ice, and kept at –80°C. Homogenates from cytosol fractions (TBS-soluble), membrane fractions (detergent-soluble), and detergent-insoluble fractions (formic acid-soluble) were prepared in buffers including protease and phosphatase inhibitors as already shown [35, 36, 50]. Briefly, tissue samples were homogenized in 8 volumes of TBS containing

a cocktail of protease inhibitors (Complete™, Roche Diagnostics, Indianapolis, IN, USA, and 10 μg/ml of pepstatin A) and phosphatase inhibitors [phosSTOP (Roche Diagnostics), 1 mM sodium orthovanadate and 50 mM sodium fluoride]. Samples were briefly sonicated (3 × 5 s) and centrifuged at 100,000× *g* for 20 min at 4°C to generate a TBS-soluble fraction (cytosol fraction). The TBS-insoluble pellets were sonicated (3 × 5 s) in 8 volumes of lysis buffer (150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, 1% Triton X-100, 0.5% SDS, and 0.5% deoxycholate) containing the same protease and phosphatase inhibitor cocktail. The resulting homogenates were centrifuged at 100,000× *g* for 20 min at 4°C to produce a lysis buffer-soluble fraction (detergent-soluble membrane fraction). Lastly, the pellet fraction was resuspended in 99% formic acid to form the insoluble fraction. Samples used for western blot analysis were resuspended in Laemmli 1X. Protein concentrations in TBS- and detergent-soluble fractions were determined using bicinchoninic acid assays (Pierce, Rockford, IL). Molecular mechanisms were investigated in the parieto-temporal cortex because the object recognition memory is highly dependent on cortical regions comprised within [51].

ELISA

Cortical soluble (TBS fraction) and detergent-insoluble (formic acid-soluble) $A\beta_{40}$ and $A\beta_{42}$, and detergent-insoluble total human tau were analyzed separately by ELISA, as described previously [5, 35, 36, 52]. The detergent-soluble protein fractions were not used for these experiments. We used high sensitivity ELISA kits from Wako (Osaka, Japan) to detect both $A\beta_{40}$ and $A\beta_{42}$ and from Life Technologies/Thermo Fisher Scientific (Carlsbad, CA) to detect total human tau in the brain of 3xTg-AD mice. ELISA was performed according to the manufacturer recommendations and the plates were read at 450 nm using a Synergy™ HT multi-detection microplate reader (Biotek, Winooski, VT).

Western blotting

Immunoblotting experiments were performed as described previously [5, 35, 36, 52]. Equal amounts of protein per sample (15 μg of total protein per lane) were added to Laemmli's loading buffer, heated to 95°C for 5 min before loading in a random order, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electroblotted onto PVDF membranes (Millipore, Billerica, MA) before blocking in 5% nonfat dry milk and 0.5% bovine serum albumin (BSA) in PBS-Tween 20 for 1 h. Membranes were immunoblotted with appropriate primary and secondary antibodies followed by chemiluminescence reagents (Lumiglo Reserve, KPL, Gaithersburg, MD). Band intensities were quantified using a KODAK Image Station 4000 MM Digital Imaging System (Molecular Imaging Software version 4.0.5f7, Carestream Health, Rochester, NY). Refer to the summary table of the different antibodies used in this study (Table 2).

Immunohistofluorescence

Hemi-brains were postfixed with 4% PFA (4°C for 48 hours), cryoprotected with 20% sucrose-PBS, snap frozen at –80°C, and microtome-sectioned into coronal (25 μm) sections. Washes in 0.1 M PBS, pH 7.4, were performed between each step of the immunohistofluorescence protocol. Free-floating brain sections from mice were blocked for 1 h in a PBS solution containing 5 % horse serum (Invitrogen, Carlsbad, CA) and 0.4% Triton X-100. Sections were then incubated overnight at 4°C with primary antibody in the blocking solution. For neurofibrillary tangle detection, mouse anti-tau antibody (clone tau13; Covance, Berkeley, CA, USA) was used as primary antibody, followed by detection with Alexa Fluor 488-labeled secondary donkey antibody (Life Technologies, Burlington, ON, Canada). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, Waltham, MA). Finally, slices were mounted on SuperFrost Plus slides (Thermo Fisher Scientific), treated with 0.5% Sudan black (in 70% methanol) for 5 min, and placed under coverslips with Mowiol mounting medium. Immunofluorescence images were acquired using an EVOS® FL Auto Cell Imaging System (Life Technologies/Thermo Fisher Scientific) with a 20X objective. Selected photographs were taken at Bregma –2.92 mm.

Characterization of Plasma Phenolic Metabolites

Phenolic metabolites were extracted from plasma and analyzed as previously described [53]. Briefly, Waters OASIS HLB μ elution plates 2 mg – 30 μm (Milford, MA, USA) were preconditioned using 250 μl of methanol and 250 μl of 0.2% acetic acid. Plasma samples (50 to 350 μl) were mixed with

Table 2
Summary table of antibodies used in this study

	IgG antibody	kDa	Immunogen	Host	Dilution	Source
Amyloid-β						
	6E10	110	a.a. N-terminus part of human Aβ peptide (1–17)	mouse	1/1000	Covance, CA
Amyloid-β mechanisms						
	ADAM10	84	a.a. 732–748 of human ADAM 10	rabbit	1/1000	Millipore, MA
	BACE	70	peptide corresponding to 17 a.a at the C-terminus of human BACE	rabbit	1/500	Thermo Scientific, IL
Tau						
	Tau 12	60	a.a. N-terminal of human Tau protein (2–23)	mouse	1/1000	Millipore, MA
	Tau 13	60	a.a N-terminal of human Tau protein (20–35)	mouse	1/5000	Covance, Berkeley, CA
	Tau C	60	a.a. C-terminal of total Tau protein (243–441)	rabbit	1/10000	Dako, Canada
	Tau AT270	60	peptide containing the phospho-T181 of human tau	mouse	1/5000	Thermo Scientific, IL
	Tau CP13	60	peptide containing the phospho-S202/T205 of human tau	mouse	1/5000	gift from Dr P. Davies
	Tau PHF1	60	peptide containing the phospho-S396/S404 of human tau	mouse	1/500	gift from Dr P. Davies
Synaptic proteins						
	Drebrin	120	a.a. C-terminal coupled to KLH (632–649)	mouse	1/500	Progen, Germany
	SNAP25	25	a.a. C-terminal of SNAP25 protein	mouse	1/20000	Covance, CA
	Synaptophysin	38	rat retina synaptophysin	mouse	1/20000	Millipore, MA
Inflammation						
	NFκB	70	a.a. C-terminal of human NFκB p65	rabbit	1/2000	Santa Cruz Bio., TX
Energy metabolism						
	SIRT1	80	His-tagged recombinant protein corresponding to human SIRT1	rabbit	1/1000	Millipore, MA
	AMPKα	62	a.a. C-terminal of human AMPKα	rabbit	1/1000	Cell Signaling, MA
	pAMPKα	62	peptide containing the phospho-Thr172 of human AMPKα	rabbit	1/1000	Cell Signaling, MA
Neurogenesis						
	BDNF	14, 32	a.a. 130–247 mapping at the C-terminus of BDNF of human origin	rabbit	1/200	Santa Cruz Bio., TX

4% phosphoric acid to disrupt phenol-protein binding, and loaded to the plates. The plates were then washed with 200 µl of ultrapure water and 200 µl of 0.2% acetic acid. The retained phenolic compounds were eluted with 75 µl of acetone/ultrapure water/acetic acid solution 70/29.5/0.5 v/v/v in presence of rosmarinic acid as internal standard (1 µg/ml final concentration), and then directly analyzed by ultra-high-pressure liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). Their separation was performed at 30°C using an Agilent Plus C18 column (2.1 mm × 100 mm, 1.8 µm) (Santa Clara, CA, USA). The mobile phase, consisting of 0.2% acetic acid in ultrapure water and acetonitrile (solvent A and B, respectively), was used with the following gradient elution: 0–8 min, 5–50% B; 8–9.10 min, 50–90% B; 9.10–10 min, 90% B; 10–10.10 min, 90–5% B; 10.10–13 min, 5% B, with a flow rate of 0.4 ml/min. The MS/MS analyses were carried out in negative mode using the following electrospray source parameters: electrospray capillary voltage: 3.01 kV, source temperature: 150°C, desolvation temperature: 400°C and cone and desolvation gas flows: 80 l/h and 800 l/h respectively. Cone voltage and collision energy parameters were optimized for each compound. The identification of metabolites was performed by comparing their retention times with those of available phenolic standards and/or analyzing their fragmentation. Data were acquired through the multiple reaction monitoring (MRM) mode, tracking the transition of parent product ion specific for each compound. The detected metabolites were quantified using standards when available. Otherwise, a relative quantitation was achieved using the calibration curve of their aglycone or most similar phenolic structure.

All chemicals analyzed (gallic acid, caffeic acid, vanillic acid, *p*-coumaric acid, 4-hydroxybenzoic acid, 4-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxyphenyacetic acid, resveratrol, catechin, and epicatechin) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Liquid chromatography grade solvents acetone, methanol, and acetonitrile were purchased from EMD Millipore Chemicals (Billerica, MA, USA). Glacial acetic acid and phosphoric acid were obtained from Anachemia (Montreal, QC, Canada). Ultrapure

water was obtained from a Millipore Milli-Q water purification system.

Statistical analyses

Data from all analyses were expressed as mean ± SEM. Statistical comparisons of data between groups were performed with Kruskal-Wallis ANOVA analysis followed by Dunn's multiple comparison tests when standard deviations were considered significantly different according to the Bartlett test. Otherwise, standard parametric ANOVA were used. Individual data were excluded for technical reasons or if determined as an outlier based on ROUT method using GraphPad Prism 6.0. Sex difference between male and female mice was statistically tested for each evaluated parameter with two-way ANOVA. Unless indicated otherwise, no sex difference was observed and data from males and females were pooled together. For the NOR test, the mean of each group was compared to the threshold of 50%, which represents an equal observation time between the old object and the new one. Coefficients of correlation and significance of the degree of linear relationship between parameters were determined with a simple regression model. The accepted level of significance was set at 0.05. Statistical analyses were done using Prism 6.0 or JMP Statistical Analysis Software (version 10.0).

RESULTS

The Neurophenols PEBG improved object recognition in 3xTg-AD mice without modifying anxiety level or motor activity

The memory of mice was evaluated using the NOR test (Fig. 2). The observation time ratio, or recognition index, was determined for each mouse and was compared with a value of 50% corresponding to the expected random selection of a mouse with equivalent interest in both objects. Younger control mice presented a recognition index of 62% ($p = 0.007$). In contrast, 15-month-old NonTg mice fed the non-supplemented diet did not interact with the new object more than the familiar one during the testing phase, as revealed by a recognition index of 53% ($p = 0.316$), highlighting a potential age effect. Next, NonTg mice fed Polyph1 or Polyph2 diets presented a recognition index significantly higher from the threshold of 50% (57%, $p = 0.012$ and 56%, $p = 0.035$, respectively). Interestingly, compared to 3xTg-AD mice under control diet, which presented a recognition index of 50%, dietary intake of PEBG led to higher recognition indexes (Polyph1 diet: 57%, $p = 0.032$; Polyph2 diet: 63%, $p < 0.001$) (Fig. 2A). Motor performance was not different between groups, regardless of diet, as revealed by the total duration of time mice spent on the rotarod device, with a tendency for 3xTg-AD to be more active than NonTg mice (Fig. 2B). These results were confirmed in the open field test where no difference of the total distance traveled by mice in one hour was observed between groups (Fig. 2C). Similarly, the dark emergence test did not reveal any effect of genotype or diet on anxiety levels (Fig. 2D), indicating that the effect of PEBG was specific to object recognition.

PEBG did not alter tau neuropathology in 3xTg-AD mice

First, we quantified total tau proteins (i.e., murine and human origin) by western immunoblotting in cytosolic fraction using a polyclonal antibody targeting both mouse and human tau C-terminus (Tau C, see Table 2). No difference was observed between genotypes and supplemented diets, regardless of the polyphenol doses (Fig. 3A). Similarly, no difference was observed for the phosphorylated forms of tau (clone CP13, tau phosphorylated at serine 202/threonine 205, see Table 2) in cytosolic fraction between groups (Fig. 3B). We confirmed these results with other specific phospho-tau antibodies (clones AT270 and PHF1; see Table 2 for phosphorylation sites and Table 4 for results). Similarly, PEBG did not significantly affect cytosolic levels (TBS-soluble) of total human tau in 3xTg-AD mice (Fig. 3C). Interestingly, despite a lack of significant difference, a strong downward trend of detergent-insoluble human tau (assessed with tau 12 antibody, see Table 2) was observed in 3xTg-AD mice fed the diet with the higher concentration of polyphenols (Fig. 4D, $p = 0.077$). Data obtained using clone tau 13 (see Table 4) were consistent with those gathered with tau 12 confirming the species-specificity of the polyphenols effect on human tau protein. Both monoclonal antibodies used here to detect human tau, clones tau 12 and tau 13, target epitopes in the N-terminal part of human tau protein [54, 55]. However, this tendency was not confirmed by ELISA analysis of human tau concentration in the formic acid fraction (Fig. 3E). Moreover, we investigated total human tau by immunofluorescence in the hippocampus of NonTg fed control diet and 3xTg-AD mice fed

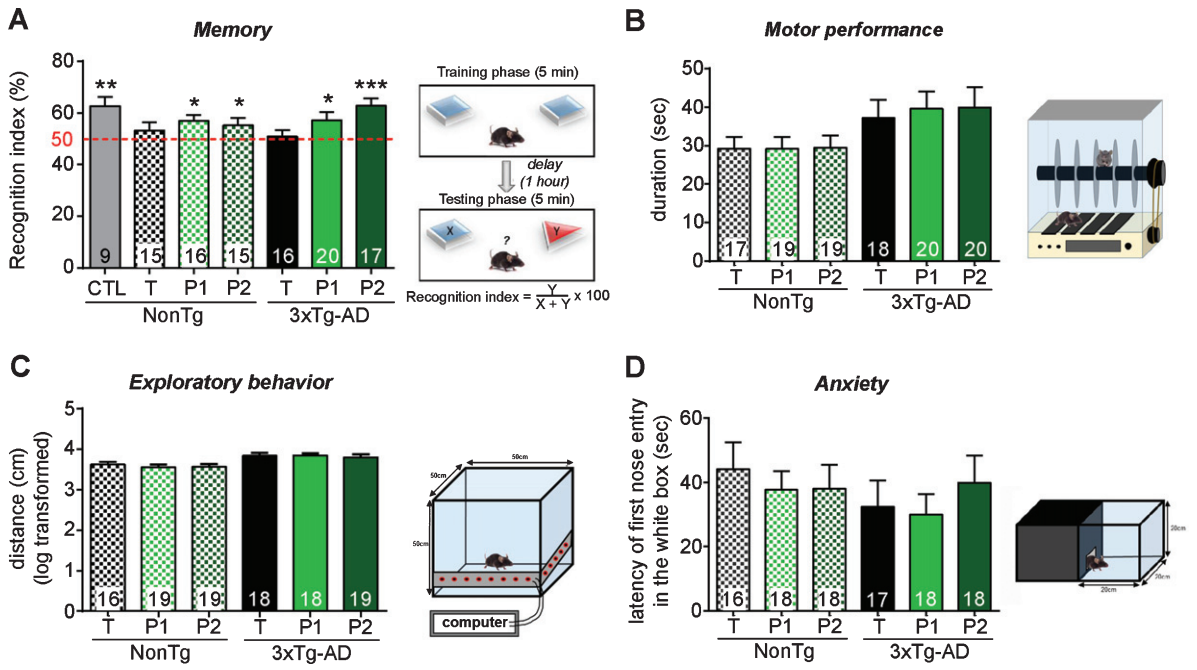

Fig. 2. PEBG improved memory performance in 3xTg-AD mice. A) Memory performance of mice was evaluated using the novel object recognition test. The observation time ratio (recognition index) was determined for each mouse by dividing the time exploring the new object by the total exploration time. B) Motor performance of each mouse was evaluated using a Rotarod device. C) Exploratory behavior, indexed by the total distance traveled, was monitored for each mouse with an open field device recording spontaneous movements during one hour. D) Anxiety level of each mouse was investigated with the dark emergence test. The latency of the first nose entry in the white compartment was used to evaluate anxiety level in each mouse. Values are expressed as means ± S.E.M. Sample size is indicated in the graph bars. Statistical analysis: A) one-sample Student's *t*-test statistical analysis was performed with a threshold of 50% which corresponds to a random observation of both objects ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$). B-D) Kruskal-Wallis ANOVA followed by Dunn's *post-hoc* tests were performed. No statistical difference was observed. 3xTg-AD, triple-transgenic mouse model of Alzheimer's disease; CTL, younger (4-month-old) control mice; NonTg, non-transgenic mouse; P1, Polyph1; P2, Polyph2; T, Tpolyph.

supplemented-diet or not. We first confirmed accumulation of human tau protein in hippocampus of 3xTg-AD compared to NonTg (Fig. 3F, G). However, none of the two diets with PEBG induced a significant decrease of total human tau in 3xTg-AD mice (Fig. 3G-I).

PEBG had no effect on cortical Aβ concentration in 3xTg-AD mice

We then investigated human soluble amyloid-β protein precursor (sAβPPα) and full-length human amyloid-β protein precursor (AβPP) levels (with clone 6E10, see Table 2) by western blot (Fig. 4A-C) and $Aβ_{42}$ and $Aβ_{40}$ levels by ELISA (Fig. 4D-I) in homogenates from the parieto-temporal cortex of 3xTg-AD animals. As previously reported, female 3xTg-AD mice displayed higher levels of human AβPP, soluble and insoluble $Aβ_{42}$ and $Aβ_{40}$ peptides than males, while no difference was observed on the ratio of TBS-soluble over detergent-soluble human AβPP and on the $Aβ_{42}/Aβ_{40}$ ratio in TBS- and formic acid-soluble fractions. Moreover, no effect of PEBG supplementation was detected on soluble and full-length AβPP levels (Fig. 4A-C) or on $Aβ_{42}$ and $Aβ_{40}$ accumulation in soluble (Fig. 4D and E, respectively) and insoluble (Fig. 4G and H, respectively) fractions, or in their respective ratios (Fig. 4F and I, respectively). Finally, PEBG, regardless of the concentration used, did not induce any change in levels of beta-secretase 1 (BACE) or of a disintegrin and metalloproteinase domain 10 (ADAM10) measured by western blotting, two proteins involved in amyloidogenic and non-amyloidogenic pathways, respectively (see Table 4 for results).

PEBG had no effect on synaptic, inflammatory, and energy metabolism proteins in the cortex of 3xTg-AD mice

Western blotting analyses revealed that PEBG supplementation did not induce any significant effect

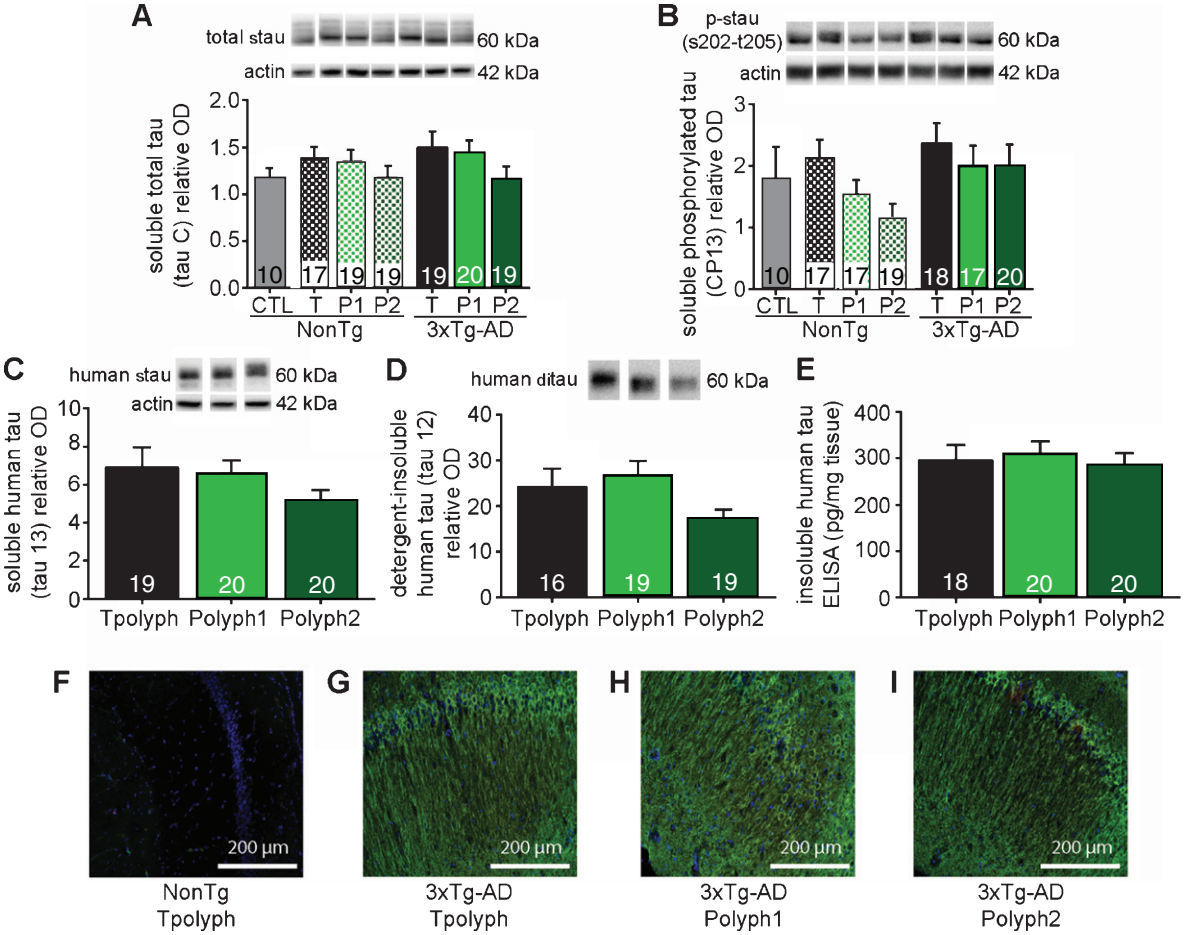

Fig. 3. PEBG did not alter tau neuropathology in 3xTg-AD mice. A-D) Tau protein levels were evaluated by western immunoblot in the parieto-temporal cortex of NonTg and 3xTg-AD mice fed with Tpolyph, Polyph1, or Polyph2 diets: (A) total tau (clone tau C), (B) a phosphorylated form of tau (CP13 antibody; s202/t205) and (C) human tau in the soluble fraction (clone tau 13); (D) human tau in the detergent-insoluble (clone tau 12). Examples of western blots are arranged in the same order as the graphs and were taken from the same immunoblot experiment. E) Aggregated human tau protein levels in the detergent-insoluble fraction were analyzed by ELISA in 3xTg-AD mice fed with diet Tpolyph, Polyph1, or Polyph2. Human tau (C-E) was measured in 3xTg-AD mice only because there is no expression of human tau in NonTg mice. F-I) Total human tau in the hippocampus was revealed by immunofluorescence in non-transgenic (NonTg) and triple-transgenic (3xTg-AD) mice at Bregma –2.92 (tau13 antibody, green; DAPI, blue; 20X). Values are expressed as means ± S.E.M. Sample size is indicated in the graph bars. No significant difference was observed. Two-way ANOVA with Bonferroni's *post-hoc* tests were performed for graphs A and B. Kruskal-Wallis ANOVA with Dunn's *post-hoc* tests were performed for graphs C to E. 3xTg-AD, triple-transgenic mouse model of Alzheimer's disease; CTL, younger (4-month-old) control mice; NonTg, non-transgenic mouse; OD, optical density; P1, Polyph1; P2, Polyph2; T, Tpolyph; stau, soluble tau; ditau, detergent-insoluble tau.

on drebrin levels in cytosolic and membrane fractions from the parieto-temporal cortex (Fig. 5A-C, respectively). Similarly, no treatment effect was observed on presynaptic proteins synaptophysin and synaptosomal-associated protein 25 (SNAP-25) (Fig. 5D and E, respectively). Moreover, levels of proteins involved in inflammatory pathway (nuclear factor kappa B) or energy metabolism (sirtuin-1) were not influenced by treatments (see Table 4).

PEBG restored mature BDNF/pro BDNF ratio in 3xTg-AD mice to NonTg level

PEBG induced an increase of the ratio of both forms of BDNF protein (mature/pro) in 3xTg-AD mice. The decrease of BDNF levels observed in 3xTg-AD mice compared to younger NonTg mice (Fig. 6; –45%, $p < 0.05$) was restored in PEBG treated-mice, at both doses (Fig. 6; +57% for

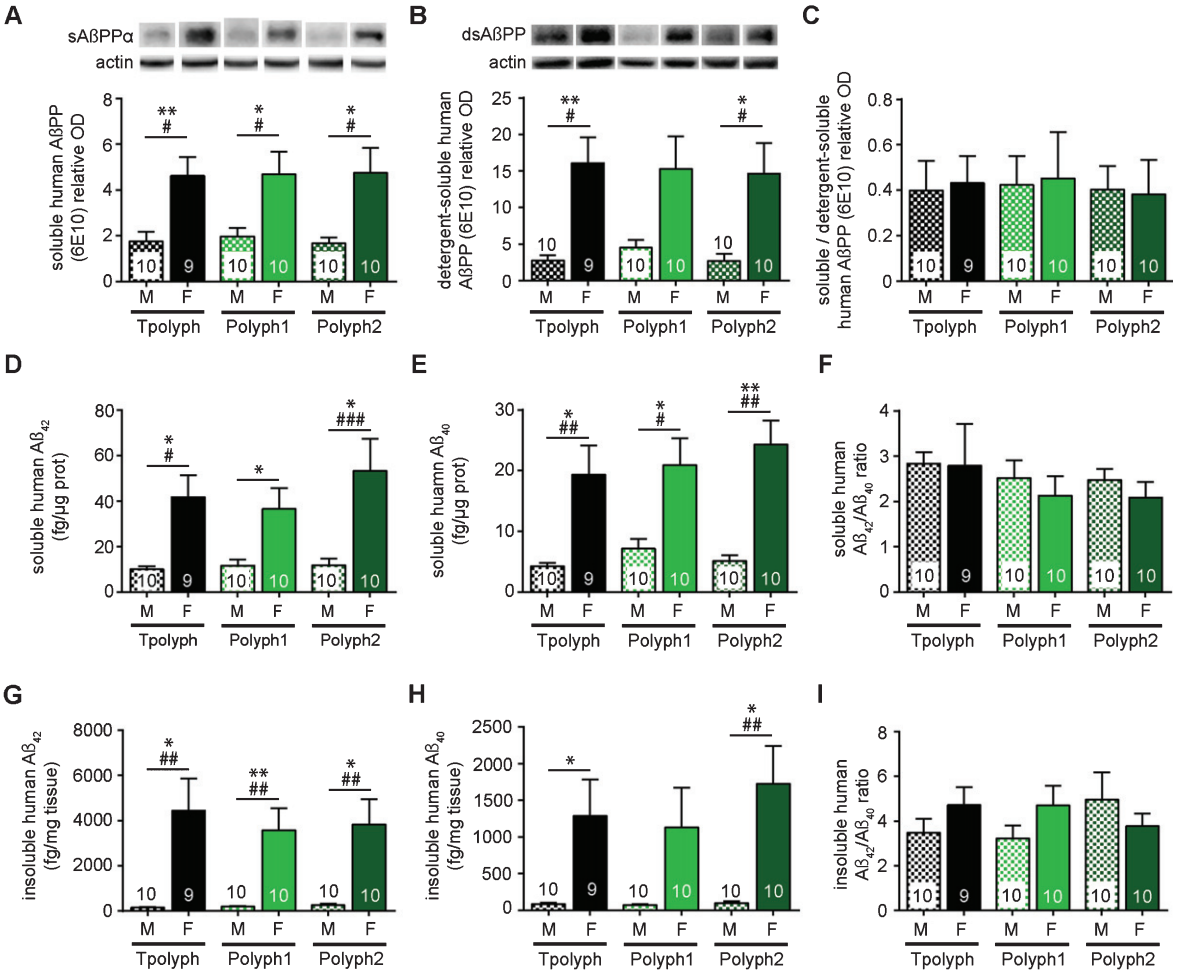

Fig. 4. PEBG had no effect on cortical Aβ concentrations in 3xTg-AD mice. A-C) Human soluble amyloid-β protein precursor (sAβPPα), full-length human amyloid-β protein precursor (AβPP) levels (clone 6E10), and the ratio of both were evaluated by western immunoblotting in 3xTg-AD mice fed with Tpolyph, Polyph1, or Polyph2 diets. Examples of western blots are arranged in the same order as the graphs and were taken from the same immunoblot experiment. D-I) Human $A\beta_{42}$ and $A\beta_{40}$ peptides levels were analyzed by ELISA in 3xTg-AD mice fed with Tpolyph, Polyph1, or Polyph2 diets. D-F) Analysis of $A\beta_{42}$ and $A\beta_{40}$ peptides and their ratio in the TBS-soluble fraction and G-I) formic acid-soluble fraction. sAβPPα, full-length AβPP, and Aβ were measured in 3xTg-AD mice only because there is no expression of human AβPP and human Aβ in NonTg mice. Values are expressed as means ± S.E.M. Sample size is indicated in the graph bars. No significant difference between mice of the same sex was observed, regardless of dietary treatment, using Kruskal-Wallis ANOVA with Dunn's *post-hoc* tests. Differences were observed between male and female mice fed the same diet using two-way ANOVA followed by Bonferroni *post hoc* tests ($^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$) and Student *t* test with Welch correction ($^{*}p<0.05$; $^{**}p<0.01$). dsAβPP, detergent-soluble human amyloid-β protein precursor; F, female 3xTg-AD mice; M, male 3xTg-AD mice; OD, optical density; P1, Polyph1 diet; P2, Polyph2 diet; sAβPPα, soluble human amyloid-β protein precursor; T, Tpolyph diet.

3xTg-AD_P1, $p=0.011$ and, +59% for 3xTg-AD_P2, $p=0.026$, compared to 3xTg-AD_T).

Plasma concentrations of phenolic metabolites correlated with cognitive performance

As revealed by UHPLC-MS/MS analysis, 18 phenolic metabolites were identified in the plasma, mainly intestinal and/or hepatic metabolites of catechin and resveratrol native compounds, as well as phenolic acids and microbial metabolites. Among them, 11 compounds were specifically found in the plasma of supplemented animals, with a dose-response effect (Table 3). Differences were observed between genotypes as catechins glucuronide, methyl catechins glucuronide, and 2-hydroxybenzoic acid were increased in the plasma of 3xTg-AD compared to NonTg mice on the same diet. On the other hand, hydroxyphenylvaleric acid was decreased in the plasma of 3xTg-AD compared to NonTg mice

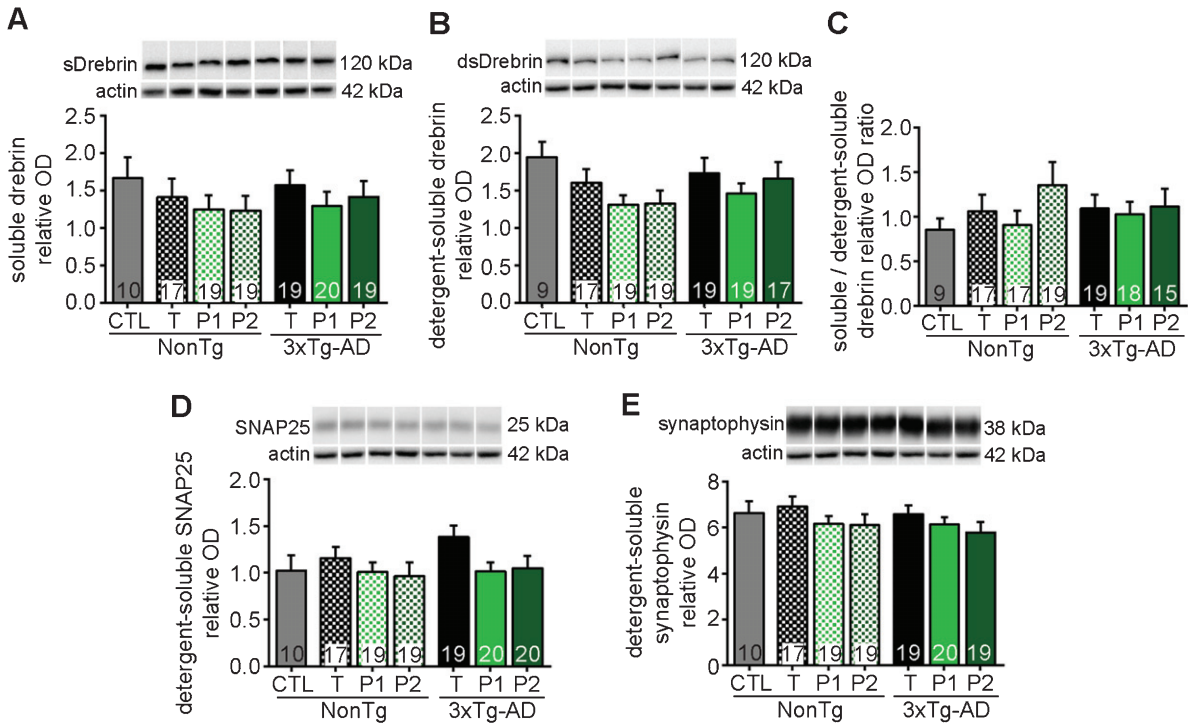

Fig. 5. PEBG had no effect on synaptic proteins in 3xTg-AD mice. A-E) Synaptic proteins levels were evaluated by western immunoblotting in 3xTg-AD mice fed with Tpolyph, Polyph1, or Polyph2 diet: cytosolic drebrin (A), detergent-soluble drebrin (B), the ratio of both fractions (C), membrane synaptophysin (D), and membrane SNAP-25 (E). Examples of western blots are arranged in the same order as the graphs and were taken from the same immunoblot experiment. Values are expressed as means ± S.E.M. Sample size is indicated in the graph bars. No significant difference was observed using either Kruskal-Wallis ANOVA with Dunn's *post-hoc* tests or two-way ANOVA with Bonferroni's *post-hoc* test. 3xTg-AD, triple-transgenic mouse model of Alzheimer's disease; CTL, younger (4-month-old) control mice; NonTg, non-transgenic mouse; OD, optical density; P1, Polyph1 diet; P2, Polyph2 diet; T, Tpolyph diet.

(Table 3). To probe the relationship between phenolic concentrations in plasma and cognitive performance, we performed linear regression analyses. Interestingly, levels of three important phenolic compounds found in plasma samples correlated positively with memory performance of mice evaluated in the NOR test. Indeed, regardless of genotype, three compounds displaying higher concentrations in the plasma of supplemented animals, and more specifically in the Polyph2 group, showed significant positive relationships with NOR performance of mice: 2-hydroxybenzoic acid (Fig. 7A, B; $r^2 = 0.2229$; $p = 0.0032$), hydroxyphenylpropionic acid (Fig. 7C, D; $r^2 = 0.1257$; $p = 0.0465$), and dihydroxyphenyl valerolactone (Fig. 7E, F; $r^2 = 0.1771$; $p = 0.0165$).

DISCUSSION

The results of this study are consistent with a potential beneficial effect of a 3-month treatment with PEBG on the memory performance of 15-month-old NonTg and 3xTg-AD mice. The PEBG-induced improvement of memory performance, as revealed in the NOR test, was not accompanied with changes in cerebral levels of main neuropathological markers of AD (i.e., Aβ, tau and synaptic proteins), or of proteins involved in inflammatory pathway or in energy metabolism regulation. However, enhanced memory performance after PEBG treatment coincided with the preservation of mature BDNF levels in the cortex and was correlated with key plasma phenolic metabolites.

Improvement of memory performance of mice after polyphenol treatment: Does the present data fit in the literature?

In the present study, we confirmed the benefit of a diet supplemented with PEBG on cognitive performance in old mice. The NOR test used here, which is based on the fact that rodents have a tendency to interact more with a novel object than with a familiar one, has become a benchmark task to study learning

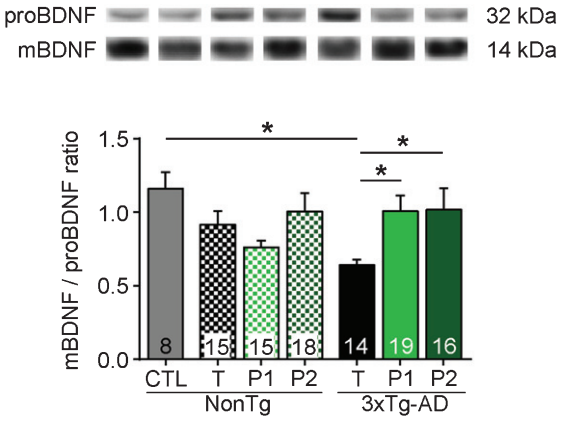

Fig. 6. PEBG prevented the decrease in mature BDNF observed in the parieto-temporal cortex of 16-month-old 3xTg-AD mice. BDNF protein levels were evaluated in the cytosolic fraction by western immunoblotting in NonTg and 3xTg-AD mice fed with Tpolyph, Polyph1, or Polyph2 diet. Since BDNF activity requires proteolytic conversion from proBDNF to mature BDNF (mBDNF), data are presented as mBDNF/proBDNF ratio. Examples of western blots are arranged in the same order as the graph and were taken from the same immunoblot experiment. Values are expressed as means ± S.E.M. Sample size is indicated in the graph bars. Kruskal-Wallis ANOVA followed by Dunn's *post-hoc* tests were performed. $^{*}p < 0.05$. 3xTg-AD, triple-transgenic mouse model of Alzheimer's disease; BDNF, brain-derived neurotrophic factor; CTL: younger (4-month-old) control mice; NonTg, non-transgenic mouse; P1, Polyph1 diet; P2, Polyph2 diet; T, Tpolyph diet.

and for assessing recognition memory [49, 51, 56]. Our data show that PEBG intake prevented the age-induced decrease in performance in the NOR test observed in both NonTg and 3xTg-AD mice fed the control diet. This effect was more prominent and dose-dependent in 3xTg-AD animals. The absence of differences between groups in exploratory and motor behavior indicates that the effect of PEBG was specific for object recognition.

Accordingly, phytochemical-rich foods, particularly those rich in flavonoids, have been shown to be effective in reversing age-related deficits in memory and learning in preclinical studies. Oral administration of quercetin, a naturally occurring flavonoid, significantly improved the behavioral performance of high-cholesterol-fed old mice in the step-through test and the Morris water maze task [57]. Dietary supplementation with a grape seed phenolic extract (GSPE) prevented both Aβ and tau neuropathologies and attenuated cognitive deterioration observed in transgenic mouse models of AD [31, 32]. Moderate consumption of red wines containing high contents of grape polyphenols also reduced Aβ neuropathology and improved cognitive performance in the Tg2576 mouse model [58, 59]. More recently, a treatment with olive oil polyphenols was shown to improve contextual memory of aged C57Bl/6J mice in the step-down test to levels similar to young animals [60].

The identification of which polyphenols display sufficient bioavailability will be instrumental in our understanding of their mechanisms of action. Whether polyphenol-induced memory improvement is exclusively mediated by direct central action or through other mechanisms such as stimulation of endothelial function and peripheral blood flow [61] remains to be determined. Nonetheless, previous studies indicate that key polyphenols, such as flavonoids, may cross the blood-brain barrier and reach therapeutically significant concentrations in cerebral tissue [62, 63]. Our plasma data confirm the presence of catechin and epicatechin in the circulation after dietary supplementation with the Neurophenols PEBG. Sulfate and glucuronide forms of catechin as well as gallic, caffeic, and hydroxyphenylvaleric acids were particularly increased in a dose-dependent fashion. Interestingly, the positive correlations observed here between plasma concentration of 2-hydroxybenzoic acid, hydroxyphenylpropionic acid, and dihydroxyphenyl valerolactone with the memory performance of mice in the NOR test are consistent with cognition enhancing properties. The present data combined with previous work should lead us to consider that polyphenols may exert their behavioral effects through bioactive metabolites.

We also observed that some phenolic metabolites, namely catechins glucuronide, methyl catechins glucuronide, and 2-hydroxybenzoic acid, were increased whereas hydroxyphenylvaleric acid was decreased in the plasma of 3xTg-AD compared to NonTg mice on the same diet. Despite the great variety of natural dietary polyphenols, the diversity of polyphenol metabolites resulting from a gut microflora-dependent metabolism is relatively small due to a convergent pattern of bioconversion [64]. Although we cannot rule out a genotype-dependent variation at this point, if this was the case, one could expect differences in a larger number of polyphenol metabolites according to genotype.

The main neuropathological markers of AD are not modified by polyphenolic extracts in 3xTg-AD mice

Given the memory improvement observed in 3xTg-AD, one would expect that PEGB would have

Table 3
Plasma concentrations of phenolic compounds in NonTg and 3xTg-AD mice fed diets supplemented with PEBG

Concentration (nM)	MRM transitions	NonTg			3xTg-AD			Statistical tests	Two-way ANOVA (*p*-values)		
		Tpolyph	Polyph 1	Polyph 2	Tpolyph	Polyph 1	Polyph 2		genotype	treatment	genot. x treat.
Gallic acid	169 > 125	n.d.	235.9 ± 127.6	171.8 ± 90.5	n.d.	4.3 ± 1.3	85.4 ± 27.1	K-W + Dunn's	n.s.	n.s.	n.s.
2-hydroxybenzoic acid	137 > 93	28.4 ± 8.1	34.4 ± 6.8	188.0 ± 62.9*	65.8 ± 22.4	69.9 ± 16.1	545.5 ± 123.6 $^{*\$\P}$	K-W + Dunn's	0.037	<0.0001	<0.0001
Caffeic acid	179 > 135	n.d.	21.4 ± 9.2	502.0 ± 188.4$^{\$}$	32.2 ± 10.2	103.0 ± 47.5	275.4 ± 94.9	K-W + Dunn's	n.s.	0.041	n.s.
Vanillic acid	167 > 152	14.4 ± 4.7	8.6 ± 3.0	48.4 ± 18.4	12.7 ± 2.8	7.8 ± 2.3	16.7 ± 5.0	K-W + Dunn's	n.s.	n.s.	n.s.
p-coumaric acid	163 > 119	60.4 ± 19.1	170.8 ± 50.9	215.8 ± 53.4	109.0 ± 27.3	94.3 ± 21.5	292.5 ± 70.9	K-W + Dunn's	n.s.	0.0173	n.s.
Hydroxyphenylvaleric acid	193 > 175	n.d.	124.4 ± 34.9	957.8 ± 263.7$^{\$}$	n.d.	118.3 ± 33.7	261.5 ± 80.8¶	K-W + Dunn's	n.s.	0.0006	<0.0001
Dihydroxyphenylacetic acid	167 > 123	19.1 ± 5.6	8.8 ± 2.5	31.8 ± 9.1	11.6 ± 2.9	15.6 ± 4.0	22.3 ± 7.2	K-W + Dunn's	n.s.	0.0447	n.s.
Hydroxyphenylpropionic acid	165 > 121	24.9 ± 7.9	342.6 ± 132.6	1610.9 ± 504.2	31.9 ± 10.8	190.6 ± 49.6	2338.4 ± 799.0*	K-W + Dunn's	n.s.	<0.0001	0.0023
2-hydroxyphenylacetic acid	151 > 107	73.6 ± 19.4	58.7 ± 11.6	90.8 ± 26.7	89.5 ± 17.8	116.7 ± 24.6	84.8 ± 17.1	K-W + Dunn's	n.s.	n.s.	n.s.
Dihydroxyphenylvalerolactone	207 > 163	n.d.	558.3 ± 255.7	2128.0 ± 694.6	n.d.	725.8 ± 129.8	3399.6 ± 756.4$^{\$}$	K-W + Dunn's	n.s.	<0.0001	<0.0001
Procyanidins Dimers B	577 > 289	n.d.	n.d.	127.7 ± 44.3	n.d.	n.d.	173.7 ± 93.5	*t*-test	n.s.	n.s.	n.s.
Methyl catechins glucuronide	479 > 303	n.d.	351.8 ± 103.3	4455.4 ± 1010.4$^{\$\$\$\$}$	n.d.	756.0 ± 157.5¶	3400.3 ± 603.8$^{\$}$	K-W + Dunn's	n.s.	<0.0001	<0.0001
Catechins glucuronide	465 > 289	n.d.	251.7 ± 98.9	4558.5 ± 1686.8$^{\$\$\$}$	n.d.	823.7 ± 159.1¶¶	4567.8 ± 950.1	K-W + Dunn's	n.s.	0.0002	0.0042
Methyl catechins sulfate	383 > 303	n.d.	89.5 ± 37.4	1437.7 ± 478.6$^{\$\$\$}$	n.d.	290.3 ± 69.1	1206.7 ± 216.4	K-W + Dunn's	n.s.	<0.0001	0.0015
Catechins glucuronide sulfate	545 > 289	n.d.	30.2 ± 12.0	589.2 ± 283.5	n.d.	26.5 ± 9.6	213.1 ± 74.2	K-W + Dunn's	n.s.	n.s.	n.s.
Catechins sulfate	369 > 289	n.d.	203.6 ± 94.7	2963.0 ± 938.9$^{\$\$\$}$	n.d.	408.3 ± 90.5	2012.6 ± 455.0$^{\$}$	K-W + Dunn's	n.s.	0.0002	0.0017
Dihydroresveratrol glucuronide	405 > 229	n.d.	11.9 ± 3.8	20.5 ± 7.8	n.d.	3.0 ± 1.3	12.9 ± 3.3	K-W + Dunn's	n.s.	n.s.	n.s.
Resveratrol sulfate	307 > 227	n.d.	7.1 ± 2.6	2.5 ± 0.9	n.d.	1.2 ± 0.3	15.5 ± 5.2$^{\$}$	K-W + Dunn's	n.s.	n.s.	n.s.

The word "catechins" refers to catechin and epicatechin compounds. 3xTg-AD, triple-transgenic mouse model of Alzheimer's disease; MRM, multiple reaction monitoring mode; n.d., non-detectable; n.s., non-significant; PEBG, polyphenolic extract from grapes and blueberries; K-W, Kruskal-Wallis. $N = 10$ / group. Values are expressed as means ± S.E.M. Statistical analysis: Kruskal-Wallis followed by a Dunn's *post hoc* test $^{**}p < 0.01$ and $^{***}p < 0.001$ versus control diet (same genotype), $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ and $^{\$\$\$\$}p < 0.0001$. Polyph 1 versus Polyph 2 diet (same genotype). Student *t*-test with the Welch correction $^{\P}p < 0.05$, $^{\P\P}p < 0.01$ versus non-transgenic mice (same diet).

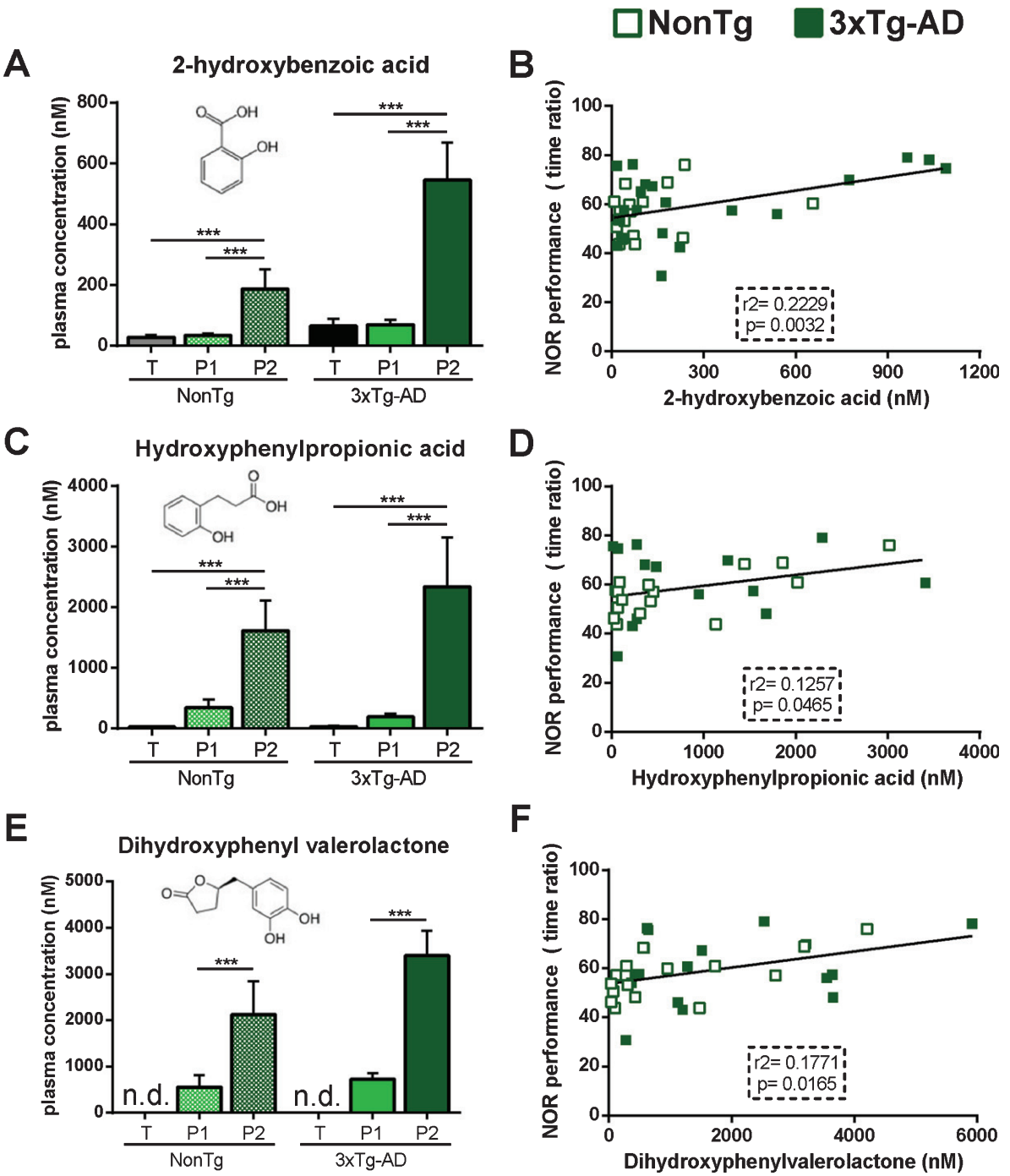

Fig. 7. Correlation between cognitive performance and concentrations of phenolic compounds in the plasma of treated mice. A, C, E) Plasmatic concentration of three phenolic compounds in the plasma of mice fed with Tpolyph, Polyph1, or Polyph2 diet: 2-hydroxybenzoic acid (A), hydroxyphenylpropionic acid (B), and dihydroxyphenyl valerolactone. B, D, F) Linear correlative analyses between the plasmatic levels of (B) 2-hydroxybenzoic acid, (D) hydroxyphenylpropionic acid, and (F) dihydroxyphenyl valerolactone and memory performances of mice evaluated in the novel object recognition (NOR) test. Values are expressed as means ± S.E.M ($n = 10$/group). Two-way ANOVA with Bonferroni's *post-hoc* test were performed in A, C, and E. $^{***}p < 0.001$. 3xTg-AD, triple-transgenic mouse model of Alzheimer's disease; n.d., not detectable; NonTg, non-transgenic mouse; P1, Polyph1 diet; P2, Polyph2 diet; T, Tpolyph diet.

improved tau or amyloid pathologies concomitantly. Indeed, a main advantage of the 3xTg-AD model is to recapitulate key features of AD, such as Aβ and tau neuropathology as well as cognitive decline with increasing age [35, 37, 38, 45]. Sex difference in Aβ levels between male and female 3xTg-AD mice observed in this study is in accordance with our previous work [36, 38] and with reports from other groups [65–67]. However, the PEBG used in our study did not induce any significant effect on main neuropathological markers of AD in supplemented 3xTg-AD mice. This contrasts with previous reports in which dietary supplementation with a GSPE, or moderate consumption of red wines containing high contents of grape polyphenols, attenuated the progression of Aβ neuropathology in the Tg2576 mouse model [31, 58, 59], and reduced tau aggregation in the brain of TMHT [32] or JNPL3 [68] mouse models of tau pathology. No effect on synaptic proteins was observed either, including drebrin, whose levels are decreased in AD [69, 70]. We previously reported that the drebrin loss in the brain cortex of 3xTg-AD was minimal compared to NonTg mice [35, 46], while no difference was observed in synaptophysin and SNAP-25 levels between NonTg and 3xTg-AD mice [35, 37, 46]. In contrast, a previous study using a specific phenolic compound, resveratrol, already showed that APP/PS1 mice, another AD mouse model, demonstrated a 2.2-fold increase in drebrin when fed resveratrol [71]. Whereas the reasons underlying these discrepancies are unclear, our data are consistent with the growing body of evidence indicating that overt modulation of Aβ, synaptic, and/or tau neuropathology is not a prerequisite for treatment-induced cognitive enhancement [72, 73]. Neuropathological markers of AD such as Aβ, particularly soluble species, are subjected to important physiological variations (i.e., clearance mechanisms [39, 74, 75] and the awake/sleep state [76]) and are not always directly associated with behavioral deficits [38]. A previous report suggests that functional modifications such as synaptic activity are more closely associated with behavioral expression of cognitive deficits [38]. Moreover, this lack of effect on AD markers is also consistent with the fact that the PEBG had beneficial effects on memory not only in 3xTg-AD mice but also in NonTg animals, suggesting that the Neurophenols consortium extract has a broader impact and might also be used in cases of age-related cognitive decline or cases without a definitive AD diagnosis.

Could polyphenolic extracts improve memory performance via BDNF-related pathways?

For more than two decades, BDNF has garnered significant attention as a potential therapeutic target for neurodegenerative diseases such as AD, but was not translated into clinical application due to its inability to cross the blood-brain barrier [77]. Indeed, BDNF-induced changes in neuronal spine density and morphology have been shown to dictate the efficiency of synaptic connections and, consequently, cognitive performance [78–80]. BDNF protein levels in the brain originate from the cleavage of inactive pro-BDNF into mature active BDNF by the tPA/plasmin enzyme system, which is expressed in synapses [81]. The reduced levels of active BDNF found here in 3xTg-AD mice compared to both young and old NonTg is in agreement with previous work in the same model [82], and suggest that Aβ or tau neuropathologies may progressively impair BDNF activity. Accordingly, abnormalities in BDNF activity have been reported in the brains of AD patients [83] and may represent a marker of cognitive deterioration [84]. The treatment with PEBG brought back active BDNF to levels comparable to non-transgenic animals on the control diet, and could thus underlie the observed improvement in object recognition in 3xTg-AD mice. Consistent with this hypothesis, intracerebral treatment with BNDF improved memory performance in 3xTg-AD [72] and in APP/PS1 mice [73], without altering Aβ or tau pathologies. Therefore, the PEBG-induced increase in mature active BDNF levels in the cortex of 3xTg-AD mice provides not only a potential mechanism of action of polyphenols, but also suggests that brain BDNF activity may be enhanced non-invasively using natural compounds in the diet.

This contention that polyphenols act on BDNF is fueled by a wealth of previously published evidence. Indeed, flavonoids can directly influence neuronal dendrite outgrowth *in vitro* [85–87] as well as BDNF levels and neuronal morphology [88]. An *in vivo* study has shown that spatial memory performance in 12-month-old Wistar rats supplemented with blueberries (mainly anthocyanins) correlates well with the activation of CREB and with the increase of BDNF in the hippocampus [89]. Similarly, a flavonol found in strawberries (named fisetin) has also been shown to improve long-term potentiation and to enhance object recognition in male C57BL/6 mice by a mechanism dependent on the activation of BDNF and CREB

Table 4
Western immunoblot quantification of proteins from the parieto-temporal cortex of NonTg and 3xTg-AD mice fed diets supplemented with PEBG

Proteins (relative O.D.)	Fraction	NonTg				3xTg-AD		
		Control 4 months $n=10$	Tpolyph $n=17$	Polyph1 $n=19$	Polyph2 $n=19$	Tpolyph $n=19$	Polyph1 $n=20$	Polyph2 $n=20$
Aβ pathways								
ADAM10	*detergent-soluble*	2.62 ± 0.70	2.56 ± 0.50	2.65 ± 0.27	2.99 ± 0.33	2.68 ± 0.43	3.36 ± 0.38	4.39 ± 0.78
BACE	*detergent-soluble*	1.22 ± 0.21	1.03 ± 0.13	1.29 ± 0.31	0.99 ± 0.13	1.16 ± 0.16	1.09 ± 0.20	1.01 ± 0.16
ADAM10 / BACE ratio	*detergent-soluble*	3.42 ± 1.34	3.92 ± 1.14	3.57 ± 0.78	4.73 ± 1.31	3.28 ± 0.67	6.55 ± 1.68	6.92 ± 1.41
Tau proteins								
ptau (AT270)	*soluble*	1.34 ± 0.33	1.52 ± 0.19	1.56 ± 0.20	1.31 ± 0.17	1.81 ± 0.27	1.96 ± 0.27	2.09 ± 0.23
ptau (PHF1)	*soluble*	1.24 ± 0.26	1.28 ± 0.12	1.21 ± 0.16	1.00 ± 0.13	1.15 ± 0.14	1.31 ± 0.15	1.49 ± 0.15
human tau (tau 13)	*detergent-insoluble*					37.36 ± 20.21	18.45 ± 4.88	12.35 ± 3.26
Inflammatory pathways								
NFkB	*soluble*	2.29 ± 0.36	2.57 ± 0.28	2.04 ± 0.22	1.91 ± 0.17	2.28 ± 0.30	1.91 ± 0.20	1.81 ± 0.16
NFkB	*detergent-soluble*	2.92 ± 0.96	3.60 ± 0.86	3.51 ± 0.84	4.42 ± 0.72	3.39 ± 0.73	4.52 ± 0.87	5.93 ± 1.31
NFkB ratio	*soluble/detergent-soluble*	4.25 ± 2.32	17.33 ± 14.88	3.65 ± 1.89	0.85 ± 0.27	12.74 ± 10.37	33.03 ± 32.34	0.74 ± 0.18
Energy metabolism								
SIRT1	*soluble*	0.53 ± 0.09	0.57 ± 0.09	0.34 ± 0.04	0.25 ± 0.04	0.55 ± 0.08	0.35 ± 0.06	0.43 ± 0.07
pAMPK	*detergent-soluble*	0.22 ± 0.09	0.43 ± 0.10	0.36 ± 0.10	0.38 ± 0.13	0.49 ± 0.12	0.32 ± 0.07	0.29 ± 0.09
AMPK	*detergent-soluble*	1.31 ± 0.20	1.13 ± 0.11	1.34 ± 0.12	1.10 ± 0.10	1.35 ± 0.12	1.38 ± 0.11	1.27 ± 0.20
pAMPK / AMPK ratio	*detergent-soluble*	0.19 ± 0.08	0.35 ± 0.07	0.26 ± 0.07	0.30 ± 0.10	0.34 ± 0.08	0.22 ± 0.05	0.19 ± 0.04
Loading controls								
actin	*soluble*	1.92 ± 0.37	1.97 ± 0.28	1.77 ± 0.26	1.89 ± 0.25	1.85 ± 0.29	1.87 ± 0.28	1.69 ± 0.22
actin	*detergent-soluble*	2.87 ± 0.27	3.03 ± 0.23	3.02 ± 0.24	2.80 ± 0.16	3.29 ± 0.28	3.16 ± 0.28	2.69 ± 0.21
GAPDH	*soluble*	4.47 ± 1.59	6.20 ± 1.65	4.93 ± 1.03	4.71 ± 0.91	5.81 ± 1.43	4.74 ± 0.97	5.23 ± 1.11

3xTg-AD, triple-transgenic mice model of Alzheimer's disease; ADAM10, A disintegrin and metalloproteinase domain 10; AMPK and pAMPK, adenosine monophosphate-activated protein kinase, phosphorylated or not; AT270, phosphorylated-tau (Thr181); BACE, beta-secretase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (loading control); NFκB, nuclear factor kappa B; NonTg, non-transgenic mice; PEBG, polyphenolic extract from grapes and blueberries; PHF1, phosphorylated-tau (Ser 396 and 404); SIRT1, NAD-dependent deacetylase sirtuin-1; tau13, total human tau (epitopes in the N-terminal part of human tau protein). Values represent relative optical density and are expressed as means ± S.E.M. Comparisons were first made between mice of the same genotype fed different diet and then, between mice under same diet but with different genotype. No statistical differences were observed.

[90]. More recently, a treatment with a 2% (w/w) blueberry diet resulted in significant improvement in spatial working memory that was associated with the activation of the ERK-CREB-BDNF pathway [62]. In addition, the effect of pure flavanol monomers (2)-epicatechin and (+)-catechin on spatial working memory in aged animals was paralleled by increased BDNF levels [91]. Altogether, previous results and ours are in accordance and strengthen the role of BDNF as a pivotal target pathway for the treatment of AD-related cognitive symptoms and emphasize a potential role of polyphenols as natural BDNF modulators in the central nervous system.

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