Food & Function

Stimulatory effects of (-)-epicatechin and its enantiomer (+)-epicatechin on mouse frontal cortex neurogenesis markers and short-term memory: Proof of concept

Journal:	*Food & Function*
Manuscript ID	FO-ART-11-2020-003084.R4
Article Type:	Paper
Date Submitted by the Author:	01-Mar-2021
Complete List of Authors:	Navarrete, Viridiana; Instituto Politécnico Nacional, Escuela Superior de Medicina Garate-Carrillo, Alejandra; University of California San Diego, ; Instituto Politecnico Nacional, Ayala, Marcos; Instituto Politécnico Nacional, Escuela Superior de Medicina; Instituto Politecnico Nacional Escuela Superior de Medicina, Rodriguez, Antonio ; Instituto Politécnico Nacional, Escuela Superior de Medicina Mendoza, Patricia; Universidad Juárez Autónoma de Tabasco Ceballos, Guillermo; Escuela Superior de Medicina,IPN, Ordoñez-Razo, Rosa; Hospital de Especialidades Centro Medico Nacional Siglo XXI Dugar, Sundeep; Cardero Therapeutics Schreiner, George; Cardero Therapeutics Villarreal, Francisco ; University of California, San Diego, USA, Department of Medicine Ramirez-Sanchez, Israel; Instituto Politecnico Nacional, Medicine

Stimulatory effects of (-)-epicatechin and its enantiomer (+)-epicatechin on mouse frontal cortex neurogenesis markers and short-term memory: Proof of concept

Viridiana Navarrete-Yañez[1], Alejandra Garate-Carrillo[1,2], Marcos Ayala[1], Antonio Rodriguez-Castañeda[1], Patricia Mendoza-Lorenzo[3], Guillermo Ceballos[1], Rosa Ordoñez-Razo[4], Sundeep Dugar[5], George Schreiner[5,] Francisco Villarreal[2,6], Israel Ramirez-Sanchez[1,*]

[1] Seccion de Estudios de Posgrado e Investigacion, Escuela Superior de Medicina, Instituto Politecnico Nacional, Mexico D.F. Mexico

[2] School of Medicine, University of California, San Diego, California U.S.A.

[3] Division Academica de Ciencias Basicas, Unidad Chontalpa, Universidad Juarez Autonoma de Tabasco. Tabasco, Mexico

[4] Laboratorio de Genetica Molecular, Hospital de Pediatria, Centro Medico Nacional Siglo XXI, Instituto Mexicano del Seguro Social, Mexico D.F. Mexico

[5] Epirium Bio, Inc., U.S.A.

[6] VA San Diego Health Care System, San Diego, California, USA

[*]**Address correspondence to:**
Israel Ramirez-Sanchez, Ph.D.
Seccion de Estudios de Posgrado e Investigacion
Escuela Superior de Medicina
Instituto Politecnico Nacional
Plan de San Luis y Diaz Miron s/n
Col. Casco de Santo Tomas
Del. Miguel Hidalgo, C.P. 11340,
Ciudad de Mexico, Mexico
israel.ramirez14@hotmail.com

Abstract

Consumption of (-)-epicatechin (Epi), a cacao flavanol improves cognition. The aim was to compare the effects of (-)-Epi or its stereoisomer (+)-Epi on mouse frontal cortex-dependent short-term working memory and modulators of neurogenesis. Three-month-old male mice (n=7/group) were provided by gavage either water (vehicle; Veh), (-)-Epi, at 1 mg/kg or (+)-Epi at 0.1 mg/kg of body weight for 15 days. After treatment, spontaneous alternation was evaluated by Y-maze. Brain frontal cortex was isolated for nitrate/nitrite measurements, Western blotting for nerve growth factor (NGF), microtubule associated protein 2 (MAP2), endothelial and neuronal nitric oxide synthase (eNOS and nNOS) and immunohistochemistry for neuronal specific protein (NeuN), doublecortin (DCX), capillary (CD31) and neurofilaments (NF200). Results demonstrate the stimulatory capacity of (-)-Epi and (+)-Epi on markers of neuronal proliferation as per increases in immunoreactive cells for NeuN (74 and 120% respectively), DCX (70 and 124%) as well as in NGF (34.4, 63.6%) and MAP2 (41.8, 63.8%). Capillary density yielded significant increases with (-)-Epi (~80%) vs. (+)-Epi (~160%). CD31 protein levels increased with (-)-Epi (~70%) and (+)-Epi (~140%). Effects correlated with nitrate/nitrite stimulation by (-)-Epi and (+)-Epi (110.2, 246.5%) and enhanced eNOS phosphorylation (Ser1177) with (-)-Epi and (+)-Epi (21.4, 41.2%) while nNOS phosphorylation only increased with (+)-Epi (18%). Neurofilament staining was increased in (-)-Epi by 135.6 and 84% with (+)-Epi. NF200 increased with (-)-Epi (116%) vs. (+)-Epi (84.5%). Frontal cortex-dependent short-term spatial working improved with (-)-Epi and (+)-Epi (15, 13%). In conclusion, results suggest that both enantiomers, but more effectively (+)-Epi, upregulate neurogenesis markers likely through stimulation of capillary formation and NO triggering, improvements in memory.

Key words: Flavanols, epicatechin, neurogenesis, nitric oxide, eNOS.

Introduction

During adulthood, neurogenesis is a limited process that mainly occurs in two regions of the healthy brain, the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) [1]. However, it has been reported that adult neurogenesis also occurs in areas such as striatum, amygdala, hypothalamus [2–4], neo and visual cortex [5,6] , evidencing that this cellular process is more widespread. *In vitro* and *in vivo* studies show that neural progenitor cells (NPCs) derived from frontal cortex of adult animals and humans can proliferate and differentiate to either neurons or glial cells depending on the external stimuli [7–10]. Endogenous cellular precursors in the adult brain, appear to be activated to produce neurons and partially repopulate brain regions as a compensatory mechanism [2].

Nitric oxide (NO) is recognized as a stimulator of neurogenesis *in vitro* [11]. In *in vivo* studies, brain endothelial cell-derived NO has demonstrated to promote neurogenesis [12] in both embryonic and adult animal models [13]. Flavonoids are recognized for their safety and beneficial effects. The flavanol (-)-epicatechin (Epi) has garnered interest since it has been widely demonstrated to improve vascular function through NO production via endothelial NO synthase (eNOS) activation [14–17]. Furthermore, (-)-Epi can traverse the blood–brain barrier, positively impacting brain function [18,19]. Using animal models of aging and chronic neurodegenerative diseases it has also been demonstrated that (-)-Epi exerts positive effects, as a neuroprotector and preventing neuronal death [20,21]. In a study performed in mice, (-)-Epi was able to improve memory, suppress neuro-inflammatory genes and stimulate neuroprotective counterparts [19]. Furthermore, a clinical trial implemented in normal older subjects using cocoa extracts, demonstrated improved memory and cognition associated with enhanced hippocampus function [22]. However, no studies have examined the effects of cacao flavanols on frontal cortical region dependent short-term memory.

In contrast to (-)-Epi, the enantiomer (+)-Epi, is found in very low amounts in cacao and fruits [23]. We reported on the pharmacokinetics of (+)-Epi and its safety in healthy and pre-diabetic subjects also noting positive effects on metabolism in mice [24]. The plasma half-life values estimated for (+)-Epi metabolites were higher than those for (-)-Epi which suggests that for potential therapeutic purposes (+)-Epi could be administered less frequently and possibly at lower doses. However, the influence that flavanols stereochemical configuration have on biological processes is unknown. Therefore, the objective of this study was to examine the effects of (-)-Epi and (+)-Epi on brain frontal cortex neurogenesis-related markers and on associated short-term working memory.

Materials and methods

Animals

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of " University of California, San Diego" , with the Code of Ethics of the World Medical Association (Declaration of Helsinki amended in 2000) the US Guidelines for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources (http://www.nal.usda.gov/awic/pubs/noawicpubs/careuse.htm), and approved by the Animal Ethics Committee of " UCSD Institutional Animal Care and Use Committee (IACUC)". Three-month-old male C57BL/6J wild type mice were housed and provided with water and regular chow *ad libitum*.

Compounds

(-)-Epi (≥90% purity) was obtained from Sigma-Aldrich Co. The compound was further purified and recrystallized in ethanol before use. (+)-Epi was a gift from Epirium Bio, Inc. The purity of both compounds (>95%) was corroborated using HPLC mass spectrometry.

Treatment

A total of 21 mice were randomly allocated into three groups (n=7 animals/group): 1) water-treated (Vehicle [Veh]), 2) (-)-Epi, and 3) (+)-Epi-treated. Solutions at a concentration of 1mg/ml of (-)-Epi and (+)-Epi were prepared in distilled water, aliquoted and frozen at -80^{o}C upon use. Animals were provided with either Veh, (-)-Epi at 1 mg/kg or (+)-Epi at 0.1 mg/kg body weight by oral gavage daily for 15 days. These doses, duration of treatment and age of mice were chosen based on our previous studies, which have demonstrated notable positive effects on mouse brain in the setting of disease [25] aging and adulthood [26] as well as for higher efficacy of (+)-Epi vs. (-)-Epi in mouse models of high fat diet induced obesity [24] and stimulation of mitochondrial biogenesis [27].

Brain sample collection and determination of epicatechin tissue levels

At the end of treatment, animals were sacrificed by decapitation after isoflurane anesthesia and brain tissue collected. Brain frontal cortex was isolated and frozen in liquid nitrogen for nitrate/nitrite measurements and Western blotting. For immunohistochemistry purposes, sections of frontal cortex were fixed during 24 h at 4°C using 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, then dehydrated with ethanol, cleared in xylene and embedded in paraffin blocks. Sagittal sections (5µm thickness) from comparable regions of the frontal cortex were obtained and mounted onto microscope slides.

Immunohistochemistry staining for NeuN, DCX and capillaries

Frontal cortex sections from all 3 groups were prepared as above and labeled using a rabbit anti mouse antibody against CD31 (Abcam ab119339) [28], rabbit anti DCX (Cell Signaling 4604S) and rabbit anti mouse against NeuN (Cell Signaling 24307), overnight (4°C) (dilution 1:1000) rinsed and incubated for 30 min with rabbit mouse peroxidase complex diluted 1:200 in 1% goat serum in PBS. Color development was performed by treating the slides with 0.05% diaminobenzidine hydrochloride (DAB) and 0.02% H_2O_2 in PBS for 8 min. Slides were counterstained with hematoxylin and dehydrated in alcohol at ascending concentrations, cleared in xylene and glass cover slips were mounted with Permaslip [29]. Negative control slides were prepared by substitution of the primary antibody with mouse IgG. Slides were analyzed by light microscopy at 40X magnification (Zeiss, Jena, Germany) and digital images of the sections were obtained. NeuN and DCX positive cells as well as capillaries were quantified by blinded operators using 12 randomly chosen digital images of the same size derived from 6 out of 30 nonconsecutive sagittal sections on a broad range of 150 µm of each frontal cortex.

Immunofluorescence

Frontal cortex sections were deparaffined using two absolute xylene washes and re-hydrated with decreasing dilutions (100-50%) of ethanol followed by washing with water. Sections were processed using the heat-induced antigen retrieval for 15 min (Declere, Biosciences). Tissue sections were treated for 15 min with freshly prepared 3% H_2O_2, (to inhibit endogenous peroxidase activity) and washed with water. Tissue sections were blocked with 10% goat serum and 0.01% triton X-100 in PBS for 30 min and then incubated overnight (4°C) with mouse anti neurofilament (NF-200, Sigma N4142) primary antibodies (dilution 1:1000). Slides were washed and incubated 1 h with AlexaFluor 546-conjugated goat anti-mouse IgG secondary antibody (dilution 1:400). Sections were mounted with the nucleus-specific 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), cover-slipped and dried at 4°C overnight in the dark, then analyzed by fluorescence microscopy. Positive staining quantification was performed using images taken at 40X with a Nikon digital camera DMX1200 mounted on an epifluorescence microscope (Nikon Eclipse E600) and analyzed using Nikon's ACT-1 imaging software. Image analysis was performed using 12 randomly chosen digital images from 6 nonconsecutive sections of frontal cortex taken from each animal (n=7 animal/group) and examined by blinded operators.

Nitrate/nitrite *measurements*

Nitric oxide (NO) products (i.e. nitrate and nitrite anions) were measured using a fluorescent nitrate/nitrite kit (Cayman Chemical 780051) as per manufacturer instructions. Briefly, 10 mg of frontal cortex was homogenized on dry ice in 0.1 ml of cold PBS (pH: 7.4) and centrifuged (10,000g) for 20 min at 4°C. Supernatant was recovered to measure total protein content (Bradford Assay Bio-Rad) then centrifuged (100,000 g) 30 min at 4°C. Supernatant was recovered and ultrafiltered through 10 kDa molecular weight cut-off (Amicon ultra-0.5 10K Millipore) by centrifugation (10,000g) 15 min at 4°C. Total NO was measured in eluted

fraction in 96-well plate using a fluorometer (FLx800 Bio-Tek instruments) as per specifications.

Western blotting

Approximately 10 mg of brain frontal cortex was homogenized with a polytron in 150 µl of lysis buffer (1% Triton X-100, 20 mM Tris, 140 mM NaCl, 2 mM EDTA, 0.1% SDS) with protease and phosphatase inhibitor cocktails (P2714 and P2850; Sigma-Aldrich), supplemented with 0.15 mM Phenylmethanesulfonyl fluoride (PMSF), 5 mM Na_3VO_4, and 3 mM NaF. Homogenates were sonicated for 15 min at 4°C and centrifuged at 12,000 g for 10 min. Total protein content was measured in the supernatant using the Bradford method. A total of 40 µg of protein was loaded onto a 4–15% precast polyacrylamide gel (Bio-Rad) and electrotransferred to a polyvinylidene difluoride membrane using a semidry system. Membranes were incubated for 1 h in blocking solution (5% nonfat dry milk in NaCl/Tris plus 0.1% Tween-20) and then overnight at 4°C with primary antibodies. To examine capillary and NO related proteins, antibodies against CD31 (Abcam 119339), endothelial nitric oxide synthase (eNOS) (ab76198 from Abcam, Cambridge, MA, USA), p-ser1177-eNOS (9570 from Cell Signaling), neuronal nitric oxide synthase (nNOS) (4236S from Cell Signaling, Danvers, MA, USA), and p-ser1417-nNOS 07-544 from Upstate were used [28]. To examine neurogenesis and neurofilament related proteins, antibodies against nerve growth factor (NGF), (sc-32300 Santa Cruz Biotechnologies, Santa Cruz, CA, USA) Microtubule Associated Protein-2 (MAP2 4542 from Cell Signaling) and Neurofilament (NF200 N4142 from Sigma-Aldrich) were used. GAPDH (Cell Signaling rabbit 5174 or mouse 97166) were used as a loading control. All primary antibodies were diluted 1:1000–2000 in blocking solution. After primary antibody incubation, membranes were washed (3X5 min) in NaCl/Tris plus 0.1% Tween-20 and incubated for 1 h at room temperature with specific

horseradish peroxidase-conjugated secondary antibodies. Membranes were washed 3X in NaCl/Tris plus 0.1% Tween-20 and the immunoblots developed using an ECL Plus detection kit (Amersham-GE, Pittsburgh, PA, USA). Band intensities were digitally quantified with IMAGEJ (http://www.nih.gov).

Y-maze working memory test

Spontaneous alternation was used as a measure of spatial working short-term memory which reflects frontal cortex function [30–33]. Two days prior the test, animals were habituated for handling in the area. At the day of the test, animals were placed in the evaluation room for 1 h. Mice were then placed at the end of the starting arm of the Y-maze and allowed to move freely through the maze for 10 min. The total number of arm entries (locomotor activity) and alternation behavior were video recorded. The percentage of alternation was calculated as (total of spontaneous alternation/total arm entries – 2) X 100.

Statistical analysis

All results are presented as mean ± standard error of the mean (SEM) and analyzed by one-way ANOVA using multiple comparison of each group means and for each variable. Post hoc Tukey's test was used to determine differences between means. p values <0.05 were considered statistically significant.

Results

Markers of neurogenesis

To evaluate the effects of (-)-Epi and (+)-Epi (Fig. 1A-B) on neuronal differentiation and maturation in brain frontal cortex, we analyzed recognized markers of such processes (NeuN, DCX, NGF and MAP2) by immunohistochemistry and Western blots. Stained sections using the NeuN and DCX antibodies are shown in Fig. 2A and 2B respectively. The number of immunolabeled cells/area was quantified (Fig. 2C) in 12 randomly chosen images from 6 nonconsecutive sections of frontal cortex from each animal (n=7/group). Representative images from Westerns for NGF and MAP2 are shown in Fig. 2D. Changes in NGF and MAP-2 protein levels were normalized by GAPDH and represented as graphs in right lower panels. A significant increase in NeuN and CDX immunoreactive cells as well as an increase in NGF and MAP-2 protein levels was observed with (-)-Epi and (+)-Epi treatments vs. Veh. A significant difference in both NeuN and NGF was also observed between the (+)-Epi vs. (-)-Epi groups (Fig. 2A, C and Fig. 2D). In preliminary experiments, we detected (-)-Epi or (+)-Epi in mouse brains after being subcutaneously applied (data not shown) thus, we believe that the effects reported here are secondary to a local action of the enantiomers.

Capillarity density

It is well known that neurogenesis in particular of the adult brain, appears to require angiogenesis [34]. We previously reported on the capacity of (-)-Epi to induce heart and skeletal muscle angiogenesis in mice [28,35]. Thus, we analyzed the effect of (-)-Epi and (+)-Epi on capillary formation in the brain frontal cortex by evaluating changes in CD31 expression. As shown in figure 3A and 3B (immunostaining and Western blot respectively), (-)-Epi and (+)-Epi were able to induce the expression of CD31. Image analyses yield a statistically significant increase in CD31 by immunostaining and Western with (-)-Epi and (+)-Epi as

compared vs. Veh (Fig. 3A and 3B; lower panel). Higher levels were observed with (+)-Epi vs. (-)-Epi.

Effects of treatment on NOS/ nitrate/nitrite/*NO pathway in brain frontal cortex*

An upstream signaling pathway involved in angiogenesis is eNOS activation [28]. We and others have demonstrated the capacity of (-)-Epi to induce nitrate/nitrite production via eNOS [17,28]. In this study, we analyzed the effect of (-)-Epi and (+)-Epi on nitrate/nitrite production as well as nNOS and eNOS protein levels and in their activation status (i.e. phosphorylation levels). As demonstrated in figure 4A, both flavanols were capable of inducing nitrate/nitrite synthesis via eNOS activation/phosphorylation (Fig. 4B left lower panel) while not affecting their protein levels (Fig. 4B right lower panel). Modest nNOS activation was observed only with (+)-Epi (Fig. 4C left lower panel) in the absence of changes in total protein (Fig. 4C right lower panel). Interestingly, (+)-Epi was also able to induce a significant 60% greater nitrate/nitrite level vs. (-)-Epi. Correlation analysis (Fig. 4D) were implemented in order to potentially identify the relative role of p-eNOS vs. p-nNOS in the production of nitrite/nitrate induced either by (-)-Epi (Fig. 4D left) or (+)-Epi (Fig. 4D right).

Neurofilament markers

As evidence for neural maturation, we evaluated the effects of treatment on the expression of NF200 a recognized marker of neurofilaments. In figure 5A (upper panel), immunostaining results and graphical representation of their quantification are shown. NF200 was also analyzed by Westerns (Fig. 5B upper and lower panels). Significant increases of neurofilament staining with (-)-Epi and (+)-Epi treatment was observed. (-)-Epi treatment also demonstrated greater quantities of immunoreactive cells and protein levels vs. (+)-Epi treatment.

Y-maze working memory test

Spontaneous alternation as a measure of frontal cortex-dependent short-term working memory was evaluated in Y-maze (Fig. 6A). (-)-Epi and (+)-Epi demonstrated increased spontaneous alternation (15% and 13% respectively) vs. Veh (Fig. 6A), while total number of arm entries was not significantly different across any of the groups (Fig. 6B). There was no correlation between spontaneous alternation rate and the number of total arm entries between groups as demonstrated by Pearson's analysis of correlation (Fig. 6C), indicating that locomotor activity did not impact the quantification of spontaneous alternation.

Discussion

Results demonstrate that in the brain frontal cortex of adult mice, (-)-Epi and its enantiomer (+)-Epi upregulate the expression of important neuronal development factors, the neuronal specific nuclear protein NeuN, doublecortin (DCX), NGF and MAP2. We also report on concurrent increases in the endothelial protein marker CD31, as well as in eNOS/nitrate/nitrite/activation/synthesis. Furthermore, our results demonstrate the overexpression of the neurofilament NF200, a hallmark molecule associated with neuronal axon maturation. These improvements appear to translate into enhanced frontal cortex-dependent short-term working memory. We also confirm our previous observations as to the greater relative potency of (+)-Epi vs. (-)-Epi [24].

Until recently it was believed that in mammals neurogenesis occurred (within limited periods) during development and quickly arrested after birth [13], since the number of proliferating progenitors and young neurons in the dentate gyrus declines sharply during the first year of life and only a few young neurons are observed in childhood [36]. Studies on cortical adult neurogenesis have reported interesting findings. Brain cortical adult neurogenesis is likely part of the neuronal network essential for relational memory organization [37]. In the healthy adult brain, cortical neurogenesis can apparently generate neural progenitor cells (NPC) and neuronal stem cells (NSC) which can migrate to repopulate SVZ and white matter [38]. Several reports also indicate that adult neurogenesis in frontal cortex is upregulated after brain injury (i.e. ischemia, epilepsy, neural degeneration or traumatic lesions), suggesting that cortical adult neurogenesis has possible key functions in brain injury. Thus, enhanced neurogenesis in the cerebral cortex may have neuroprotective[39].

Furthermore, it has been recently demonstrated that adult neurogenesis can be activated in selected areas of the adult nervous system in response to a variety of stimuli such as environmental, physical activity [40], neuroendocrine, neurochemical [41] and with brain injury [42,43]. Nutritional components including dietary flavonoids, can also act as neurogenic factors [22]. Flavonoids, contained in cacao have been shown to increase adult hippocampal neurogenesis in chronically stressed rats, an effect that is mediated by nerve growth factors [44].

Adult neurogenesis is known to be influenced by NGF, a member of the nerve growth factor/neurotrophin family [45]. NGF is necessary for neuronal survival, proliferation, facilitates morphological differentiation (outgrowth process by stimulation of cellular metabolism) and can as well, improve function in several areas of the brain [46]. NGF also increases the expression of neurofilament subunits in PC12 cells [47].

Neuronal microtube associated protein doublecortin DCX has emerged as an important marker of neurogenesis in adulthood [48]. The nuclear antigen NeuN has been widely used as a marker for neurons in a variety of species including humans whereas MAP2, a neuron-specific cytoskeletal protein, has been used as a marker for a "growing" neuronal phenotype [49]. We demonstrate that (-)-Epi and (+)-Epi treatment can stimulate NGF in the frontal cortex simultaneously with the upregulation of the NeuN and DCX suggesting that the flavanols may effectively stimulate the neurogenic processes. These results are consistent with those of Valente et al., 2009 where they demonstrated that a polyphenol and polyunsaturated fatty acids rich diet can induce neurogenesis in the SVZ and SGZ of adult mice and that the same diet protects cortical and hippocampal neurons against oxidative stress [50].

Using an *in vivo* model of diabetes Al-Gayyar et al., 2011, examined the neuroprotective effects of (-)-Epi. Treatment of diabetic animals with (-)-Epi (100 mg/kg/day, orally for 3

weeks) induced neuronal survival rather than neurogenesis [20]. This effect appeared related mainly to a neuroprotective action of (-)-Epi. The effects noted in diabetic rats vs. our results may be related with the (-)-Epi dose administrated to the animals and their disease status. Whereas we treated normal mice with a lower dose of (-)-Epi (1 mg/kg/day) and for a shorter period of time (15 days), Al-Gayyar et al., used higher doses (100 mg/kg/day) in diabetic rats for a longer period of time (21 days). It is also possible that the use of high (-)-Epi doses may limit neuronal proliferative responses. Further studies would be needed to unmask the precise dose/response effects of (-)-Epi on neurogenesis.

Adult human neurogenesis and angiogenesis are regulated in parallel [51]. Exercise enhances cerebral blood volume, resulting in more adult neurogenesis in mice and better cognitive performance in humans [52]. In the adult central nervous system, the vasculature of several neurogenic niches can regulate neuron development (i.e. proliferation and differentiation) by providing circulating nutrients and endothelium-secreted factors (i.e. NGF and NO) [53]. Angiogenesis is also directly related with neurogenesis since blood supply is necessary for new neuronal survival and development [54]. There is evidence suggesting that (-)-Epi can increase hippocampal angiogenesis in young mice [19] possibly through regulation of NOS as reported with other polyphenols [55]. This response has been linked to neurogenic signaling activation of serotonin receptors and to increases in factors such as NGF [41,56]. The capacity of (-)-Epi to induce vascular NO/nitrites/nitrates production is well established [17,28]. Interestingly, there is a link between endothelial cell derived NO and the ensuing processes of angiogenesis and neurogenesis [57–60] highlighting the role that the NO/angiogenesis pathway may play as a required process for neuronal development in the adult brain [11]. We previously reported the effects of (-)-Epi treatment on mouse skeletal muscle [35] and cardiac angiogenesis [28], which was associated with the activation of the VEGF/eNOS/NO pathway.

Substantial experimental evidence supports the participation of NO in neurogenesis [61,62]. However, its specific role remains unclear. Nonetheless, data presented here indicates that (-)-Epi and (+)-Epi treatment stimulates NO/nitrite/nitrate production in the frontal cortex concomitantly with increases in indicators of angiogenesis and neurogenesis. However, correlation analysis failed to demonstrate a relationship between (-)-Epi or (+)-Epi treatment with nitrite/nitrate production and the phosphorylation of eNOS or nNOS suggesting that the angiogenesis and neurogenesis processes are likely complex.

It has been demonstrated that nNOS expression increases in several neuronal structures during embryonic neurogenesis and decreases its expression during adulthood [13]. This is consistent with findings of this study in which we observed an apparent low basal level of nNOS as compared with eNOS in the frontal cortex. Interestingly, treatment with (+)-Epi significantly stimulated nNOS phosphorylation levels, which may be related to its overall greater capacity to stimulate NO. However, there is limited information regarding the manner by which such stimulation occurs. We have published evidence for the activation of cell membrane receptors (GPER/GPER1/GPER30), which are associated with pathways known to be linked with the stimulation of NOS [63]. We can speculate that such receptors may be present in brain endothelial cells and/or possibly in neurons triggering NOS responses. It is also worth noting that even though nitrate/nitrite levels were increased by (-)-Epi and (+)-Epi, it is likely not due to the activation of the pro-inflammatory isoform of NOS (inducible NOS [iNOS]). iNOS and eNOS often have opposite actions in promoting cell survival. For instance, if increases of nitrate/nitrite levels would have been due to iNOS activation, the concentration (Fig. 4A) would have likely increased to micromolar range. Consequently, we would have observed the opposite effect on markers of neurogenesis. According to Carreira et al., 2015, when iNOS is activated after a brain injury it promotes an inflammatory milleu

where new neurons formed at an earlier stage decreased due to the very large increase in NO. In fact, high NO levels can promote the maintenance of neuroinflammation which can provide an injurious environment for newborn cells, which ultimately fail to survive [64].

Findings from this study also provide evidence for increases in neurofilaments (as evidenced by NF200 staining along the axonal body) a structure whose development is associated with late stage neuronal differentiation and maturation [47,65] leading to the establishment of synapses. Results indicate that (-)-Epi and (+)-Epi treatment can significantly increase the expression of NF200, indicating that flavanol treatment may also support neuronal differentiation and maturation. Interestingly, a relationship between NO and neuronal proliferation/differentiation, appears to develop in a dose dependent manner. Large amounts of nNOS-derived nitrate/nitrite/NO can induce neuronal proliferation while modest nitrate/nitrite/NO amounts (mainly eNOS-derived) appear to be both proliferative and differentiating [11]. Results shown in this study suggest an apparent fine stereochemical modulation by the flavanols since (-)-Epi induced a 25% lower eNOS activation, 40% lower nitrate/nitrite, less NGF and NeuN levels but greater NF200 levels (i.e. differentiation).

Past research has revelated that the consumption of flavonoids improves cognitive capabilities through cellular processes such as enhanced of capillary/vascular flow, inhibition of cholinesterases, modulation of free radicals and signaling pathways implicated in cognitive and neuroprotective functions (i.e. ERK and PI3K/AKT) [66]. Flavonoid effects on cognitive performance may also be secondary to the stimulation of neurogenesis as suggested by our results. Increases in Y-maze spontaneous alternation in (-)-Epi and (+)-Epi mice demonstrate improved frontal cortex working memory function. Since the total number of arm entries was similar between groups, we eliminated the confounding influences of locomotor hyperactivity. Studies have demonstrated that frontal cortex memory contributes

importantly to the organization and integration of information that supports episodic memory [67] and its capability to use information from past events.

Conclusion

In conclusion, we provide provocative evidence for the stimulatory effects of (-)-Epi and (+)-Epi on adult mouse frontal cortex neurogenesis, a phenomenon that may be dependent on nitrate/nitrite production. Results support the idea that (+)-Epi and (-)-Epi positively impact brain function as spatial working memory is improved. These results set the stage for the future testing of the flavanols in the setting of normal and impaired brain function. Furthermore, both compounds have been tested in humans with no apparent adverse effects [24] and thus, may be considered in the implementation of long-term clinical trials to evaluate their effects on cognitive function.

Author contributions

I. R. S. designed and implemented studies, analyzed data, wrote the manuscript and prepared figures, V. N-Y., A. G-C., M. A., A. R-C., P. M.-L., implemented studies, analyzed data and prepared manuscript, G. C. and F. V. conceived the study, designed protocols, analyzed data, prepared figures and wrote manuscript. All authors reviewed the manuscript.

Conflicts of Interest

Dr. Villarreal (co-founder) and Dr. Ceballos are stockholders of Epirium Bio, Inc. The other authors report no conflict of interests.

Funding:

This study was partly supported by the Instituto Politecnico Nacional grant 20195888 and CONACyT 283938

References

1. Lledo PM, Alonso M, Grubb MS. Adult neurogenesis and functional plasticity in neuronal circuits. Nat Rev Neurosci. 2006;7(3):179–93.

2. Mitchell BD, Emsley JG, Magavi SSP, Arlotta P, Macklis JD. Constitutive and induced neurogenesis in the adult mammalian brain: Manipulation of endogenous precursors toward CNS repair. Dev Neurosci. 2004;26(2–4):101–17.

3. Gould E. How widespread is adult neurogenesis in mammals? Nat Rev Neurosci. 2007;8(6):481–8.

4. Fowler CD, Liu Y, Wang Z. Estrogen and adult neurogenesis in the amygdala and hypothalamus. Brain Res Rev. 2008;57(2):342–51.

5. Dayer AG, Cleaver KM, Abouantoun T, Cameron HA. New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. J Cell Biol. 2005;168(3):415–27.

6. Kaplan MS. Neurogenesis in the 3-month-old rat visual cortex. J Comp Neurol. 1981;195(2):323–38.

7. Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, et al. Neural stem cells in the adult mammalian forebrain: A relatively quiescent subpopulation of subependymal cells. Neuron. 1994;13(5):1071–82.

8. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science (80-). 1992;255(5052):1707–10.

9. Kempermann G, Kuhn HG, Gage FH. More hippocampal neurons in adult mice living in an enriched environment. Nature. 1997;386(6624):493–5.

10. Åberg MAI, Åberg ND, Hedbäcker H, Oscarsson J, Eriksson PS. Peripheral infusion of IGF-I selectively induces neurogenesis in the adult rat hippocampus. J Neurosci. 2000;20(8):2896–903.

11. Sülz L, Astorga G, Bellette B, Iturriaga R, Mackay-Sim A, Bacigalupo J. Nitric oxide regulates neurogenesis in adult olfactory epithelium in vitro. Nitric Oxide - Biol Chem. 2009;20(4):238–52.

12. Faraci FM. Protecting the brain with eNOS: Run for your life. Circ Res. 2006;99(10):1029–30.

13. Sulz L, Bacigalupo J. Role of nitric oxide during neurogenesis in the olfactory epithelium. Biol Res. 2006;39(4):589–99.

14. Piotrkowski B, Calabró V, Galleano M, Fraga CG. (-)-Epicatechin prevents alterations in the metabolism of superoxide anion and nitric oxide in the hearts of L-NAME-treated rats. Food Funct. 2015;6(1):155–61.

15. West SG, McIntyre MD, Piotrowski MJ, Poupin N, Miller DL, Preston AG, et al. Effects of dark chocolate and cocoa consumption on endothelial function and arterial stiffness in overweight adults. Br J Nutr. 2014;111(4):653–61.

16. Loke WM, Hodgson JM, Proudfoot JM, McKinley AJ, Puddey IB, Croft KD. Pure dietary flavonoids quercetin and (-)-epicatechin augment nitric oxide products and reduce endothelin-1 acutely in healthy men. Am J Clin Nutr. 2008;88(4):1018–25.

17. Ramirez-Sanchez I, Maya L, Ceballos G, Villarreal F. (-)-Epicatechin activation of endothelial cell endothelial nitric oxide synthase, nitric oxide, and related signaling pathways. Hypertension. 2010;55(6):1398–405.

18. Abd El Mohsen MM, Kuhnle G, Rechner AR, Schroeter H, Rose S, Jenner P, et al. Uptake and metabolism of epicatechin and its access to the brain after oral ingestion. Free Radic Biol Med. 2002;33(12):1693–702.

19. Van Praag H, Lucero MJ, Yeo GW, Stecker K, Heivand N, Zhao C, et al. Plant-derived flavanol (-)epicatechin enhances angiogenesis and retention of spatial memory in mice. J Neurosci. 2007;27(22):5869–78.

20. Al-Gayyar MMH, Matragoon S, Pillai BA, Ali TK, Abdelsaid MA, El-Remessy AB. Epicatechin blocks pro-nerve growth factor (proNGF)-mediated retinal neurodegeneration via inhibition of p75 neurotrophin receptor proNGF expression in a rat model of diabetes. Diabetologia. 2011;54(3):669–80.

21. Mohamed RH, Karam RA, Amer MG. Epicatechin attenuates doxorubicin-induced brain toxicity: Critical role of TNF-α, iNOS and NF-κB. Brain Res Bull. 2011;86(1–2):22–8.

22. Brickman AM, Khan UA, Provenzano FA, Yeung LK, Suzuki W, Schroeter H, et al. Enhancing dentate gyrus function with dietary flavanols improves cognition in older adults. Nat Neurosci. 2014;17(12):1798–803.

23. Kofink M, Papagiannopoulos M, Galensa R. Enantioseparation of catechin and epicatechin in plant food by chiral capillary electrophoresis. Eur Food Res Technol. 2007;225(3–4):569–77.

24. Moreno-Ulloa A, Nájera-García N, Hernández M, Ramírez-Sánchez I, Taub PR, Su Y, et al. A pilot study on clinical pharmacokinetics and preclinical pharmacodynamics of (+)-epicatechin on cardiometabolic endpoints. Food Funct. 2018;9(1):307–19.

25. Estrada-Mena FJ, Rodriguez A, Mendoza-Lorenzo P, Neri-Gomez T, Manjarrez-Gutierrez G, Perez-Ortiz AC, et al. Effects of (−)-epicatechin on frontal cortex DAPC and dysbindin of the mdx mice. Neurosci Lett. 2017;658:142–9.

26. Navarrete-Yañez V, Garate-Carrillo A, Rodriguez A, Mendoza-Lorenzo P, Ceballos G, Calzada-Mendoza C, et al. Effects of (-)-epicatechin on neuroinflammation and hyperphosphorylation of tau in the hippocampus of aged mice. Food Funct. 2020;11(12):10351–61.

27. Dugar S, Villarreal F, Hollinger FH, Mahajan D, Ramirez-Sanchez I, Moreno-Ulloa A, et al. 11-β-hydroxysterols as possible endogenous stimulators of mitochondrial biogenesis as inferred from epicatechin molecular mimicry. Pharmacol Res. 2020;151(104540).

28. Ramirez-Sanchez I, Nogueira L, Moreno A, Murphy A, Taub P, Perkins G, et al. Stimulatory effects of the flavanol (-)-epicatechin on cardiac angiogenesis: Additive effects with exercise. J Cardiovasc Pharmacol. 2012;60(5):429–38.

29. Lopez-Lopez C, LeRoith D, Torres-Aleman I. Insulin-like growth factor I is required for vessel modeling in the adult brain. Proc Natl Acad Sci U S A. 2004;101(26):9833–8.

30. Li B, Arime Y, Hall FS, Uhl GR, Sora I. Impaired spatial working memory and decreased frontal cortex BDNF protein level in dopamine transporter knockout mice. Eur J Pharmacol. 2010;628(1–3):104–7.

31. Miedel CJ, Patton JM, Miedel AN, Miedel ES, Levenson JM. Assessment of spontaneous alternation, novel object recognition and limb clasping in transgenic mouse models of amyloid-β and tau neuropathology. J Vis Exp. 2017;123(e55523):1–8.

32. Mamiya T, Ukai M. [Gly14]-Humanin improved the learning and memory impairment induced by scopolamine in vivo. Br J Pharmacol. 2001;134(8):1597–9.

33. Ma MX, Chen YM, He J, Zeng T, Wang JH. Effects of morphine and its withdrawal on Y-maze spatial recognition memory in mice. Neuroscience. 2007;147(4):1059–65.

34. Chen J, Li Y, Zhang R, Katakowski M, Gautam SC, Xu Y, et al. Combination therapy of stroke in rats with a nitric oxide donor and human bone marrow stromal cells enhances angiogenesis and neurogenesis. Brain Res. 2004;1005(1–2):21–8.

35. Nogueira L, Ramirez-Sanchez I, Perkins GA, Murphy A, Taub PR, Ceballos G, et al. (-)-Epicatechin enhances fatigue resistance and oxidative capacity in mouse muscle. J Physiol. 2011;589(18):4615–31.

36. Sorrells SF, Paredes MF, Cebrian-Silla A, Sandoval K, Qi D, Kelley KW, et al. Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. Nature. 2018;555(7696):377–81.

37. DeVito LM, Kanter BR, Eichenbaum H. The hippocampus contributes to memory expression during transitive inference in mice. Hippocampus. 2010;20(1):208–17.

38. Ohira K. Regulation of Adult Neurogenesis in the Cerebral Cortex. Vol. 3, J Neurol Neuromedicine. 2018.

39. Ohira K, Takeuchi R, Shoji H, Miyakawa T. Fluoxetine-induced cortical adult neurogenesis. Neuropsychopharmacology. 2013;38(6):909–20.

40. Boldrini M, Fulmore CA, Tartt AN, Simeon LR, Pavlova I, Poposka V, et al. Human Hippocampal Neurogenesis Persists throughout Aging. Cell Stem Cell. 2018;22(4):589–99.

41. Pinnock SB, Herbert J. Brain-derived neurotropic factor and neurogenesis in the adult rat dentate gyrus: Interactions with corticosterone. Eur J Neurosci. 2008;27(10):2493–500.

42. Terpolilli NA, Moskowitz MA, Plesnila N. Nitric oxide: Considerations for the treatment of ischemic stroke. J Cereb Blood Flow Metab. 2012;32(7):1332–46.

43. Kokaia Z, Lindvall O. Neurogenesis after ischaemic brain insults. Curr Opin Neurobiol. 2003;13(1):127–32.

44. An L, Zhang Y-Z, Yu N-J, Liu X-M, Zhao N, Yuan L, et al. The total flavonoids extracted from Xiaobuxin-Tang up-regulate the decreased hippocampal neurogenesis and neurotrophic molecules expression in chronically stressed rats. Prog Neuro-Psychopharmacology Biol Psychiatry. 2008;32(6):1484–90.

45. Zhu W, Cheng S, Xu G, Ma M, Zhou Z, Liu D, et al. Intranasal nerve growth factor enhances striatal neurogenesis in adult rats with focal cerebral ischemia. Drug Deliv. 2011;18(5):338–43.

46. Neeper SA, Gómez-Pinilla F, Choi J, Cotman CW. Physical activity increases mRNA for brain-derived neurotrophic factor and nerve growth factor in rat brain. Brain Res. 1996;726(1–2):49–56.

47. Schimmelpfeng J, Weibezahn KF, Dertinger H. Quantification of NGF-dependent neuronal differentiation of PC-12 cells by means of neurofilament-L mRNA expression and neuronal outgrowth. J Neurosci Methods. 2004;139(2):299–306.

48. Balthazart J, Ball GF. Doublecortin Is a Highly Valuable Endogenous Marker of Adult Neurogenesis in Canaries. Brain Behav Evol. 2014;84(1):1–4.

49. Tanapat P. Neuronal Cell Markers. Mater Methods. 2013;3.

50. Valente T, Hidalgo J, Bolea I, Ramirez B, Anglés N, Reguant J, et al. A diet enriched in polyphenols and polyunsaturated fatty acids, LMN diet, induces neurogenesis in the subventricular zone and hippocampus of adult mouse brain. J Alzheimer's Dis. 2009;18(4):849–65.

51. Boldrini M, Hen R, Underwood MD, Rosoklija GB, Dwork AJ, Mann JJ, et al. Hippocampal angiogenesis and progenitor cell proliferation are increased with antidepressant use in major depression. Biol Psychiatry. 2012;72(7):562–71.

52. Pereira AC, Huddleston DE, Brickman AM, Sosunov AA, Hen R, McKhann GM, et al. An in vivo correlate of exercise-induced neurogenesis in the adult dentate gyrus. Proc Natl Acad Sci U S A. 2007;104(13):5638–43.

53. Katsimpardi L, Litterman NK, Schein PA, Miller CM, Loffredo FS, Wojtkiewicz

GR, et al. Vascular and neurogenic rejuvenation of the aging mouse brain by young systemic factors. Science (80-). 2014;344(6184):630–4.

54. Slevin M, Kumar P, Gaffney J, Kumar S, Krupinski J. Can angiogenesis be exploited to improve stroke outcome? Mechanisms and therapeutic potential. Clin Sci. 2006;111(3):171–83.

55. Simão F, Pagnussat AS, Seo JH, Navaratna D, Leung W, Lok J, et al. Pro-angiogenic effects of resveratrol in brain endothelial cells: Nitric oxide-mediated regulation of vascular endothelial growth factor and metalloproteinases. J Cereb Blood Flow Metab. 2012;32(5):884–95.

56. Binnington JC, Kalisch BE. Nitric oxide synthase inhibitors modulate nerve growth factor-mediated regulation of amyloid precursor protein expression in PC12 cells. J Neurochem. 2007;101(2):422–33.

57. Louissaint A, Rao S, Leventhal C, Goldman SA. Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. Neuron. 2002;34(6):945–60.

58. Angels Font M, Arboix A, Krupinski J. Angiogenesis, Neurogenesis and Neuroplasticity in Ischemic Stroke. Curr Cardiol Rev. 2010;6(3):238–44.

59. Tang EHC, Vanhoutte PM. Gene expression changes of prostanoid synthases in endothelial cells and prostanoid receptors in vascular smooth muscle cells caused by aging and hypertension. Physiol Genomics. 2008;32(3):409–18.

60. Newton S, Duman R. Regulation of Neurogenesis and Angiogenesis in Depression. Curr Neurovasc Res. 2005;1(3):261–7.

61. Zhu DY, Liu SH, Sun HS, Lu YM. Expression of inducible nitric oxide synthase after focal cerebral ischemia stimulates neurogenesis in the adult rodent dentate gyrus. J Neurosci. 2003;23(1):223–9.

62. Luo CX, Zhu XJ, Zhou QG, Wang B, Wang W, Cai HH, et al. Reduced neuronal nitric oxide synthase is involved in ischemia-induced hippocampal neurogenesis by up-regulating inducible nitric oxide synthase expression. J Neurochem. 2007;103(5):1872–82.

63. Moreno-Ulloa A, Nogueira L, Rodriguez A, Barboza J, Hogan MC, Ceballos G, et al. Recovery of Indicators of Mitochondrial Biogenesis, Oxidative Stress, and Aging With (-)-Epicatechin in Senile Mice. Journals Gerontol - Ser A Biol Sci Med Sci. 2015;70(11):1370–8.

64. Carreira BP, Santos DF, Santos AI, Carvalho CM, Araújo IM. Nitric Oxide Regulates Neurogenesis in the Hippocampus following Seizures. Oxid Med Cell Longev. 2015;2015.

65. Chen J, Maiwulanjiang M, Lam KYC, Zhang WL, Zhan JYX, Lam CTW, et al. A standardized extract of the fruit of Ziziphus jujuba (Jujube) induces neuronal

differentiation of cultured PC12 cells: A signaling mediated by protein kinase A. J Agric Food Chem. 2014;62(8):1890–7.

66. Bakoyiannis I, Daskalopoulou A, Pergialiotis V, Perrea D. Phytochemicals and cognitive health: Are flavonoids doing the trick? Biomed Pharmacother. 2019;109:1488–97.

67. Farovik A, Dupont LM, Arce M, Eichenbaum H. Medial prefrontal cortex supports recollection, but not familiarity, in the rat. J Neurosci. 2008;28(50):13428–34.

Figures

Figure 1

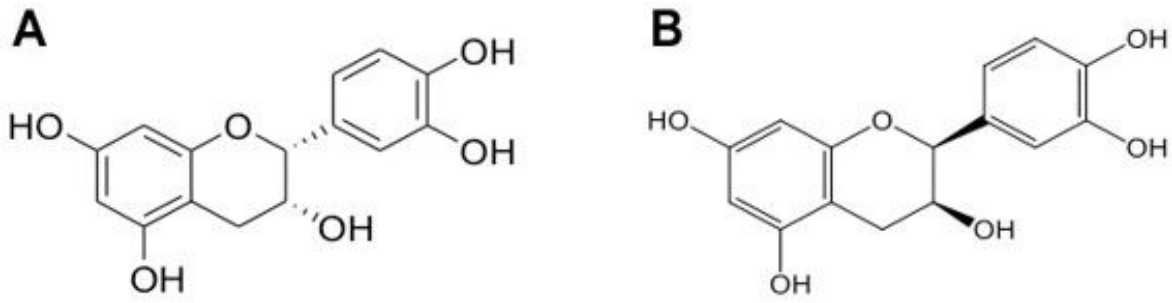

Figure 2

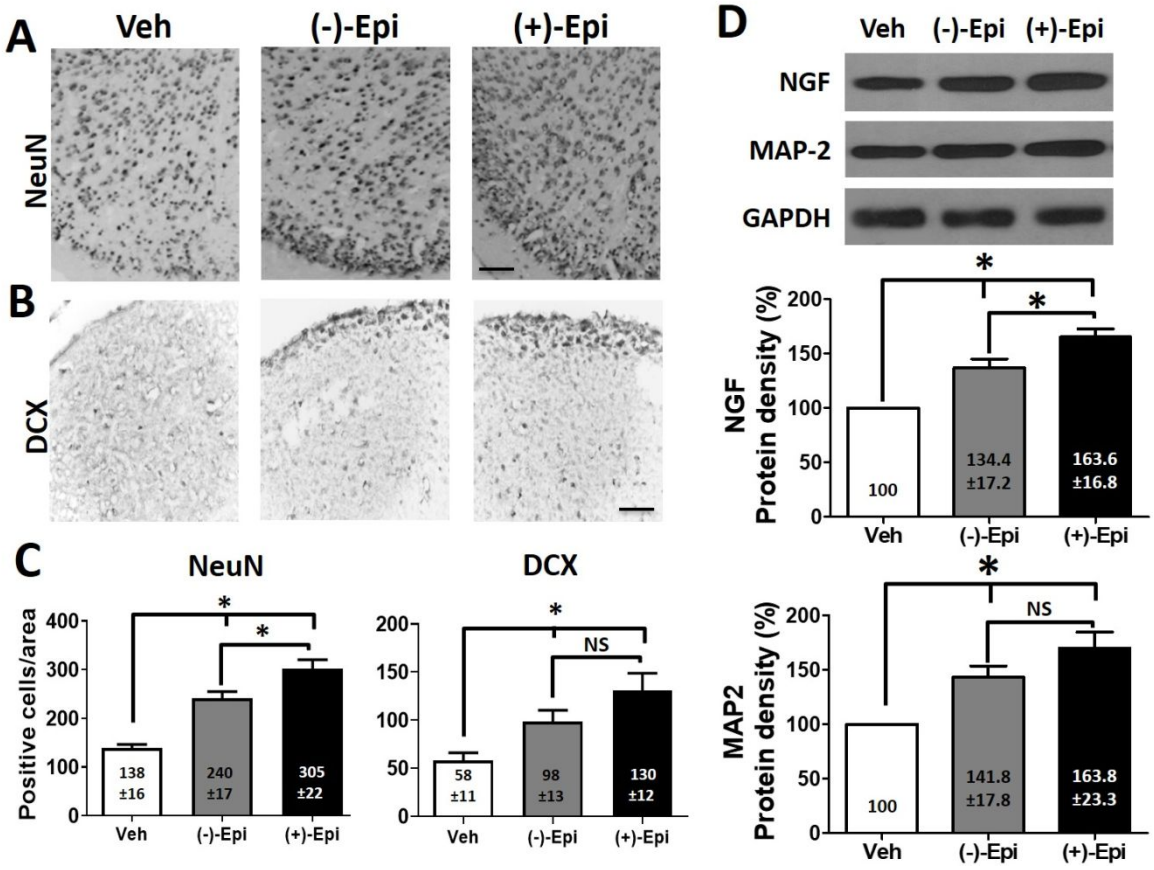

Figure 3

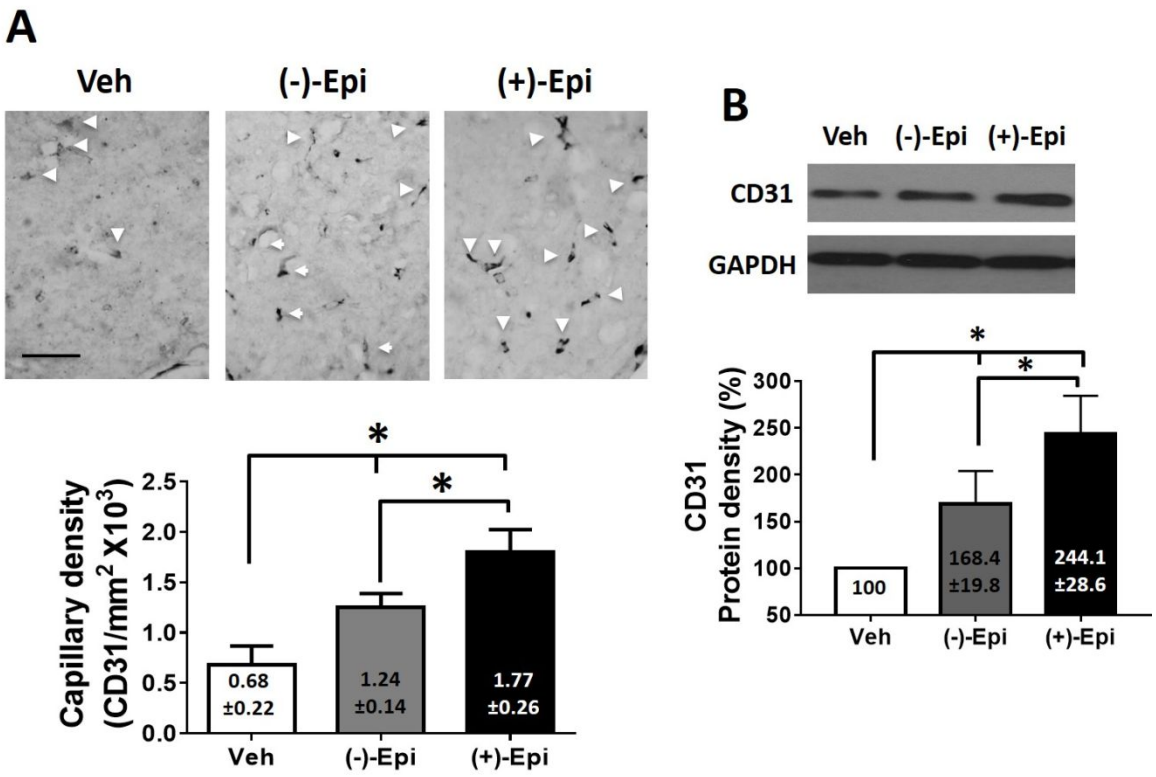
A
Veh
(-)-Epi
(+)-Epi
Capillary density (CD31/mm^2 X10^3)
0.68 ±0.22
1.24 ±0.14
1.77 ±0.26
B
Veh (-)-Epi (+)-Epi
CD31
GAPDH
CD31 Protein density (%)
100
168.4 ±19.8
244.1 ±28.6

Figure 4

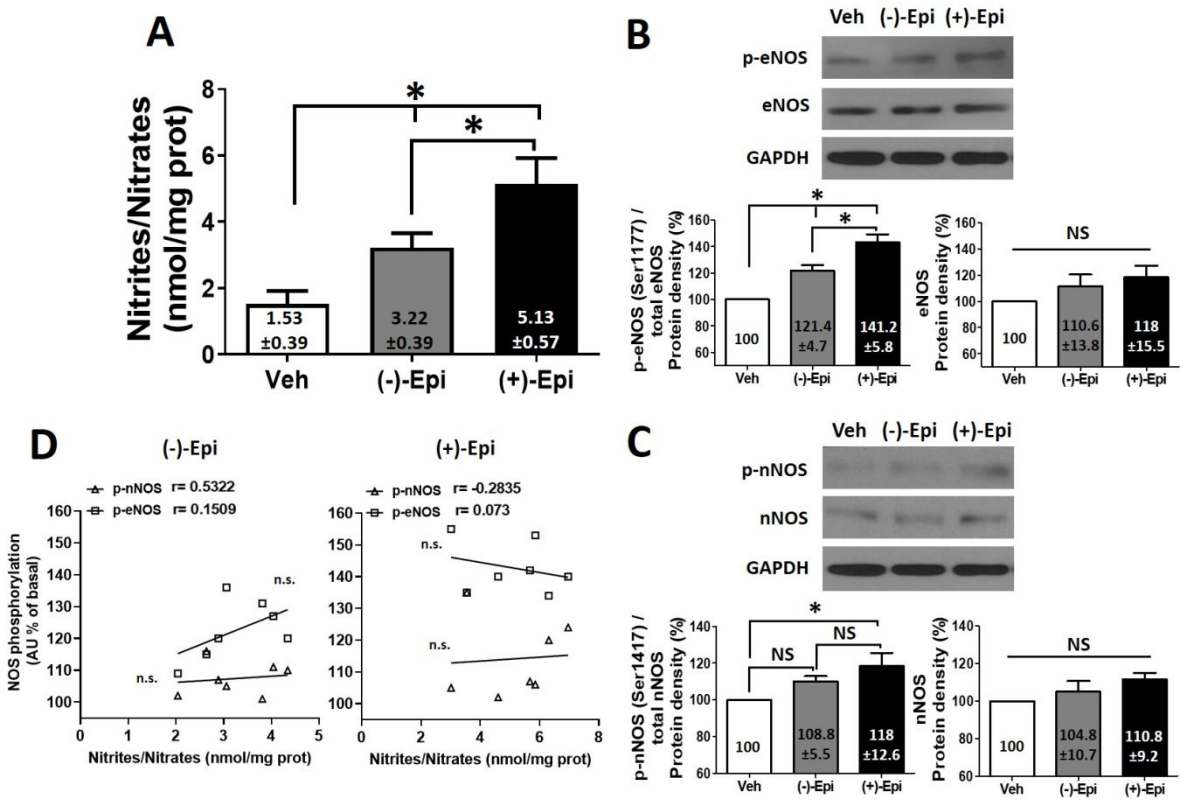
A
Nitrites/Nitrates (nmol/mg prot)
1.53 ±0.39
3.22 ±0.39
5.13 ±0.57
Veh
(-)-Epi
(+)-Epi
B
Veh (-)-Epi (+)-Epi
p-eNOS
eNOS
GAPDH
p-eNOS (Ser1177) / total eNOS Protein density (%)
100
121.4 ±4.7
141.2 ±5.8
eNOS Protein density (%)
NS
110.6 ±13.8
118 ±15.5
C
Veh (-)-Epi (+)-Epi
p-nNOS
nNOS
GAPDH
p-nNOS (Ser1417) / total nNOS Protein density (%)
NS
108.8 ±5.5
118 ±12.6
nNOS Protein density (%)
104.8 ±10.7
110.8 ±9.2
D
(-)-Epi
(+)-Epi
p-nNOS r= 0.5322
p-eNOS r= 0.1509
p-nNOS r= -0.2835
p-eNOS r= 0.073
n.s.
NOS phosphorylation (AU % of basal)
Nitrites/Nitrates (nmol/mg prot)

Figure 5

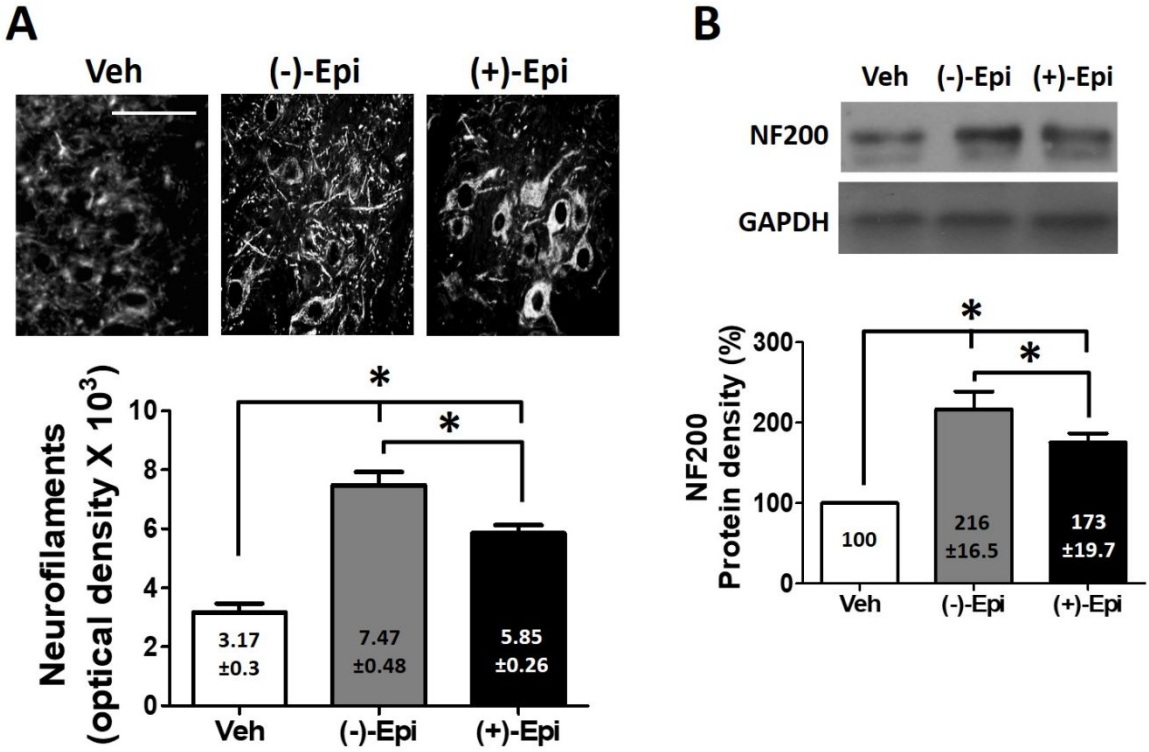
A
Veh
(-)-Epi
(+)-Epi
Neurofilaments (optical density X 10^3)
10
8
6
4
2
0
*
*
3.17 ±0.3
7.47 ±0.48
5.85 ±0.26
Veh
(-)-Epi
(+)-Epi
B
Veh
(-)-Epi
(+)-Epi
NF200
GAPDH
NF200 Protein density (%)
300
200
100
0
*
*
100
216 ±16.5
173 ±19.7
Veh
(-)-Epi
(+)-Epi

Figure 6

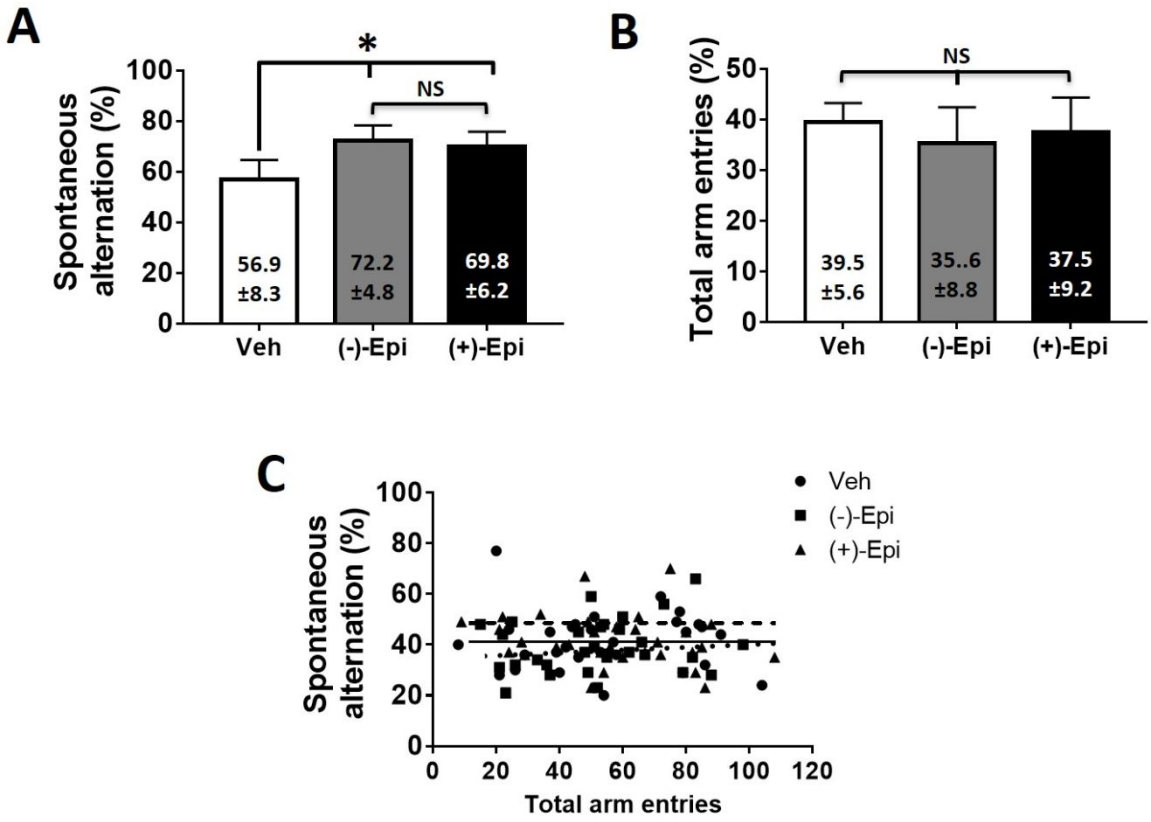
A
Spontaneous alternation (%)
100
80
60
40
20
0
*
NS
56.9
±8.3
72.2
±4.8
69.8
±6.2
Veh
(-)-Epi
(+)-Epi
B
Total arm entries (%)
50
40
30
20
10
0
NS
39.5
±5.6
35..6
±8.8
37.5
±9.2
Veh
(-)-Epi
(+)-Epi
C
Spontaneous alternation (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Total arm entries
Veh
(-)-Epi
(+)-Epi

Figure legends

Figure 1. Chemical structures of (-)-epicatechin (A) and its enantiomer (+)-epicatechin (B).

Figure 2. Effects of (-)-Epi and (+)-Epi treatment (1 mg/kg, or 0.1 mg/kg 15 days) on frontal cortex neurogenesis markers. A) neuronal specific nuclear marker (NeuN), B) Doublecortin (DCX) assessed by immunostaining and C) Graphical representation of positive cells stained either with NeuN (left) or DCX (right), D) representative nerve growth factor (NGF) and microtubule associated protein (MAP-2) Western blot images. For immunohistochemistry, each column represents mean ± SEM of 12 digital images from 6 nonconsecutive sections of 5 animals/group. For Westerns, GAPDH protein levels were used for normalization purposes. Statistical differences were assessed using ANOVA and Tukey post hoc analysis. n=7/group, * $p< 0.05$.

Figure 3. Effect of (-)-Epi and (+)-Epi (1 mg/kg, or 0.1 mg/kg 15 days) on frontal cortex capillarity as reflected by CD31 immunostaining density and Western blot. In the representative images A) (upper panel), arrows indicate examples of polygonal-shape positive staining. In the quantitative analysis of CD31 (lower panel), each column represents mean ± SEM of 12 digital images from 6 nonconsecutive sections of 5 animals/group. B) representative CD31 Western blot images (n=7 animals/group). For Westerns, GAPDH protein levels were used for normalization purposes. Statistical differences were assessed using ANOVA and Tukey post hoc analysis, * $p< 0.05$.

Figure 4. Effects of (-)-Epi or (+)-Epi (1 mg/kg, or 0.1 mg/kg 15 days) on Nitrites/Nitrates production in the brain frontal cortex of adult mice. A) Nitrites/Nitrates levels evaluated fluorometrically. B) Representative Western blot images of eNOS protein levels, phosphorylation of Ser1177 (left lower panel) and their quantification. C) Representative Western blot images and analysis of nNOS protein levels, phosphorylation at Ser1417 (left lower panel) and their quantification. GAPDH protein levels were used for normalization purposes. D) Correlation analysis of p-eNOS/p-nNOS levels vs. nitrites/nitrates with either (-)-Epi (n=7 left) or (+)-Epi (n=7 right) treatment. Data are expressed as mean ± SEM. n=7/group, * $p<0.05$.

Figure 5. Effects of (-)-Epi and (+)-Epi (1 mg/kg, or 0.1 mg/kg 15 days) on brain cortex neurofilament markers. A) Immunofluorescence images of the neurofilament marker NF-200 and their quantitative assessment (lower panel) are shown. B) Representative Western blot images and analysis of NF200 protein levels. For immunostaining, each column represents mean ± SEM of 12 digital images from 6 nonconsecutive sections of 5 animals/group. For Westerns, GAPDH protein levels were used for normalization purposes. Statistical differences were assessed using ANOVA and Tukey post hoc analysis. n=7/group * $p< 0.05$.

Figure 6. Effects of (-)-Epi and (+)-Epi (1 mg/kg, or 0.1 mg/kg 15 days) on spatial work memory. A) Comparison of spontaneous alternation and B) total number of arm entries in Y-maze in vehicle vs. (-)-Epi and (+)-Epi treated mice. Columns represent mean ± SEM of an 8-min session of 6 animals/group. C) Pearson's analysis showing no correlation between spontaneous alternations and total arm entries in each group. Statistical differences were assessed using ANOVA and Tukey post hoc analysis. * $p< 0.05$. n=7/group * $p< 0.05$.