AGATHISFLAVONE, A FLAVONOID DERIVED FROM *POINCIANELLA PYRAMIDALIS* (Tul.), ENHANCES NEURONAL POPULATION AND PROTECTS AGAINST GLUTAMATE EXCITOTOXICITY

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Abstract

Flavonoids are bioactive compounds that are known to be neuroprotective against glutamate-mediated excitotoxicity, one of the major causes of neurodegeneration. The mechanisms underlying these effects are unresolved, but recent evidence indicates flavonoids may modulate estrogen signaling, which can delay the onset and ameliorate the severity of neurodegenerative disorders. Furthermore, the roles played by glial cells in the neuroprotective effects of flavonoids are poorly understood. The aim of this study was to investigate the effects of the flavonoid agathisflavone (FAB) in primary neuron-glia co-cultures from postnatal rat cerebral cortex. Compared to controls, treatment with FAB significantly increased the number of neuronal progenitors and mature neurons, without increasing astrocytes or microglia. These pro-neuronal effects of FAB were suppressed by antagonists of estrogen receptors (ER α and ER β). In addition, treatment with FAB significantly reduced cell death induced by glutamate and this was associated with reduced expression levels of pro-inflammatory (M1) microglial cytokines, including TNF α , IL1 β and IL6, which are associated with neurotoxicity, and increased expression of IL10 and Arginase 1, which are associated with anti-inflammatory (M2) neuroprotective microglia. We also observed that FAB increased neuroprotective trophic factors, such as BDNF, NGF, NT4 and GDNF. The neuroprotective effects of FAB were also associated with increased expression of glutamate regulatory proteins in astrocytes, namely glutamine synthetase (GS) and Excitatory Amino Acid Transporter 1 (EAAT1). These findings indicate that FAB acting via estrogen signaling stimulates production of neurons in vitro and enhances the neuroprotective properties of microglia and astrocytes to significantly ameliorate glutamate-mediated neurotoxicity.

Key words: Neuroprotection, flavonoid, anti-inflammatory, phytoestrogen

Introduction

With the increase in life expectancy over the last century, the prevalence of age-related disorders, such as neurodegenerative diseases continues to rise. This is the case of Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and other neurodegenerative diseases (Procaccini et al., 2016). In addition, ischemic stroke is one of the main causes of death in the world and occurs due to the significant decrease or occlusion of blood flow in a particular brain area, which may be temporary or permanent (Jean et al., 2012). A key cause of neuronal loss is glutamate-mediated excitotoxicity, which is known to be involved in the pathogenesis of AD, HD, PD and ischemic stroke (Xu et al., 2016, Pallo et al., 2016, Douaud et al., 2009, Xing et al., 2012). Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS), but elevated levels of glutamate result in neuronal excitotoxicity (Olney, 1969). Astrocytes are responsible for the removal and recycling of extracellular glutamate via the glutamate transporter EAAT1 and glutamine synthetase (GS) (Hertz, 2014). This is essential to maintain spatial and temporal resolution of synaptic signaling and to prevent excitotoxicity (Danbolt, 2001, Meeker et al., 2015). Hence, astroglial glutamate regulation is a potential therapeutic target in multiple neuropathologies. Furthermore, oxidative stress and neuroinflammation have been implicated in the progression of AD, HD, PD and ischemic stroke (Agostinho et al., 2010, Amor et al., 2010, Uttara et al., 2009, Chen et al., 2016, Xing et al., 2012). Neuroinflammation constitutes a beneficial process involved in the maintenance of organ homeostasis and the brain response to infection or injury (Glass et al., 2010). However, sustained neuroinflammatory processes contribute to the cascade of events leading to the progressive neuronal damage observed in ageing (Barrientos et al., 2015). In this context, as the resident immune cells of the brain, microglia is central to neuroinflammation and controlling their responses is relevant to most neuropathologies (Salter and Stevens, 2017).

Flavonoids are polyphenolic compounds that are ubiquitously present in plants and have biological effects on animal cells (Pandey and Rizvi, 2009). Plants containing these bioactive compounds have been used for centuries because of their beneficial

effects on human health, reducing inflammation, promoting cognition and preventing cancer (Scalbert et al., 2005, Spencer et al., 2008, Williams and Spencer, 2012, Spencer et al., 2012, Sokolov et al., 2013, Busch et al., 2015). Moreover, dietary flavonoids can be neuroprotective and anti-inflammatory and successfully reduce the risk or delay the onset or progression of AD (Dai et al., 2006). The effects of flavonoids are generally related to their antioxidant properties and modulation of intracellular signaling pathways, such as ERK1–ERK2 and PI3K neuroprotective signalling. In addition, flavonoids may act via estrogen signaling (Lehart et al., 2005), although at present this is poorly defined in neuropathology. Nonetheless, estrogen has been shown to affect neural development, maturation, function, and plasticity, in particular influencing synaptogenesis, being anti- apoptotic (Brinton, 2013, Sehara et al., 2013) and stimulating neurite outgrowth (Rozovsky et al., 2002, Islamov et al., 2002). Estrogens induce their effects through estrogen receptors (ER), which exist mainly as ER α and ER β forms. In particular, it has been discovered that ERs coordinate multiple neuroprotective signaling cascades, either directly or through interactions of ERs with the receptors for other neuroprotective factors (Arevalo et al., 2015). For example, studies on primary cortical neurons have shown that the synthetic estrogen estradiol activates ERK1–ERK2 and PI3K neuroprotective signaling in parallel in the same neurons (Mannella and Brinton, 2006).

Notably, estradiol is used therapeutically in humans, but its therapeutic use in controlling neurodegeneration is limited because of the increased risk of some estrogen-dependent cancers. Therefore, the role of ERs in the activation of neuroprotective mechanisms has led researchers to assess the neuroprotective potency of different ER ligands, such as selective ER modulators (SERMs) (Arevalo et al., 2015). The possibility that flavonoids can act as SERMs represents a potential alternative to estrogen that avoids its side-effects for the treatment or the prevention of neurodegeneration, since these compounds may activate multiple neuroprotective mechanisms of action. Here, we have investigated the neuroprotective and anti-inflammatory effect of agathisflavone a biflavonoid extracted from *Poincianella pyramidalis* (Tul.), an abundant plant at the northeast of Brazil, in primary cultures of neurons and glial cells. The results indicate agathisflavone acts as an SERM to promote generation of neurons *in vitro* and is a potent neuroprotective agent against glutamate-mediated excitotoxicity, acting at least in part by polarizing microglia

towards an anti-inflammatory and neuroprotective M2 phenotype and enhancing glutamate regulation in astrocytes.

Methods

Neuron/Glial cell primary co-cultures

Cell cultures were prepared from cerebral hemispheres from Wistar rats, obtained from the Department of Physiology of the Institute of Health Sciences of the Federal University of Bahia (Salvador, BA, Brazil). All experiments were performed in accordance with the local Ethical Committee for Animal Experimentation of the Health Sciences Institute (protocol n°027/2012).

Glial cell primary cultures were obtained from cerebral hemispheres of Wistar rats as previously described (Silva et al., 2013). Cerebral hemispheres from one-day-old postnatal Wistar rat pups were isolated aseptically, and the meninges were mechanically removed. The cerebral cortex was dissected out and then gently forced through a sterile 70-µm Falcon™ Cell Strainer. Cells were suspended in DMEM HAM F12 medium (Gibco®), supplemented with 2 mM L-glutamine, 0.011 g/l pyruvate, 10% FBS, 3.6 g/L Hepes, 33 mM glucose (Cultilab, SP, Brazil), 100 IU/mL penicillin G and 100 µg/ml streptomycin, and cultured in 100mm Ø plates in a humidified atmosphere with 5% CO₂ at 37°C. Culture medium was changed every two days, and cells were cultured for 15 days (Fig. 1A). Cells were then washed 3X with PBS, detached with trypsin (Trypsin EDTA) and plated at a density of 1x10⁵ cell/cm² and maintained in culture for 48 h. After incubation, neurons obtained from cerebral hemispheres of 15 day-old Wistar rat embryos, using the same method described above for glial isolation, were suspended in supplemented DMEM/HAM F12 and seeded at half the amount of glial cells (5x10⁴ cells/cm²) onto the astroglial monolayer. Cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C for 8 days, when treatments were performed.

Microglial Cultures

Microglial cultures were obtained from the cerebral cortex of new born Wistar rats as described previously (Mecha et al., 2011). The brain meninges were carefully stripped off, and cerebral tissues were washed in PBS with 0.6 % glucose (Sigma

Aldrich, St. Louis, MO, USA), dissociated mechanically and resuspended in DMEM (Cultilab, SP, Brazil), supplemented with 10% FBS (Gibco®), 10% HS, 4 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were cultured on poly-D-lysine (25 µg/mL)-coated flasks. The cultures were incubated at 37°C in humidified 5% CO₂ upon reaching confluence (7-10 days). The microglia were isolated from astrocytes at 13 DIV by shaking for 3 h at 37°C at 165 rpm. Isolated microglia were seeded at a density of 3x10⁴/cm², and experiments performed after 24 h. For LPS and IL4 treatment, microglial cells were incubated for an additional 24 h with DMEM medium without FBS (control), with LPS (1 µg/mL, Sigma Aldrich, St. Louis, MO, USA) and IL4 (20ng/mL, Sigma Aldrich, St. Louis, MO, USA).

Treatments

Agathisflavone was extracted from *Poincianella pyramidalis* (Tul.) leaves as previously described (Mendes et al., 2000), stored at 100 mM in dimethyl sulfoxide (DMSO; Sigma Chemical Co), and kept out of light at -20°C until use. 17β-estradiol (EST, the primary estrogen hormone) was obtained from Tocris (2824) stored at 100 mM in dimethyl sulfoxide (DMSO; Sigma Chemical Co), and kept out of light at -20°C until use.

To determine the effects of agathisflavone on neuronal and neuronal progenitor number, neuron-glia co-cultures were treated for 72 h with 10 µM of agathisflavone, diluted in culture medium; control cultures were treated with DMSO the vehicle of dilution of FAB (Fig. 1A).

To induce excitotoxicity, co-cultures were treated with 1 mM glutamate (Glut; Sigma Chemical Co) for 4 h, then the medium was removed and replaced with medium containing agathisflavone (0.1-10 µM), 17β-estradiol (100 nM) or vehicle. The experiments were performed after 24 h and 72 h after treatment (Fig. 1B).

Fig.1. Experimental design. (A) Co-cultures of neuron and glial cells were obtained from cerebral hemispheres from Wistar rats. Co-cultures were treated with agathisflavone (FAB, 10 μ M) for 72 h when the analyses for neurogenesis were performed. (B) Excitotoxicity: Co-cultures of neuron and glial cells were pretreated with 1mM of glutamate for 4 h, after glutamate treatment cells were treated with FAB (0.1-10 μ M) or 17 β Estradiol (100 nM) for 24 and 72 h.

ER Antagonists Treatments

To establish if the effects of agathisflavone were mediated through estrogen receptors (ER), neuron-glial co-cultures were treated with specific ER antagonists starting 2 h before agathisflavone treatment through 72 h (during FAB treatment). In this study, we used selective antagonists for ER- α 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP dihydrochloride; 10 nM, from Sigma), or for ER- β 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) at 1 μ M (Tocris). Control cells were treated with the vehicle of dilution of agathisflavone (DMSO).

Immunostaining

For immunostaining, cells were washed with phosphate-buffered saline (PBS) three times and fixed with 4% paraformaldehyde for 15 min at room temperature (RT). Cultures were washed three times with PBS, incubated with 0.3% Triton X-100 in PBS (Sigma) for 5 min and blocked by incubation with PBS containing 5% bovine serum albumin (BSA) (Sigma) for 1 h. After blocking, samples were incubated with primary antibodies diluted in PBS containing 1% of BSA overnight. Cells were washed with PBS three times. Then, secondary antibodies were added to cells and incubated for 2 h. The cells were washed with PBS three more times and incubated

with 1.0 µg/mL 4,6-diamidino-2-phenylindole (DAPI) for nuclear staining. Staining was visualized on a confocal microscope (Leica, TCS-SP5). Images were captured with either a 40x objective or a 63x oil immersion objective. The following primary antibodies were used at the indicated dilutions: anti-Tubulin β 3 (mouse, 1:1000; BioLegend, 801201), anti-doublecortin (rabbit, 1:1000; Abcam, ab18723), anti-MAP2 (Mouse, 1:500; Sigma, M1406), anti-neurofilament (mouse, 1:400; Abcam, AB24574), anti-vGlut2 (mouse, 1:500; Abcam, AB79157), anti-GFAP (rabbit, 1:300; DAKO, Z0334), anti-Glutamine Synthetase (GS, rabbit, 1:500; Abcam, ab49873), anti-EAAT1 (rabbit, 1:200; Abcam, ab416), anti-Iba1 (rabbit, 1:200; Wako, 019-19741), anti-CD11b/c [OX42] (mouse, 1:200; Abcam, ab1211), anti-CD68 (rat, 1:100; Abcam, ab53444), anti-CD206 (mouse, 1:100; BioRad, MCA2235GA), anti-active caspase-3 (rabbit, 1:300; Chemicon, ab3623). The following secondary antibodies were used at the indicated dilutions: Alexa Fluor 488-conjugated goat anti-mouse IgG conjugated (1:400; Molecular Probes, A11001), Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:400; Molecular Probes, A11037), Alexa Fluor 555-conjugated goat anti-rat IgG (1:400; Molecular Probes, A21434), Alexa Fluor 488-conjugated goat anti-rabbit IgG conjugated (1:400; Molecular Probes, A11008). All experiments included cultures where the primary antibodies were not added, unspecific stained was not observed in such negative controls.

Fluoro-Jade B staining

Fluoro-Jade B (FJ-B) stain (Schmued and Hopkins, 2000) was used to investigate neuronal loss. Neuron-glia co-cultures were cultivated in 96 well black plates, 1.5×10^4 cells/cm², and were treated with 1 mM glutamate (Glut) for 4 h, then the medium was removed and replaced with medium contain agathisflavone or 17 β -estradiol, and kept for 72 h. After treatment, the cultures were fixed in ethanol at 4°C for 10 min. Cultures were washed three times with PBS then incubated with 0.3% Triton X-100 in PBS (Sigma) for 10 min. After washes in PBS three times, cells were incubated with 0.001% Fluoro-jade B in PBS for 30 min at RT, under agitation and protected from the light. After incubation, the cells were washed three times with PBS and incubated for 5 min at RT in the dark with 1.0 µg/mL 4,6-diamidino-2-phenylindole (DAPI) for nuclear staining, and then washed 3 times with PBS. Analyses were performed in a spectrophotometer (Varioskan™ Flash Multimode Reader, Thermo Plate), and the fluorescence intensity of each sample was

measured at 480 nm for fluoro-Jade B and 350 nm for DAPI. The values of absorbance of fluoro Jade B of each well were normalized to the DAPI absorbance in the same well.

RNA extraction and qPCR

After 24 h of treatment, cells were processed for qPCR. Total RNA was isolated using Trizol reagent (Ambion™ 15596018) and subjected to DNase treatment using TURBO DNA-free™ Kit (Invitrogen, AM1906), following manufacturer's instructions. The concentration of the RNA samples was determined spectrophotometrically. Complementary DNA was generated from 2.5 µg total RNA using the SuperScript® VILO™ Master Mix according to manufacturer recommendations. Expression of mRNA of target genes and the endogenous controls genes Actin B and HPRT1 were assessed by real-time PCR (with TaqMan Gene Expression Assay products, Applied Biosystems), according to the manufacturer's recommendations. Expression levels for each gene of interest were calculated by normalizing the quantified mRNA amount to Actin b and HPRT1. Relative gene expression was determined and used to test significance between different groups. Real-time PCR was performed in QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, CA, USA) using TaqMan Universal PCR Master Mix II (Applied Biosystems™ 4440044), TaqMan probes and primers provided by Applied Biosystems. The assay ID provided by the manufacturer are the following: IL-1b (Rn00580432_m1), IL-6 (Rn01410330_m1), TNF (Rn01525859_g1), IL-10 (Rn00563409_m1), TGFB1 (Rn00572010_m1), Arg1 (Rn00691090_m1), BDNF (Rn02531967_s1), NGF (Rn01533872_m1), NTF4 (Rn00566076_s1), GDNF (Rn00569510_m1), CDNF (Rn01765001_m1), HPRT1 (Rn01527840_m1), Actin B (ACTB; Rn00667869_m1).

Flow Cytometry Analysis

After treatments, the cells were analyzed by flow cytometry. Cells were dissociated with trypsin, washed with PBS, and fixed with 4% paraformaldehyde for 15 min. Cell permeabilization was achieved with PBS containing 0.3% Triton X-100 for 5 min and blocking was performed in PBS with 5% BSA (Sigma). Following 3 washes with PBS, cells were incubated overnight with anti-β-tubulin III (mouse, 1:2000, BioLegend 801202), anti-doublecortin (rabbit, 1:2000; Abcam, ab18723),

anti-MAP2 (Mouse, 1:500; Sigma, M1406), anti-vGlut2 (Mouse, 1:1000; Abcam, AB79157), anti-GFAP (rabbit, 1:500; DAKO, Z0334), anti-Iba1 (rabbit, 1:500; Wako, 019-19741) antibodies in PBS with 1% BSA. Cells were washed with PBS three times and hybridized with Alexa 488- or Alexa 633-conjugated secondary antibodies for 2 h (1:1000; Invitrogen), after which three more PBS washes were performed. Flow cytometry was performed using a FACSCalibur cytometer (Beckman Coulter, Brea, CA). Data analyses were performed using FlowJo or WinMDI software (v.2.9). Fluorescence thresholds and immune positive cell rates were calculated using the same program. For all experiments, the isotype antibodies were used as controls.

Western Blot

After treatments, neuron-glia co-cultures were collected and lysis buffer was added, total protein was extracted with buffer (4M urea, 2% SDS, 2 mM EGTA, 62,5mM Tris – HCl pH 6,8, 2 mM EDTA, 0,5% Triton X-100) supplemented with protease inhibitor cocktail (Sigma-Aldrich, P8340). Thirty micrograms of protein per lane were electrophoretically separated on a 10% polyacrylamide gel. After separation, proteins were transferred into a polyvinylidenedifluoride (PVDF) membrane (Hybond, Amersham, Piscataway, USA) in a semi-dry system (Bio-Rad, Hercules, USA) for 120 min at a constant current of 0.15 mA. Membranes were blocked with 5% nonfat milk in tris-buffered saline containing 0.05% Tween-20 (TBS-T) for 1 h at RT under agitation. After blocking, membranes were incubated with primary antibodies for either Anti-Glutamine Synthetase (GS, rabbit, 1:10000; Abcam, ab49873) or Anti-Cyclophilin B (Rabbit 1:5000 Abcam, ab178397) overnight at 4°C. Afterward, membranes were washed with TBS-T three times and incubated for 1 h at RT under agitation with goat anti-rabbit peroxidase-conjugated (1:10000; Molecular probes, G21234) secondary antibody diluted in 5% nonfat milk in TBS-T. Membranes were then washed three times under agitation in TBS-T. Blots were developed using Amersham ECL Prime (GE). After protein detection, densitometric analyses were performed using ImageJ software. The values of each protein were normalized to the Cyclophilin B amount in the same lane.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 5 and validated with Student's t-tests or one-way analysis of variance (ANOVA) when more than 2

groups were compared. Confidence intervals were defined at 95% confidence level ($p < 0.05$ was considered to be statistically significant). Fold change was calculated by dividing the average (mean) value of the experimental group to that of the control group. In all figures, error bars represent SEM, of at least 3 independent experiments.

Results

The flavonoid agathisflavone enhances generation of neurons

The effects of agathisflavone (FAB) on the neuronal population in primary cortical co-cultures of neurons and glial cells were investigated by quantification of the number of neuronal cells after 72 h of treatment. Compared to controls, cultures treated with agathisflavone showed significant increases in the proportion of cells expressing the neuronal markers DCX (from 33% in controls to 52% in agathisflavone, $p < 0.05$; Fig. 2A- C), β Tubulin III (37% in controls to 58% in agathisflavone, $p < 0.05$; Fig. 2D- F), MAP2 (40% in controls to 58% in agathisflavone, $p < 0.05$; Fig 2G, H and I) and vGlut2 (36% in controls to 47% in agathisflavone, $p < 0.05$; Fig 2P-R). We also observed an apparent increase in the expression of Neurofilament 200, although this was not quantified (Fig. 2M, N). On the other hand, agathisflavone had no significant effect on the percentage of Iba+ microglia (12.5% in control and 13% in agathisflavone, $p > 0.05$; Fig 2F-O) or GFAP+ astrocytes (53 % in control and 46% in agathisflavone, $p > 0.05$; Fig 2J-L).

Fig.2. Agathisflavone (FAB) increases neuronal population in neuron-glia co-cultures. Representative immunofluorescence images for detection of neuronal markers doublecortin (DCX⁺; red, A and B), β TubIII⁺ (green, D and E), MAP2⁺ (green, F and H), vGlut2 (green, P and Q), Neurofilament 200 (Neurof, green, M and N) and glial markers, Iba1 (red, F and H) and GFAP (red, J and K) counterstained with DAPI (blue); scale bars = 100 μ m (A-H, P and Q) and 50 μ m (J-N). Neuron-glia co-cultures that were treated with 10 μ M FAB for 72 h showed increased immunostaining for (B) DCX, (E) β TubIII, (H) MAP2, (Q) vGlut2 and (N) Neurof, but not (K) GFAP or (H) Iba1. Bar graphs showing the percentage of DCX (C), β TubIII (F), MAP2 (O), vGlut2 (R), Iba1 (I) and GFAP (L) positive cells in each treatment group; cultures treated with FAB displayed significantly more cells immunopositive for neuronal markers than in controls (values are mean $\pm$ SEM, $n=3$, * $p<0.05$, ** $p<0.01$ unpaired t-tests).

The pro-neuronal effects of agathisflavone involve estrogen receptors

To investigate the involvement of Estrogen receptor (ER) signaling on the effects of agathisflavone on neuronal and neuronal progenitor number, we carried out experiments with antagonists to ER α and ER β subtypes. Antagonists were added to cultures 2h before and concomitant with agathisflavone treatment and the number of neurons was measured after 72 h. Pharmacological antagonism of ER α with methyl-piperidinopyrazole (MPP), a selective and widely used antagonist of this receptor, markedly reduced the number of β TubIII⁺ cells compared with agathisflavone-treated cultures, from 50% in agathisflavone-treated cultures to 21% in MPP + agathisflavone (Fig. 3B, C and E; $p < 0.05$, unpaired t-test). Similarly, blocking ER β with pyrazolo[1,5-a]pyrimidine (PHTPP) resulted in a significant decrease in the number of β TubIII⁺ cells, from 50% in agathisflavone to 32% in PHTPP + agathisflavone (Fig. 3B, D and E; $p < 0.05$, unpaired t-test).

Fig.3. Agathisflavone (FAB) increases neurons through activation of estrogen receptors.

Representative immunofluorescence images for the neuronal marker β TubIII⁺ (green, A-D), counterstained with DAPI (blue). Neuron-glia co-cultures were treated with: (A) vehicle DMSO, (B) 10 μ M FAB for 72 h, and in the presence of (C) FAB (10 μ M) and the ER α antagonist methyl-piperidinopyrazole (MPP, 10 nM), and (D) FAB (10 μ M) and the ER β antagonist pyrazolo[1,5-a]pyrimidine (PHTPP, 1 μ M); ER antagonists were applied 2 h prior to and throughout FAB treatment. The proportion of β TubIII⁺ neurons induced by FAB (see Fig. 2D-F for comparison with FAB compared to normal control medium) was decreased by ER antagonists (A-D; scale bars = 50 μ m). (E) Bar graph showing the percentage of β TubIII⁺ cells in each treatment group; the values are expressed as the mean $\pm$ SEM, $n=3$; significant differences are indicated with * $P < 0.05$ when compared with control and # $P < 0.05$ when compared with FAB treatment, (one-way ANOVA).

Agathisflavone protects against glutamate-mediated excitotoxicity

Excitotoxicity is a pathological event characterized by neuronal excitation through overstimulation of neurons by excitatory glutamate receptors contributing to neuronal degeneration in many acute and chronic CNS diseases. To investigate the neuroprotective potential of agathisflavone against glutamate-mediated excitotoxicity, neuron-glia co-cultures were exposed to glutamate (1mM) for 4 h and then treated with agathisflavone for 24h or 72h; 17 β estradiol (EST) has known neuroprotective effects (Arevalo et al., 2015) and was used as a positive control.

First, we used Fluoro Jade B (FJ-B) to stain neurons undergoing degeneration and compared the effects of different concentrations of agathisflavone (0.1-10 μ M) on glutamate-mediated excitotoxicity (Fig.4B). As expected, we observed a significant increase in FJ-B Fluorescence Intensity after treatment with 1mM of glutamate (0.60, Fig. 4B), when compared with control treated with DMSO vehicle (0.31, Fig. 4B). In contrast, treatment with agathisflavone after exposure to glutamate significantly reduced FJ-B fluorescence intensity at all concentrations of agathisflavone tested (0.33 agathisflavone 0.1 μ M, 0.35 agathisflavone 1 μ M, 0.37 agathisflavone 10 μ M, Fig. 4B), significantly less than glutamate treated cells (0.60, Fig. 4B); similar results were observed in cells treated with EST after exposure to glutamate (0.42; Fig. 4B). The FJ-B Fluorescence Intensity was similar in the experimental groups treated with agathisflavone or EST alone when compared with control groups (Fig. 5B), suggesting that agathisflavone and EST have no effect on cell death in cortical co-cultures in the absence of a cytotoxic insult (0.33 agathisflavone 0.1 μ M, 0.27 agathisflavone 1 μ M, 0.39 agathisflavone 10 μ M, 0.39 EST 0.1 μ M, Fig. 4B). In addition, we examined whether agathisflavone (10 μ M) affected glutamate-mediated apoptosis, using TUNEL staining, and the results indicated that agathisflavone decreased significantly the amount of TUNEL-positive cells (Fig. 4A).

To evaluate whether agathisflavone was protecting specifically by reducing caspase 3, we also evaluated by immunofluorescence the amount of cleaved caspase-3 in co-culture (Fig. 4C). Cultures exposed to glutamate (Glut), showed a significant increase in the percentage of caspase-3⁺ cells (14.5% Fig. 4C and D), compared with controls (4.4% Fig. 4C and D). Compared to the glutamate treated group (14.5%), agathisflavone treatment significantly reduced the percentage of Caspase 3 positive cells (5.7% Fig. 4C and D), and similar results were observed in cells treated with EST (5.4% Fig. 4C and D); agathisflavone or EST treatment did not

significantly alter the proportion of Casp3⁺ cells in normal medium (Fig. 4C and D). These data show that agathisflavone protects against glutamate-mediated neuronal excitotoxicity.

Fig.4. Agathisflavone protects neurons against glutamate-mediated excitotoxicity. (A) Confocal photomicrographs of neurons in neuron-glia co-cultures after the fluorescent TUNEL assay (green); neurons are labeled for β -tubulin III (red) and nuclei are counterstained with DAPI (Grey). Cells were incubated with 0.01% of vehicle (DMSO) or treated with 1mM Glutamate (Glu), 10 μ M of agathisflavone (FAB), 0.1 μ M of 17 β Estradiol (EST) or the combination of 1 mM Glu plus 10 μ M FAB (Glu + FAB) or the combination of 1 mM Glu plus 0.1 μ M EST (Glu + EST). (B) Bar graph of the proportion of neurons undergoing degeneration measured by the FJ-B Fluorescence Intensity/DAPI Fluorescence Intensity stain; "Glu" means glutamate. (C) Representative immunofluorescence images for detection of apoptotic marker caspase 3 (Casp 3⁺, red C), nuclei were counterstained with DAPI (blue) by confocal microscope. (D) Bar graph showing the percentage of Caspase 3⁺ cells in each treatment group; the values are expressed as the mean $\pm$ standard error mean (SEM); n=3. Significant differences between control are indicated with *P $\leq$ 0.05 and #P<0.05 when compared with glutamate treatment (##p<0.05, ###p<0.01). Scale bar: 50 μ m.

Agathisflavone reduces neuroinflammation induced by glutamate

Immunofluorescence analyses of microglia was performed in neuron-glia co-cultures 72 h after treatment to investigate the activation of microglia after glutamate-mediated excitotoxicity (Fig. 5A). A significant increase in the percentage of Ox42⁺ microglial cells was observed after glutamate treatment (10.1%) compared with controls (2.0%). In contrast, agathisflavone significantly reduced microglial activation induced by glutamate, as observed by the reduction in the percentage of Ox42⁺ cells (3.6%), and similar results were observed following treatment with 17 β Estradiol (4.8%). We did not observe differences in the percentage of Ox42⁺ cells in groups treated only with agathisflavone or EST (Fig. 5B).

In order to evaluate if glutamate-mediated excitotoxicity induces distinct cytokine expression, qPCR was performed in neuron-glia co-cultures 24 h after treatments. Glutamate (Glut) induced an increase of mRNA expression of TNF (1.4), IL1 β (2.0), IL6 (2.4), arginase1 (1.46) and IL10 (1.35), compared with control (1.0); however, the expression levels of TGF- β was not altered. In contrast, agathisflavone reduces the expression of the inflammatory cytokines TNF (1.2), IL1 β (1.5), IL6 (1.4), induced by glutamate, whereas expression of anti-inflammatory markers arginase1 (2.26) and IL10 (4.31) were increased by agathisflavone (Fig. 5C). EST also reduced the levels of TNF (1.2), IL1 β (1.7), IL6 (1.3) and increased the expression of arginase1 (2.2) and IL10 (5.3) when compared with glutamate. Treatment with agathisflavone or EST alone showed increased expression of IL6 (1.2 and 1.4 respectively) and IL10 (1.2, 1.4) but not other targets (IL1 β , TNF, IL1 β , arginase and TGF β), when compared with control group.

To investigate the molecular mechanisms involved in the neuroprotection induced by agathisflavone, qPCR of neurotrophins was performed in neuron-glia co-cultures 24 h after treatments. Glutamate induced an increase in mRNA expression of GDNF (1.3), but reduced the levels of BDNF (0.7), when compared with control (1.0); no differences were observed in the expression of NGF, NT4 and CDFN. Treatment with glutamate plus agathisflavone increased the levels of BDNF (2.2), NGF (1.7), NT4 (2.4) and GDNF (2.2), but not CDFN (1.05), when compared with glutamate treated cells. Similarly, treatment with EST plus glutamate elevated the levels of BDNF (1.5), NGF (1.4), NT4 (4.0) and GDNF (1.9), but not CDFN (1.12), compared to glutamate treated cells. Treatment with agathisflavone alone increased the expression of BDNF (1.2), NT4 (1.2, 1.4) and CDFN when compared with control

medium group; EST alone elevated the levels of BDNF (1.5) and GDNF (1.3) (Fig. 5D).

Fig.5. Agathisflavone reduced microglia activation induced by glutamate. (A) Representative immunofluorescence images staining for GFAP (green) and OX42 (red) counterstained with DAPI (blue) in co-cultures. (B) Bar graph showing the percentage of Ox42⁺ cells in each treatment group. (C) FAB and EST modulate cytokine expression after glutamate treatment. qPCR analysis showing that cells treated with FAB or EST reduced expression of the pro-inflammatory cytokines TNF α , IL1 β , and IL6, whereas there was increased expression of the anti-inflammatory markers IL10 and ARG1, but not TGF β . (D) FAB modulates the expression of neurotrophins: qPCR analysis showing that cells treated with FAB or EST increased expression the neurotrophins BDNF, NGF, NT4 and GDNF, but not CDFN after glutamate induced excitotoxicity. (E) Confocal photomicrographs of isolated microglia cultures immunostained for the microglial marker Iba1 (green) and the M1 microglial marker CD68 (red); nuclei were counterstained with DAPI (blue). Cells were incubated with 0.01% of vehicle (DMSO) or treated with 1mM Glutamate (Glu), 1 μ g/ mL of LPS, 20ng/mL of IL4, 10 μ M of agathisflavone (FAB), 100nM of 17 β Estradiol (EST) or the combination of 1 mM Glu plus 10 μ M FAB (Glu + FAB) or the combination of 1 mM Glu plus 100nM EST (Glu + EST). (F) Confocal photomicrographs of isolated microglia cultures, immunostained for the microglial marker Iba1 (red) and the M2 microglial marker CD206 (green); nuclei were counterstained with DAPI (blue). Values in B-D are expressed as the mean $\pm$ SEM, n=3; significant differences are indicated with *P $\leq$ 0.05 when compared with control, #P<0.05 when compared with glutamate treatment and &p<0.05 when compared with Glut +FAB treatment, one way ANOVA. Scale bar: 50 mm (A) and 100mm (E and F).

To confirm the effects of agathisflavone on the apparent change in the M1/M2 polarization of microglial cells after glutamate-induced excitotoxicity, isolated microglial cultures were pre-treated with glutamate and then treated with agathisflavone and EST and processed for immunofluorescence for Iba1, a general marker for microglia, plus CD68 and CD206 for the M1 and M2 phenotypes, respectively. Cultures treated with 1 µg/mL of LPS were used as a positive control of the M1 phenotype, and cultures treated with 20 ng/mL IL4 were used as a control of the M2 phenotype. We observed an increase in the levels of the M1 marker CD68 in cells treated with LPS and EST, when compared with control, whereas agathisflavone and glutamate did not change the levels of CD68 in these conditions (Fig. 5E). In comparison, the M2 marker CD206 was increased in IL4 treated groups and reduced in cells treated with LPS or glutamate, when compared with control (Fig. 5F). Notably, agathisflavone and EST treatment alone and after pre-treatment with glutamate resulted in an increase in CD206 expression. Together, these results show that glutamate-mediated excitotoxicity induces microglial activation and that agathisflavone reduces inflammation induced by glutamate and polarizes microglia towards an M2 phenotype.

Agathisflavone increases the levels of EAAT1 and GS in astrocytes

Since one of the most important functions of astrocytes in the brain is their control of glutamate clearance, whose accumulation in the extracellular space can trigger excessive activation of glutamatergic receptors and lead to excitotoxicity, a characteristic of many neurodegenerative diseases (Murphy-Royal et al., 2017). Based on these observations, we postulated that astrocytes could have a role in the neuroprotective effects of agathisflavone. To investigate this, we performed immunofluorescence and western blot analysis of EAAT1 and GS expression on co-cultures 72 h after treatments. Cultures exposed to glutamate (Glut), showed a decrease in the intensity EAAT1 fluorescence (7.61) compared with control groups (9.45, Fig. 6A and B). agathisflavone and EST treatment increased the levels of EAAT1 (13.52 and 13.56 respectively) when compared with control treated with the vehicle and when compared with glutamate treated group. Treatment with agathisflavone after glutamate induced excitotoxicity markedly increased the levels of EAAT1 (21.85), compared with control (9.45, a) and glutamate (7.61); EST also

elevated the levels of EAAT1 after glutamate induced excitotoxicity (17.99). Furthermore, EAAT1 expression was increased to a larger extent in agathisflavone than EST after glutamate induced excitotoxicity (Fig. 6A and B).

Fig.6. Agathisflavone increases the levels of *Excitatory Amino Acid Transporter 1* (EAAT1) and *Glutamine synthetase* (GS) in astrocytes. Cells were incubated with 0.01% of vehicle (DMSO) or treated with 1mM Glutamate (Glut), 10 μ M of agathisflavone (FAB), 0.1 μ M of 17 β Estradiol (EST) or the combination of 1 mM Glut plus 10 μ M FAB (Glut + FAB) or the combination of 1 mM Glu plus 0.1 μ M EST (Glut + EST). (A) Representative immunofluorescence images stained for β Tubulin III (green) and EAAT1 (red), counterstained with DAPI (blue) in neuron-glia co-cultures. (B) Bar graph of the fluorescence intensity as a percentage of the total number of cells showing significantly increased EAAT1 staining in Glut + FAB and Glut + EST-treated cells compared to glutamate. (C) Representative immunofluorescence images stained for β Tubulin III (green) and GS (red), counterstained with DAPI (blue), in neuron-glia co-cultures. (D) Bar graph of the fluorescence intensity as a percentage of the total number of cells showing significantly increased GS staining in Glut + FAB and Glut + EST-treated cells compared to glutamate. (E) Western blot showed increased expression of GS in treated cells, the results were normalized relative to control group considered as 100% and at least three independent experiments. The values are expressed as the mean $\pm$ SEM; n=3; significant differences are indicated with *P $\leq$ 0.05, ***p<0.001 when compared with control, #P<0.05, ###p<0.001 when compared with glutamate treatment and &p<0.05 when compared with Glut +FAB treatment, one way ANOVA. Scale bar: 50 μ m.

Glutamine synthetase (GS) is an ATP-dependent enzyme found in most species that synthesizes glutamine from glutamate and ammonia. In the CNS, GS is exclusively located in astrocytes where it serves to maintain the glutamate-glutamine cycle (Jayakumar and Norenberg, 2016). We evaluated the levels of GS in co-cultures by immunofluorescence and western blot. The immunofluorescence showed a tendency of reduction in the levels of GS in cultures exposed to glutamate (Glut, Fig. 6C and D), which was confirmed by western blot that showed a significantly decreased level of GS (23.99%), when compared with control groups (100%, Fig. 6E). The treatment with agathisflavone alone did not change significantly the levels of GS (84%, Fig. 6E), whereas agathisflavone after glutamate induced excitotoxicity resulted in an increase of the levels of GS showed by immunofluorescence and confirmed by western blot (87% Fig. 6E) when compared with glutamate (23.99%); the results of agathisflavone after glutamate induced excitotoxicity was similar to control levels. Western blot showed that treatment with EST alone reduced the levels of GS (65.9%, Fig. 6E), when compared with the control (100%, Fig. 6E), but increased the level of EAAT1 after glutamate induced excitotoxicity (70.5%), when compared with glutamate (23.99%). These data show that agathisflavone modulates the astrocyte response against glutamate-mediated excitotoxicity.

Discussion

Glutamate-mediated excitotoxicity is a major factor in neuronal loss in neurodegenerative diseases. Notably, there is evidence that estrogens are neuroprotective, but their therapeutic use in humans is limited by the increased risk of cancer. Here, we provide evidence that the flavonoid agathisflavone acts as a modulator of estrogen receptors (ER) to promote the generation of neurons *in vitro* and protects against glutamate-mediated neurotoxicity as effectively as the synthetic estrogen estradiol. In neuron-glia co-cultures, we show that the protective effects of agathisflavone against glutamate-mediated neurotoxicity are mediated at least in part by polarizing microglia towards an anti-inflammatory M2 phenotype and increased expression of neuroprotective trophic factors. In addition, agathisflavone treatment maintained the expression of the glutamate regulatory proteins EAAT1 and GS in astrocytes, which is likely to be important in protecting neurons against elevated levels of glutamate. The neuroprotective and anti-inflammatory potency of

agathisflavone suggest it may provide a potential alternative to estrogens for the treatment of neurodegeneration.

The increased neuronal population in cortical cultures after agathisflavone treatment gave rise to the population of doublecortin-(DCX)-expressing cells that comprises the late intermediate progenitor cells as well as immature neurons. Agathisflavone also increased the population of neurons (β TubIII⁺ and MAP2⁺ Cells). This effect was dependent, at least partly, on estrogen receptor (ER) activation, since ER antagonists were capable of reducing the generation of these cells. Natural compounds and their potential effect on brain plasticity have become particularly interesting for their beneficial effects to both the general public and scientists (Ortiz-Lopez et al., 2016). Preclinical studies, *in vitro* and *in vivo* suggest that natural compounds such as polyphenols modulate neuroplasticity (Dias et al., 2012). Several natural compounds reportedly favored the generation of new neurons (Dias et al., 2012). For example, epigallo-catechin-3-gallate (EGCG), a polyphenol compound mainly found in green tea leaves (*Camellia sinensis*) increased the population of doublecortin-(DCX)-expressing cells and also significantly increased net neurogenesis in the adult hippocampus (Ortiz-Lopez et al., 2016). On the other hand, some flavonoids, in particular apigenin, have been shown to activate estrogen receptors (Mak et al., 2006), which affect the development, maturation, function, and plasticity of the nervous system. Furthermore, it has been previously shown that the flavonoid apigenin induces neural differentiation of human pluripotent stem cells and this neural conversion was dependent, at least partly, on ER activation (Souza et al., 2015). We previously demonstrated that agathisflavone, a product of the oxidative coupling of an apigenin dimer, enhances neural differentiation induced by retinoic acid (RA) in murine pluripotent stem cells by increasing the expression of all trans retinoic acid (RA) receptors (RAR) (Paulsen et al., 2011). The results of the present study show that agathisflavone also modulates ER signaling to promote the generation of neurons *in vitro*.

The influence of the agathisflavone on cell death induced by glutamate has not been explored in co-culture previously. Glutamate is one of the most abundant excitatory neurotransmitters in the CNS and is particularly involved in the cortical and hippocampal regions, which deal especially with synaptic plasticity, memory and learning among other functions (Collingridge and Lester, 1989, Esposito et al.,

2013). Excess levels of glutamate and other excitatory molecules results in over excitation of the ionotropic glutamatergic receptors N-methyl-d-aspartate (NMDA) and 2-amino-3- (3-hydroxy-5-methylisoxazol-4-yl) propionate (AMPA), and the release of other excitotoxins followed by excessive calcium levels in cytosol (Essa et al., 2013). Excitotoxicity describes the pathological event characterized by neuronal excitation through overstimulation of neurons by excitatory amino acids receptors of glutamate and aspartate (Olney, 1969). Glutamate induced excitotoxicity is considered a direct cause of cell degeneration in several neurological disorders, such as HD (Douaud et al., 2009, Malkki, 2016), PD (Rodriguez et al., 1998, Marti et al., 2000) and AD (Bezprozvanny and Mattson, 2008, Zhang et al., 2016). Here, we demonstrate that the flavonoid agathisflavone reduced neuronal cell death induced by glutamate excitotoxicity in neuron-glia co-cultures. The reduction of neurodegeneration by agathisflavone is consistent with previous results that show that flavonoids can reduce cell death (Zong et al., 2016, Cong et al., 2016). Our results show that agathisflavone reduced the percentage of cells expressing caspase 3, a member of the caspase family of proteases that play a pivotal role in apoptosis. In healthy cells, caspase-3 is present in the cytoplasm as an inactive proenzyme, pro-caspase-3. During apoptosis, pro-caspase-3 is hydrolyzed to the active-caspase-3 (D'Amelio et al., 2011).

Flavonoids are widely known as antioxidants and the mechanisms by which they protect against cell death induced by glutamate are likely to be multifarious. Recently, much interest has focused on the suggested anti-inflammatory and neuroprotective effects of dietary derived polyphenols (Vauzour et al., 2015). We demonstrated that the flavonoid agathisflavone (bis-apigenin) reduces the neuroinflammation induced by glutamate, by modulating microglial activation and reducing the expression of pro-inflammatory cytokines TNF, IL6 and IL1 β , and increasing the expression of anti-inflammatory markers. The anti-inflammatory effects of flavonoids are well established (Rahimifard et al., 2017). For example, in an *in vivo* model, apigenin showed a significant reduction in severity of experimental autoimmune encephalomyelitis (EAE) progression and in relapses observed in C57BL/6 (progressive) and SJL/J (relapse-remitting) mouse models of MS, where apigenin modulated microglial activation via inhibition of STAT1-induced CD40 expression (Rezai-Zadeh et al., 2008). In addition, a recent study using the human induced pluripotent stem cell (hiPSC) model of familial and sporadic AD to assess

the neuroprotective activity of apigenin, demonstrated that hiPSC-derived AD neurons exhibited a hyper-excitable calcium signalling phenotype, elevated levels of nitrite, increased apoptosis, reduced neurite length and increased susceptibility to inflammatory stress challenge from activated murine microglia, in comparison to neurons derived from healthy controls. In this study, they identified that the flavonoid apigenin had potent anti-inflammatory effects with the ability to protect neurites and cell viability by promoting a down-regulation of cytokine and nitric oxide (NO) release in inflammatory cells (Balez et al., 2016).

Neurotrophins (NTFs) are endogenous peptides secreted from neurons and glial cells, and are associated with regulating brain function, survival, and development of individual cells and neuronal networks across the entire brain. Specifically, NTFs regulate synaptic plasticity, protect neurons from apoptosis, and stimulate neurogenesis (Skaper, 2012, Leal et al., 2015, Kuipers et al., 2016, Wurzelmann et al., 2017). The neuroprotective effect of agathisflavone was associated with the increase of the NTFs BDNF, NGF, CDNF, NT4 and GDNF. Survival signaling is important to suppress apoptosis and counterbalance death signaling in the nervous system. Several studies have shown that polyphenolic compounds enhance neuronal survival and increase the expression levels of NTFs (Moosavi et al., 2016). For example, apigenin reverses depression-like behavior induced by chronic corticosterone treatment in mice. In addition, the administration of apigenin ameliorated the levels of BDNF in corticosterone-treated mice. The antidepressant-like effects of apigenin have been suggested to be mediated, at least partly, by up-regulation of BDNF levels in the hippocampus (Weng et al., 2016). BDNF, the main neurotrophin expressing in the central nervous system, has an important role in promoting survival, growth and synaptic plasticity in the CNS (Huang and Reichardt, 2001).

Astrocytes, earlier presumed to serve as supportive roles for the neuronal network, have recently been shown to play an active role in the synaptic dysfunction, impairment of homeostasis, inflammation, as well as excitotoxicity in relation to several neurological disorders (Dezsi et al., 2015). The role of astrocytes in the neuroprotective effects of agathisflavone against glutamate-induced excitotoxicity was evaluated. In physiological conditions to prevent over-stimulation, glutamate is removed from the synaptic cleft by astrocytes through EAAT located in the plasma membrane and converted through the action of GS to L-glutamine, which is released

to the extracellular fluid and taken up by neurons (Danbolt, 2001, Hazell et al., 2001, Walton and Dodd, 2007). We demonstrated that treatment with agathisflavone after excitotoxicity induced by glutamate increased the levels of EAAT1 and GS in astrocytes, suggesting that an important neuroprotective effect of agathisflavone is via glutamate uptake and recycling in astrocytes.

In conclusion, we showed that treatment of co-cultures of neurons and glial cells with agathisflavone increases the neuronal population at least in part via ER. Furthermore, we demonstrate that agathisflavone has broad neuroprotective effects against glutamate induced excitotoxicity, associated with anti-inflammatory effects on microglia and increased expression of neuroprotective cytokines and trophic factors. Taken together, these data suggest that agathisflavone could be a potential agent for treatment of excitotoxicity-related diseases.

Conflict of interest and funding

The authors report no conflicts of interest, including personal or financial.

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