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The Seed Extract of *Cassia obtusifolia* Offers Neuroprotection to Mouse Hippocampal Cultures

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Abstract. The precise causative factors in neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's disease remain elusive, but mechanisms implicated comprise excitotoxicity, mitochondrial dysfunction, and in the case of AD, the amyloid beta peptide (Aβ). Current therapeutic strategies for such disorders are very limited; thus, traditional herbal medicines currently receive increased attention. The seeds of *Cassia obtisufolia* have long been used in traditional eastern medicine and more recently the ethanolic fraction of the seeds (COE) has been shown to attenuate memory impairments in mice. In this study, we set out to determine the effect of COE (range: 0.1 – 10 μg/ml) on calcium dysregulation and cell death models in mouse primary hippocampal cultures implicated in general neurodegenerative processes and in the pathogenesis of AD: excitotoxicity, mitochondrial dysfunction, and Aβ toxicity. It was found that treatment with COE attenuated secondary Ca^{2+} dysregulation induced by NMDA (700 μM), while a pre-application of COE also reduced NMDA-induced cell death. Furthermore, COE was neuroprotective against the mitochondrial toxin 3-NP (1 mM), while having no significant effect on cell death induced by incubation with naturally-secreted oligomers of Aβ (8.2 pg/ml). Collectively, these results are important for the therapeutic use of COE in the treatment of neurodegenerative disorders.

Keywords: Alzheimer's disease, amyloid, excitotoxicity, mitochondria, hippocampus

Introduction

Neurodegenerative diseases represent a large group of neurological disorders with heterogeneous clinical and pathological expressions that affect specific subsets of neurons in defined functional anatomic systems. The precise causal factors involved in the progression of neurodegeneration remain often unknown, and in the few cases where they have been identified (e.g., the mutated Huntingtin gene in HD), the mechanisms by which they initiate cell death remain elusive.

One of the central processes implicated is excitotoxicity, caused by excessive release of excitatory amino acids such as glutamate (for a review, see ref. 1). Glutamate, the major excitatory neurotransmitter in the mammalian brain, acts via ionotropic AMPA, kainate, and NMDA receptors and metabotropic receptors (2), thus playing a vital role in fast neurotransmission as well as plastic events. Conversely, the continuous or over-activation of NMDA and group I mGluR receptors by glutamate eventually leads to an overload of cellular calcium and subsequent cell death.

Neuronal function and survival, and indeed susceptibility to excitotoxicity, depend heavily on a continuous supply of ATP generated from glucose and oxygen via mitochondrial respiration, essential for the maintenance of high metabolic activity and the requirement to sustain ionic homeostasis (for a review, see ref. 3). The age-

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related decline in energy metabolism has been directly linked to neuronal loss during normal aging and in neurodegenerative disorders (4, 5) and thus is likely to be the basis for ageing as the main risk factor in a range of neurodegenerative disorders.

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder (6), and its pathological hallmarks include *β*-amyloid (A*β*) plaques (7), dystrophic neurites associated with plaques (8), and neurofibrillary tangles composed of hyperphosphorylated tau protein (9). The gradual degeneration of nerve cells and the resultant loss of specific synaptic connections in the brain is the key pathological change associated with the emergence and progression of AD, with the amygdala, hippocampus, parahippocampal, and other adjacent cortical regions being particularly susceptible to such degeneration (for a review, see ref. 10). In vivo imaging of AD brains has indicated that glucose utilization is lower than in age-matched controls, while in vitro studies have shown that energy metabolism inhibition can elevate the levels of *β*-secretase and increase amyloidogenic processing of the amyloid precursor protein (11 – 14). Furthermore, the A*β* peptide itself has been shown to induce cell death in neurons in vitro (15 – 17). Soluble, naturally secreted forms of the peptide are reportedly more toxic and induce synaptic dysfunction and deficits in hippocampal long-term potentiation at low nanomolar concentrations in vivo and in vitro (18, 19).

While providing temporary alleviation of the symptoms associated with neurodegenerative disorders, the treatments currently available fail to target neurodegeneration directly, which has led to traditional herbal medicines receiving increased attention. The seeds of *Cassia obtusifolia* (Leguminosae), a plant widespread across North, Central, and South America; Asia; Africa; and Oceania, have been used in traditional Korean, Japanese, and Chinese medicine to treat eye inflammation, photophobia, and lacrimation (20), in addition to dysentery, headache, and dizziness (21). Furthermore, *Cassia obtusifolia* extract (COE) has been reported to have an anti-*Helicobacter pylori* effect, inhibitory actions on the growth of *Clostridium perfringens* and *Escherichia coli*, estrogenic effects, and inhibitory effects on histamine release from mast cells and platelet aggregation (22 – 25). A recent study also reported that *Cassia obtusifolia* can attenuate memory impairments in mice induced by scopolamine administration or transient bilateral common carotid artery occlusion and that these effects were mediated via acetylcholinesterase inhibition (26).

To date, no studies have been undertaken to investigate the cellular actions and potential neuroprotective properties of COE. The present study investigated the protection offered by the ethanolic fraction of COE in cell death models in mouse primary hippocampal cultures implicated in general neurodegenerative processes and in the pathogenesis of AD: excitotoxicity, mitochondrial dysfunction, and A*β* toxicity.

Materials and Methods

Compounds

The seeds of *Cassia obtusifolia* were obtained from a herbal supplier in Seoul (Korea), and voucher specimens (KHUOPS-04-31) were deposited at the herbarium of the College of Pharmacy, Kyung Hee University (Seoul, Korea). The material was authenticated by Emeritus Professor Chang Soo Yook of the Department of Oriental Pharmaceutical Science, College of Pharmacy, Kyung Hee University.

COE was the same as used in a previous study (26), where chemical profiling and standardization of COE had been performed. The extract was dissolved in 1% TWEEN-80, resulting in 0.001% or 0.01% solvent in working solutions. BACE inhibitor II (1 μM, *N*-benzyloxycarbonyl-Val-Leu-Leucinal; Calbiochem, Nottingham, UK) was dissolved in DMSO, while memantine (memantine hydrochloride; Tocris, Bristol, UK) and cycloheximide (Ascent Scientific, Weston-super-Mare, UK) were water soluble. All other reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

Cell culture

Hippocampal cultures: Hippocampal cultures were prepared from 3 – 4-day-old C57Bl/6 mice as described previously (27). Briefly, the brain was quickly removed, hippocampi were dissected and placed in ice-cold HEPES-buffered solution [HBS; composition: 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 25 mM D-glucose; (pH 7.4)]. The tissue was chopped and treated with protease solution (type XIV only, at 1 mg/ml HBS; Sigma) for 30 min, room temperature. Then it was subsequently washed in HBS and repeatedly triturated before double centrifugation. The resulting cell pellet was resuspended in Neurobasal medium (NB; Gibco, Paisley, UK) supplemented with 1% fetal bovine serum (Helena Biosciences, Sunderland, UK), 1% 2 mM L-glutamine, and 2% B27 (Invitrogen, Paisley, UK). This solution was then plated onto poly-L-lysine coated (0.02 mg/ml, Sigma) coverslips within 35-mm culture dishes (Gibco) and incubated for 60 min (37°C, 5% CO_2 / 95% O_2). The cultures were allowed to mature for 2 days prior to replacement of their medium with the previously

described supplemented NB medium plus 0.025% 25 μM L-glutamate. This tissue culture procedure produces mixed cultures that contain glia (approx. 60%, of these: oligodendrocytes <10%, microglia <10%, and astrocytes 80 – 90%) and neurons (approx. 40%).

N2a cells: Mouse Neuroblastoma N2a cells stably expressing an APP isoform (APP695) containing the NFEV sequence for increased BACE cleavage were cultured in standard Dulbecco's modified Eagles medium (DMEM, Gibco), supplemented with L-glutamine, penicillin, and streptomycin, at 37°C in a humidified incubator (5% CO_2 / 95% O_2). After 5 days in vitro (DIV), the medium was removed from the cultures, centrifuged to remove debris, and frozen at –20°C.

Acute excitotoxicity

Fura-2 AM Ca^{2+} imaging: Experiments were conducted using hippocampal cultures at 3 – 10 DIV. Cultures were incubated for 60 min in the dark at room temperature with 10 μM of the cell-permeable fluorescence Ca^{2+} indicator, Fura-2-AM (Cambridge Bioscience, Cambridge, UK), in HBS and perfused at a rate of approx. 2 ml/min. Under the microscope (40× objective; Olympus, Tokyo), a suitable field of cells was selected and images captured (Orca-ER CCD camera; Hamamatsu Photonics, Hamamatsu) using Openlab software (V5; Improvision, UK). Ratiometric imaging was conducted using alternating wavelengths of 340 and 380 nm, from a Xenon lamp regulated by a monochromator (DG-4 illumination system; Sutter Instruments Company, CA, USA) and an emission filter (wavelength 510 nm). Background levels of fluorescence were subtracted on line. The image acquisition rate was set to 5 s, and ratio values were plotted against time for multiple regions of interest (ROIs, neurons, and glia determined by morphological analysis and by fast neuronal responses to application of NMDA).

Drug application

Low-Mg^{2+} HBS (as HBS above but with 0.1 mM $MgCl_2$) was the standard perfusion solution used. After assessing neuronal recovery at various concentrations of NMDA, 700 μM NMDA applied for 5 min was considered suitable since a spectrum of neuronal recovery was apparent from complete recovery to secondary calcium dysregulation. The protective action of co-applied COE was studied at 1 and 10 μg/ml, with the extract present during an initial 5-min baseline stabilizing period, throughout the NMDA-insult (5 min), and for a further 40-min recovery period. For comparison, 10 μM memantine was also applied throughout an NMDA insult experiment.

Imaging data analysis

As in previous studies, graphical illustration and statistical analysis of Fura-2 fluorescence values were performed as % change in fluorescence relative to baseline fluorescence (% dF/F; e.g., in ref. 28). Initial NMDA peak $[Ca^{2+}]_i$ responses were measured in the presence and absence of COE or memantine. Calcium dysregulation, defined as a secondary or sustained rise in $[Ca^{2+}]_i$ after NMDA application, was also determined. Disrupted Ca^{2+} homeostasis may lead to apoptosis and neuronal degeneration (29) and is therefore an early key indicator of neuronal damage. The level of recovery of $[Ca^{2+}]_i$ post-NMDA was classified (as % of the total number of neurons) as either full recovery (i.e., $[Ca^{2+}]_i$ within ±10% of the original baseline value), up to 50% recovery (recovery to baseline of less than half the initial NMDA peak value), or no recovery (values above 50% of the peak value), determined 40 min after washout. The percentage of neurons showing early (from drug application to 10 min) or late (secondary, 20 to 40 min) calcium dysregulation (i.e., a secondary rise in $[Ca^{2+}]_i$) was also determined and compared across experimental groups.

Statistical analyses were performed by the GraphPad Prism statistics package (GraphPad Software, Inc., La Jolla, CA, USA) with a Kruskal-Wallis test due to the non-parametric distribution of data, followed by a Dunn's multiple comparison post-hoc test. Experimental groups were compared with controls for statistical significance using a Mann-Whitney test, in each category of analysis. Percentage responder rates were calculated as responders from total neurons selected as regions of interest (ROIs). These responding neurons were then used in order to calculate the percentages for each recovery stage (full, 50% or none), which were compared using the above methods.

Cell death protocols

Hippocampal cells from cultures between 3 – 10 DIV were selected and subdivided into treatment group categories, maintaining uniform culture quality, composition, and cell density. Cultures were treated with a variety of solutions containing test reagents applied to fresh standard media for a predetermined time span. All treatment solutions were allowed to temperature and gas equilibrate in the aforementioned incubated environment for a period of at least 30 min and were adjusted to physiological pH immediately prior to sterile-filtered application to cultures. To assess any adverse effects the extract may have alone, control dishes were incubated with COE for 72 h, as this represented the longest exposure protocol utilized in the study.

NMDA incubation

In addition to the assessment of acute NMDA effects on neuronal Ca homeostasis (see above), we assessed the cell death rates resulting from NMDA exposure 48 h after insult. The treatment medium consisted of 700 μM NMDA, 5 μM glycine, and 0.1, 1, or 10 μg/ml COE in low-Mg^{2+} HBS, to relieve the Mg^{2+} block of the NMDA receptors. COE was replaced with a similar volume of 1% TWEEN-80 solution in negative controls and memantine (10 μM) in positive controls. In the sterile, laminar airflow cabinet, the existing culture medium was removed and the dishes washed with a 1-ml application of the treatment medium, emptied, followed by a further 2-ml application to each dish. Cells were then incubated for a period of 1 h, before the treatment medium was washed off and replaced with minimum essential medium (MEM, Gibco) for a further 48 h before imaging commenced. Experiments were carried out with co-application and a 1-h pre-application of COE with the toxic dose of NMDA.

3-Nitropropionic acid (3-NP)

The treatment medium consisted of 1 mM 3-NP in supplemented NB medium (described above) with COE (0.1, 1, or 10 μg/ml), 1% TWEEN-80, memantine (10 μM), or cycloheximide (2 μM) depending on the experimental group. In the sterile, laminar airflow cabinet, the existing culture medium was removed and the dishes washed with a 1-ml application of the treatment medium, emptied, followed by a further 2-ml application to each dish. The cultures were then incubated for 72 h before staining.

β-Amyloid

Similar to previous studies highlighting the high potency of naturally excreted amyloid (18, 19), dishes were treated with 500 μl of Aβ-containing N2a medium in 2 ml of supplemented NB medium for 72 h. Analysis of the N2a medium via an ELISA (Biosource-Europe S.A., Nivelles, Belgium; n = 3) indicated an Aβ 1-40 level of 8.2 pg/ml and negligible Aβ 1-42 content. COE (1/10 μg/ml), vehicle (1% TWEEN-80), or memantine (10 μM) were also co-applied, depending on the experimental group.

To further confirm that Aβ production was responsible for hippocampal cell death, the medium of N2a cells cultured in the presence of BACE inhibitor II (1 μM, *N*-benzyloxycarbonyl-Val-Leu-Leucinal) was also tested. As a further control, the effect of unconditioned standard DMEM was also investigated. Additionally, in order to assess any effect of COE on Aβ production, N2a cells were cultured in the presence of the extract.

Cell viability determination

To assess cell viability, cultures were stained using a Calcein-AM/Propidium Iodide (PI) double cell staining kit (Sigma), as in previous investigations (30). This procedure allows the simultaneous staining of viable and dead cells, respectively, with calcein-AM generating a green fluorescence in viable cells, and PI labeling dead or dying cells with compromised cell membranes red by intercalating with DNA.

In darkness (to prevent bleaching), the treatment medium was removed from the dishes, the dishes washed 3 times with HBS, and calcein-AM/PI solution (10 and 2 μl, respectively, in 5 ml HBS) added. Covered, the dishes were left for 20 min before the calcein-AM/PI solution was removed and the dishes washed briefly with HBS. Finally, a further 2 ml of HBS was added to each dish for imaging.

Image acquisition and data analysis

As in previous investigations (30), cells were visualized using an Axioskop 2 plus microscope (Carl Zeiss, Germany) with a 40× phase contrast water immersion objective, and images captured using an AxioCam HRc camera controlled by AxioVision software. Neurons were readily distinguishable from glia as they appeared phase-bright, with smooth, rounded somata and distinct processes, and in a focal plane above that of the glial layer. A brightfield image was initially captured to allow identification of the cellular composition, followed by further images using FITC (for calcein-AM) and Rhodamine (for PI) filters (Fig. 3). Images were taken from three separate regions per dish and each experiment was conducted in triplicate in three different cultures, to ensure reproducibility. A manual count of Calcein-AM stained cells allowed determination of the number of live cells, while a count of PI-stained cells indicated the number of dead cells.

Cell viability, according to cell type, was calculated as relative survival. Statistics were calculated using GraphPad Prism® (Version 4.01, GraphPad Software). Mean survival rates and S.E.M.s were calculated for each group and cell type. Absolute survival rates were calculated for each control group, and both one- and two-way analysis of variance (ANOVA) were performed for within and between-group comparisons, respectively, followed by *post hoc* analyses (Tukey). Resultant *P* values were rendered as significant ($P<0.05$) or non-significant (ns). Degrees of significance were assessed by three different rating values: $P<0.05$ = *(significant), $P<0.01$ = **(highly significant), and $P<0.001$ = ***(extremely significant). For clarity, data in figures are expressed relative to their respective controls.

Results

The present study investigated cellular and neuroprotective properties offered by the ethanolic extract of the seeds of *Cassia obtusifolia* (COE) to primary mouse hippocampal cultures exposed to three models of cell death implicated in neurodegeneration: acute and endpoint studies related to excitotoxicity induced by NMDA, a model of mitochondrial dysfunction induced by incubation with 3-NP (31) and a Aβ-induced cell death resulting from incubation with medium from β-amyloid-producing N2a cells. Memantine, a drug currently licensed for AD treatment based on its ability to antagonize NMDA receptors, was used as a reference compound throughout, with the anti-apoptotic agent cycloheximide used as an additional positive control in the 3-NP experiments.

To probe for potentially damaging effects of COE itself, controls were conducted for all three concentrations used, over 72 h (the longest exposure protocol used throughout these studies). COE had no significant effect on cell survival or cellular morphology at any of the concentrations tested compared to non-treated control cultures (all P's>0.05). Neuronal survival was measured at 92.1%, 98.0%, and 94.6% for 0.1, 1, and 10 μg/ml respectively; and the corresponding glial survival was 96.5%, 97.7%, and 96.1%, respectively.

Excitotoxicity

Acute Ca^{2+} dysregulation: The application of 700 μM NMDA induced an initial NMDA response followed by varying degrees of recovery, alongside both primary and secondary dysregulation in hippocampal cultures. In controls, neurons (n = 97) showed initial peak NMDA responses of 246.2 ± 24.6 ΔF/F. During wash-out, 48.5% neurons failed to recover, 29.9% underwent at least 50% recovery, and the remaining 21.6% fully recovered to within 10% of the original baseline (Figs. 1 and 2). Among the controls, 92.2% underwent primary and 35.7% underwent late, secondary calcium dysregulation.

In the presence of COE (1 and 10 μg/ml), the amplitude of the maximum peak calcium response to 700 μM NMDA was slightly raised (Fig. 2A), but this change was not statistically significant (P's>0.05). As expected, the NMDA receptor channel–blocker memantine (Fig. 2C) significantly reduced the amplitude of the NMDA peak calcium response at a concentration of 10 μM (P<0.05).

A comparison of positive recovery parameters (full and 50% recovery, Fig. 2B.) indicated no significant difference between controls, the herbal extract COE or memantine (P's>0.05). Memantine-treated neurons (n = 56) had a trend for a higher recovery rate, both full and 50% compared with controls, although not statistically significant. The 1 μg/ml COE (n = 68) experiment

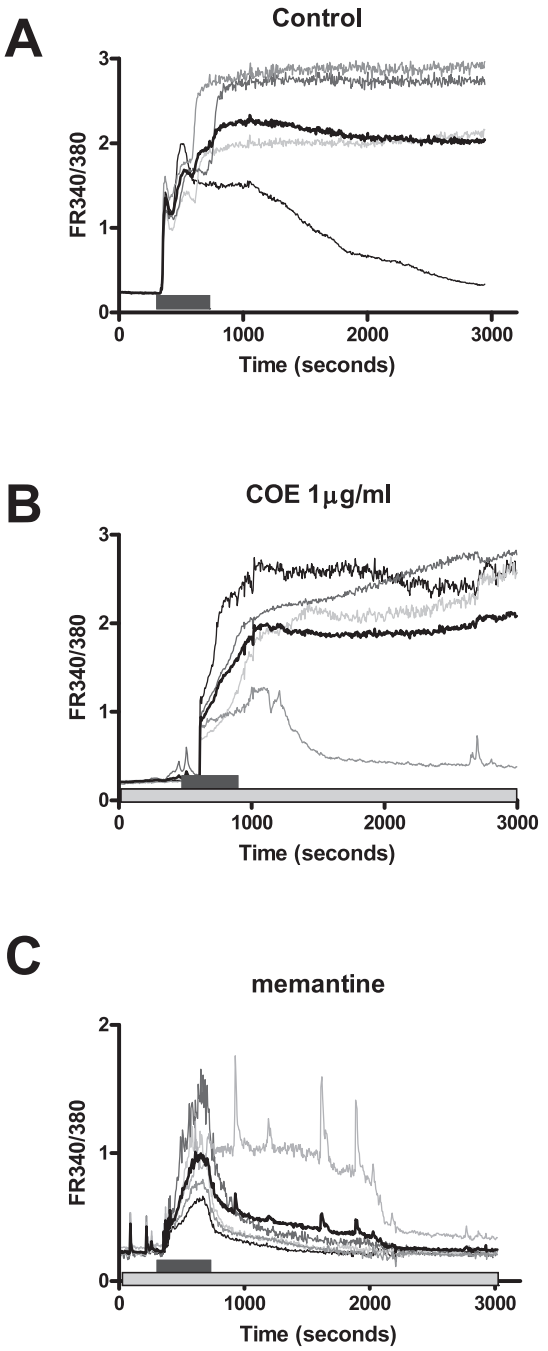

Fig. 1. NMDA insult and Ca^{2+} dysregulation: Acute application (5 min) of 700 μM NMDA to hippocampal cultured neurons. Sample timecourses in controls (A, n = 4 ROIs); for NMDA applied in the presence of COE (B, 1 μg/ml, n = 4 ROIs); and in the presence of 10 μM memantine (C, n = 5 ROIs). Each trace shows representative $[Ca^{2+}]_i$ levels (in greyscale) as % ΔF/F versus time, along with the mean for an overall experiment (black line). Application of NMDA is indicated by a black bar, and the continuous presence of COE and memantine, respectively, is illustrated by a grey bar.

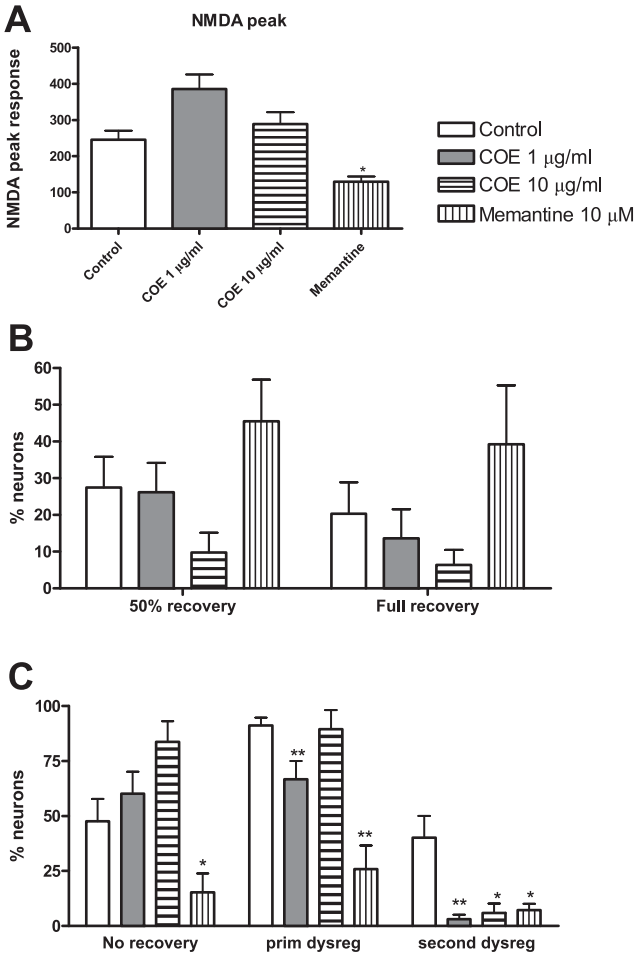

Fig. 2. Mean effects of COE and memantine on neuronal NMDA-induced calcium responses, neuronal recovery rates, and incidence of dysregulation. A: NMDA peak response across experiments, expressed as percentage of the original baseline (% $\Delta F/F$). B: Pooled data from neurons recovering to below 50% of the maximum response and neurons returning to within 10% of the original baseline (full recovery). C: Percentage of cells that fail to recover after a 700-μM NMDA insult, and undergo primary or secondary calcium dysregulation. **P*<0.05, ***P*<0.01. Control, n = 97; COE, 1 μg, n = 68; COE, 10 μg, n = 61; memantine, n = 56.

displayed comparable values to controls with 13.6% undergoing full recovery and 26.2% showing >50% recovery, while there was a non-significant trend for reduced recovery in the 10 μg/ml COE group (n = 61).

Figure 2C displays negative parameters measured in this paradigm. The beneficial effects of 10 μM memantine are clearly seen with a statistically significant reduction in cells showing no recovery (24.3%) compared with the control (*P*<0.05). Memantine also reduced primary (*P*<0.01) and secondary calcium dysregulation (*P*<0.05). Interestingly, although the COE extract did not alter the proportion of neurons failing to recover, 1 μg/ml COE treatment significantly reduced the level of primary dysregulation (62.8%) compared to controls (92.2%, *P*<0.01). Furthermore, secondary dysregulation, assumed to be a pre-apoptotic event (32), was reduced by memantine (*P*<0.05), COE 1 (*P*<0.01), and 10 μg/ml (*P*<0.05), compared with the occurrence rates in control neurons.

NMDA-induced cell death study

NMDA was applied to cultures in low-Mg^{2+} HBS to relieve the voltage-dependent Mg^{2+} block of the NMDA-receptor ion channel and in the presence of the receptor co-agonist glycine to aid channel opening. Compared to control dishes stained with PI and Calcein-AM (Fig. 3A), incubations with NMDA (Fig. 3B) resulted in a cell survival rate of 62.33% in neurons and 77.26% in glial cells, significantly lower than non-treated sister cultures where survival rates were 90.5% and 92.4%, respectively (Fig. 4: A and B, both *P*<0.001). Incubation with low-Mg^{2+} HBS alone had no significant effect on survival of either cell type (both *P*'s>0.05), indicating that NMDA was responsible for the cell death seen. Initially, COE was co-applied with the toxic dose of NMDA (Fig. 4: A and B), and under these conditions the extract was found to provide no significant protection compared to the control at concentrations of 0.1, 1, or 10 μg/ml. Survival rates were 69.0%, 67.8%, and 65.8% in neurons and 80.4%, 70.6%, and 77.1% in glia for 0.1, 1, and 10 μg/ml respectively (all *P*'s>0.05). In comparison, the non-competitive NMDA-receptor antagonist memantine offered protection from NMDA-mediated cell death, with survival rates of 81% in neurons (*P*<0.01) and 87.2% in glia (*P*<0.05).

Since lipophilic compounds such as the extract used here have slow access to relevant binding sites, and putative cellular targets may even be found intracellularly, it was next investigated whether a 1-h pre-application of the extract would offer better protection against cell death (Fig. 4: C and D). Control-treated dishes were not significantly different from the co-application experiment; cell survival was 62.2% in neurons and 75.5% in glia. A pre-application of the cultures with 1 μg/ml COE provided significant neuroprotection with 77.9% survival observed (*P*<0.01) and also slightly, albeit insignificantly, enhanced survival rates for glia (survival of 81.72%, *P*>0.05). However, at the higher concentration of 10 μg/ml and lower concentration of 0.1 μg/ml, COE offered no significant protection against excitotoxic cell death; the survival was 62.7% and 67.9% in neurons and 80.0% and 77.0% in glia respectively (all *P*'s>0.05). In the positive control, memantine provided significant protection to neurons (survival of 77.5%, *P*<0.01) and glia (survival of 87.7%, *P*<0.05), with the level of neuroprotection identical to levels in dishes treated with 1 μg/ml COE.

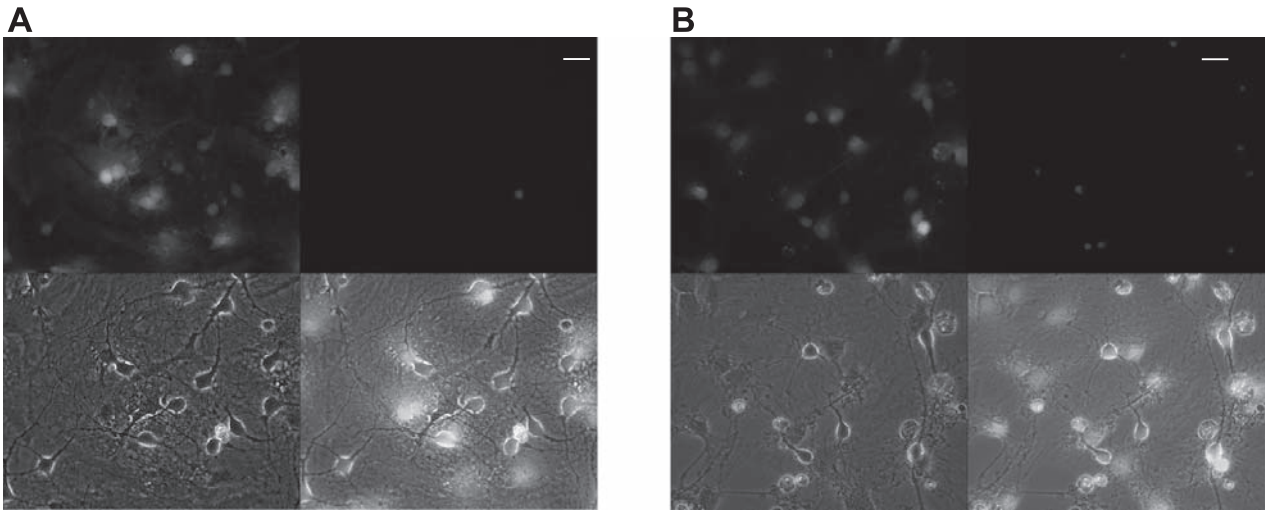

Fig. 3. Sample images from a non-treated control dish (A) and NMDA-treated dish (B). Images show calcein-AM–stained cells (top left), PI-stained cells (top right), a brightfield image of the dish (bottom left), and an image of all three channels superimposed (bottom right). Scalebar indicates 50 μm.

Mitochondrial dysfunction

Incubations with the mitochondrial succinate dehydrogenase inhibitor 3-NP in mouse hippocampal cultures (1 mM for 72 h) resulted in a mean survival rate of 67.5% in neurons and 88.7% in glia (Fig. 5: A and B). Neuronal survival was significantly lower than in non-treated dishes (survival: 90.6%, $P<0.001$), while glial cell survival not statistically different to that in non-treated dishes (survival 92.4%, $P>0.05$), indicating that 3-NP at this dose was selectively toxic to neurons. Co-application of 1 μg/ml COE with 3-NP provided complete neuroprotection ($P<0.001$) with a survival rate of 87.9% observed, a value not significantly different to non-treated controls ($P>0.05$). At 94.0%, glial cell survival was not significantly different from the control. Co-application of 0.1 μg/ml COE had no significant effect on cell survival, neuronal survival was 70.8% and glial survival was 89.4% (both P's>0.05). Treatment with the NMDA-receptor antagonist memantine also failed to significantly affect survival of both neurons and glia, with survival rates of 76.5% and 92.7% recorded, respectively (both P's>0.05). The level of neuronal survival in memantine-treated dishes was also significantly lower than in dishes treated with 1 μg/ml COE ($P<0.01$). Co-application of the anti-apoptotic agent cycloheximide (2 μM) with 3-NP provided complete neuroprotection ($P<0.001$), with 91.9% of neurons and 96.7% of glia surviving. The level of cell survival was not significantly different from non-treated controls or dishes treated with 1 μg/ml COE (all P's>0.05). Application of 2 μM cycloheximide alone had no significant effect on cell survival (data not shown; $P>0.05$, compared to non-treated controls).

β-Amyloid toxicity

Similar to 3NP, Aβ-containing medium was selectively toxic towards neurons compared to glia in control dishes, with mean survival rates of 71.4% and 93.7%, respectively (Fig. 6: A and B). ELISA analysis of this medium indicated a Aβ 1-40 content of 8.2 pg/ml and a Aβ 1-42 content below detectable limits (Fig. 6C). Application of standard DMEM to the primary cultures did not result in any significant effect on neuronal or glial survival (survival of 96.4% and 98.5%, respectively), while incubation of the N2a cells with the commercially available BACE inhibitor II (1 μM) significantly reduced the neurotoxicity of the resultant medium (survival of 89.5%; $P<0.001$, compared to amyloid control), positively identifying Aβ as the toxic moiety in the N2a supernatant responsible for the neuronal death witnessed. This was again confirmed by an ELISA, with levels of Aβ below detectable limits in medium from BACE inhibitor II–incubated N2a cells (Fig. 6C). COE had no significant effect on Aβ toxicity at either 1 or 10 μg/ml. Neuronal survival was measured to be 75.6% and 77.2%, respectively, while glial survival was 88.11% and 95.78%, respectively (all P's>0.05). Similarly, memantine had no significant effect on cell death rates, with a mean survival rate of 74.33% recorded in neurons and 93.22% in glia.

In an attempt to assess any potential effect of the extract on secretases or any other aspect of Aβ production, N2a cells were grown in the presence of COE (1 and 10 μg/ml) and the levels of cell death in the mouse hippocampal cultures investigated after subsequent application of the N2a medium. Neither neuronal nor glial cell survival (72.47% and 94.55%,

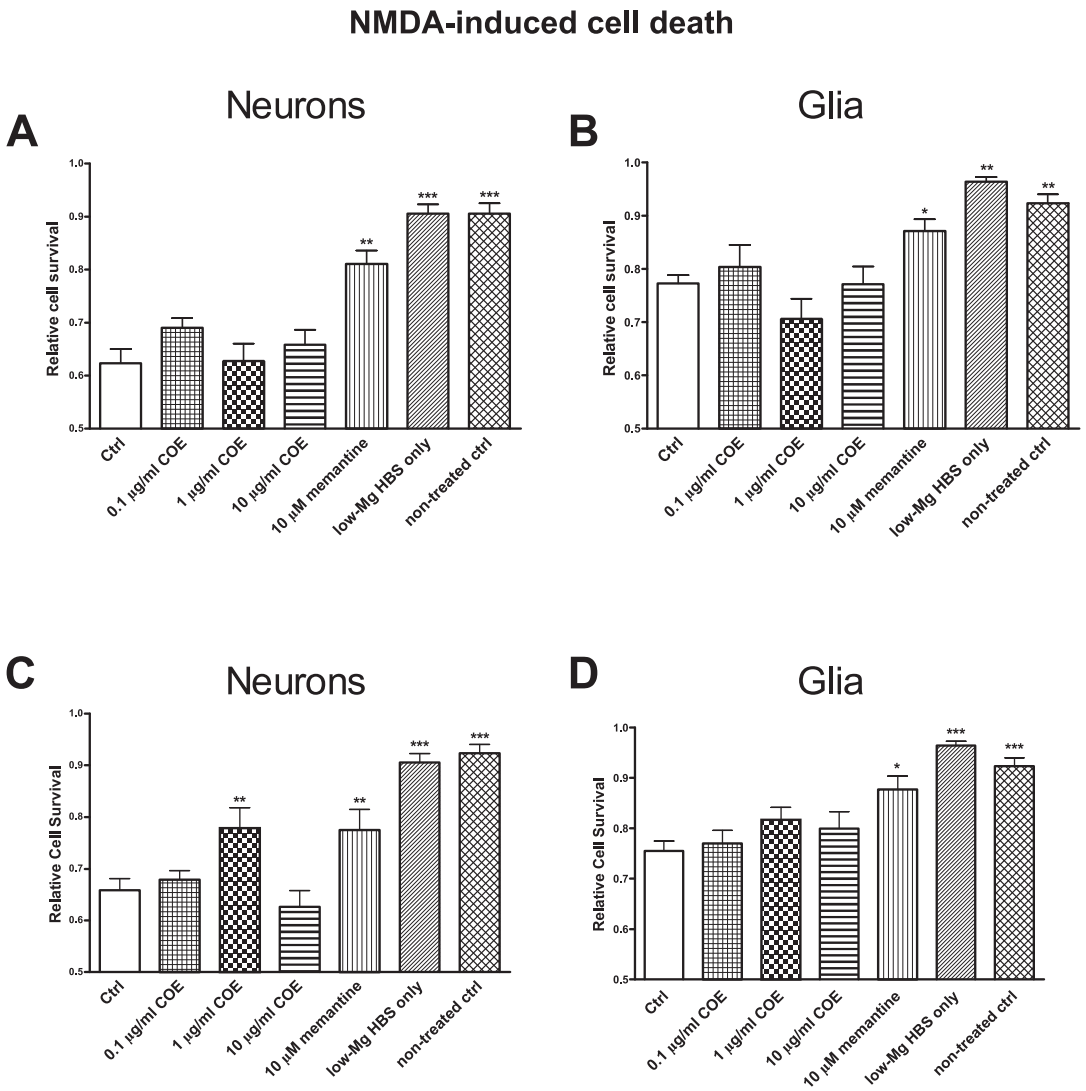

Fig. 4. Relative cell survival of neurons and glia in response to 700 μM NMDA (control n = 26) with co-application (A and B) and pre-application (C and D) of COE (0.1, 1, and 10 μg/ml, all n = 9) or memantine (10 μM, n = 9). When co-applied with NMDA, COE failed to provide any significant protection to either neurons or glia (all P>0.05). Only memantine significantly increased neuronal survival (**P<0.01). When present for 1 h prior to NMDA application, 1 μg/ml COE and 10 μM memantine significantly increased neuronal survival (**P<0.01). Memantine (10 μM) also significantly increased glial survival compared to the control (*P<0.05).

respectively) was statistically different from their control, indicating that COE had no effect on Aβ production (both P's>0.05). ELISAs of the N2a medium confirmed that COE did not significantly attenuate Aβ production with levels of 8.5 and 9.3 pg/ml Aβ 1-40 in cultures treated with 1 and 10 μg/ml, respectively (Fig. 6C).

Discussion

The present study investigated for the first time the neuroprotective properties of COE in mouse primary hippocampal cultures. It was found that treatment of hippocampal cultures with COE attenuated cell death and secondary Ca^{2+} dysregulation induced by NMDA and 3-NP, while having no significant effect on cell death induced by incubation with naturally secreted Aβ. Collectively, these results are relevant with regards to a potential therapeutic use of COE in the treatment of neurodegenerative disorders. They also highlight important differences between amyloid-induced cell death versus pathways triggered by excitotoxicity and mitochondrial stress.

Traditionally, the seeds of *Cassia obtusifolia* have been used to treat eye inflammation, headache, dizziness, and dysentery and more recently have been shown to possess larvicidal activity and potent peroxynitrite

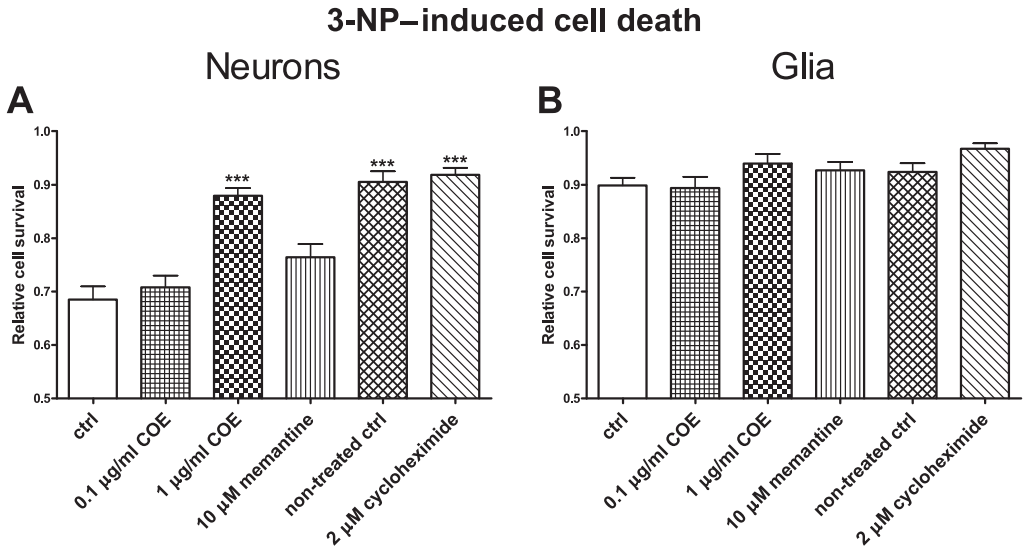

Fig. 5. Relative cell survival of neurons (A) and glia (B) in response to the mitochondrial toxin 3-NP (1 mM, control: n = 20) with co-application of COE (0.1 and 1 μg/ml, both n = 9), memantine (10 μM, n = 9), and cycloheximide (2 μM, n = 9). 1 μg/ml COE provided significant neuroprotection (***P<0.001) as did 2 μM cycloheximide (***P<0.001). No treatment significantly affected glial survival compared to the control.

scavenging activities (21 – 25, 33). Furthermore, it has recently been reported that COE attenuated memory impairment induced by scopolamine and carotid artery occlusion in passive avoidance, Y-maze, and Morris water maze tests in mice (26); and the purgative resin, emodin, contained in *Cassia* species can ameliorate tau protein aggregation (34).

Here, we tested cell death models specific to AD but also of relevance in a range of CNS disorders. Excitotoxic cell death due to over-activation of neuronal NMDA receptors has been implicated in the widespread neurodegeneration witnessed in AD, as well other neurodegenerative disorders and ischemia (35, 36). In the present study, COE significantly protected neurons from excitotoxic cell death to a level comparable with the NMDA-receptor antagonist memantine and significantly reduced the percentage of neurons undergoing secondary calcium dysregulation, while having no significant effect on peak NMDA responses or neuronal recovery. It is therefore unlikely that COE has a direct effect on NMDA receptors; rather, it functions downstream of NMDA-receptor activation, in line with the hypothesis that Ca^{2+} dysregulation involves intracellular stores and signaling cascades (29).

The toxic action of 3-NP is also of interest since mitochondrial dysfunction may contribute to age-related neurodegenerative disorders, excitotoxic events, and the amyloidogenic processing of the amyloid precursor protein (4, 5, 11 – 14). Previous studies indicate that in neurons 3-NP induces three interacting processes: metabolic impairment, excitotoxicity, and oxidative stress that combine to result in cell death via apoptotic and necrotic pathways (31, 37 – 39). Under our conditions, 3-NP resulted in significant neuronal (but not glial) death which was completely reversible by the anti-apoptotic agent cycloheximide but not preventable by co-incubation with the NMDA-receptor antagonist memantine, suggesting that 3-NP was inducing cell death predominantly by apoptosis. Here, COE provided complete neuronal protection when co-applied with 3-NP, indicating that COE can either act directly on mitochondria or inhibit pathways downstream of mitochondrial dysfunction, or act to inhibit apoptotic cell death. This finding is particularly surprising when considering a previous report that COE had a detrimental effect on muscle mitochondria (40), which may indicate cell type specificity, or a concentration-dependent toxicity effect (see below).

Elucidating the exact cellular mechanism of action of COE is outside the scope of the present study, and it can only be speculated whether COE acts at a common point downstream of NMDA-receptor activation and succinate dehydrogenase inhibition or whether multiple active ingredients within the extract are inhibiting distinct pathways involved in cell death. Indeed, the very nature of herbal extracts means it is impossible to attribute actions seen to a specific compound, given the number of pharmacologically active substances in such preparations and their potentially complex interactions. Thin layer chromatography analysis of COE has shown it to be a complex mixture of compounds, with over 50 constituents isolated (41). Like other *Cassia* species,

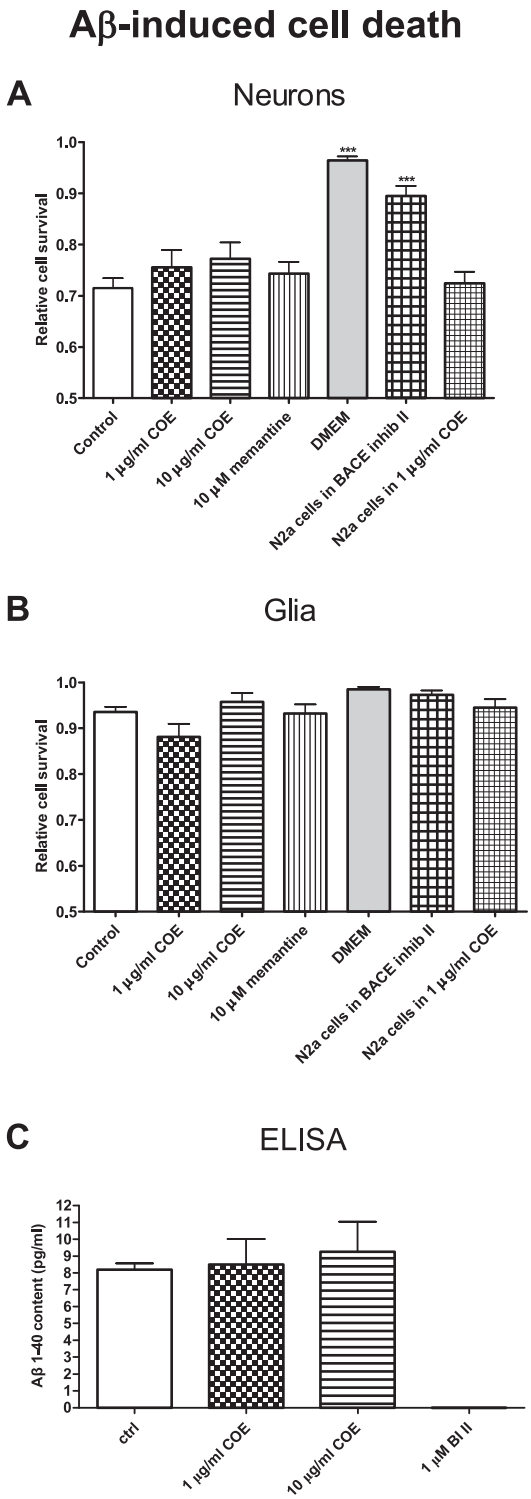

Fig. 6. Relative cell survival of neurons (A) and glia (B) in response to treatment with Aβ produced by N2a cells (control, n = 23) with the co-application of COE (1 and 10 μg/ml, both n = 9) and memantine (10 μM, n = 9). Neither COE nor memantine had any significant effect on the survival of neurons or glia (all *P*'s>0.05). When N2a cells were grown in the presence of BACE inhibitor II (1 μM, n = 9) the medium was not significantly toxic when subsequently applied to hippocampal cultures. Similarly, DMEM (N2a medium) was not in itself toxic (n = 9, both *P*'s<0.001, cf. untreated N2a medium). COE (1 and 10 μg/ml, both n = 9) also had no significant effect on the production of Aβ by N2a cells (both *P*>0.05). C: Aβ 1-40 content of N2a medium used to induce cell death as measured by ELISA. The peptide was present at a mean concentration of 8.2 pg/ml in control medium and was below detectable limits when N2a cells were incubated in the presence of BACE inhibitor II. Growing of N2a cells in the presence of COE did not alter the production of Aβ 1-40 at concentrations of 1 or 10 μg/ml (*P*'s>0.05).

COE contains a large amount of anthraquinones including chrysophanic acid, physcion, obtusifolin, emodin, and questin, the vast majority of which exist as di- or tri-glycosides. Interestingly, emodin derivatives have previously been shown to provide neuroprotection from NMDA and glutamate in retinal ganglion cells and rat cortical neurons via antioxidant mechanisms (42, 43). Furthermore, the antraquinone alaternin from the related *Cassia tora* species has been shown to be a potent peroxynitrite and hydroxyl radical scavenger (33, 44, 45). Other major constituents of COE include chlorophyll-related compounds, β-sitosterol, caffeic acid, and other as-yet-unidentified flavonoids (41). Flavanoids are phenolic compounds with anti-mutagenic, anti-carcinogenic, and anti-ageing properties thought to stem from their anti-oxidant and free-radical scavenging activities (46 – 49). Thus, unidentified flavanoids could be responsible for the neuroprotective properties of COE witnessed in the present study.

It should also be noted that only a concentration of 1 μg/ml COE provided significant protection against excitotoxicity and 3-NP. Lower concentrations of COE were ineffective in these models, while in cultures treated with 10 μg/ml COE, there was even an increased proportion of neurons that were unable to recover from treatment with NMDA in calcium imaging experiments, indicative of detrimental effects of COE at higher concentrations. Although treatment with COE alone had no significant effect on cell survival, such non-linear dose-response curves have been described for other herbal compounds such as Kava and Ginkgo biloba extract, with pro-oxidant and pro-apoptotic effects observed at higher concentrations (50, 51). While such effects are also typical of pure antioxidative drugs such as vitamin C and R-apomorphine (52, 53), the complex pharmacology of component mixtures found in extracts calls for an analysis of its individual components.

With regards to cell death induced by Aβ, we confirmed here the highly toxic nature of naturally secreted, soluble Aβ compared to the synthetic peptide and fibrillary forms of Aβ (54 – 60). In the present study, naturally secreted Aβ was used to induce cell death in primary hippocampal cultures, in line with previous

studies where Aβ in this form was highly neurotoxic, induced synaptic dysfunction, and deficits in hippocampal long-term potentiation at concentrations similar to those used here (18, 19). Although the exact mechanism of Aβ toxicity is not fully understood, increased intracellular calcium concentration, reactive oxygen species production, altered signalling pathways, mitochondrial dysfunction, and excitotoxicity have all been implicated (61 – 65). The magnitude of the damage contributed by each of these factors and the extent of their interaction remain unresolved. In the present study, COE failed to significantly attenuate the toxicity of Aβ to neurons in culture. Considering that COE provided significant neuroprotection from excitotoxicity and complete protection from mitochondrial dysfunction, their respective contribution to the mechanism of Aβ toxicity under our conditions are likely to be negligible.

The hippocampal cultures utilized in the current study were of mixed cell type, containing neuronal and glial cells. Interestingly, incubation of cultures with 3-NP or Aβ did not result in significant toxicity to glial cells, while incubation with NMDA did. In the adult brain, glia function to provide support and protection to neurons, supplying nutrients and oxygen, destroying pathogens and removing dead cells. Furthermore, more recent studies have indicated that glia are also active participants in synaptic transmission, regulating clearance of neurotransmitter from the synaptic cleft, releasing factors such as ATP that modulate presynaptic function, and even releasing neurotransmitters themselves. Following 3-NP administration, there was no significant glial cell death in the present study as reported previously (ref. 38, but also see refs. 66 – 70). Due to their relatively higher metabolic rate, neurons are heavily reliant on functional mitochondria and therefore are exquisitely sensitive to metabolic impairment. Glial cells contain a higher concentration of glutathione, a major antioxidant in tissue defense against oxidative stress (71, 72) and are assumed not to possess functional NMDA receptors (73). Thus, they are overall more resistant to insults such as hypoxia/ischemia than neurons (74) and survive exposures to NMDA and AMPA at concentrations that completely destroy cortical or hippocampal neurons (75 – 77). Here, the death of these support cells is likely to be secondary to the release of glutamate, calcium, and free radicals by compromised neurons. Indeed, application of memantine proved to be protective to both neurons and glia and previous studies attribute glial NMDA responses to indirect glutamate release following activation of neuronal NMDA receptors (78).

In summary, the present study demonstrates an ability of COE to significantly attenuate toxicity in models of excitotoxicity and particularly in mitochondrial dysfunction in hippocampal cultures, pointing to a role in the regulation and maintenance of cellular homeostasis and apoptosis. Our findings are relevant for future therapeutic considerations related to COE or one of its active ingredients in the treatment of neurodegenerative disorders.

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