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Neuroprotective Properties of *Valeriana officinalis* Extracts

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Valeriana officinalis **have been used in traditional medicine for its sedative, hypnotic, and anticonvulsant effects. There are several reports in the literature supporting a GABAergic mechanism of action for valerian. The rationale of the present work is based on the concept that by decreasing neuronal network excitability valerian consumption may contribute to neuroprotection. The aim of our investigation was to evaluate the neuroprotective effects of *V. officinalis* against the toxicity induced by amyloid beta peptide 25-35 [$A\beta_{(25-35)}$]. Cultured rat hippocampal neurons were exposed to $A\beta_{(25-35)}$ (25 μM) for 24-48 h, after which morphological and biochemical properties were evaluated. The neuronal injury evoked by Aß, which includes a decrease in cell reducing capacity and associated neuronal degeneration, was prevented by valerian extract. Analysis of intracellular free calcium ($[Ca^{2+}]_i$)indicated that the neuroprotective mechanisms may involve the inhibition of excess influx of Ca^{2+} following neuronal injury. Moreover, membrane peroxidation in rat hippocampal synaptosomes was evaluated, and our data indicate that valerian extract partially inhibited ascorbate/iron-induced peroxidation.**

In conclusion we show evidence that the signalling pathways involving $[Ca^{2+}]_i$ and the redox state of the cells may play a central role in the neuroprotective properties of *V. officinalis* extract against Aß toxicity. The novelty of the findings of the present work, indicating neuroprotective properties of valerian against Aß toxicity may, at the long-term, contribute to introduction of a new relevant use of valerian alcoholic extract to prevent neuronal degeneration in aging or neurodegenerative disorders.

Keywords: Valeriana officinalis; Amyloid-beta peptide; GABA; Intracellular calcium; Synaptosomes; Neuronal degeneration; Aging; Hippocampal neurons; Cell culture; Neuroprotection;

INTRODUCTION

The research of physiological and pathological brain aging is a special relevant scientific and economical issue with particular preponderance in Western developed countries. As a consequence of the increase in the life span, a better understanding of the physiological and pathological mechanisms involved in brain aging may aid in preventing neurological diseases and thereby increase the quality of life in the elderly.

Alzheimer's disease is among the most prevalent neurological diseases in aged patients. Important landmarks of the disease include deposit of amyloid plaques, oxidative stress and neuronal degeneration (Behl and Moosmann, 2002). The biological cause of the initiation and progress of the disease has not been identified but consensus exists that it may involve abnormal proteolytic cleavage of the amyloid precursor protein and the formation of amyloid beta peptides [1-40 and 1-42: $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$] (Behl and Moosmann, 2002). It is well known that these amyloidogenic peptides induce neuronal degeneration. Consequently a toxic sequence of the peptides (25-35) [$A\beta_{(25-35)}$] has been used for several years in the investigation of mechanisms involved in Aß peptide toxicity (Pereira *et al.*, 2000).

As a consequence of the increasing prevalence of the aged population the identification of neuroprotective drugs or products able to prevent both physiological and Aß-induced neuronal degeneration may represent important health tools and economic resources. In recent years many research groups have been investigating neuroprotective properties of plant extracts and their active compounds. Some of the most popular neuroprotective plant extracts and derivatives include *Ginkgo biloba* (Luo, 2001; Cheung *et al.*, 2002; Lee *et al.*, 2002; Ahlemeyer and Krieglstein, 2003), Ginseng (Salim *et al.*, 1997), *Hypericum perforatum* (Cheung *et*

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al., 2002; Silva *et al.*, 2004), green tea (Levites *et al.*, 2003; Mandel *et al.*, 2003) and red wine (Virgili and Contestabile, 2000).

Valerian root extracts have been used for centuries in popular medicine for the treatment of anxiety, epilepsy, and sleep disorders (Hadley and Petry, 2003). Part of the biologically active properties of valerian extracts may include increased release of γ-aminobutyric acid (GABA) and increased activation of $GABA_A$ receptors (Santos *et al.*, 1994; Marder *et al.*, 2003). By decreasing the overall excitability of the neuronal brain circuits and consequently by preventing excitotoxic neuronal death, the increase in function of inhibitory neurotransmitter systems may result in neuroprotection. A similar rationale was used by our group to identify neuroprotective effects of neuropeptide Y activation against excitotoxic insults (Silva *et al.*, 2003).

In the present work we investigated the putative neuroprotective and antioxidant properties of valerian extracts against neurotoxicity induced by $A\beta_{(25-35)}$ in cultured embryonic rat hippocampal neurons.

METHODS

Rat Embryonic Hippocampal Neurons in Culture

Animals were used and handled in accord with the ethical guidelines from the European Union. Rat embryonic hippocampal neurons were isolated as previously described (Brewer *et al.*, 1993) with some modifications (Ambrósio *et al.*, 2000). In brief, hippocampal neurons were dissociated from hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin (2.0 $mg.ml^{-1}$, 15 min, 37ºC) and deoxyribonuclease I (0.15 $mg.ml^{-1}$) in Ca^{2+} and Mg^{2+} free Hank's balanced solution (137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM $Na_2HPO_4.2H_2O$, 4.16 mM $NaHCO_3$, 5 mM glucose, supplemented with 0.001% phenol red, 1 mM pyruvate, and 10 mM HEPES, pH 7.4). The cells were cultured in B-27 supplemented serum-free Neurobasal medium (Gibco), containing glutamate (25 μM), glutamine (0.5 mM) and gentamicin (0.12 $mg.ml^{-1}$). Cultures were kept at 37ºC in a humidified incubator in 5% CO_2/95% air, for 7 days, the time required for maturation of hippocampal neurons. For morphology studies with cresyl violet and Hoescht 33342 staining and cobalt uptake, and for viability tests with Syto 13/propidium iodide (PI) or for single cell Ca^{2+} imaging studies, cells were plated at a density of 45 x 10^3 $cells.cm^{-2}$ on poly-D-lysine-coated (0.1 $mg.ml^{-1}$) coverslips.

Isolation of Synaptosomes

For the isolation of synaptosomes (Malva *et al.*, 1996), briefly, brain cortex of male Wistar rats (1.5 month-old) were manually homogenized in a cold solution of 0.32 M sucrose, 10 mM HEPES-Na, pH 7.4 and centrifuged at 3000*g*, for 2 min. The pellet obtained (P1) was re-suspended, followed by sedimentation at the same speed. The combined supernatants were centrifuged for 12 min at 14600*g*. The pellet (P2) was re-suspended in 2 ml of sucrose medium. Protein concentration was determined by using the biuret method.

Evaluation of Cell Viability - MTT Assay

Assessment of neuronal injury was performed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Ambrósio *et al.*, 2000). Krebs buffer with MTT (0.5 mg/ml) was added to the cell cultures and incubated for 1 h at 37ºC in the incubation chamber. MTT, when taken up by cells that remain alive, is converted from yellow to a water-insoluble blue-colored product. The precipitated dye was dissolved in 0.04 M HCl in isopropanol, for 5 min, and optic density (absorbance at 570 nm) was quantified. All experiments were performed in triplicate.

Evaluation of Cell Viability - Syto13/PI Staining

The viability of hippocampal neurons, following incubation with valerian extracts and/or drugs, was evaluated with Syto-13/PI, two dyes with different membrane permeability that bind to nucleic acids. Syto-13 is membrane permeable green fluorescent probe, used to identify cells that remain alive and cells that have entered the first steps of apoptosis. PI is a non-permeable membrane red dye, used to identify cells with altered membrane integrity, in advanced stages of apoptosis or necrosis. Briefly, after incubation, cells were washed three times with Krebs buffer (37ºC), incubated with 20 μl of marker solution (1 μM de Syto-13, 0.002 mg/ml PI, 37ºC) in Krebs buffer for 2-3 min (i.e., the time required for entry of the fluorescent probes into the cells). Cells were observed in a fluorescence microscope (triple filter - Omega Optical XF63), and counted: the number of viable, apoptotic (primary and secondary), and necrotic cells was recorded (Rego *et al.*, 2003). A total of 300 neurons in each preparation was counted. Each cell was identified as viable (green and regular nucleus), apoptotic (green or red condensed nucleus) or necrotic (red enlarged nucleus). Red (stained by PI) condensed nuclei originated from neuronal death caused by the isolation procedure, present in control cultures, were not taken into account for the viability of the cultures.

Nissl Staining

After incubation of cultured hippocampal neurons with

the extract and $A\beta_{(25-35)}$ for 48 h, the culture medium was removed, the cells were washed two times with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), and fixated with paraformaldehyde (4% paraformaldehyde w.v^{-1}, and 4% sucrose w.v^{-1}) in PBS, at room temperature. After 30 min, the cells were washed two times with PBS (5 min each), and then dehydrated in a gradient of ethanol/PBS (50%, 70%, 80%, 95% and 100% ethanol) and ethanol/acetone 1:1. After this procedure, the cells were re-hydrated in the same gradient, in the reverse order, and then washed two times with PBS. The cells were stained with 0.5% cresyl violet for 5 min, washed two times with water, and air-dried. Finally, the coverslips containing the cells were mounted with Entellan (Merck) and photographs were taken (Ambrósio *et al.*, 2000).

Analysis of Nuclear Morphology by Hoescht Staining

Cultured rat hippocampal neurons cultured on 2 cm^2 round glass coverslips were incubated with 200 µl of Hoescht 33342 (Molecular Probes) dissolved in PBS at 15 µg /ml, for 5 minutes. Digital photographs were taken by using a Zeiss Axiovert 200 (Zeiss) fluorescence microscope with a coupled Coll SNAP digital camera (Roper Scientific). Bright-field images with Nomarsky light interference and fluorescence images obtained with Xenon light-source (Lambda DG4, Sutter Instruments Company) with a Carl Zeiss filter (ref. 02) were obtained.

Cobalt Uptake and Neuronal Staining

A method for neuronal staining of a sub-population of cells expressing AMPA-permeable Ca^{2+} receptors was used (Malva *et al.*, 2003a,b). After incubation of hippocampal neurons with the extract and $A\beta_{(25-35)}$ for 48 h, the cultured medium was removed, the cells were washed two times with HEPES buffer-solution (146 mM NaCl, 4.2 mM KCl, 0.5mM $MgCl_2$, 0.8mM $CaCl_2$, 55.6 mM glucose, 20 mM HEPES, pH 7.4), and incubated with 5 mM $CoCl_2$ in HEPES-buffer solution, with 100 µM kainate plus 30 µM cyclothiazide. After 30 min of incubation, cells were washed two times (5 min and 10 min) with 2 mM EDTA in HEPES-buffer solution, and incubated with Na_2S (0.12% w.v^{-1}) for 5 min. After this procedure, the cells were washed with HEPES-buffer solution, and fixated with paraformaldehyde (4% paraformaldehyde w.v^{-1}, and 4% sucrose w.v^{-1}) in PBS for 30 min, at room temperature. The cells were washed with development solution (292 mM sucrose, 15.5 mM hydroquinone and 42 mM citric acid, 50ºC), and revealed with a silver nitrate solution, (1 mg.ml^{-1}) made in development solution, two times (30 min each time) at 50ºC. The cells were washed once more with development solution, incubated with sodium thiosulfate (5% w.v^{-1}) for 10 min (room temperature) and then washed with water. Finally, the coverslips were mounted with Entellan and photographs were taken.

Single Cell Calcium Imaging

Analysis of single cell Ca^{2+} changes in hippocampal neuronal cultures (Ambrósio *et al.*, 2000) either in the absence or after pre-treatment with Aß was evaluated. In brief, cells were loaded in Krebs buffer containing 5 µM Fura 2/AM (Molecular Probes) in the presence of 0.02% pluronic acid F-127 (Molecular Probes) and 0.1% fatty acid-free bovine serum albumin, for 45 min at 37ºC. After a 10 min post-loading period at room temperature the glass coverslip was mounted on RC-20 chamber in a PH3 platform (Warner Instruments), at 37ºC, on the stage of an inverted fluorescence microscope Axiovert 200 (Zeiss). The cells were continuously washed with Krebs solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose, 10 mM $NaHCO_3$, 10 mM HEPES, pH 7.4) and stimulated by applying high-potassium Krebs solution (containing 34 mM KCl, isosmotic substitution of NaCl), Krebs solution containing 100 µg/ml valerian extract or high-potassium Krebs supplemented with valerian extract. Solutions were added to the cells by a fast-pressurized (95% air, 5% CO_2 atmosphere) system (AutoMate Scientific, Inc). The cells were alternately excited at 340 and 380 nm (750 msec) using a Lambda DG4 apparatus (Sutter Instruments Company), and a 510 nm band-pass filter was used (Carl Zeiss ref. 21) before fluorescence acquisition with a 40x objective and a Coll SNAP digital camera (Roper Scientific). Acquired values were processed using the MetaFluor software (Universal Imaging Corporation). The results are presented as ratio of fluorescence intensities after excitation at 340 nm and at 380 nm.

Membrane Peroxidation of Rat Hippocampal Synaptosomes

The synaptosomal fraction was diluted to obtain a final concentration of 0.5 mg protein.ml^{-1}. Lipid peroxidation was assayed after incubation of synaptosomes with 800 µM ascorbic acid and 2.5 µM $FeSO_4$, in a final volume of 1.2 ml of Krebs buffer (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose, 10 mM HEPES-Na, pH 7.4) in the presence of valerian extract (37ºC, 15 min). The reaction was stopped after

placing the sample solutions in ice, and 0.5 ml of the sample solution was added to 0.5 ml trichloroacetic acid (TCA, 40%, w.v^{-1}) and 2.0 ml thiobarbituric acid (TBA, 0.67%, w.v^{-1}). The solution was then heated at 100ºC for 10 min. Controls were made with synaptosomes, without the oxidant pair. After centrifugation for 10 min at 3000*g* to remove the precipitated protein, the colour of lipid peroxidation-TBA complex was detected at 530 nm (Pereira *et al.*, 2000).

Valerian officinalis Extract

The alcoholic extract of valerian root (*Valeriana officinalis*) (3-6:1 w/v in ethanol 70%), containing 0.52% valeneric acids, was obtained from Solvay Pharmaceuticals (Hannover, Germany).

Statistical Analysis of the Results

The results were treated with ANOVA test of variance followed by Bonferroni's *post-hoc* test, using the GraphPad 3.0 software from Prism (USA). Asterisks indicate the level of significance between test and control condition; (***) $p < 0.001$, (**) $p < 0.01$, (*) $p < 0.05$.

RESULTS

Neurotoxicity Caused by Valerian Extract

The neurotoxicity induced by exposure of cultured rat hippocampal neurons to valerian extract was evaluated following 48 h exposure to increasing concentrations of the extract. Neuronal viability was assessed at the end of the exposure period (48 h) by following neuronal morphology (Nissl staining).

We observed that increasing concentrations of valerian extracts from 10 ng/ml to 100 µg/ml were not significantly toxic. Concentrations above 1 mg/ml extract caused significant neuronal degeneration, as determined by a decrease in the number of healthy neuronal neurites and cell bodies (not shown).

Valerian Extract and Neuroprotection Against Aß$_{(25-35)}$

The exposure of cultured rat hippocampal neurons to 25 µM Aß$_{(25-35)}$ for 48 h caused a decrease in the capacity of cells in reducing the MTT, to 51.0 ± 1.09% of the control value. In the presence of valerian extract, 10 or 100 µg/ml, the neurotoxicity caused by the simultaneous exposure to 25 µM Aß$_{(25-35)}$ was significantly reduced to 72.08 ± 4.61 or 62.73 ± 4.14% of the control, respectively (FIG. 1A). Both concentrations of valerian extract tested (10 and 100 µg/ml) were not significantly toxic.

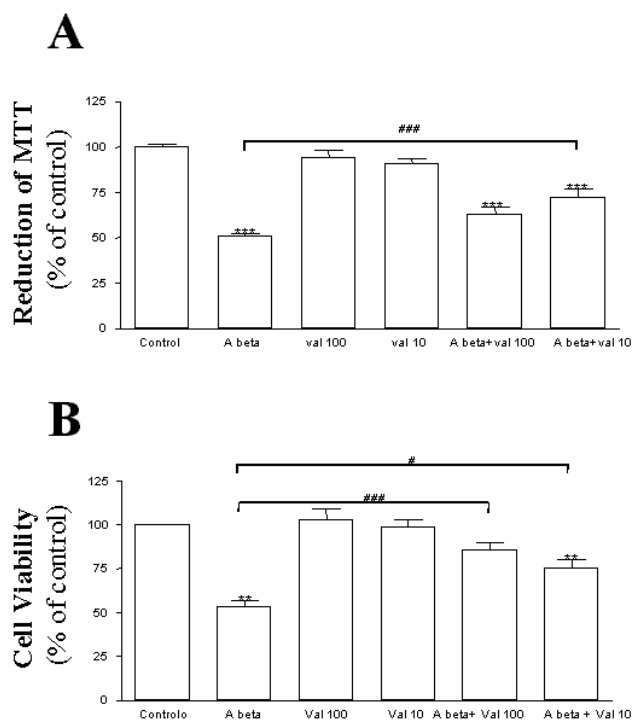

FIGURE 1 Evaluation of the neuroprotective properties of valerian extract against Aß$_{(25-35)}$ toxicity in cultured rat hippocampal neurons by using the MTT assay (**A**) or Syto 13/propidium iodide staining (**B**). Concentrations of 10 and 100 µg/ml of valerian extract are not toxic to cultured hippocampal neurons and could partially prevent the neurotoxicity induced by the exposure to 25 µM Aß$_{(25-35)}$. Data correspond to the mean ± S.E. of 4-5 independent experiments preformed in triplicate.

The neuroprotective action of valerian extract was further investigated by determining the pattern of neuronal degeneration involving apoptotic and necrotic-like cell degeneration, by using the cell life/death assay with Syto 13/PI staining.

The exposure of the cultures to 25 µM Aß$_{(25-35)}$ for 48 h caused a decrease in the number of viable neurons to 53.43 ± 3.71% of the total cells in the preparation. In agreement with the results obtained with the MTT assay, the toxicity caused by the Aß peptide could be partially prevented by 10 or 100 µg/ml valerian extract and the number of viable cells determined was 75.64 ± 4.50 or 85.32 ± 4.31% of the total number of cells (FIG. 1B). Again, the exposure of the neuronal cultures to valerian extract was not toxic by itself. Moreover, we could observe that the exposure of the neuronal cultures to 25 µM Aß$_{(25-35)}$ for 48h caused a significant neuronal degeneration with apoptotic-like morphology (not shown).

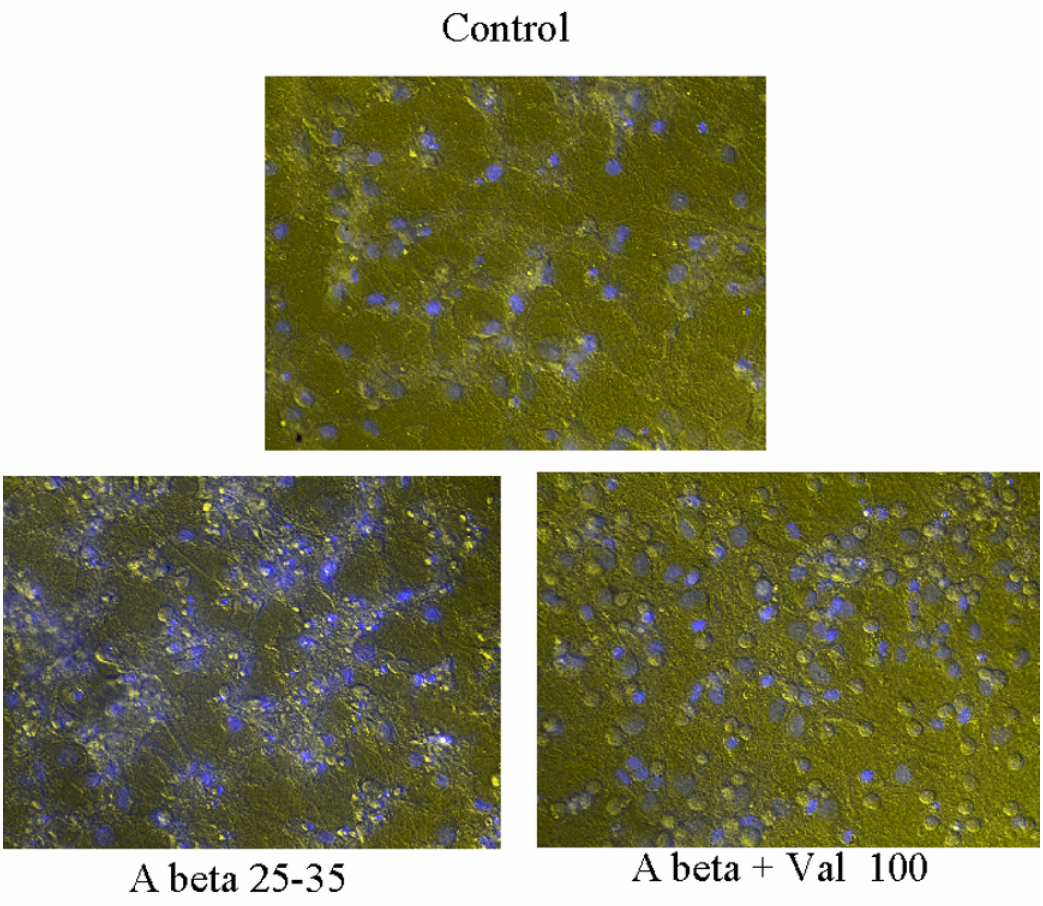

FIGURE 2 Evaluation of neuronal morphology in neurodegeneration and neuroprotection. Cultured embryonic rat hippocampal neurons were stained with Hoescht 33342 to reveal nuclear morphology. Note that insult with $A\beta_{(25-35)}$ induced increased nuclear condensation (evident by the presence of smaller and brighter nuclei) and fragmentation. Moreover, treatment of the neuronal cultures with 25 μM Aß in the presence of valerian extracts (100 μg/ml) resulted in a very good preservation of the neuronal morphology, comparable with the control.

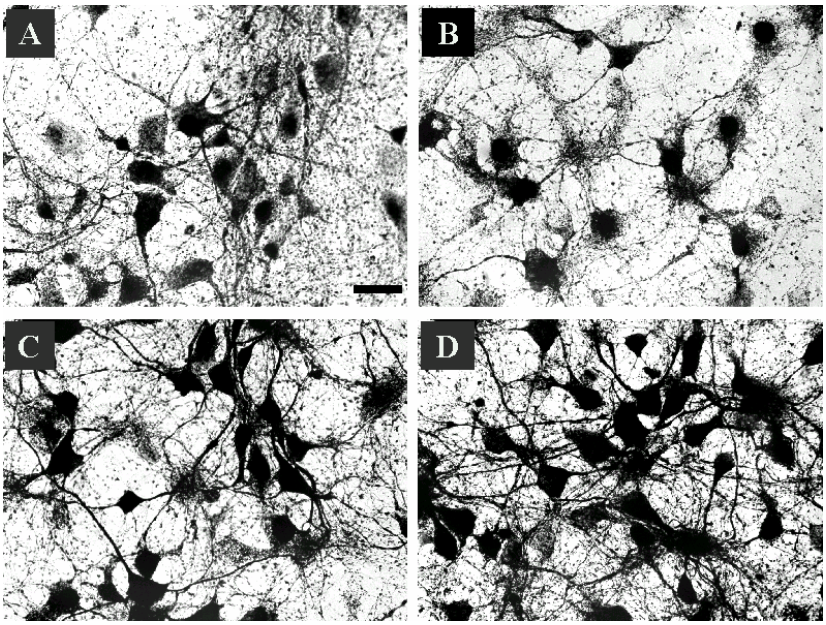

FIGURE 3 Evaluation of dendritic morphology of cultured rat hippocampal neurons exposed to 25 μM $A\beta_{(25-35)}$ for 48 h in the absence or in the presence of valerian extract. Neurons were loaded with cobalt following non-desensitizing activation of AMPA receptors, and the cobalt precipitates were visualized following treatment with silver nitrate. **A-** Control culture - note the fine ramified processes of a healthy culture; **B-** Following treatment with $A\beta_{(25-35)}$ a significant reduction in the dendritic network was observed (dendrites are shorter and thicker); **C** and **D-** Co-application of Aß peptide together with 10 and 100 μg/ml of valerian extract. The bar in the control figure corresponds to 10 μm.

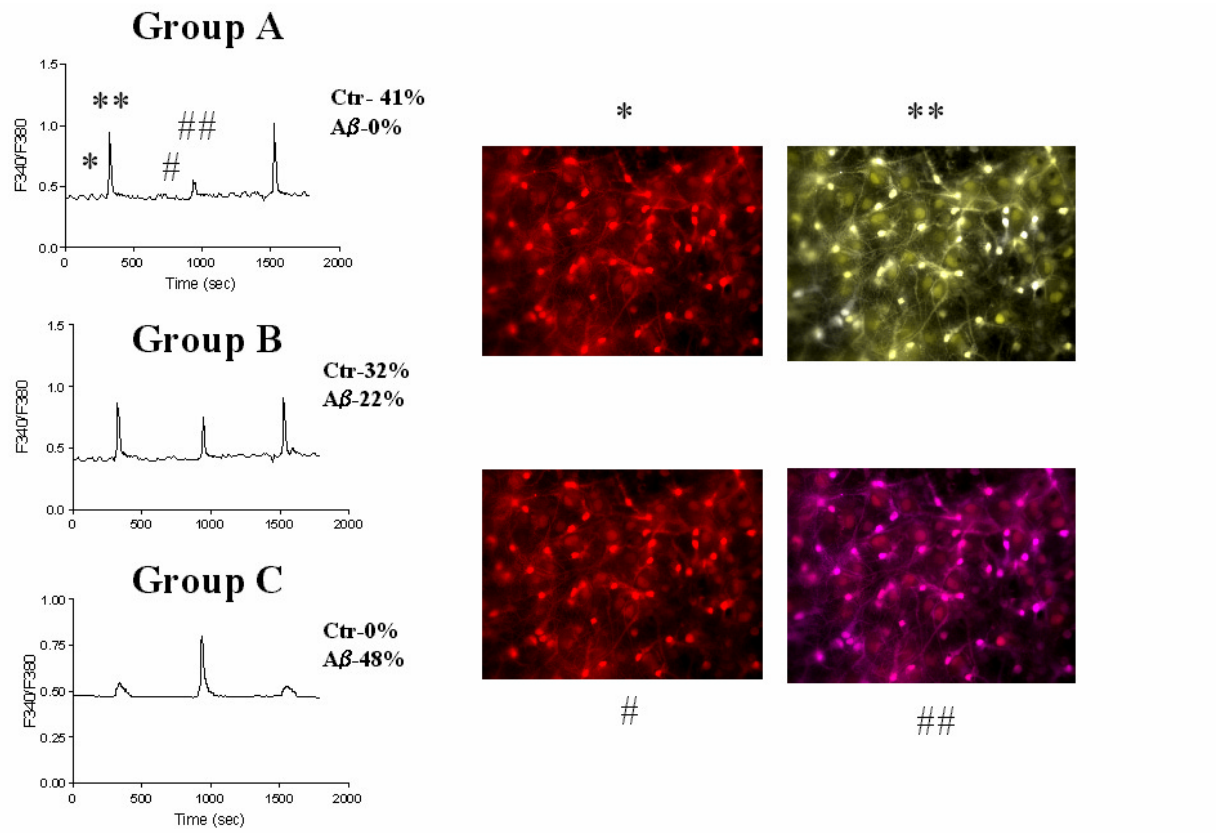

FIGURE 4 Inhibition of the KCl-evoked increase in the intracellular free calcium concentration ($[Ca^{2+}]_i$) by valerian extracts. Cultured rat hippocampal neurons were stimulated with 30 mM KCl at minute 5 and 25, in the absence of valerian extract, or stimulated with 30 mM KCl in the presence of valerian extract (100 µg/ml) at min 15. Two major group of cells were identified: Group A cells were cells strongly inhibited (more than 50% of the response) by valerian extract (41% of cells); Group B were identified as cells where the inhibition of the KCl-induced increase in the $[Ca^{2+}]_i$ was between 0 and 50% (32% of cells). In the cultures exposed to 25 µM $Aß_{(25-35)}$ for 24 h the most common profile of neuronal response included: higher basal $[Ca^{2+}]_i$; low amplitude increase in the $[Ca^{2+}]_i$ evoked by KCl, and potentiated response in the presence of valerian extract (Group C, 48% of the cells). The results were obtained by single cell analysis of 108 neurons (per experimental condition) in three different preparations. Left pseudocolour panels correspond to (*) basal resting $[Ca^{2+}]_i$; (#) basal resting $[Ca^{2+}]_i$ in the presence of valerian extract; (**) KCl-evoked high $[Ca^{2+}]_i$; (##) KCl-evoked increase in the $[Ca^{2+}]_i$ in the presence of valerian extract.

The evaluation of neuronal morphology following insult with Aß peptide and neuroprotection caused by the simultaneous exposure to valerian extract was performed by Nissl (with cresyl violet), Hoescht 33342 staining and by cobalt uptake staining.

We could observe that the healthy neuronal morphology observed in the control conditions was significantly affected following the exposure to 25 µM Aß peptide for 48 h. A significant decrease in neuronal volume and nuclear condensation was observed in the majority of the cultured neurons when compared to the control. In the presence of valerian extract the overall evaluation of the neuronal morphology and nuclear condensation indicates a moderate neuroprotection mediated by the plant extract (FIG. 2).

Following stimulation of AMPA receptors in the presence of cobalt a sub-population of the cultured neurons can be loaded with cobalt. Following treatment with silver the accumulated cobalt can be transformed in a dark precipitate inside the cytoplasm. This technique is particularly useful to visualize the integrity of the dendritic network of the neurons expressing Ca^{2+}-permeable AMPA receptors (Malva *et al.*, 2003a). The analysis of neuronal morphology of control neurons revealed a complex dendritic network where fine and long dendrites can be identified. Following exposure to 25 µM $Aß_{(25-35)}$ for 48 h the neuronal dendrites retract and become shorter and thicker, indicating a strong dendritic degeneration. The co-exposure of Aß peptide with valerian extract resulted in a moderate preservation of the dendritic network (FIG. 3), which may eventually be attributed to strong neuroprotection at the synaptic level.

Effect of Valerian Extracts on $[Ca^{2+}]_i$

The effect of valerian extract on the evoked increase in the intracellular free calcium concentration ($[Ca^{2+}]_i$) was investigated by single cell recording of Fura 2 flu-

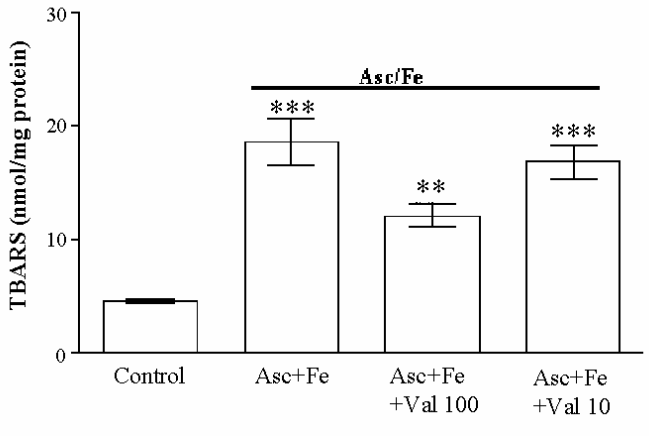

FIGURE 5 Membrane peroxidation in rat hippocampal synaptosomes evaluated by the formation of thyobarbituric acid reaction species (TBARS). The reaction was started by addition of 800 µM ascorbic acid and 2.5 µM $FeSO_4$ to 0.5 mg of synaptosomal protein, and the reaction occurred for 15 min at 37ºC. Following a 5 min pre-incubation of the synaptosomes with valerian extract, at 100 µg/ml, a significant inhibition in the formation of TBARS was observed, suggesting antioxidant properties of the valerian extract.

orescence. The application of 100 µg/ml of valerian extract consistently inhibited the 30 mM KCl-evoked increase in $[Ca^{2+}]_i$ (FIG. 4). In control cultures the majority of the analysed neurons were either significantly inhibited (more than 50%, Group A, 41%, *n*=108, three independent experiments) or slightly inhibited (less than 50%, Group B, 32%, *n*=108, three independent experiments) by the presence of valerian extract (FIG. 4). In control cultures 27% of those analysed had a basal fluorescence ratio above 0.6 and were excluded from further analysis. Following treatment with 25 µM $Aß_{(25-35)}$ for 24 h the cells showed an increased fluorescence ratio (excitation 340/380 nm), indicating an increased basal $[Ca^{2+}]_i$ and a deficient response following stimulation with 30 mM KCl (FIG. 4). Interestingly, in Aß peptide-treated cultures the exposure to valerian extract resulted in a weaker inhibition of the KCl-induced response (without detected group A cells) in some cells and a strong potentiation of the response to application of KCl in the majority of the cells analyzed (FIG. 4 - Group C, 48% of analyzed cells). In Aß cultures 30% of analysed cells had a basal fluorescence ratio above 0.7 and were excluded from further analysis.

Antioxidant Properties of Valerian Extract

Membrane peroxidation caused by reactive oxygen species (ROS) was evaluated by the formation of thiobarbituric acid reactive species (TBARS) following incubation of rat hippocampal synaptosomes with thiobarbituric acid in the presence of the oxidant pair ascorbate/iron. The putative antioxidant properties of valerian extract was investigated by following the inhibition of the formation of TBARS caused by pre-incubation (5 min) with valerian extract (10-100 µg/ml). The basal peroxidation determined corresponded to the production of 4.51 ± 0.18 nmol TBARS/mg protein. Following incubation with ascorbate/iron (800 µM ascorbic acid and 2.5 µM FeSO4) the observed peroxidation increased significantly to 18.59 ± 2.04 nmol TBARS/mg protein. In the presence of 100 µg/ml of valerian extract the lipid peroxidation induced by ascorbate/iron could partially be prevent to 12.12 ± 1.02 nmol TBARS/mg protein (FIG. 5).

DISCUSSION

Valerian extracts have been used for centuries in popular medicine to treat sleep disorders, anxiety and epilepsy. The popularity of the widely used valerian extracts makes it plausible that the pharmacological effects of valerian must involve molecular and cellular targets in the central nervous system (CNS), but until now there are no definitive evidences for the identification of these targets (Houghton, 1999).

The total aqueous extract of valerian root is enriched in GABA, which may account in part for the peripheral pharmacological effects attributed to valerian consumption (Cavadas *et al.*, 1995). However, it is not expected that GABA present in the extract may be responsible for the biological effect of valerian in the CNS, since it is not likely that GABA can cross the blood-brain barrier. Other candidates that may act in the CNS may include volatile oils, like valeneric acid or valeranone, or the valepotriates group (Houghton, 1999). Valeneric acid was shown to inhibit GABA metabolism (reviewed in Houghton, 1999) and by this mechanism may contribute to increased GABAergic transmission in the brain, resulting in sedation. It is also possible that not-yet identified biological compounds present in the extract of *Valeriana officinalis* are able to cross the blood-brain barrier and may be responsible for the pharmacological function of valerian in the CNS.

Several recently published papers attribute to flavonoids strong neuroprotective properties (Youdim *et al.*, 2002; Ahlemeyer *et al.*, 2003; Dajas *et al.*, 2003; Silva *et al.*, 2004). We, and others, have previously shown that neuroprotection attributed to flavonoids may, in part, involve antioxidant properties (Areias *et al.*, 2001; Prior, 2003; Silva *et al.*, 2004). The relevance of these findings highlights the contribution of natural research products in the search of new biologically

active products with neuroprotective properties. In this context, several plant extracts, including extract of valerian root are endowed with flavonoids (Wasowski *et al.*, 2002; Marder *et al.*, 2003) making this popular and very well tolerated plant attractive for the search of a daily-used neuroprotective product for human consumption. In the present work we decided to investigate the presence of putative neuroprotective properties of valerian root extract.

After challenging the neuronal cultures with $A\beta_{(25-35)}$, the identified toxic sequence of the Aß peptide, we could observe significant neuronal degeneration with patterns of apoptotic cell death, including neuritic apoptosis described by several authors (Mattson *et al.*, 1998). Interestingly, we could observe significant neuroprotective properties caused by simultaneous exposure of $A\beta_{(25-35)}$ and valerian extract at 10-100 µg/ml. The effect of valerian extract was evident in protecting the overall cell viability of the neuronal cultures, inhibiting nuclear condensation and fragmentation and inhibiting dendritic degeneration, specifically in the subpopulation of neurons expressing AMPA permeable Ca^{2+} receptors (Malva *et al.*, 2003a,b).

It was previously reported that treatment of neuronal cultures with $A\beta_{(25-35)}$ induces a partial loss of the cells unable to handle a low basal $[Ca^{2+}]_i$ (Agostinho and Oliveira, 2003). We now report that after exposure of the hippocampal neuronal cultures to 25 µM $A\beta_{(25-35)}$ the 340/380 nm excitation ratio of fluoresce of Fura 2 was increased from 0.4 to 0.5 in the majority of the cells analysed (FIG. 4). This small increase in fluorescence excitation ratio represents a significant increase in the free $[Ca^{2+}]_i$, indicating that neurons, in these conditions, were affected by the Aß peptide. Moreover, these neurons show a very limited KCl-evoked increase in the $[Ca^{2+}]_i$ (FIG. 4) that may give an indication of pre-depolarization. Interestingly, valerian extract could significantly inhibit the KCl-evoked increase in the $[Ca^{2+}]_i$ in control cultures (FIG. 4). However, from these data, the mechanism responsible for the valerian extract-induced inhibition of the increase in the $[Ca^{2+}]_i$ was not evident. Interestingly, when neurons were exposed to Aß peptide the KCl-evoked increase in the $[Ca^{2+}]_i$, in a significant number of neurons, was significantly higher in the presence than in the absence of valerian extract (Fig. 4 - group C). This may be considered a good indication that the mechanism of valerian-induced inhibition of the KCl-evoked increase in the $[Ca^{2+}]_i$ (in neurons not exposed to Aß peptide) involves neuronal hyperpolarization and does not represent inhibition of Ca^{2+} channels.

It was previously reported by our group that GABA is present in high concentration in valerian aqueous extracts (Santos *et al.*, 1994). However, in the alcoholic extract used in the present work the presence of water-soluble aminoacids is significantly lower, compared to the higher concentration in volatile oils. One possibility is that the high content of valeneric acid may contribute to increased tonic GABAergic neurotransmission, due to inhibition of GABA metabolism (reviewed in Houghton, 1999). Furthermore, the increase in the GABA concentration may result in hyperpolarization mediated by chloride influx through $GABA_A$ receptors (Ortiz *et al.*, 1999). A similar mechanism of action was reported, on the basis of the neuroprotective properties of melatonin in chick retina neuronal cultures (Paula Lima *et al.*, 2003). It is reasonable to expect that stimulation of the GABAergic system by valerian consumption may reduce neuronal excitability, and by this mechanism may be neuroprotective against excitotoxic insults and contribute to the putative anticonvulsant properties of valerian.

It is also possible that undetermined compounds present in the valerian extract can account for the observed inhibitory effects in the evoked-increase in the $[Ca^{2+}]_i$. Accordingly to the latter concept, the recent identification of 6-methylapigenin and hesperidin flavonoids on the valerian extracts able to interact with GABA receptors (Ortiz *et al.*, 1999; Wasowski *et al.*, 2002; Marder *et al.*, 2003) may open additional lines of research for the neuroprotective properties of valerian extracts.

It is evident that dysfunction of the neuronal capacity in handling resting basal $[Ca^{2+}]_i$ may cause mitochondrial dysfunction and ROS production (Ward *et al.*, 2000). The increase in the production of ROS can cause lipid peroxidation (Pereira *et al.*, 1999) and cell death (Alvarez *et al.*, 2003). We evaluated the effect of valerian extract in protecting rat hippocampal synaptosome membranes from peroxidation induced by ascorbate/iron. Since the biological effects of the Aß peptide involve ROS production (Pereira *et al.*, 1999) we may speculate that this process can be involved in valerian extract-mediated neuroprotection against amyloid toxicity. Comparable neuroprotective and antioxidant properties were identified in a flavonoid fraction of Hypericum perforatum extract (Silva *et al.*, 2004).

The present work used rather simple biological models to tackle putative neuroprotective properties of the alcoholic extract from the valerian root. In these models, the access of the compounds to the neurons, or to the synaptosomes, is direct and does not take into consideration the *in vivo* effects of absorption and metabolism that may play a critical role in tissue access of

some active compounds present in these assays. Moreover, the permeability of the blood-brain barrier cannot be taken into consideration in these models. It is clear that a direct correlation between these *in vitro* results and the *in vivo* effects of the valerian extract cannot be used. However, it is plausible to consider that some biologically active compounds like some essential oils and flavonoids (as discussed above) can cross the blood-brain barrier and account for the potential neuroprotective, *in vivo* effect of valerian extract.

Additional characterization of the described neuroprotective effect of valerian extract against Aß-induced neurotoxicity using models of increasing complexity, like organotypic cultures of rat hippocampal slices and *in vivo* analysis of the neuroprotective effect of chronic consumption of valerian extracts in models of aging in rats, may reinforce the importance of these first findings. Moreover, the search of new components of this extract that can be able to rescue neurons from physiological or pathological aging is of critical relevance for human health.

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