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Original Article

An alkaloid extract obtained from *Phlegmariurus Saururus* induces neuroprotection after *status epilepticus*

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ABSTRACT

Background: The brain is exposed to many excitotoxic insults that can lead to neuronal damage. Among these, Epilepsy is a neurological disease that affects a large percentage of world population and is commonly associated with cognitive disorders and excitotoxic neuronal death. Most experimental strategies are focused on preventing *Status Epilepticus* (SE), but once it has already occurred, the key question is whether it is possible to save neurons.
Purpose: The aim of this study was to determine if a purified alkaloid extract (AE) obtained from *Phlegmariurus saururus*, a genus of Lycophyte plants (sometimes known as firmossesor fir club mosses) could induce neuroprotection following SE.
Methods: *In vitro* and *in vivo* techniques were applied for this purpose. Protein levels were measured by western blotting procedures. Neuronal death analysis was performed by calcein-ethidium staining and the presence of the NeuN protein as a marker for presence or absence of cells (*in vitro* experiments) and by Fluoro Jade B staining for the *in vivo* experiments.
Results: The effect of AE in the hippocampal neurons culture was the first determination, where we found an increase in neuronal survival and in the level of pErk and TrkB activation, 24 h after the addition of AE. In a well-established *in vitro* model of SE, we found that 24 h after being added to the hippocampal neuron-astrocyte co-culture, the AE induces a significant increase in neuronal survival. In addition to this, in the *in vivo* Li-pilocarpine model of SE, the AE induced a remarkable neuroprotection in areas such as the entorhinal cortex and hippocampal CA1 area.
Conclusion: These results make the AE an excellent candidate for potential clinical use in neurological disorders where memory impairment and neuronal death occurs.

Introduction

Brain pathologies (such as ischemic stroke, epilepsy, Alzheimer's disease, etc.) can cause brain damage due to an increase in neuronal death and, in consequence, impairments in learning, memory and cognitive functions. It is important to find neuroprotective agents that can be used to protect neurons from different types of damage. In this context, there are many studies focused on the capacity of natural compounds to prevent certain brain injuries. This research was therefore conducted on *Phlegmariurus saururus* (Lam.) B.Øllg.[ex *Huperzia saururus*(Lam.) Trevis.] (Lycopodiaceae), a species commonly known in Argentine folk medicine as "*cola de quirquincho*", which is generally consumed as an infusion and popularly used as an aphrodisiac or for memory improvement (Martinez et al., 1981). We previously demonstrated as well, that the purified alkaloid extract (AE) of *P. saururus* shows activity as acetyl cholinesterase inhibitor, improving memory retention and hippocampal synaptic plasticity (Ortega et al., 2006; Vallejo et al., 2009, 2013).

Neurotrophins (NT) are secretory proteins that bind to target receptors, influencing survival activity, CREB activation and protein

Abbreviations: AE, alkaloid extract; SE, Status Epilepticus; pTrkB, Phosphorylated Tropomyosin related kinase type B; Erk, Extracellular related kinase; BDNF, Brain derived neurotrophic factor; Li, Lithium; AChEI, acetyl cholinesterase inhibitors; NT, Neurotrophins; Cdk5, Cyclin dependent kinase5; EC, Entorhinal Cortex; HRP, Horseradish Peroxidase; CREB, cAMP response element-binding; p75ntr, p75 neurotrophin receptor; AraC, arabino furanosyl cytidine; DMEM, Dulbecco's Modified Eagle Medium; NB, Neurobasal Medium; CNS, Central Nervous System; i.p., Intraperitoneal

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synthesis, among other functions (Skaper, 2012). One of these NT is the Brain Derived Neurotrophic Factor (BDNF), which has two principal isoforms: proBDNF, which is released and binds to p75ntr and sortilin to induce neuronal death, only when the level of tyrosine kinase receptor B (TrkB) receptor is decreased (Unsain et al., 2009); and BDNF, which also is released and binds to TrkB and p75ntr to induce, mainly, different signaling cascades leading to neuron survival and plasticity (Blum and Konnerth, 2005). Epilepsy is a common neurological disorder that affects 1–2% of the population worldwide (Hesdorffer et al., 2013). In animals, prolonged seizures induced by pilocarpine pretreated with lithium lead to *Status Epilepticus* (SE) resulting in neuronal death (Covolan and Mello, 2000). SE induced in rodents serves as a model of Temporal Lobe Epilepsy (TLE) with hippocampal sclerosis, the most common epilepsy among humans. Current studies are focusing on the development of better antiepileptic drugs to treat seizures and antiepileptogenic drugs that can prevent the disease (Curia et al., 2008). However, it is also important to find new compounds that could have neuroprotective effects once the damage has occurred.

Until now, no detailed studies have been conducted to test the possibility that an AE obtained from *P. saururus* may induce neuroprotection in different pathologies of the Central Nervous System (CNS). Therefore, the aim of this study was to determine whether the AE obtained from *P. saururus* has the potential to protect neurons after an excitotoxic event.

We were able to demonstrate that the AE had a remarkable neuroprotective effect on hippocampal neurons in two well-known SE models, and suggesting that this effect could be mediated by the activation of TrkB receptors.

Materials and methods

Plant material

P. saururus aerial parts were collected in October 2012, in Pampa de Achala, San Alberto Department, Province of Cordoba, Argentina (31° 42′ 11″ South, 65° 1′ 8″ West). The plant was identified by Dr. Gloria Barboza, Instituto Multidisciplinario de Biologia Vegetal, Universidad Nacional de Cordoba. A voucher specimen is deposited at the herbarium of the Museo Botánico de Córdoba (CORD) as CORD 684.

Extraction and isolation of purified alkaloid extract (AE)

P. saururus aerial parts (2.45 kg) were dried in the shade, ground, and mixed with NaOH and reduced to a powder (200 g). The mixture was hydrated with distilled water and extracted in a Soxhlet extractor with $CHCl_3$. The organic solvent was evaporated under reduced pressure until acquiring half of its original volume and partitioned twice with 0.01 N HCl. These acidic (pH 2) aqueous extracts were combined and then alkalized with 0.1 N NaOH to pH 12 and subsequently partitioned with $CHCl_3$ in a liquid-liquid extractor. The chloroform extract obtained was purified by passage over Sephadex LH-20 in a glass column, employing $CHCl_3$-EtOH (1:1) as mobile phase. All fractions positive to Dragendorff's reagent were combined and evaporated under reduced pressure and 2.41 g of an AE was obtained.

Identification and quantification of alkaloids in AE

The identification of the major constituents of **AE** was performed on GC–MS using a Shimadzu QP5050A apparatus, and a capillary column VF-5 ms (5% phenyl and 95% dimethyl-polisiloxane), 30 m x 0.25 mm x 0.25 μm. The injection volume was 0.1 μl with He as carrier, at flow rate 1 ml/min. Temperature program: 60 °C (3 min), 60–250 °C at 30 °C/min, 250 °C (1 min), 250–280 °C at 10 °C/min, 280 °C (4.67 min). The temperatures of injector, interface and ion source were 280 °C, 300 °C, and 280 °C, respectively and the ionization energy was 70 eV The alkaloid identifications were realized considering our reference library of mass spectra obtained from authentic samples of our own Lycopodium alkaloid collection and also by comparing breakdown patterns with those found in the literature (Ortega et al., 2004; Vallejo et al., 2013).

In order to quantify the major constituents of the AE, a GC–MS equipment (described above) was utilized, and the internal standard methodology was applied using ciclohexanone (Chex, analytical grade, Sigma) at a concentration of 1 μl/ml. The methodology of quantification was validated considering the linearity, accuracy, precision and specificity according to European Medicines Agency (EMEA), 1995. European Medicines Agency, ICH Topic Q 2 (R1) Validation of Analytical Procedures: Text and Methodology, Ref: CPMP/ICH/381/95.

In order to assess the linearity, a calibration curve was obtained by injecting five solutions of Sauroxine (Sx, a majority alkaloid present in AE, 99% of purity by GC–MS). The Sx solutions were prepared with the internal standard Chex in triplicate at 0.12–0.84 μg/ml of concentration, diluting a stock solution serially. Each analysis was done by triplicate, and the calibration curve was obtained applying the linear regression method. The limits of detection (LOD) and limits of quantification (LOQ) values were obtained from the standard deviation of the y-intercept (σ) and the slope of the standard calibration curve (S). For this reason, Eq. (3.3) σ/S allowed us to obtain the LOD value and LOQ was calculated by Eq. (10) σ/S. Accuracy was calculated as average of recovery with its relative standard deviation (%RSD) from three solutions of AE (2–4 mg/ml, 80–120% levels of alkaloid concentration) by triplicate. Regarding precision, the intra-day analysis (repeatability) was carried out using a working solution of the AE (3 mg/ml) that was analyzed six times in the same day, and the %RSD was informed. Specificity of the method was determined by the analysis of the breakdown patterns of each alkaloid identified in the AE.

Pure hippocampal neuron culture

Primary cultures of embryonic rat hippocampal neurons were prepared as described by Kaech and Banker (2006), with minor modifications. In brief, the hippocampi, after being dissociated, were plated in individual dishes. 35,000–40,000/cm^2 cells were plated into 35 mm plastic dishes or on glass coverslips coated with poly-L-lysine (0.1 mg/ml) containing Neurobasal medium (Invitrogen) plus B27 supplement Thermo Fisher Scientific, 17504-044 (Invitrogen). Ara C (dilution 1:10,000) was added to the culture on the second day after plating to prevent glial cells proliferation. Cultures were maintained for 10–11 DIV at 37 °C in a 5% CO_2/95% air atmosphere.

Hippocampal neuron-astrocyte Co-culture

Hippocampal astrocyte cultures were prepared from P0-P2 animals. Hippocampi were chopped into small sections and incubated in 0.05% trypsin/EDTA (Invitrogen) for 15 min at 37 °C, 5% CO_2. Trypsin was quenched by adding 2 ml of growth media (DMEM, 10% fetal bovine serum, 0.5% streptomycin/penycilin) and the tissue was transferred to 1 ml of fresh growth media and triturated 10–20 times through a fire-polished Pasteur pipette before plating in tissue culture dishes. Once the astrocytes reached confluence, they were shaken for 12 h to eliminate non-astrocyte cells. Then, the astrocytes culture was trypsinized and plated on glass coverslips. The astrocyte culture was maintained for 6–7 days in growth media (DMEM + 10%SFB + 0.5% Pen/Strep). Once the astrocytes reached confluence again, hippocampal neurons from E18 embryo were added to the astrocyte monolayer. The media was changed to Neurobasal medium containing B27 supplements, glutaMAX (Invitrogen) and 0.5%Pen/Strep.

In vitro and in vivo extract assay

AE (1 mg) was dissolved in 6 μl of DMSO and resuspended in 994 μl of Neurobasal medium for the *in vitro* experiments, and in saline for the

in vivo experiments. Aliquots of 1, 10 and 100 µl were taken up again to a volume of 1000 µl with NB/saline. The AE was added at different times depending on each experiment, as described in the Results section. Cannulated rats received 1.5 µl of saline or extract in a concentration of 10 µg/ml.

In vitro model of SE

After 10–11 DIV, cultures were utilized for experimentation and SE was induced as described previously (Deshpande et al., 2007). Maintenance medium was replaced with recording solution with or without $MgCl_2$ containing (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 2 $CaCl_2$, 10 glucose, and 0.002 glycine, pH 7.3. Continuous epileptiform high-frequency bursts were induced by exposing neuronal cultures to physiological recording solution without added $MgCl_2$(low Mg^{2+}). The SE continued until maintenance medium was added back to the cultures.

Animals and induction of SE

Adult male Wistar rats (Instituto Ferreyra, Córdoba, Argentina), aged 2–2½ months weighing 200–250 g were used and housed under environmentally controlled conditions. Animals received food and water *ad libitum* and were maintained in a 12 h light/dark cycle. The experimental protocol for this study followed the guidelines of the USA National Research Council Guide for the Care and Use of Laboratory Animals and was approved by the Committee of the Institute of Biological and Technological Research, RES 2015. SE was induced by the i.p. administration of lithium chloride (3 meq/kg) 12 h before pilocarpine hydrochloride (40 mg/kg, i.p.; Sigma, St Louis, MO, USA). The peripherally acting anticholinergic, methyl-scopolamine (1 mg/kg i.p.) was administered 30 min prior to the pilocarpine injection as described by Curia et al. (2008). Following this procedure, the animals were monitored to determine the onset of SE. The validity of relying on behavioral monitoring to assess seizure activity has been demonstrated previously (Unsain et al., 2008).

Surgery and general infusion procedure

Male Wistar rats were implanted under deep ketamine-xylazine (100:15 mg/kg) anesthesia with 22 gauge guide cannulas in the dorsal CA1 region of the left hippocampus as described before (Unsain et al., 2009). Briefly, a screw was placed in the skull and the cannulae were fixed to the skull with dental acrylic. After 4–5 days of recovery from the surgery, the animals were subjected to SE, before receiving the infusions. Infusion cannulae (30 gauges) passed the end of the guide cannulae by 1.8 mm, making the final ventral coordinate of the infusion 3 mm. Infusions were in all cases performed at a rate of 1 µl/min with a Hamilton precision pump. And the infusion cannulae were left *in situ* for 2 min. Histological examination of infusion cannulae placements were performed by analyzing serial Nissl-stained sections. At least 3–4 animals were used in each experimental condition.

In vitro and in vivo neuronal death assay

In the cultures with only hippocampal neurons, neuronal death was assessed 24 h after adding the AE to the culture, using calcein-AM and ethidium homodimer-1 (Cat. # L3224, Invitrogen). In the SE *in vitro* experiments, neuronal death was assessed 12 h and 24 h later, respectively, after ending the excitotoxic insult. Two different protocols were utilized for this procedure: calcein/ethidium protocol and the presence of the NeuN protein as a marker of presence/absence of cells (Liu et al., 2013) and immunolabeling Neurofilament-M protein (1:1000, Milipore AB 1987). The number of dying neurons after SE *in vivo* was assessed by labeling with FluoroJade B (FJB, Sigma-Aldrich) and Cresyl violet staining. In some experiments and brain areas (such as piriform cortex or hippocampus), we had to compare a wide array of neuronal injury features, including sections with small damage and sections with holes and fractures due to extensive neuronal damage. Due to this event, counting FJB+ neurons was impossible to perform and therefore was not a good tool in these cases. For this reason, we used a semi-quantitative Neuronal Injury Scale, as previously published (Unsain et al., 2009).

Electrophoresis and western blot

Control and AE treated cultures were homogenized at the indicated time points, in radio-immunoprecipitation assay buffer (RIPA) containing protease and phosphatase inhibitors (protease inhibitor cocktail Sigma P8340 and phosphatase inhibitor cocktail Sigma P5726). Homogenates were cleared by centrifugation at 5000 g twice for 7 min and protein concentration was determined using the Bradford protocol (Bradford, 1976); samples were then boiled in gel-loading buffer and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 8% for TrkB and pTrkB, 12% for Erk and pErk). Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories Inc., PA, USA) and blocked with 5% non-fat milk in Tris-buffered saline with 0.05% Tween, for 1 h at 25 °C. Membranes were then incubated with the primary antibody of interest. Rabbit polyclonal antibody against TrkB (sc:8316) was obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA; rabbit monoclonal antiphospho-TrkBY817 antibody was obtained from Abcam, USA; rabbit polyclonal antibody against Erk and mouse monoclonal antibody against pErk were obtained from Cell Signaling Technology, USA. Membranes were then incubated with appropriate HRP secondary antibodies, followed by enhanced chemiluminescence detection (ECL), and then exposed to films (Kodak, Rochester, NY, USA). Membranes were re-probed with a monoclonal antibody against anti-tubulin (Santa Cruz Biotechnology) to control protein loading. Images were scanned and gel bands were analyzed using gel-scanning integrated optical density software (FIJI, NIH, USA).

Statistical analysis

Results are expressed as percentage of control (mean ± SEM). In the quantification of FJB-positive cells, an ANOVA test with a nested model was used, considering each slice of the same animal as a pseudo-replicate. The ANOVA was followed by Tukey-*post hoc* comparisons, with $p \leq 0.05$ considered significant. Protein levels were quantified in a minimum of 2 different cultures per group. A one-way ANOVA was used to compare relative protein levels between groups. The ANOVA was followed by Tukey *post hoc* comparisons, with $p \leq 0.05$ considered significant. When necessary, the Kruskal–Wallis test was used for non-normal distributions. In the quantification of Calcein-ethidium or NeuN positive cells, an ANOVA test with a nested model was used, considering each field of the same dish as a pseudo-replica.

Results

Identification and method validation

As a result of the AE preparation, a DER of 1017:1 was obtained. Validation was carried out employing a GC–MS methodology with Chex as internal standard. In order to assure specificity, the analysis of the fragmentation pattern of each alkaloid present in the extract was performed by CG-MS, identifying unequivocally the presence of these Lycopodium alkaloids. Thus, AE showed the presence of Sauroine, Sx and 6-hydroxylycopodine, the majority alkaloids present in this species (Ortega et al., 2004; Vallejo et al., 2013) (See Supplementary Figs. 1–6). Linearity showed a good correlation coefficient of $R^2 = 0.9912$ for Sx in the 0.12–0.84 µg/ml range, with a regression equation of $y = 3.2422x–0.1348$. The LOD and LOQ were 0.032 and 0.096 µg/ml, respectively. The %RSD value was obtained to test the precision, which

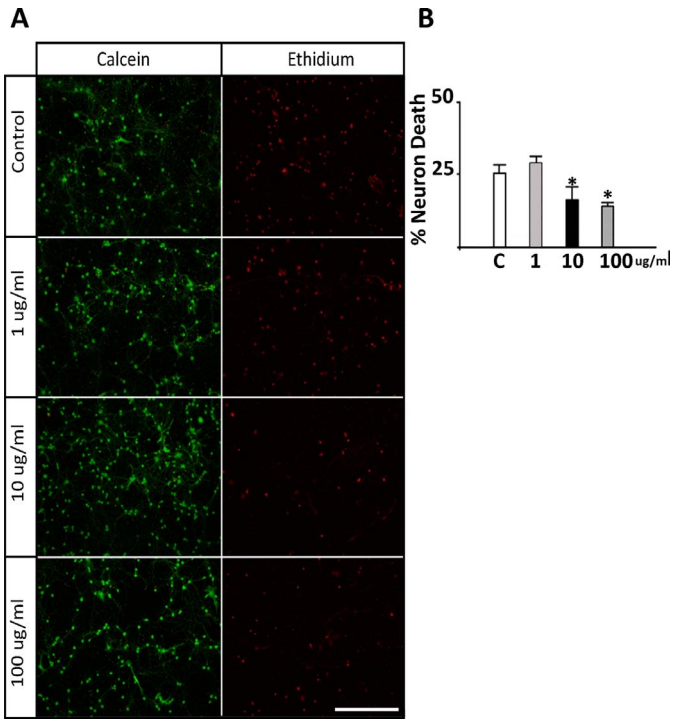

Fig. 1. *The addition of AE increases neuronal survival in vitro.* (a) Micrographs of hippocampal neurons stained with calcein-etidium show representative treatments. (b) Dose response showing that a concentration of 1 μg/ml failed to increase neuronal survival on the day analyzed but 10 μg/ml and 100 μg/ml significantly increases neuronal survival at 10–11 DI*V. Scale bar 200 μm.* Mean ± SEM are shown. * indicates significant differences compared with control ($p < 0.05$).

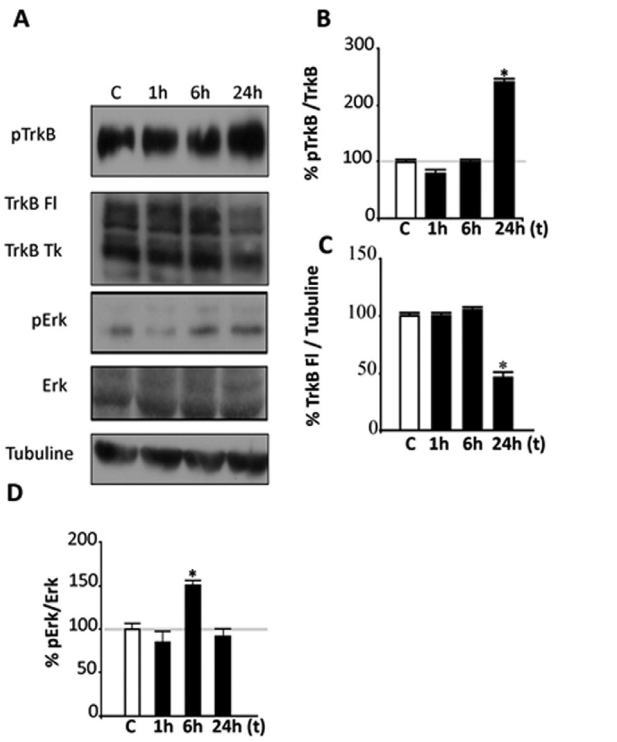

Fig. 2. *The addition of AE increases the levels of pTrkB and pErk.* (a) Representative Western Blot shows pTrkB, TrkB-fl, TrkB-Tk, pErk and Erk levels at 1, 6 and 24 h after the addition of the AE. (b) A significant increase in pTrkB levels 24 h after being added. (c) Quantification of TrkB-fl over tubuline showed a decrease in TrkB-fl levels 24h after the addition of AE. (d) The levels of pErk increase after 6h of AE addition. Mean ± SEM are shown. * indicates significant differences compared with control ($p < 0.05$).

was 3.8%. For accuracy, the average recovery rate was 104.5% (RSD = 4.1%) for 80% and 97.7% (RSD = 2.1%) for 120%. According to the parameters evaluated, the CG-MS methodology showed a good precision, accuracy and specificity, allowing a suitable quantification of the alkaloid content in AE of 0.77 ± 0.02 mg/ml, expressed as Sx.

AE increases neuronal survival in hippocampal neuron cultures

It has been demonstrated that the AE obtained from *P. saururus* improves several hippocampal functions (Ortega et al., 2006; Vallejo et al., 2009). Because many neurological diseases affect this region of the brain it was important to test if AE could also have neuroprotective properties.

It is well-established that due to culture conditions, hippocampal neurons experience death from the day of plating (Stevens, 1991).To test whether AE could have a neuroprotective effect a dose-dependent curve was used to evaluate this attribute. The AE was added at three different concentrations (1 μg/ml, 10 μg/ml and 100 μg/ml) to the culture for 24 h As can be observed in Fig. 1, doses of 10 μg/ml or 100 μg/ml induced a significant increase in hippocampal neuronal survival. Taking into account this result, 10 μg/ml concentration was used in all of the following experiments.

The AE increases Erk and TrkB activation in hippocampal neuron cultures

It is well-known that TrkB receptor activation is associated with a plethora of functions and one of the most important is neuronal survival (Skaper, 2012). To determine possible molecular mechanisms that may explain this neuroprotection, we evaluated TrkB and its phosphorylation state after the addition of AE to the hippocampal neuron culture. We found that the AE induced a decrease in TrkB levels and an increase in phosphorylated TrkB 24 h after being added (Fig. 2). Also, we evaluated the modification in the levels of pErk, another well-known pro-survival protein. We found that 6 h after the addition of AE, the levels of pErk increased significantly (Fig. 2). These findings show that the AE is able to activate pro-survival signaling pathways in hippocampal neurons.

To assess if the pro-survival effects found of AE was TrkB dependent, we performed an experiment using k252a, a well-established Tyrosin kinase inhibitor. AE was unable to induce protection of the neurons in the presence of k252a, suggesting the participation of TrkB receptor in the AE action (Fig. 3).

The AE induces neuroprotection in a model of SE in vitro

To test if the AE can induce neuroprotection in a model of neuronal degeneration, we used an *in vitro* model of SE (Deshpande et al., 2007). The AE was added 24 h before the onset of the excitotoxic insult, or immediately after the excitotoxic insult. In both cases, AE did not have a pro-survival effect (Fig. 4).

Since it has been demonstrated that astrocytes are critically involved in many pathological conditions, it is possible that AE only has a pro survival effect in this SE model when astrocytes are present. To test this hypothesis, a co-culture of neurons and astrocytes was used. A remarkable neuroprotection was found when AE was added in this co-culture before the onset of the excitotoxic insult (Fig. 5).

The AE induces neuroprotection in a model of SE in vivo

It was fundamental to examine if this pro-survival effect could also be observed *in vivo*. To assess this hypothesis, we utilized the lithium-pilocarpine SE animal model. This model induces neuronal death in several brain areas, including the hippocampus and the entorhinal cortex (EC). A group of cannulated implanted animals were unilaterally infused with AE in the left dorsal CA1 hippocampus region immediately

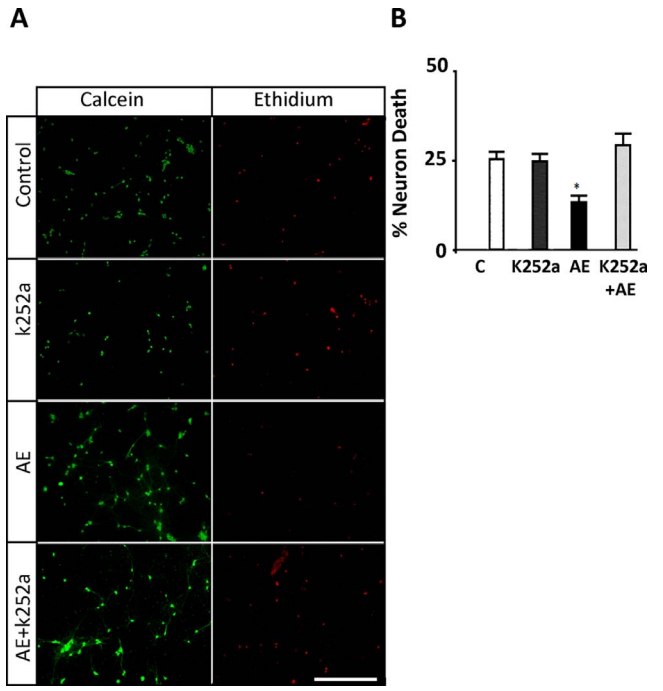

Fig. 3. *K252a abolished the neuroprotective effect induced by AE.* (a) Micrographs of hippocampal neurons stained with calcein-etidium show representative treatments. (b) Tyrosin kinase inhibitor (k252a) was added at the same time with the AE. The inhibition of tirosin kinases blocked the pro-survival effect of AE. *Scale bar 200 μm.* Mean ± SEM are shown. * indicates significant differences compared with control ($p < 0.05$).

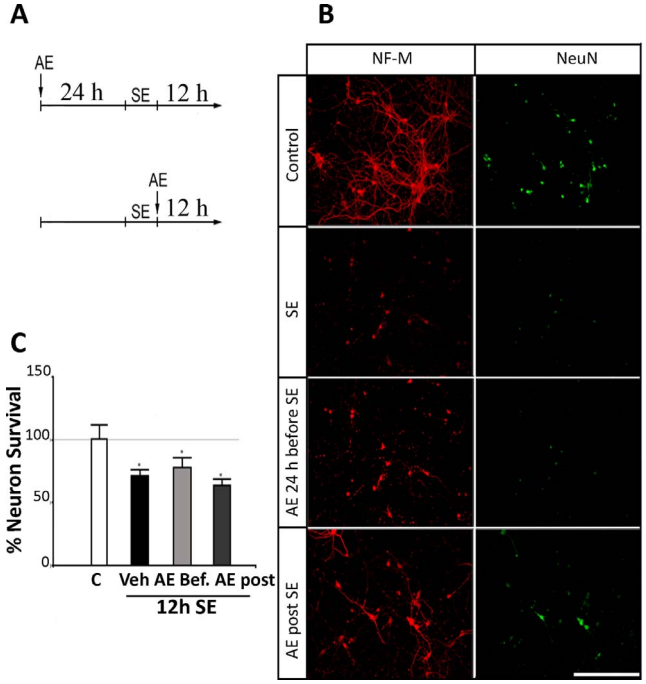

Fig. 4. *The addition of AE does not induce neuroprotection in a culture of pure hippocampal neurons following SE.* (a) The AE was added to control or SE cultures of hippocampal neurons, 24 h before or immediately after the excitotoxic insult. (b) Micrographs of hippocampal neurons stained with Neurofilament M (left) and NeuN (right) show representative treatments. Neuronal death was analyzed 12 h later. (c) The AE failed to protect hippocampal neurons even when added 24 h before the excitotoxic insult. *Scale bar 200 μm.* Mean ± SEM are shown. * indicates significant differences compared with control ($p < 0.05$).

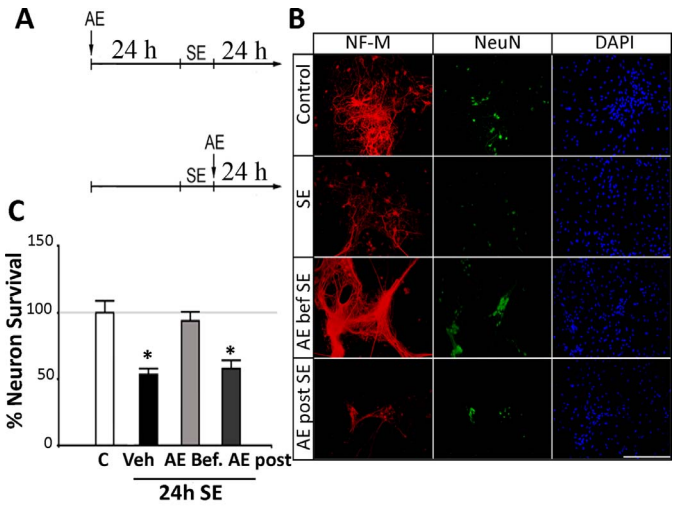

Fig. 5. *The addition of AE induces neuroprotection in a co-culture of hippocampal neurons and astrocytes following SE.* (a) The AE was added to control or SE cultures of hippocampal neurons, 24 h before or immediately after the excitotoxic insult. (b) Micrographs of hippocampal neurons stained with Neurofilament M (left), NeuN (middle) and DAPI (right) show representative treatments. Neuronal death was analyzed 24 h later (c) In the co-culture, the AE only protect hippocampal neurons when was added 24 h before the insult. *Scale bar 200 μm.* Mean ± SEM are shown. * indicates significant differences compared with control ($p < 0.05$).

after the finalization of the SE. Twenty-four hours later, brain slices were analyzed for neuronal damage by Nissl and FJB stains (Fig. 6).

Considering that the number of dead neurons in this model is almost the same in both hemispheres, FJB positive neurons counted in the left (infused side) were expressed as a percentage of those found in the right (non-infused hippocampus). The animals that received the AE showed significant decrease in neuronal death (73 ± 1% relative to the contralateral hippocampus, $p \leq 0.01$). Control animals receiving vehicle infusion showed no differences in neuronal death between hemispheres (Fig. 6). Interestingly, when the AE was infused in the hippocampal CA1 we also found a remarkable decrease in the number of FJB positive cells in the EC compared to the contralateral side (Fig. 7).

In contrast with the neuroprotection observed in the CA1 and EC area, there was no difference in neuronal damage in the hilus of the dentate gyrus between both hippocampi in all treatments (Fig. 7), even when the AE was infused into the hilus (data not shown).

Discussion

The administration of the AE induced a remarkable neuroprotection both *in vitro* and *in vivo* models of SE. Temporal correlations between neuroprotection and increases in pTrkB suggest that NT may be involved in the AE effect. Although the behavior of this receptor is complex and can induce several cellular functions, its physiological activation is associated with neuronal survival (Skaper, 2012). We did not evaluate the release of BDNF *in vitro,* but the fact that AE induced increases the TrkB phosphorylation and that K252 blocked the pro-survival effect, suggest that the AE induced BDNF release from neurons and consequently, activates TrkB. Previously it was demonstrated that another alkaloid extract promotes neuroprotection and release of NGF, a well-known NT associated with neuron survival (Yu et al., 2013). However, it cannot be discarded that the activation of TrkB receptor is not exclusively linked to BDNF. Recently published data shows that Cdk5 or an increase in zinc levels may also activate TrkB in a BDNF independent manner (Huang and McNamara, 2012; Lai et al., 2012). Future experiments will be conducted to determine the exact mechanism by which the TrkB receptor is activated after addition of AE.

Given these results, it was important to determine whether the same neuroprotective effect can be acquired in models of neurodegeneration,

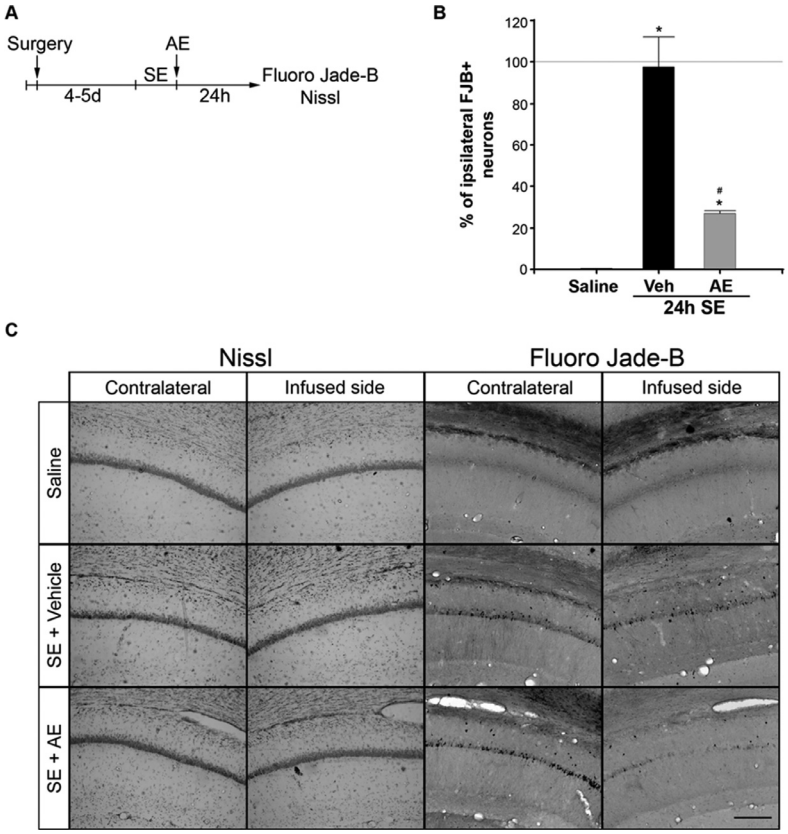

Fig. 6. *AE infused after SE decreased neuron death in hippocampal CA1.* (a) Control or SE animals received an infusion of AE or vehicle after 2 h SE and the tissue was analyzed after 24 h by FJB and Nissl. (b) The infusion of AE after SE markedly prevented neuronal death in the infused hippocampus, while vehicle caused no effect. (c) Micrographs of hippocampal CA1 stained with Nissl (left) and FJB (right) show representative animals from each treatment. *Scale bar 200 μm.* Mean ± SEM are shown. * indicates significant difference compared with control (saline) animals, $p < 0.05$. # indicates significant differences compared with vehicle (SE) animals. $p < 0.05$.

both *in vitro* and *in vivo* excitotoxic events. In the *in vitro* experiments, AE only induced neuroprotection if it was added 24 h before the onset of the excitotoxic insult in the co-culture situation. This result demonstrates that astrocytes are key players in the AE neuroprotection effect. The presence of astrocytes may incorporate the excess on proBNDF released during excitotoxic insults, (Bergami et al., 2008; Vignoli et al., 2016,) or on the other hand, may release several NT factors that could promote neuronal survival (Xu et al., 2013).

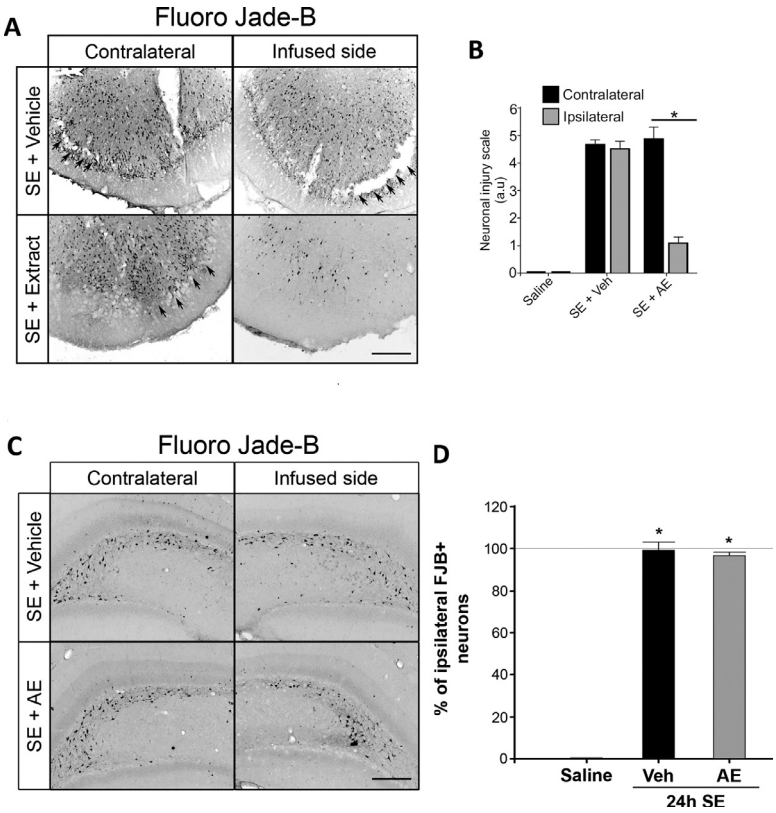

Fig. 7. *Effect of AE in the Entorhinal Cortex and hilus after SE.* (a) The infusion of AE into hippocampal CA1 protects against neuronal death in EC after SE. Micrographs of EC stained with FJB show representative animals from each treatment. (b) The infusion of AE into hippocampal CA1 prevent cell death in the entorhinal cortex. (c) Micrographs of hippocampal hilus stained with FJB show representative animals from each treatment. (d) The infusion of AE after SE did not prevent neuronal death in the hippocampal hilus *Scale bar 200 μm.* Mean ± SEM are shown. * indicates significant difference compared with control (saline) animals, $p < 0.05$.

In the *in vivo* model of epilepsy, the AE was administered after SE, to discard the possibility that AE could interfere with normal epileptiform activity. The administration of AE in the hippocampal CA1 induced a remarkable protection against neuronal death after SE. Surprisingly, we also found an extensive and remarkable neuroprotection in the amygdala-EC area, even though the infusion was far away this region. This may be due to the circuit connections between hippocampus and amygdala-EC (Witter, 1993). In this model of SE, the limbic system is severely damaged. In particular, there is evidence showing that the hippocampal region plays an important role as initiator and controller of epileptic activity for both EC and amygdala (Stoop and Pralong, 2000). To our knowledge, this is the first study demonstrating neuroprotection in the Amygdala-EC area when a compound is administered in the hippocampus after the SE has been developed.

The lack of neuroprotection in the hilus may be due to different processes determining neuronal death in this area. We previously demonstrated that infusion in the hippocampal CA1 of a blocking function BDNF antibody was not able to protect the neurons of the hilus (Unsain et al., 2009). It is known that SE induces necrotic and apoptotic neuronal death (Fujikawa, 2005), and it was demonstrated that neuronal death occurs earlier in the hilus than in the CA1 region. It is possible that different mechanisms of cell death occurred in different cerebral regions (Unsain et al., 2008). These differences in mechanisms between both regions could be related to timing of cellular death. It is also possible that the AE had no effect because the neurons in the hilus are already dead before its administration. Future experiments will be conducted to test if the AE, systemically administrated, may also have a neuroprotective effect in the hippocampal hilus.

In this extract ten Lycopodium alkaloids were identified (Ortega et al., 2004; Vallejo et al., 2013). Sauroine, Sauroxine and 6-OH lycopodine are the majority constituents, the others appear in a very small concentration or at trace level (Ortega et al., 2007; Vallejo et al., 2013). To distinguish which of them are responsible for the AE activity, further experiments will be carried out.

Together the *in vitro* and *in vivo* results demonstrate that the purified AE has neuroprotective activity and therefore has a potential use in neurological disorders involving neuronal death and memory impairment.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support that could have influenced its outcome.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phymed.2017.05.010.

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