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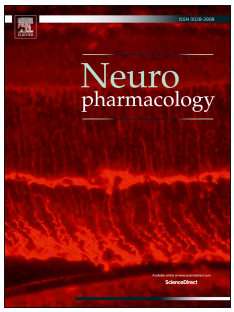

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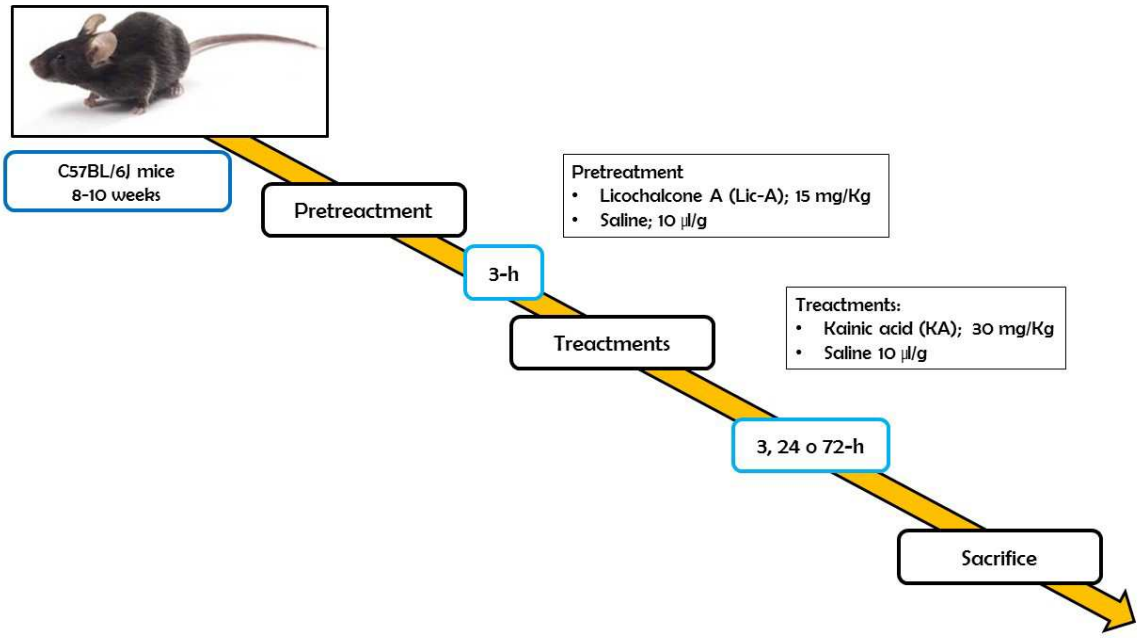

JNK1 inhibition by Licochalcone A leads to neuronal protection against excitotoxic insults derived of kainic acid

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Abstract

The mitogen-activated protein kinase family (MAPK) is an important group of enzymes involved in cellular responses to diverse external stimuli. One of the members of this family is the c-Jun-N-terminal kinase (JNK). The activation of the JNK pathway has been largely associated with the pathogenesis that occurs in epilepsy and neurodegeneration. Kainic acid (KA) administration in rodents is an experimental approach that induces status epilepticus (SE) and replicates many of the phenomenological features of human temporal lobe epilepsy (TLE).

Recent studies in our group have evidenced that the absence of the JNK1 gene has neuroprotective effects against the damage induced by KA, as it occurs with the absence of JNK3. The aim of the present study was to analyse whether the pharmacological inhibition of JNK1 by Licochalcone A (Lic-A) had similar effects and if it may be considered as a new molecule for the treatment of SE. In order to achieve this objective, animals were pre-treated with Lic-A and posteriorly administered with KA as a model for TLE. In addition, a comparative study with KA was performed between *wild type* pre-treated with Lic-A and single knock-out transgenic mice for the *Jnk1* $^{-/-}$ gene.

Our results showed that JNK1 inhibition by Lic-A, previous to KA administration, caused a reduction in the convulsive pattern. Furthermore, it reduced phosphorylation levels of the JNK, as well as its activity. In addition, Lic-A prevented hippocampal neuronal degeneration, increased pro-survival anti-apoptotic mechanisms, reduced pro-apoptotic biomarkers, decreased cellular stress and neuroinflammatory processes. Thus, our results suggest that inhibition of the JNK1 by Lic-A has neuroprotective effects and that; it could be a new potential approach for the treatment of SE and neurodegeneration.

Introduction

Temporal lobe epilepsy (TLE) is the most common form of human epilepsy (Levesque M et al., 2013). It is characterized by the occurrence of unpredictable and recurrent focal seizures (Lucke-wold BP et al., 2015) Currently, although there are effective treatments for this pathology, there is the problem of drug-resistant epilepsy in certain patients, a condition defined by the *International League against Epilepsy,* as the persistence of epileptic seizures despite using at least two treatments with appropriate antiepileptic applications. In this case, the only therapeutic alternative in such patients is the surgical resection of the brain's epileptic tissue. For this reason, the development of new therapeutic approaches is necessary.

Although there are no preclinical models that reproduce all the features of TLE, some experimental models have been used over the past decades due to their high capacity of reproduction of human epilepsy (Jefferys J et al., 2016). One of these well-known, thoroughly described models is the administration of kainic acid (KA, 2-carboxy- 4-isopropenylpyrrolidin-3-ylacetic acid). This model, which was originally reported by Ben-Ari (Ben-Ari and Lagowska, 1979), features TLE allowing for a deep study of this pathology.

The neurotoxin KA was first isolated in the red alga *Digenea simplex* which is found in tropical and subtropical waters (Fernández-Espejo, 1996). KA is an analogue of glutamate, thus, local or systemic administration of KA in rodents leads to a pattern of repetitive limbic seizures that lasts for several hours and is followed, after a variable latency period, by the chronic phase, which is characterized by the occurrence of spontaneous limbic seizures (Hammer J et al., 2008). These seizures cause extensive brain damage, often associated with aberrant axonal reorganization, an increase in reactivity of the glia (increased proliferation and hypertrophy of astrocytes and microglia), as well as dysregulation of cellular homeostasis (Dudek et al., 2002). The c-Jun N-terminal kinases (JNKs), also called stress-activated protein kinases (SAPK), belong to the family of mitogen-activated protein kinases (MAPK). These enzymes are activated by KA and are involved in stress responses and cell death (Derijard et al., 1994; Kallunki et al, 1994 and 1996; Kyriakis et al., 1994 Auladell et al., 2017). Moreover, JNKs are also involved in the processes of neuronal cell loss in cerebral ischemia-induced neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease (Dudek, 2002; Lerma & Marques, 2013). There are different JNK isoforms, encoded by three different genes, *Jnk1*, *Jnk2*, and *Jnk3*. In mammals, JNK1 (MAPK8) and JNK2 (MAPK9)

proteins are expressed ubiquitously, whereas JNK3 (MAPK10) is mostly expressed in the brain and, to a lesser extent, in the heart and testes (Yuan et al., 2003; Brecht et al., 2005).

Several synthetic compounds have shown to be able to inhibit this pathway. Among them, the best evaluated molecules at a preclinical level are SP600125 and AS601245, both unspecific inhibitors of all JNK isoforms (Bennet BL et al., 2001, Carboni S et al., 2008). However, it is necessary to develop specific inhibitors for each isoform to avoid possible side effects derived of the different roles that the three isoforms play within the tissues. The JNK3 isoform is the one that has been classically related on the development of neurotoxic responses after KA administration and, several groups, have reported interesting results using a competitive inhibitor of the JNK3, the D-JNK-1. Specifically, this inhibitor has effect on the pool of this protein found in the mitochondria (Borsello T et al., 2003, Repici M et al., 2007, Spigolon G et al., 2010 and Zhao Y et al., 2012). In addition, our group recently demonstrated that the JNK1 would also have high importance in the regulation of brain damage induced by KA (de Lemos L et al., 2017). One promising pharmacological inhibitors of the JNK1 belongs to the group of chalcones, which are found in many natural products (fruits, vegetables, spices, tea …). The roots of liquorice (Glycyrrhiza inflata) have various pharmacological properties, including anti-inflammatory, anti-oxidative and anti-carcinogenic activities (Shibata; Zasshi, 2000; Funakoshi-Tago et al., 2008). One of these chalcones, Licochalcone-A (Lic-A), has been reported to target JNK1 and JNK2 but only has inhibitory effect on the activity of the JNK1 (Yao et al., 2014).

Since the JNK1 is involved in neuronal death induced by KA, and Lic-A selectively inhibits JNK1, it was hypothesized that a pre-treatment with Lic-A, previous to KA, would reduce the consequences of epileptic seizures compared to mice only treated with KA. The results revealed a decrease in the severity of seizures and neuronal damage, along with reductions in apoptotic biomarkers and neuroinflammation. Moreover, in order to confirm that the neuroprotective effect of Lic-A against KA is, in fact, a consequence of the inhibition of the JNK1, several biomarkers that are usually altered by KA were studied versus mice pre-treated with Lic-A before KA injection, and single knock-out transgenic mice for the *Jnk1*$^{-/-}$gene also treated with KA. The analyses revealed similar effects in both experimental models. So, for the first time, it has been demonstrated that in vivo pharmacological inhibition of JNK1 by Lic-A has neuroprotective effects in mice. Therefore, this compound may constitute a new potential drug that should be evaluated in additional experimental models for the treatment of epilepsy and also other neurodegenerative pathologies.

Materials and Methods

Animals

Evaluation of the potential application of Lic-A as a neuroprotectant

C57BL6/J *wild-type* mice of two months of age were used in this study (n=15 for each experimental group). Four experimental groups were established; animal distribution can be seen in **Table 1**.

Comparative evaluation of the effects of Lic-A inhibition and Jnk1 -/- transgenesis against KA insults

Two months old C57BL6/J *wild-type* and *Jnk1* $^{-/-}$ single knock-out transgenic mice were used in this study (n=4 for each experimental group). The generation and characterization of the *Jnk1*$^{-/-}$ single knockout mouse has been previously described (Dong et al., 1998). Animal distribution for this study can be seen in **Table 1**.

Throughout all the experiments, mice were housed in a temperature and humidity controlled environment, with regular 12-h light/dark cycle, food and water were available *ad libitum*. The experiments were conducted in accordance with the Council of Europe Directive 2010/63. The procedure was registered at the *Department of Agricultura, Ramaderia i Pesca* of the *Generalitat de Catalunya*. Ref. Number order 8852.

Pre-treatment, Treatment and Sample Extraction

The different administrations established in **Table 1** were all done intraperitoneally as follows: Saline volume administration was standardized at 10 µl/g in order to keep a similar blood volume affectation on all animals. KA was administered in a 30 mg/kg dosage (Sigma-Aldrich, K-2389, USA)(Junyent et al., 2009) and Lic-A at 15 mg/kg (Calbiochem®, 435800-50MG, Denmark).

In both studies (Study 1 and 2) the animals were pre-treated 3-h before KA treatment with either saline or Lic-A. 3, 24 or 72-h after KA administration, the animals were sacrificed in the appropriate method. In study 2 the samples were limited to the 3-h period.

Evaluation of seizure-related behaviour

After the exposure to KA, the evaluation of seizures was performed according to Morrison et al. 1996. Briefly, highest stage of seizures was evaluated for 2-h after injection. Each seizure score was determined depending on the overall seizure-related behaviour for each 5-min period. Each seizure score was multiplied by the number of 5-min periods in which the animal

received that score. These numbers were added together for a raw score, and the final seizure ratings were derived from these raw scores. Raw scores less than 50 were assigned a rating of 1, those between 50 and 79 received a rating of 2, and those 80 and above received a rating of 3. Animals exhibiting no seizure activity received a rating of 0.

Immunoblot analysis

Protein extraction for western blot analysis was performed in fresh tissue samples obtained from animals sacrificed through neck dislocation. Previous to the actual protein extraction, the samples were pulverized with liquid nitrogen. Posteriorly, samples were homogenized on a lysis buffer (Tris HCl 1M pH 7.4, NaCl 5M, EDTA 0.5M pH 8, Triton, distilled H_2O), a protease inhibitor (Complete Mini, EDTA-free; Protease inhibitor cocktail tablets, 11836170001, Roche Diagnostics GmbH, Germany) and a phosphatase inhibitor (Phosphatase inhibitor cocktail 3, P0044, Sigma-Aldrich, USA). After a 30-min incubation at 4 °C, the samples were centrifuged at 14000 rpm for 10-min at 4 °C and the supernatant was recovered and frozen at -80 °C until use.

After protein extraction, protein concentration was evaluated through a Pierce™ BCA Protein Assay Kit (#23225; Thermo Scientific, USA). Sequentially, 10 µg per sample were denatured at 95 °C for 5-min in a sample buffer (0.5M Tris HCl, pH 6.8, 10% glycerol, 2%(w/v) SDS, 5%(v/v) 2-mercaptoethanol, 0.05% bromophenol blue). Electrophoresis was performed on acrylamide gels at 100 V and they were transferred to polyvinylidene difluoride sheets (Immobilon®-P Transfer Membrane; IPVH00010; Merk Millipore Ltd., USA) (200 mA). Then, membranes were blocked for 1-h with 5% non-fat milk dissolved in TBS-T buffer (0.5mM Tris; NaCl, Tween® 20 (P1379, Sigma-Aldrich, USA), pH 7.5), washed with TBS-T 3 times for 5-min and incubated with the appropriate primary antibody (**Table 2**) overnight shaking at 4 °C. Subsequently, blots were washed thoroughly in TBS-T buffer and incubated at room temperature for 1-h with the appropriate secondary antibody (**Table 2**). Ultimately, blots were exposed to a chemoluminescence detection agent (Pierce® ECL Western Blotting Substrate; #32106; Thermo Scientific, USA). Protein levels were determined using Image Lab version 5.2.1 (Bio-Rad Laboratories). Measurements were expressed as arbitrary units and all results were normalized to the corresponding GAPDH used as a loading control.

Kinase activity assay

The kinase activity assay was performed after an immunoprecipitation of the P-JNK enzyme from a protein extraction sample (200 µg were used per sample). To minimize the detection of cross-reactive proteins a pre-cleaning procedure was performed: protein samples were

incubated with 50 µl Protein A/G PLUS -Agarose (sc-2003, Santa Cruz Biotechnology, USA) for 1-h at 4 °C with gentle shaking in a rotor. This step was followed by a centrifugation at 3500g for 5-min to remove the supernatants to clean tubes. Next, samples were incubated with 3 µl of antibody plus 30 µl of Protein A/G PLUS-Agarose overnight at 4 °C with gentle shaking. On the next day, samples were centrifuged at 3500g for 5-min and the supernatant was discarded. Several cleaning steps were performed afterwards with STEN buffer (0.5M NaCl, 50 mM Tris, pH 7.6, 2mM EDTA, 0.2% NP-40) before the samples were deemed ready for the activity assay.

This procedure was performed following the SAPK/JNK Kinase Assay Kit instructions (Nonradioactive) (#9810, Cell Signaling, USA).

Immunofluorescence

Mice used for immunofluorescence studies were anesthetized by intraperitoneal injection of ketamine (100mg/kg) and xylazine (10 mg/kg) and perfused with 4% paraformaldehyde (PFA) diluted in 0.1M phosphate buffer (PB). Brains were removed and stored in the same solution overnight at 4^{o}C and 24-h later, they were cryoprotected in 30% sucrose-PFA-PB solution. Coronal sections of 20 µm of thickness were obtained by a cryostat (Leica Microsystems).

On the first day, free-floating sections were washed three times with 0.1 mol/L PBS pH 7.35 and after, five times with PBS-T (PBS 0.1 M, 0.2% Triton X-100). Then, they were incubated in a blocking solution containing 10% fetal bovine serum (FBS), 1% Triton X-100 and PBS 0.1M + 0.2% gelatin for 2-h at room temperature. After that, slices were washed with PBS-T five times for 5-min each and incubated with the primary antibody overnight (**Table 3**). On the second day, brain slices were washed with PBS-T 5 times for 5 minutes and incubated with the appropriate secondary antibody for 2-h at room temperature (**Table 3**). Later, sections were co-stained with 0.1 µg/ml Hoechst 33258 (861405; Sigma-Aldrich, St Louis, MO, USA) for 15-min in the dark at room temperature and washed with PBS 0.1M. Finally, slides were mounted using Fluoromount G (#19984-25; Electron Microscopy Sciences, USA). Image acquisition was performed with an epifluorescence microscope (Olympus BX61 Laboratory Microscope, Melville, NY-Olympus America Inc.).

Fluoro-Jade B Staining

Neurodegeneration was assessed using Fluoro-Jade B staining (AG310, Millipore, USA) (Schmueda L et al., 2000). Slides were rinsed with phosphate buffer solution (PBS), followed by two washes in distilled water. Afterwards, slides were immersed in 0.6 g/L potassium permanganate (KMnO4) for 15-min in the dark. Then, after two washes in distilled water, the

slides were transferred to the staining solution containing 0.1 mL/L acetic acid and 0.004 mL/L of the fluorophore Fluoro-Jade B for 30 min in the dark. Slides were rinsed in distilled water, and then submerged directly into xylene and mounted in DPX medium (06522, Sigma-Aldrich, USA). Slides were analysed using an epifluorescence microscopy (Olympus BX61 Laboratory Microscope, Melville, NY-Olympus America Inc.).

Evaluation of labelled cells

In order to obtain results on cellular numbers, sections corresponding to the hippocampal areas between Bregma -1.34 to -2.46 mm, in accordance with the Atlas reported by Paxinos and Franlin, (2012), were used to determine the number of positively-labelled cells in 40 mm^2 areas of each section (4-6 animals/genotype and age, 4-8 sections /animal), in both the CA3 and CA1. The density of counted cells was expressed as number of cells per square millimetre of tissue examined, using a conversion factor ($3{,}5 \cdot 10^4$) calculated for 100X magnification. Counts used to determine numerical densities of cells were made with a 25-point eyepiece morphometric grid (NE35, Electron Microscopy Sciences, Hatfield, PA) attached to the ocular of an optical microscope (Optical Microscope Olympus BX61) (Olloquequi J et al., 2010).

Statistical Analysis

All results were represented as MEAN±SD. Statistical analysis was performed trough different methods:

- Evaluation of the potential application of Lic-A as a neuroprotectant: The saline experimental group was compared against KA and Lic-A groups through one-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Tuckey post-test was performed. The KA group was compared against the Lic-A group through t-test analysis ($ $p < 0.05$, $$ $p < 0.01$, $$$ $p < 0.001$).
- Comparative evaluation of the effects of Lic-A inhibition and Jnk1 -/- transgenesis against KA insults: The KA experimental group was compared against the Lic-A + KA and *Jnk1* $^{-/-}$ + KA groups through %-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Tuckey post-test was performed.

All analyses and graph representations were performed in the program Graph Pad Prism for Windows version 6.01; Graph Pad Software, Inc.

Results

Evaluation of the potential application of Lic-A as a neuroprotectant.

Lic-A attenuates the induction of seizure-related behaviours induced by KA.

The pre-treatments with Lic-A caused a reduction of seizures compared to mice treated only with KA (**Figure 1**). Specifically, animals in the KA experimental group showed a rating value of 2.46±0.16, while those pre-treated with Lic-A had a rating of 1.83±0.17 (reduction on a 25.6%) These evaluations were performed as it's described in the corresponding subsection of the *Materials and Methods*.

Lic-A reduces JNK phosphorylation levels and activity

In previous studies, it has been demonstrated that KA administration on rodents induces the activation of MAPK (de Lemos L et al., 2010; Ettcheto M et al., 2015). Accordingly, we examined in the hippocampus of all our experimental groups, the phosphorylation levels of JNK at various time points after KA administration (3, 24 and 72-h).

The results obtained in Western blot revealed a significant increase of JNK phosphorylation levels (P-JNK) in the hippocampal tissues at 3-h after KA injection. Contrarily, the animals pre-treated with Lic-A before KA showed a phosphorylation decrease compared with the KA experimental group (69.7% of decrease). Moreover, Lic-A prevented the increase of JNK phosphorylation even against the control group (69.2% of decrease). The time course response of animals treated with Lic-A and Lic-A + KA would also suggest a reversible inhibition of the JNK seeing how, over time, it restores back to the response levels of the saline group (**Figure 2A**). Because it has been reported that JNK1 could be and specific target of Lic-A, we evaluated the effects of KA on this specific isoform 3-h after the exposition to KA. The phosphorylation values of P-JNK1 showed high correlation with the ones observed with the total P-JNK protein (**Figure 2B**).

To corroborate these observations, the levels of total and phosphorylated forms of cJUN were evaluated after 3-h of treatment. cJUN is one of the targets phosphorylated downstream of JNK1 (Sabapathy K et al., 2004). In fact, exposure to KA increased significantly the levels of total and phosphorylated forms of cJUN. By contrast, the exposure to Lic-A reduced this stress-associated response in the P-cJUN, both Lic-A and Lic-A + KA experimental groups (61.9% and 28.4% of decrease respectively of saline and KA groups) **(Figure 2B)**.

Further demonstration of JNK inhibition by Lic-A was evaluated through the assay of SAPK/JNK activity (**Figure 2C**). Results showed a significant increase in the kinase activity in

mice exposed to KA compared to control, while it was significantly reduced in mice pre-treated with Lic-A (42.3% of reduction).

Lic-A protects against neurodegeneration and induces cell survival responses

Neurodegeneration is an indicator of brain damage, clearly detected in the CA1 and CA3 hippocampal areas 24-h past the KA treatment. Fluoro-Jade B stain was used as a method of detection of neurodegenerating neurons. Only the animals in the KA experimental group showed a typical pattern of high density of degenerating cells (Schmueda L et al., 2000); those that had been pre-treated with Lic-A had no labelling (**Figure 3**). Concurrently, an evaluation on the number of surviving neurons was performed. Using the NeuN antigen in an immunofluorescence assay as an indicator of alive mature neurons, it was detected how there was a significant reduction on them in the KA experimental group versus all others (**Figure 4**).

Lic-A reduces neural proliferation responses after KA damage.

When studying the damage caused by KA in the hippocampus in mice, the subsequent proliferation of neural cells was also evaluated. The granule cell layer of the hippocampus is preserved after the exposure to KA. After the damage, it leads to the development of neurogenesis mechanisms in the subgranular zone (SGZ). To test if there were changes in this process, with Lic-A pre-treatment before KA, an immunofluorescence assay using an antibody against NESTIN, as a marker for stem cells, was performed. The results obtained evidenced a significant reduction of nestin immunopositive cells in mice pre-treated with Lic-A before KA compared to mice only treated with KA (**Figure 5**).

Lic-A decreases the need for induction of survival pathways after KA treatment

The molecular pathways involved in the response against cell cytotoxicity, caused by the exposure to KA, include the survival signalling pathway. The main effector under the regulation of the JNKs is the AKT protein, which is modulated by phosphorylation on the Ser473 residue. Thus, 3-h after the exposure to KA there was a significant increase in the phosphorylated status of AKT (**Figure 6**). Lic-A pre-treatment caused for a significant reduction of this phosphorylated status of the AKT (45.6% of reduction). Correspondingly, the cAMP responsive element-binding protein (CREB), that is found downstream of AKT in the survival pathway, showed the same pattern levels of phosphorylation as the previously described protein (41% of reduction in the Lic-A + KA experimental group). Further analysis of AKT and CREB 24-h after the exposure to KA revealed no significant variations of the protein status in any experimental group.

Lic-A promotes anti-apoptotic responses and reduces pro-apoptotic biomarkers

In order to evaluate further the data obtained in the previous section, other biomarkers associated with the development of apoptotic mechanisms were analysed. The study of biomarkers through western blot was limited to the time period 3-h after the administration of KA in order to evaluate the early response signals. As it was expected, KA promoted the increase of protein levels of B-cell lymphoma 2 (BCL-2), BCL-2-like protein 4 (BAX), BCL-2 like protein 11 (BIM) and the products of α-spectrin (aS): cleaved α-spectrin by calpain (CaSBC) and cleaved α-spectrin by caspase 3 (CaSBC3) (**Figure 7**). Also, Lic-A pre-treatments reduced significantly the levels of these same proteins; interestingly BCL-2 was increased further (increase of BCL-2 by 14%, decrease of BAX (31%), BIM (48.9%), CaSBC (38.7%) and CaSBC3 (49.1%)).

Next, immunofluorescence assays against BCL-2 and BAX on the tissue samples extracted 24-h after the administration of KA were performed. The results were very similar to those discussed for the western blot. BAX had increased its levels in the KA experimental group and Lic-A had reduced its response (**Figure 8**). In addition, BCL-2 had also increased its levels with KA but, Lic-A had promoted further its levels (**Figure 9**).

Lic-A decreases oxidative stress and neuroinflammatory responses

Oxidative stress is one of the key factors that causes damage derived of KA. Several studies by western blot with protein samples of animals exposed to KA for 3-h were performed. Significant increases in the levels of the peroxisome-proliferator-activated receptor-gamma-coactivator-1-alpha (PGC1α) were detected, a mitochondrial protein associated with the development of stress responses. In addition, significant increases in pro-inflammatory biomarkers: tumour necrosis factor α (TNFα), induced nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) were detected. All these biomarkers that found themselves increased due to the stress and damage caused by KA, were significantly reduced when the animals had undergone a pre-treatment with Lic-A (9.5, 33.5, 38.49% and 25.8% of decrease respectively). Interestingly, Lic-A also seems to decrease the presence of proADAM10 and ADAM10 (40.31% and 24.8% of decrease respectively), a metalloprotease associated with the activation of soluble TNFα (**Figure 10**).

Given the oxidative stress results, several immunofluorescence assays to evaluate its consequences on the long term were performed. Immunolabeling of the glial fibrillary acidic protein (GFAP) 72-h after the administration of KA (**Figure 11**), and the ionized calcium-binding adapter molecule 1 (IBA1) 24-h after the administration of KA (**Figure 12**), in the DG of the

hippocampus, revealed how the Lic-A pre-treatment had also caused for a significant reduction of the reactivity of these two cellular types, being highly reactive for the KA experimental group.

Comparative evaluation of the effects of Lic-A inhibition and Jnk1 -/- transgenesis against KA insults.

To confirm that the neuroprotective action of Lic-A is mediated through the inhibition of JNK1, the levels of several proteins (P-JNK/JNK, P-AKT/AKT, BCL-2, BAX and aS and its cleaved products CaSBC and CaSBC3) that had already been analysed previously in the 3-h after KA time period were evaluated. The results evidenced that Lic-A caused a reduction of these proteins as occurred in the $Jnk1^{-/-}$ mice (**Figure 13**).

Discussion

The data obtained in the present work demonstrates the neuroprotective effect of in vivo pharmacological selective inhibition of JNK1 by Lic-A, specifically in a KA-induced TLE mice model. Furthermore, the specificity of this molecule was studied further comparing the effects of Lic-A versus single knock-out transgenic mice for the *Jnk1* gene.

First of all, it was evidenced how a pre-treatment with Lic-A before KA was able to reduce seizure-related behaviours. This was obtained through an evaluation of the convulsive response of animals after KA administration. Since Lic-A is a specific inhibitor of JNK1, as it has been demonstrated in in vitro models (Yao et al., 2014), the results obtained point out to the fact that JNK1 has a role on diminishing seizures, but the pathway through which this occurs is still unknown. Considering the basis of convulsions, associated with high calcium levels in the cytoplasmic environment, it could be possible that the JNKs have some control over the contractibility of muscular cells or the activity of calcium-dependent enzymes. Tai T et al. 2017 has reported similar data referring to the anticonvulsive effect of the inhibition of the JNKs. But, they do not report the mechanism through which this would occur.

TLE has been associated many times with high values of activating phosphorylation of the JNKs in mice, rats and humans (Jeon S et al. 2000, Sabapathy et al., 2012), which eventually leads to neuronal death (Harper S et al. 2001, Verdaguer E et al., 2003). In our results, this data was confirmed through a time course, treating animals for 3, 24 and 72 h with KA. It was observed how KA caused a significant increase in JNK phosphorylation at 3h, as it was reported before in others studies from our group (Auladell C et al., 2017). Lic-A reduced the phosphorylated state of the JNKs. Specifically, significant reductions in the phosphorylation levels of total JNK as well as JNK1 after being treated with KA for 3-h were observed. The

inhibition of the JNK1 phosphorylated status, supports the data reported by Yao et al., 2014 demonstrating the specificity of Lic-A. Additionally, the results of the Lic-A experimental group points out to a mechanism of reversible inhibition when observing the time course treatment. Furthermore, the analysis of cJUN, a JNK downstream early transcription factor, added more evidences to the importance of the JNK1 in KA induced neuronal death. The role of JNK1 in this process was also supported when neuronal degeneration was evaluated by Fluoro-Jade B stain 24 hours after KA treatment. The animals that had undergone a pre-treatment with Lic-A showed no neurodegenerating neurons in the main areas of the hippocampus typically affected by KA (Nadler JV et al., 1980, Nadler JV et al., 1980 and French ED et al., 1982). A posterior analysis, in the same time period, on the number of survival neurons through the detection of the NeuN protein, a typical biomarker of mature neurons, increased the amount of evidence on the neuroprotectant effect of Lic-A through its inhibition of the JNK1. It must be noted, that our group has already published data demonstrating how the deletion of the *Jnk1* gene has neuroprotective effects in models of KA (de Lemos et al., 2017).

Moreover, following KA treatment, there is increase in the proliferation on neural cells. This situation becomes a harmful event and induces alterations in the network circuits due to the formation of aberrant synaptic contacts (Parent JM et al., 2008, Jessber S et al., 2007). This abnormal network is the cause for the development of spontaneous seizures in the so called chronic epilepsy (Mathern G et al, 1993). The reduction of nestin immunopositive cells observed in mice pre-treated with Lic-A before KA, in comparison to mice only treated with KA, suggested there was a lower chance for the formation of aberrant synaptic contacts and therefore a reduction of spontaneous seizures though a chronic status.

After the exposition to KA, many molecular pathways involved in the degeneration of neuronal cells are activated like cell survival responses. One of these is the phosphoinositide 3-kinase (PI3K)/AKT pathway (Dunleavy et al., 2013). The activation of this pathway, specifically, through the phosphorylation of AKT in the Ser473 residue, allows for the initiation of survival responses. This phosphorylation was highly increased in those animals that had been treated with KA for 3-h, Lic-A reduced significantly this phosphorylation. This data together with the rest obtained in this study, backs up our hypothesis that JNK1 inhibition is able to reduce the stress and damage that the cells suffer and, consequently, there is a lesser need for the activation of survival mechanisms. These results correlate perfectly with the data already obtained of less convulsive responses and a decrease in neuronal degeneration in the group of animals pre-treated with Lic-A. Moreover, CREB, a transcription factor downstream of AKT, which has been reported to modulate synaptic plasticity (Benito E et al., 2010) showed similar patterns of response and, as a result, the data adds up on the conclusion that Lic-A protects

neurons and prevents the formation of defective synaptic contacts (Parent JM et al., 2008, Jessber S et al., 2007) reinforcing the role of JNK1 in KA-induced neuronal damage.

It has already been described that the JNKs have a role in the regulation of apoptosis (Liou AKF et al., 2003), in the present paper it was demonstrated how the inhibition of JNK1 diminishes apoptotic responses caused by KA. Several apoptotic signals were analysed 3-h after treatment. KA effects and the subsequent activation of the JNKs lead to a promotion of pro-apoptotic biomarkers like BAX, responsible for the activation of BIM which inhibits BCL-2, an anti-apoptotic protein. Also, it has been demonstrated that JNK activation is able to inhibit BCL-2 further (Dhanasekaran, D N et al., 2008). The experiments performed in mice pre-treated with Lic-A before KA administration, showed lower activation of this pathway, decreasing the presence of BAX and BIM and promoting highly the presence of BCL-2, thus modifying the balance between apoptotic and anti-apoptotic proteins. Interestingly, in the experimental group of animals only with Lic-A, there was already an increase in anti-apoptotic protein BCL-2, corroborating the efficiency of Lic-A and its inhibition of JNK1 in preventing the activation of the apoptotic pathway. Moreover, this data is supported by the analysis on Lic-A pre-treated animals on the generation of products of aS, obtained by the activation of pro-apoptotic enzymes caspase 3 and calpain. These results are very interesting since Lic-A has also been reported to be a pro-apoptotic molecule in carcinogenic cell cultures (Park MR et al., 2015), thus arising hypothesis on its possible applications.

Moreover, linked to cellular stress, mitochondrial function alteration was evaluated, a reduction of PGC1α was observed. PGC1α is a transcriptional coactivator that regulates mitochondrial biology and is an important mediator against reactive oxygen species (ROS) (St-Pierre J et al., 2006). In our analysis, 3-h after the administration of KA, the levels of PGC1α had increased significantly, being reduced by Lic-A. KA administration causes excitotoxicity that is responsible for the seizures described in the model of TLE and a posterior inflammatory process as it has been demonstrated detecting appearance of gliosis by IBA1 and GFAP reactive immunofluorescence in the hippocampus of animals treated with KA. Early inflammatory targets were also detected 3-h after KA administration, specifically iNOS, nNOS and TNFα protein levels. The results showed similar pattern of response increasing their levels 3-h after KA administration, and as it was expected, Lic-A was able to reduce the levels of both proteins. The metalloproteinases TACE (ADAM17) and ADAM10 are the primary enzymes that catalyse the release of membrane anchored proteins from the cell surface. Then, they would be responsible for the cleavage of TNFα from its transmembrane to its soluble form. The soluble form of TNFα is the one that binds much more efficiently with the TNFαR1 receptor, related to inflammatory and apoptotic pathways (Zheng Y et al., 2004). While ADAM17 has

been the main protein described as the one responsible for this release, the focus of this study was on the levels of ADAM10 since it is more specific of neuronal tissues. Also, it is found in myelinic structures and has a role in development and growth of the neuronal network, making it an interesting target for the study of the well-being of the neuronal tissue (Huovila et al., 2017). Surprisingly, Lic-A also reduced the protein levels of ADAM10. Nonetheless, it is important to note that the anti-inflammatory property of Lic-A could come through the inhibition of JNK1 and also from its properties as a chalcone.

In order to corroborate further that JNK1 has important role in excitotoxicity and that Lic-A could be a potential pharmacological drug for the treatment of diseases where JNK1 is involved, a study comparing the specific inhibition of the JNK1 by Lic-A against single knock-out transgenic mice for the $Jnk1^{-/-}$ was performed. Both groups showed high correlation between them, the levels of P-JNK/JNK were reduced, as well as P-AKT/AKT ratio and other proteins related to apoptosis such as BAX and aS. The only difference encountered between the two JNK1 inhibition methods was in the fact that transgenic mice always had a much more significant response, this would be due to the complete ablation of the gene compared to the reversible and partial inhibition obtained by the drug.

In conclusion, our results suggest that the inhibition of JNK1 can prevent neuronal degeneration in a mice experimental model of TLE, and that Lic-A, a specific inhibitor of JNK1, is a potential drug with neuroprotectant applications for epilepsy.

Conflict of interest

All authors don't have any actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations. All authors have reviewed the contents of the manuscript being submitted, approve of its contents and validate the accuracy of the data.

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(000091, PNPC, CONACyT). The authors are grateful to Roxanne Rowles for the English revision of the manuscript.

Figure Legends

Table 1. Schematic of animal distribution, experimental groups description, pre-treatments, treatments and sacrifices timing. A. Animal distribution for initial evaluation of the effects of Lic-A as a neuroprotectant. B. Animal distribution for the comparative assays of effect of KA between the JNK1 inhibition by Lic-A and the single knock-out transgenic for *Jnk1* -/-.

Table 2. Primary and secondary antibodies for western blotting.

Table 3. Primary and secondary antibodies for immunofluorescence.

Figure 1. Evaluation of seizure-related behaviours. The quantification of the responses was performed according to the one described by Morrison et al., 1996. $ p<0.05 KA vs Lic-A + KA.

Figure 2. A. Detection of protein levels for JNK and P-JNK 3, 24 and 72-h after KA administration. B. Western blot analysis for JNK1, P-JNK1, cJUN and P-cJUN at 3-h after KA administration. C. Results of the SAPK/JNK kinase activity assay on protein samples extracted a 3-h treatment of KA. Results were represented as MEAN±SD. One-way ANOVA: * p < 0.05 Saline vs KA and Lic-A, ** p < 0.01 Saline vs KA and Lic-A and *** p < 0.001 Saline vs KA and Lic-A. Student t-test: $ p < 0.05 KA vs Lic-A + KA, $$ P < 0.01 KA vs Lic-A + KA and $$$ p< 0.001 KA vs Lic-A + KA.

Figure 3. Fluoro-Jade B staining (green) of mouse brains for the detection of degenerating neurons in the CA3 (Images A-D) and CA1 (Images E-H). Images I and J show magnifications of both CA3 and CA1 images for the KA experimental group. Image K presents a graphical representation of the results of quantification of the number of Fluoro-Jade B positively-labelled cells / 40 mm2. Results were represented as MEAN±SD. *** p < 0.001 Saline vs KA and Lic-A and $$$ p < 0.001 KA vs Lic-A + KA. Samples were obtained 24-h after the appropriate pre-treatment and treatment. All images have a scale bar of 100 µm. Abbreviations: so = stratum oriens, sp = stratum pyramidal and sr= stratum radiatum.

Figure 4. Identification and quantification of the number of surviving neurons 24 h after KA insult in the CA3 (Images A-D) and CA1 (Images E-H). Detection by NeuN immunofluorescence. Image I is a graphical representation of the results of quantification of the number NeuN positively-labelled cells / 40 mm2. Results were represented as MEAN±SD. ** p < 0,01 Saline vs KA and Lic-A, *** p < 0.001 Saline vs KA and Lic-A and $$$ p < 0.001 KA vs Lic-A + KA. All

images have a scale bar of 100 µm. Abbreviations: so = stratum oriens, sp = stratum pyramidal and sr= stratum radiatum.

Figure 5. Immunofluorescence labelling of NESTIN in the DG of the hippocampus (green) on samples after 24-h of appropriate treatment. All images have a scale bar of 100 µm. Abbreviations: h = hilus, gl = granular layer and mol = molecular layer.

Figure 6. Analysis of the phosphorylation levels of AKT and CREB survival proteins through western blot 3-h after KA-induced damage. Results were represented as MEAN±SD. * $p < 0.05$ Saline vs KA and Lic-A, *** $p < 0.001$ Saline vs KA and Lic-A and $$ $p < 0.01$ KA vs Lic-A + KA.

Figure 7. Western blot quantification of apoptotic cascade biomarkers: BCL-2, BAX, BIM, aS, CaSBC and CaSBC3. Analysis 3-h after KA administration. Results were represented as MEAN±SD. * $p < 0.05$ Saline vs KA and Lic-A, ** $p < 0.01$ Saline vs KA and Lic-A, *** $p < 0.001$ Saline vs KA and Lic-A, $ $p < 0.05$ KA vs Lic-A + KA and $$ $p < 0.01$ KA vs Lic-A + KA.

Figure 8. BAX immunofluorescence detection in the CA3 (Images A-D) and DG (Images E-H) regions of the hippocampus 24-h after exposure to KA (red). Nucleus of cells have been stained in blue using Hoechst. All images have a scale bar of 100 µm. Abbreviations: so = stratum oriens, sp = stratum pyramidal, sr= stratum radiatum, h = hilus, gl = granular layer and mol = molecular layer.

Figure 9. Immunofluorescence against BCL-2 detection in the CA3 (Images A-D) and DG (Images E-H) regions of the hippocampus 24-h after exposure to KA (green). Nucleus of cells have been stained in blue using Hoechst. All images have a scale bar of 100 µm. Abbreviations: so = stratum oriens, sp = stratum pyramidal, sr= stratum radiatum, h = hilus, gl = granular layer and mol = molecular layer.

Figure 10. Evaluation of PGC1α, iNOS, nNOS, TNFα, proADAM10 and ADAM10 protein levels through western blot with samples extracted 3-h after KA administration. Results were represented as MEAN±SD. * $p < 0.05$ Saline vs KA and Lic-A, ** $p < 0.01$ Saline vs KA and Lic-A, *** $p < 0.001$ Saline vs KA and Lic-A, $ $p < 0.05$ KA vs Lic-A + KA and $$ $p< 0.01$ KA vs Lic-A + KA.

Figure 11. GFAP immunodetection with fluorescence in the DG of the hippocampus (red). Assay performed on samples 72-h after KA treatment. Nucleus of cells have been stained in blue using Hoechst. All images have a scale bar of 100 µm. Abbreviations: h = hilus, gl = granular layer and mol = molecular layer.

Figure 12. Detection of IBA1 through immunofluorescence in the DG of the hippocampus (red). Assay performed on samples 24-h after KA-induced damage. Nucleus of cells have been stained in blue using Hoechst. All images have a scale bar of 100 µm. Abbreviations: h = hilus, gl = granular layer and mol = molecular layer.

Figure 13. Comparative evaluation of the effects of Lic-A inhibition and *Jnk1 -/-* transgenesis against KA insults. Western blot analysis of protein levels of P-JNK/JNK, P-AKT/AKT, BCL-2, BAX, aS, CaSBC and CaSBC3 3-hafter KA administration. Results were represented as MEAN±SD. * $p < 0.05$ and ** $p < 0.01$.

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Table 1

A

		Pre-treatment		Elapse	Treatment		Sacrifice
		Saline	Lic-A		Saline	KA	
Experimental groups (n=15 for each)	Saline	x		3-h	x		3, 24, 72-h
	KA	x				x	
	Lic-A		x		x		
	Lic-A + KA		x			x	

B

		Pre-treatment		Elapse	Treatment	Sacrifice
		Saline	Lic-A		KA	
Experimental groups (n=4 for each)	KA	x		3-h	x	3-h
	Lic-A + KA		x		x	
	Jnk1 -/- + KA	x			x	

Table 2

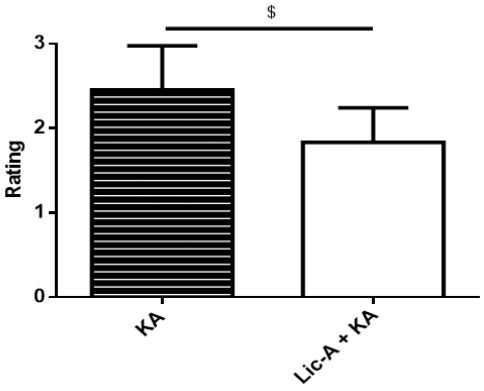
Primary Antibody	Reference	Company
Anti-cJUN N-Terminal Kinase 1 (phospho Thr183)**; P-JNK1**	ab47337	Abcam
Anti-**cJUN** (E254)	ab32137	
Anti-cJUN (phospho Ser73); **P-cJUN**	ab30620	
Anti-Neuronal nitric oxide synthase; **nNOS**	ab1376	
Anti-**ADAM10**	ab39177	
Anti-BCL-2 like protein 11; **BIM**	AB17003	Millipore
Anti-**α-Spectrin**	MAB1622	
Anti-Glyceraldehyde-3-phosphate dehydrogenase (6C5); **GAPDH**	MAB374	
Anti-cJUN N-Terminal Kinase; **JNK**	9252	Cell Signaling Technology
Anti-cJUN N-Terminal Kinase (phospho Thr183/Tyr185) (G9); **P-JNK**	9255	
Anti-cJUN N-Terminal Kinase 1 (2C6); JNK1	3708	
Anti-**AKT**	9272	
Anti-AKT (phospho Ser473) (D9E); **P-AKT**	4060	
Anti-cAMP response element binding (48H2); **CREB**	9197	
Anti-cAMP response element binding (phospho Ser133); **P-CREB**	9198	
Anti-B-cell lymphoma 2 (50E3); **BCL-2**	2870	
Anti-Peroxisome proliferator-activated receptor gamma coactivator 1 alpha; **PGC1α**	sc-13067	Santa Cruz Biotechnology
Anti-Tumor necrosis factor alpha; **TNFα**	sc-133192	
Anti-BCL-2 like protein 4 (B9); **BAX**	sc-7480	

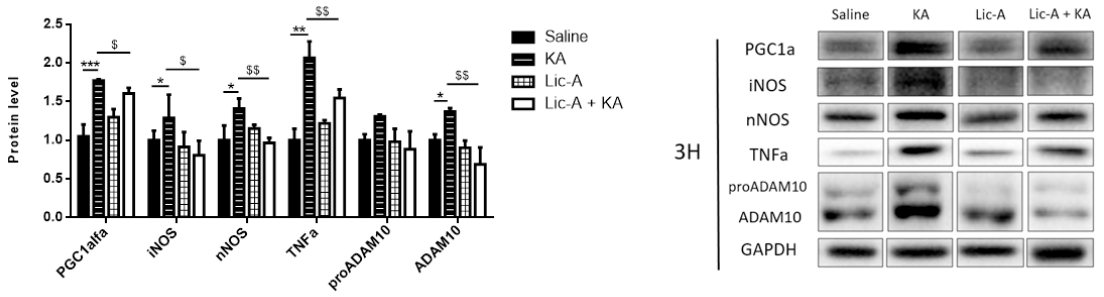
Secondary Antibody	Reference	Company
Goat Anti-Mouse IgG (Horseradish peroxidase conjugate) - Pierce™ Antibody	31430	Thermo Scientific
Goat Anti-Rabbit IgG (Horseradish peroxidase conjugate) - Pierce™ Antibody	31460	

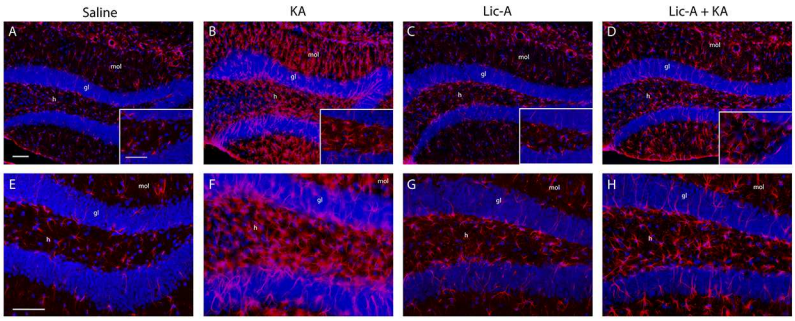
Table 3

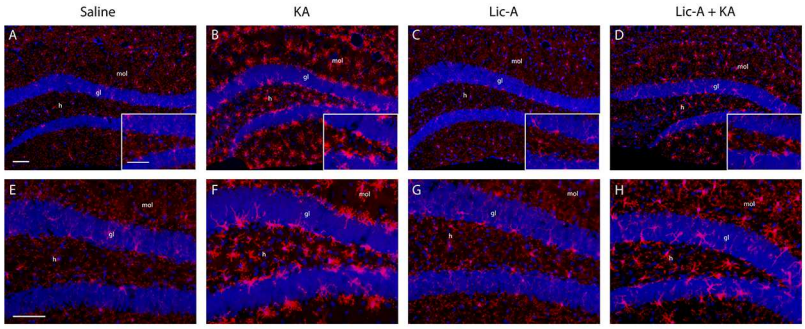
Primary Antibody	Reference	Company
Anti-Glial Fibrillary Acidic Protein; **GFAP**	Z0334	Dako
Anti-Ionized calcium-binding adapter molecule 1; **IBA1**	019-19741	Wako
Anti-**NESTIN**	MAB353	Millipore
Anti-B-cell lymphoma 2 (N-19); **BCL-2**	2870	Cell Signaling Technology
Anti-BCL-2 like protein 4; **BAX**	sc-7480	Santa Cruz Biotechnology

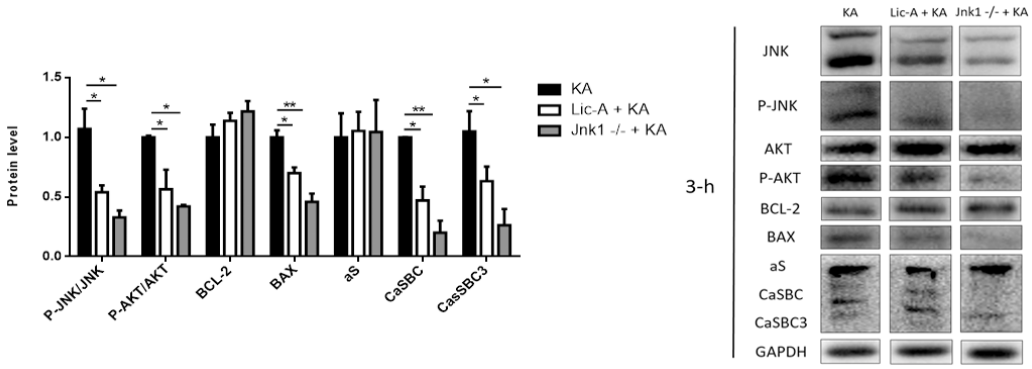
Secondary Antibody	Reference	Company
AlexaFluor 488 Donkey Anti-Rabbit	A21206	Life Technology
AlexaFluor 594 Goat Anti-Rabbit	A11012	
AlexaFluor 488 Donkey Anti-Mouse	A21202	
AlexaFluor 594 Goat Anti-Mouse	A11005	

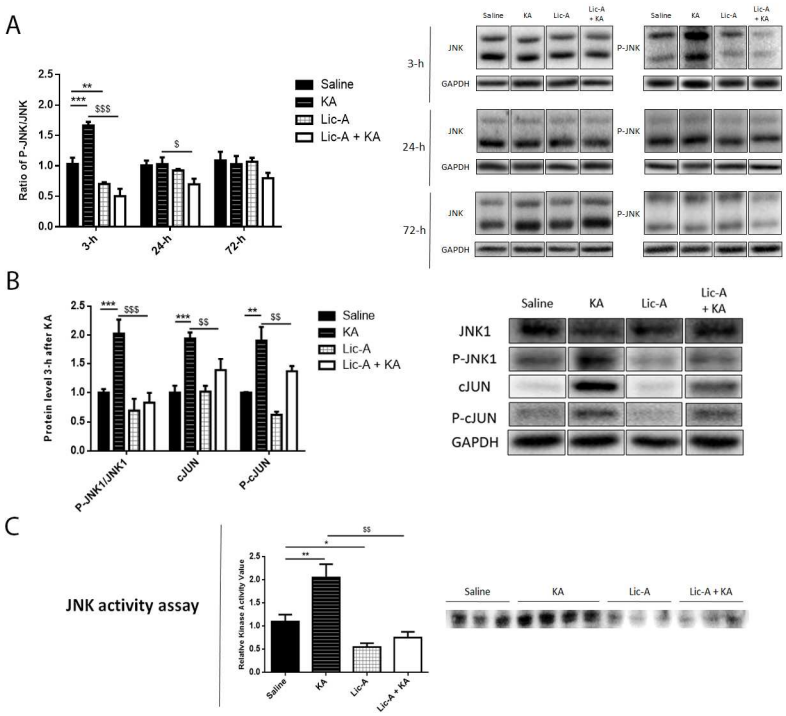


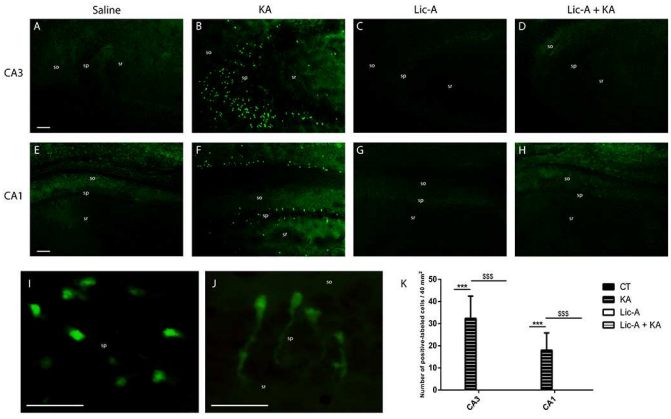


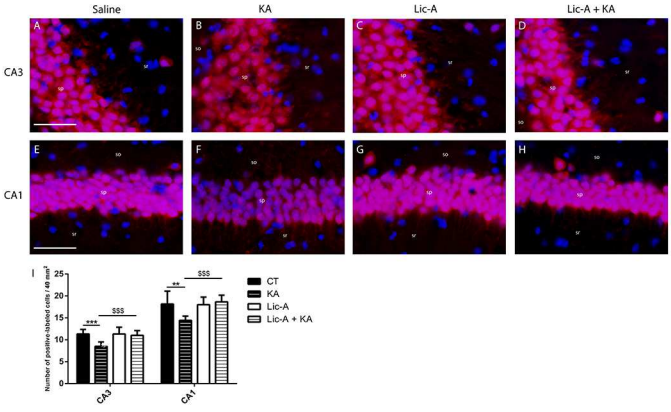


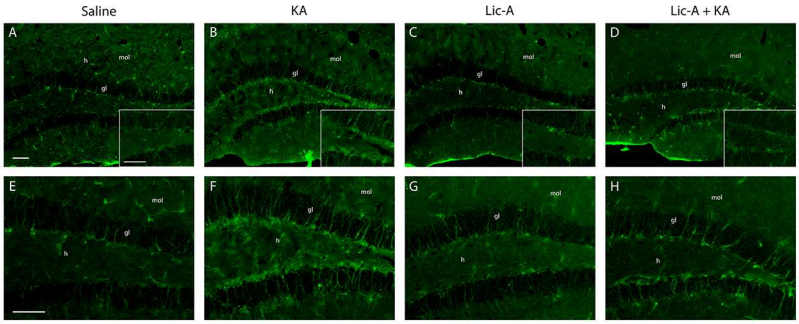


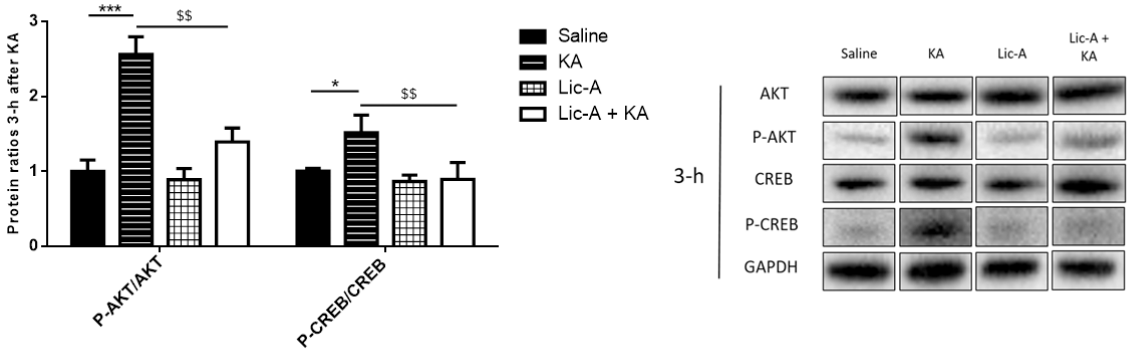


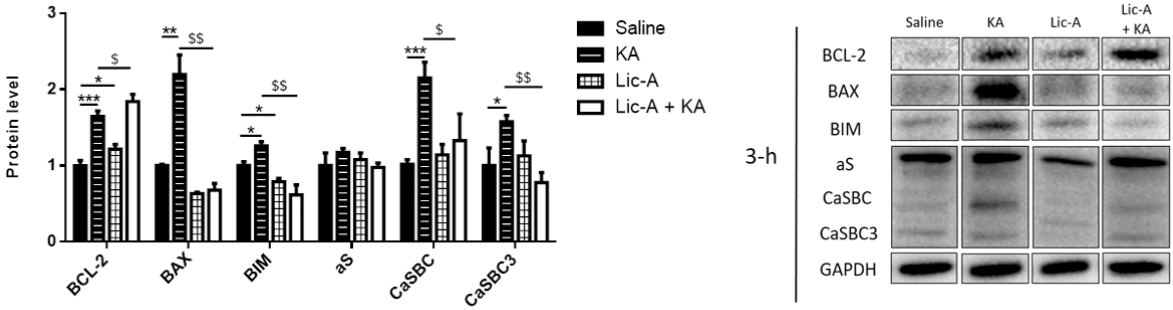


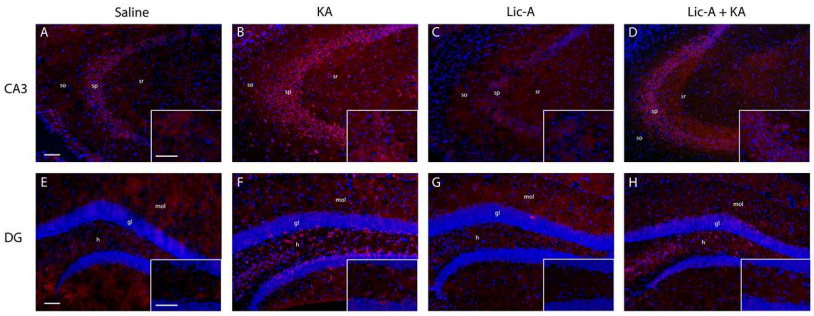


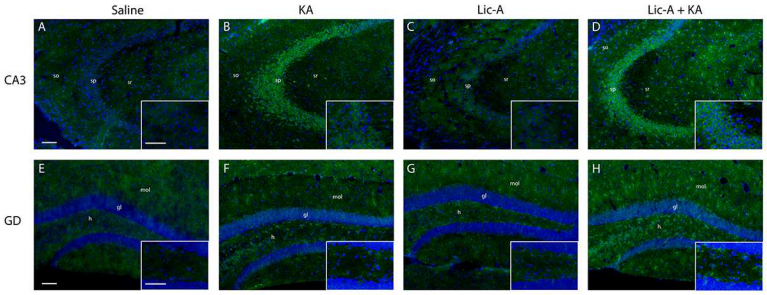












Licochalcone A

Reduces the severity of seizures

Protects against neuronal neurodegeneration associated with KA.

Promotes the increase in anti-apoptotic protein bcl-2, thus protecting neurons from apoptosis

Decrease the levels of cellular stress markers and decrease neuroinflammatory process

Conflict of interest

All authors don’t have any actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations. All authors have reviewed the contents of the manuscript being submitted, approve of its contents and validate the accuracy of the data.