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Role of JNK isoforms in the kainic acid experimental model of epilepsy and neurodegeneration

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1. ABSTRACT

Chemoconvulsants that induce status epilepticus in rodents have been widely used over the past decades due to their capacity to reproduce with high similarity neuropathological and electroencephalographic features observed in patients with temporal lobe epilepsy (TLE). Kainic acid is one of the most used chemoconvulsants in experimental models. KA administration mainly induces neuronal loss in the hippocampus. We focused the present review inthe c-Jun N-terminal kinase-signaling pathway (JNK), since it has been shown to play a key role in the process of neuronal death following KA activation. Among the three isoforms of JNK (JNK1, JNK2, JNK3), JNK3 is widely localized in the majority of areas of the hippocampus, whereas JNK1 levels are located exclusively in the CA3 and CA4 areas and in dentate gyrus. Disruption of the gene encoding JNK3 in mice renders neuroprotection to KA, since these animals showed a reduction in seizure activity and a diminution in hippocampal neuronal apoptosis. In light of this, JNK3 could be a promising subcellular target for future therapeutic interventions in epilepsy.

2. INTRODUCTION

According to the latest publication of the ILAE (International League Against Epilepsy), epilepsy is defined as a brain disorder characterized by an enduring predisposition to generate seizures and a hyper-synchronous firing of neurons, that leads to neurobiological, cognitive and social consequences (1). The gradual process by which the brain develops epilepsy is known as epileptogenesis and it is divided into three stages: the acute event (the triggering insult or initial seizure), a latent period (clinically silent) and spontaneous

seizures. In humans, the latent period can last for months or years (2-5). Therefore, epileptogenesis is a dynamic process by which the brain becomes epileptic and begins to generate spontaneous recurrent seizures (5).

There are several causes of epilepsy, such as tumors, trauma, metabolic dysfunction, infection and vascular disease, among others (5). However, some forms of epilepsy can occur due to a genetic predisposition (6). Few genes related to this neurological disorder have been identified. One group of these genes are related to ion channels, transporters and receptors, while other genes correlate with synaptic inhibition mediated by GABA neurotransmitter (6).

The signs and symptoms of seizures depend on the type, being the convulsive one the most common (60%) (4-6). These seizures derive in a systematic metabolic derangement, including hypoxia, hypotension and hypoglycaemia, leading to a reduction in high-energy phosphatases in the brain and thus potentially causing devastating effects in brain tissue. Systemic complications, such as cardiac arrhythmias, pulmonary edema, hyperthermia and muscle breakdown can also occur (3). The convulsive status epilepticus (SE) is clinically defined when the patient suffers a prolonged electrical and clinical convulsive seizure activity longer than five minutes, or more than one seizure within a five-minute period, without returning to the normal state between them. It is a neurological emergency, associated with a high mortality rate, particularly if treatment is delayed.

Two-thirds of convulsive seizures are focal (or partial) and one third is general. Focal convulsive seizures are circumscribed in a specifically brain area and are originated by abnormal electrical activity in a small group of neurons that have the ability to enhance excitability (epileptiform activity) to neighboring regions (5). The symptoms depend on the location of the focus. The intense firing of neurons may be the result of different factors such as altered cellular properties or altered synaptic connections, caused by a local scar, blood clot or tumor, frequently accompanied by unusual behaviours or consciousness alteration (7-10). Unlike the focal seizure, a primary generalized seizure disrupts normal activity in both cerebral hemispheres simultaneously. They are a 40% of non-convulsive seizures, depending on whether the seizures are associated with tonic (the person's muscles initially stiffen and they lose consciousness) or clonic movements (the individual's muscles begin to spasm and jerk).

Temporal lobe epilepsy (TLE) is the most common form of human epilepsy with focal seizures, covering 40% of all cases of this neurological disorder. A common subtype is the mesial temporal lobe epilepsy (MTLE), characterized by the presence of seizures originated in limbic areas of the mesial temporal lobe, particularly in the hippocampus, amygdala, and in the parahippocampal gyrus and its connections (11-15). This type of epilepsy is associated with an "early first insult", such as febrile seizures, a prolonged focal seizure, infection of the central nervous system (CNS), or head trauma, among others, most often occurring in the first five years of life (2-12). Patients frequently suffer from cognitive impairment, especially related to memory, and behaviour disturbances.

Its pathophysiological substrate is usually hippocampal sclerosis, the most common epileptogenic lesion found in patients with this type of epilepsy (MTLE-HS). Familial forms of MTLE-HS have been recognized, but no causal gene or linkage has been identified so far. This subtype of epilepsy is the most common cause of surgical and refractory epilepsy in adulthood (11,12).

In most cases, it takes years for any noticeable damages to appear in MTLE-HS, but it is still very important to treat the disease early and as effectively as possible, since it is often refractory to the drug treatment. Nonetheless, MTLE-HS can be abolished in most patients by surgical treatment.

In reference to the cellular basis of the epilepsy, the current axiom in epileptic research states that whereas a fine balance between excitatory (depolarizing) and inhibitory (hyperpolarizing) signals characterize normal brain function, a change in this balance leads to seizures. This imbalance is achieved by either increased excitation, reduced inhibition (i.e. des-inhibition) or both. These results in an abnormal neuronal discharge associated with a large release of the excitatory neurotransmitter glutamate, which is ultimately responsible for seizures. One of the goals of both basic research and therapeutic applications, during recent years, has been to counter-balance the sustained excitation by developing drugs that would trigger repolarization (i.e. impairment of GABAergic inhibition in epilepsy). Several lines of evidence support this approach: i) GABA is the main inhibitory neurotransmitter in the neuronal structures involved in MTLE; ii) enhancers of GABAergic inhibition (such as benzodiazepines or barbiturates) are commonly used as antiepileptic drugs; and iii) discharges can be detected following the pharmacological blockade of GABA receptors.

3. CURRENT TREATMENT OF EPILEPSY

The currently used antiepileptic drugs are heterogeneous, since they have different mechanisms of action, even though they are not completely well understood (13-16). One group interacts mainly through the regulation of sodium channels (blockade). This is the case of phenytoin, carbamazepine, oxcarbamazepine, valproate, lamotrigine, topiramate, lacosamide and also zonisamide (13,14). *In vitro* pharmacological studies evidenced that zonisamide and lacosamide enhance slow inactivation of voltage dependent sodium channels

and reduce calcium entry through voltage dependent calcium channels, thereby stabilizing hyperexcitable neuronal membranes (14-16).

Another group of drugs used for the treatment of epilepsy activates the gamma-amino-butyric acid ($GABA_A$) receptor. This leads to an increase in the intracellular Cl^- influx (ion concentration), which induces hyperpolarization of the membrane and inhibition of action potentials, hence rendering the neuron unresponsive for a period. This group includes benzodiazepines, barbiturates, topiramate, felbamate, tiagabine a potent and selective GABA reuptake inhibitor in neuronal and glial cells and gabapentin, which inhibits the release of monoamine neurotransmitters and increases GABA turnover in several brain areas (13-15). Another group of antiepileptic drugs includes pregabalin, gabapentin, topiramate and lamotrigine, which block high voltage calcium channels.

Retigabine is a different antiepileptic drug from all those currently approved. It acts primarily as a neuronal potassium channel opener, stabilizing the resting membrane potential and controlling neuronal electrical excitability. Thus, retigabine prevents the onset of discharge of epileptogenic potential action (14,16).

Felbamate blocks the NMDA receptor, a specific type of ionotropic glutamate receptor that under local membrane depolarization becomes permeable to Ca^{++}. The influx of Ca^{++} further depolarizes the neuron and contributes to Ca^{++}-mediated neuronal injury under conditions of excessive neuronal activation (16).

Perampanel is a selective non-competitive antagonist of ionotropic AMPA-type (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, a subtype of non-NMDA glutamatergic receptor) glutamate receptors in postsynaptic neurons. Although the exact mechanism by which it exerts its antiepileptic effect in humans is not yet fully elucidated, its administration is associated with a decreased frequency of seizures (13,16).

Interestingly, topiramate has more than one mechanism of action. Thus, in addition to blockade of Na^+ channels, increases $GABA_A$ receptor activation and blocks the AMPA receptors (13,14). This drug was fisrt used as an anticonvulsant and now is used to treat epilepsy in children and adults. It is also used for the treatment of Lennox-Gastaut-Syndrome, a severe form of epilepsy that shows seizure activity before the 4 years of age.

4. EXPERIMENTAL ANIMAL MODELS USED TO STUDY EPILEPSY

The understanding of epileptic disorders has derived from appropriate animal models that have allowed the study of this disease (17).

The status epilepticus defined as 5 minutes or more of continuous seizures can be induced by systemic or intracerebral administration of different convulsive agents, such as kainic acid (KA**)** (2-carboxy-4-isopropenylpyrrolidin-3-ylacetic acid), a potent neuroexcitatory amino acid that binds and activates receptors for glutamate or pilocarpine, which acts through the activation of the M1 muscarinic receptor (17-27). Prolonged seizure activity cause permanent neuronal damage and synaptic reorganization; these conditions are often associated to the development of “chronic” epilepsy (17-19).

The procedures for KA and pilocarpine injection are well established in rodents (18-33). However, the effect of these chemoconvulsants differs among animal strains (17,23). After the acute status epilepticus is induced, there is a period of free crisis known as the latent phase which can last for weeks. Finally, the chronic phase, characterized by spontaneous and recurrent seizures (17-23), emerges. The anatomical defects induced are very similar to those developed in humans with MTLE-HS. Therefore, these experimental models allow us to evaluate the pathological changes that occur in the chronic phase, but also to assess those ones appearing in the early stages and during epileptogenesis. In a recent publication (17), a comparison between the mechanisms of action of the two abovementioned pro-convulsants was performed, showingthat pilocarpine provokes a rapid status epilepticus when compared to KA. Moreover, pilocarpine has a higher mortality than KA in rodents (17,23). Nonetheless, experimental studies suggest that both models are very useful in assessing anticonvulsant drug effectivity. Anti-epileptic drugs that are already on the market, such as phenobarbital or carbamazepine, show an anti-convulsant effect in animal models treated with both neurotoxins (22). Another interesting point is that both compounds favour the neuronal loss, being pilocarpine a more quickly neuronal loss inducer, whichs explains its more lethal effects in rodents (17). There areother experimental models that induce seizures and are also useful to evaluate the efficacy of anticonvulsants, such as pentylenetetrazole (PTZ) which cause convulsions and the repeated subconvulsive electrical stimulations (kindling) in high doses (22).

The systemic administration of KA could be intraperitoneal, intravenous or subcutaneous. On the other hand, KA can be administered locally or directly in the brain (intrahippocampal, intraamygdala or intracortical) with the advantage that the brain injury effects are more localized and provides a lesser animal mortality (17-23). The main advantage of intraperitoneal injection as compared to intracranial administration, is probably the simplicity and feasibility of the procedure. In all cases, KA triggers a pattern of repeated seizures for several hours, followed by a latency period and a subsequent spontaneous occurrence of seizures (20-25).

A single systematic injection (30 mg/kg in mice) of a convulsive dose of KA results in a limbic (SE), inducing features similar to those observed in MTLE-HS adult human epilepsy, although followed by high mortality rates (17-23). Recently, Umpierre *et al*. (2016) evidenced that a systematic low dose of KA produces SE without acute degree of mortality. Interestingly, the caused pathology had the same features observed when common doses were applied, including hippocampal neurodegeneration and astrogliosis (27-33).

5. THE HIPPOCAMPUS, THE FIRST KA TARGET

The brain region most affected by seizures, both in animal models and humans, is the hippocampus. It is a small region of the brain that forms part of the limbic system and it is involved in processes such as memory and learning (34,35). Particularly, the hippocampus seems to play a major role in declarative memory, the type of memory involving concepts or events that can be purposely recalled (36). It also participates in the detection of novel stimuli, the conduct of search and in stress response. Therefore, it is critical in habituation processes like awareness and classical conditioning (37). Together with the amygdala, the thalamus, the hypothalamus, the septal area, the olfactory cortex and other CNS structures, the hippocampus is part of the limbic system (37,38).

In reference to the hippocampal architecture in rodents, the hippocampus is a complex alocortical cylindrical structure composed of different subfields. One is the dentate gyrus, a separate structure that is a tightly packed layer of small granule cells wrapped around the end of the hippocampus proper (37-39). Next, there is the *Cornu Ammonis* area (CA) considered the "hippocampus proper". This CA area is subdivided in CA4, CA3, CA2 and CA1. The CA4 area underlies the dentate gyrus and is also called the hilus or hilar region. It contains mossy cells, thus it is not filled with densely packed pyramidal cells as occurs in the other CA areas. CA4 is followed by CA3, then there is a very small zone called CA2 and after there is the CA (13, 41). Next to the CA1 there is the subiculum, followed by a pair of ill-defined areas, the presubiculum and parasubiculum. Finally, there is the enthorhinal cortex. The CA subfield, the dentate gyrus and the subiculum configurate the "hippocampal formation" (38-41).

One crucial question is whether the hippocampal-parahippocampal network dysregulation and memory deficits observed in epilepsy are state or trait characteristics of the disorder. The parahippocampal gyrus, a grey matter cortical region of the brain that surrounds the hippocampus, transmits its signals through the enthorinal cortex to the dentate gyrus via granule cell fibers, known collectively as the perforant path (39-41). The mossy fibres, which project from the granular neurons of dentate gyrus and establish synapses with the proximal dendrites of CA3 pyramidal neurons, play a crucial role during the status epilepticus, as demonstrated by the formation of novel aberrant mossy fibres on the hyppocampal neurons, as well as by an increase in the density of KA receptors and, therefore, to the reduction of seizure threshold (39-41). CA3 neurons then fires to CA1 neurons via Schaffer collaterals, which synapse in the subiculum, the main output of the hippocampus. It also receives input from the entorhinal cortical layer III pyramidal neurons. Collectively, the dentate gyrus, CA1 and CA3 areas of the hippocampus compose the trisynaptic loop.

6. MECHANISMS OF NEURODEGENERATION INDUCED BY KA

The neurotoxin KA was first isolated in the red alga, *Digenea simplex*, which is found in tropical and subtropical waters (41). KA is a non-degradable analogue of glutamate, and a potent neurotoxin that acts through glutamate receptors present in the mossy fibres in the hippocampus. Excessive stimulation of glutamate receptors induces excitotoxicity, which is the main cause of cell death in CNS diseases (42-44).

There are two groups of glutamate receptors: ionotropic (iGluR) and metabotropic receptors (mGluR) (44-49). The first group (iGluR) regulates the passage of ions through the neuronal membrane. When glutamate binds to iGluRs, conformational changes resulting in the opening of the channel occur, which increase the flow of Ca^{++} and/or Na^{+} into the cytosol, and K^{+} into the extracellular environment. iGluRs include three main type of receptors, the NMDAR (N-Methyl-D-aspartate receptor), the AMPAR (Amino Methylphosphoric Acid Receptor) and KARs (Kainic Acid Receptors) (45-49). AMPAR and KARR are also known as non-NMDA receptors, since they are not sensitive to selective antagonists of NMDAR, have lower affinity for glutamate and their activation kinetics is much faster than those of NMDAR (23,38). The KARs family is divided into two subfamilies, the GluR5-7 and KAR1-2 (4,5). These receptors are expressed in the amygdala, entorhinal cortex, basal ganglia, cerebellum, cortex and hippocampus, where high levels of KAR1 and KAR2 have been detected (45-51). It has been shown that KAR are located in both presynaptical and postsynaptic areas, being KAR1 predominant in CA3 pyramidal neurons, while KAR2 is detected in CA3 and CA1. This distribution pattern makes the hippocampal region the most susceptible to KA excitotoxicity (45-48). Several studies suggest that KA epileptogenic effect on pyramidal neurons of CA3 is caused by activation of their KARs, preferentially expressed in synaptic mossy fibers of the stratum lucidum region (slm), to promote the release of glutamate in the synaptic cleft (19,25). Thus, this is the area where seizures begin

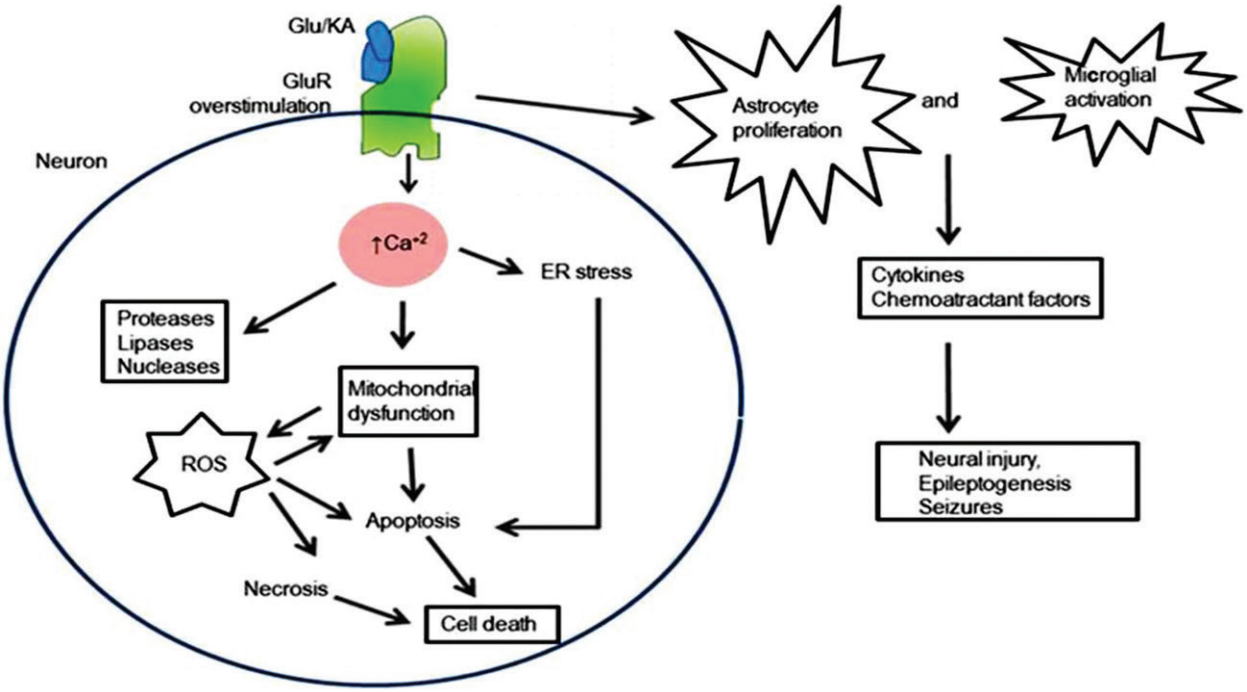

Figure 1. Kainic acid treatment induce excitability with a high increase in calcium levels an inflammation trough the activation of microglia and astrocyte.

and develop (50). Also GluR5 receptors, which are expressed in GABAergic interneurons of the CA1, as well as GluR6 receptors expressed predominantly in CA3, are involved in the excitatory action of KA (49-60). The second group (mGluR), modifies their interaction with other cytosolic proteins upon binding of glutamate. This leads to the activation of specific intracellular signaling cascades (46-49).

The excitation produced upon binding of KA to ionotropic glutamate receptors induces many cellular events, including massive influx of Ca^{2+}, leading to depolarization of the cell membrane (56-61). These conditions favour the production of ROS, which, in turn, induce structural and functional changes in proteins and DNA damage and consequently cell death. In addition, high levels of intracellular calcium trigger the release of nitric oxide synthetase (NOS), which modulates the glutamatergic transmission and can induce changes in mitochondrial function, generating more free radicals (61-65). Finally, there is also a glial activation related to an inflammatory response and neuronal death (23-25,50,54). All these events contribute to neuronal damage, and consequently to cell death (Figure 1).

During seizures, death receptors TNFR1 (tumour necrosis factor receptor 1) and FasR (Fas receptor) are activated, leading to the extrinsic pathway of apoptosis (63-66). Moreover, calcium entry also activates the intrinsic pathway of apoptosis. Hence, the altered mitochondrial function, triggers the mobility of apoptosis regulator Bax (bcl-2-like protein 4) to mitochondrial outer membrane, the cytochrome c release and subsequent activation of cysteine proteases proteins, such as caspase 9. Finally, this activates caspase 3, the main executor of the apoptotic processes (65-72).

Both *in vivo* and *in vitro* studies demonstrated that KA induces microglia activation (Figure 1), releasing high levels of proinflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-12 (IL-12), which together with Interleukin 18 (IL-18), are involved in upregulating Interferon-gamma production (IFNγ) (72-78). Moreover, the administration of KA induces the expression of high levels of other cytokines, like the tumor necrosis factor (TNFa, which could have both neuroprotective and neurotoxic properties, in contrast to the role of other proinflammatory cytokines (73-78).

Interestingly, one of the earliest changes occurring in conditions of KA-induced neuronal hyperexcitability, is an immediate gene overexpression response. Thus, an increase in the expression of mRNAs encoding heat shock proteins such as *hsp27*, *hsp70* and *hsp72* is induced (78-80). *In vivo* studies have shown that, while high expression of *hsp72* can be harmful to cells, overexpression of *hsp27* and *hsp70* have a protective function against excitotoxic damage, although it fails to rescue cells from excitotoxic death (67,77-80). It has also been detected an overexpression of *fos*, *jun* and *Erg1* genes in different hippocampal regions (79).

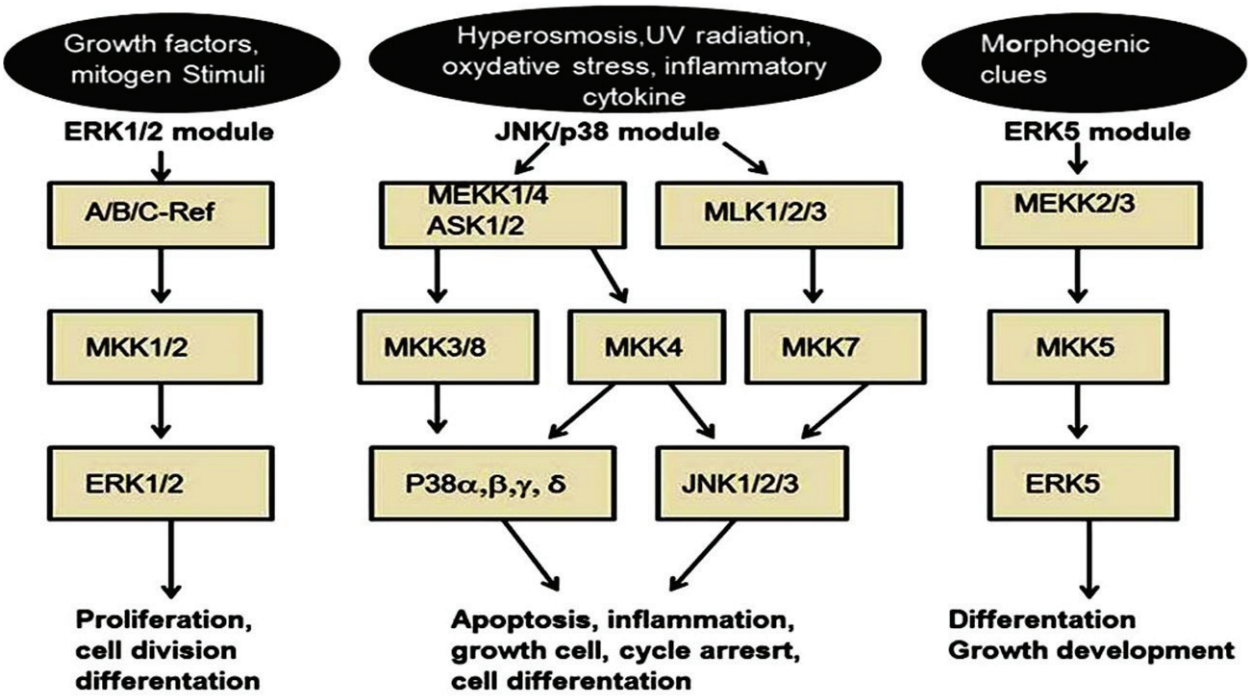

Figure 2. Simplified overview of mammalian MAPK cascade.

The proto-oncogene *jun* and *fos* encode proteins that form the complex AP1 (activator protein 1), regulating the expression of other genes (77,78). Likewise, following KA administration, there is an increase in neuropeptides, like somatostatin (SOM) and neuropeptide Y (NPY), among others (79).

Therefore, the KA experimental model provides a chance to understand the mechanisms of neuronal death. Considering that this process is present in neurodegenerative diseases, such as AD, PD or amyotrophic lateral sclerosis (ALS), the use of KA experimental model could be an important tool in epilepsy knowledge but in biomedical research in general(49-54). Furthermore, activation of glutamate receptors by KA induces the activation of Mitogen Activated Protein Kinases (MAPKs). In this review, we will focus on the c-Jun N-terminal kinase pathway (JNKs), which is clearly involved in the process of apoptotic neuronal death (22,27,28,30).

7. MITOGEN ACTIVATED PROTEIN KINASES (MAPKS)

MAPKs are a family of cytosolic protein kinases that regulate cellular processes such as cellular proliferation, differentiation and apoptosis (81-87). They are activated in response to various extracellular signals or stimuli, such as hormones, growth factors, cytokines or several stresses (83-86). There is a high correlation between the type of stimulus that activates the MAPK pathway and the specific role they develop.

MAPKs pathway is regulated through three sequential phosphorylation kinases (Figure 2). Thus, the first step in this pathway of signal transduction is the phosphorylation of threonine (Thr) and tyrosine (Tyr) residues that occurs in conserved motifs Thr-X-Tyr, located in the kinase subdomain VIII of the four MAPKs (85). This phosphorylation is gradual and starts in the membrane receptors associated with other G protein kinases or adapters, which induce the activation of MAPKKK or MAP3K (MAPK Kinase Kinase Kinase). These, in turn, phosphorylate the MAPKK or MAP2K (MAPK Kinase Kinase) that finally generates the physiological response by the activation of this biochemical pathway (Figure 2) (84-87). This activation system is highly conserved in eukaryotes. Besides, the activation of MAPK pathways through phosphorylation is regulated by the engagement and interaction with regulatory molecules called anchor proteins (Scaffold Proteins) (83-90).

Currently, four groups of MAPKs that regulate different cellular processes have been described: ERK1/2 -Extracellular Signal Regulated Kinase 1/2- with an important role in proliferation and differentiation, JNK1-3 -c-Jun N-terminal kinase 1-3-, also called SAPKs -Stress-Activated Protein Kinases-, p38MAPKα, β, γ, δ, which are activated by cellular stressand ERK5, which is activated by stress and mitogenic signals (83-95). Each one of these MAPKs has its specific MAP2Ks and

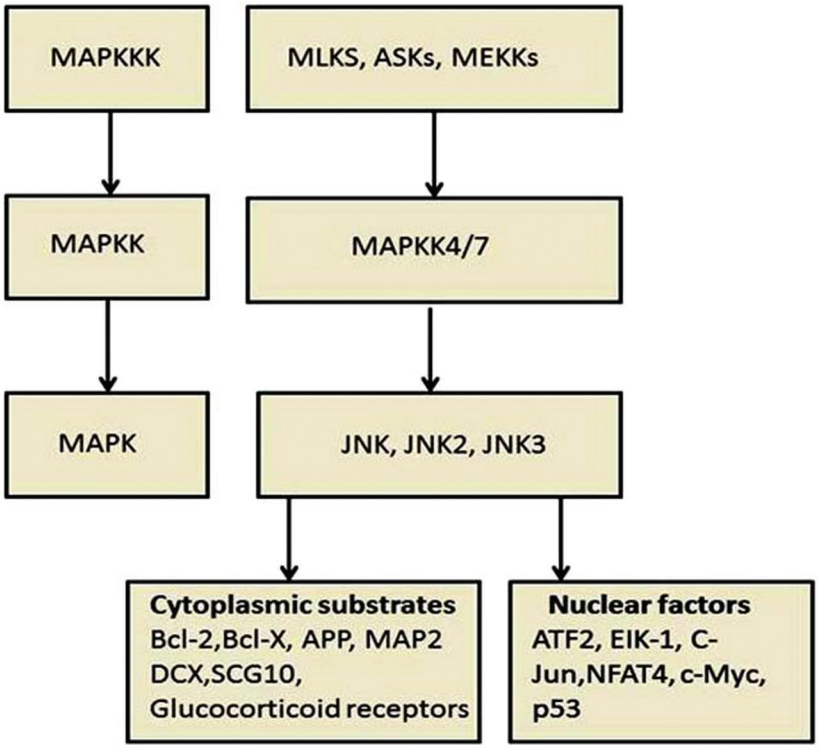

Figure 3. Simplified representation of JNK pathway.

MAP3Ks that, in turn, regulate different cellular processes (Figure 2).

7.1. The c-Jun N-terminal kinase signaling pathway (JNK)

The c-Jun N-terminal Kinase (JNK) is a member of MAPK signaling proteins (95-97).It was initially identified and purified in the liver of rodents exposed to cycloheximide by Kyriakis *et al*., (1994) as an active protein (96). JNK proteins are sensitive to stress and apoptosis-related receptors, and are activated by specific ligands of GPCRs and RTKs receptors (96-99). Many extracellular stimuli activate JNK, such as the cytokines TNF-α and IL-1β. Other cellular stress conditions that activate JNK include ultraviolet light, heat shock, osmotic shock, growth factor withdrawal and a number of toxins including hydrogen peroxide, anisomycin and cyclohexamide (96). Kinases that activate JNK include MKK-4 and -7, and upstream of these kinases there are, in turn, a number of kinases including TAK (Tat-associated kinase), MLK-3 (Mixed Lineage Protein-3), MEKK-1 and -4, and ASK-1 (Apoptosis Signal-Regulating Kinase). Specifically, MEKK1-4 and ASK1-2 are mostly induced by environmental stresses and activate MKK4, while MKK7 activated by cytokines is phosphorylated by MLK1/2/3 (96-100). Both MKK4 and MKK7 activate JNK by dual phosphorylation in the TPY (Thr-Pro-Tyr) motif. The cooperation of the two kinases is required for full activation of JNK (Figure 3) (97-101).

Many substrates, both in the cytoplasm and nucleus, are phosphorylated by JNK. Nuclear substrates include AP-1 from Jun family (c-Jun, junB, JunD), ATF-2 (Activating Transcription Factor 2), ELK-1 protein, NFAT (nuclear factor of activated T cells), c-Myc oncogene, the tumor suppressor p53 (Figure 3). Cytoplasmic substrate include Bcl-2 (B-cell lymphoma 2), Bcl-xL (B-cell lymphoma-extra-large), glucocorticoid receptors, membrane proteins such as APP (Amyloid Precursor Protein), MAP2 (Microtubule-Associated Protein 2), DCX (Doublecortin) and SCG10, a microtubule destabilizing factor (Figure 3) (85,86,94). MARCKSL1 (MARCKS-like protein 1) is another substrate phosphorylated by JNK, which is widely expressed in nervous tissue and has a key role in neural tube formation. The phosphorylation on C-terminal residues (S120, T148, and T183) enables MARCKSL1 to bundle and stabilize F-actin, increase filopodium numbers and dynamics, and retard migration in neurons (86,94). The neurofilament heavy chain is also phosphorylated by JNK, implicating this kinase in neurite outgrowth and regeneration. Moreover, different studies evidence that JNK directly targets chromatin modifiers, driving histone phosphorylation and acetylation. Thus, JNKs can exert an influence on

gene expression not only in stress responses but also during neuronal differentiation (98-103).

However, the first identified and better described JNK substrate is c-Jun, which increases its stability and its transcriptional activity when phosphorylated at residues of Ser63 and Ser73 (8,6,89,94). The c-Jun protein can form homodimers or heterodimers with other proteins, such as transcription factor ATF2, or components of the c-Fos family. This latter heterodimerization is followed by the AP-1 complex formation, which activates the transcription of various genes involved in processes related to proliferation, differentiation, apoptosis or immune response (50). High expression of *c-jun* and high levels of protein precede periods of cell death, such as those that occur during embryonic development, in trauma situations, in cerebral ischemia and after convulsions (80,95-100). A similar induction of this gene was detected in neurodegenerative diseases such as AD, in ALS and PD (43).

There are different JNK isoforms, encoded by three different genes, *jnk1*, *jnk2*, and *jnk3*. Specifically, ten isoforms of JNK having a homology of 85% have been identified from alternative splicing of the gene products: JNK1α1, JNK1α1, JNK1α2, JNK1α2, JNK2α1, JNK2α1, JNK2α2, JNK2α2, JNK3α1 and JNK3α2 (102-104). These isoforms have a molecular weight ranging from 46 to 54 kDa. In many cases, the *jnk1* gene generates a 46 kDa protein product, while the *jnk2* gene produces a protein of 54 kDa and *jnk3* gene produces a variant of 46 and 54 kDa (103).

In mammals JNK1 (MAPK8) and JNK2 (MAPK9) proteins are expressed ubiquitously, whereas JNK3 (MAPK10) is preferentially expressed in the brain and, to a lesser extent, in the heart and testes (104-106).Studies by *in situ* hybridization in rodents suggested that *jnk3* is widely expressed in neocortex, hippocampus, thalamus, and midbrain followed by *jnk1* and *jnk2 (*104-107). In rodents, JNK1 protein levels are high during brain development, in contrast to JNK2 and JNK3; however, they decrease in postnatal stages, maintaining high levels in the olfactory bulb of adults. Specifically, in the hippocampus, the JNK3 is widely dispersed in the majority of hippocampal areas, whereas JNK1 is found exclusively in the CA3 and CA4 areas, as well as in dentate gyrus (103-106). The subcellular distribution of JNK isoforms differs, thus JNK1 is located largely in the cytosol, whereas JNK3 is in the nucleus. Regarding JNK2, it is distributed both in the cytosol and in nucleus.

There are differences among the isoforms in reference to the activation of transcription factors. Thus, some studies suggest a greater ability of JNK2 in phosphorylating c-Jun substrate as compared to nuclear JNK1 (104-106). JNK1 isoform is more involved in the phosphorylation of cytosolic substrates as MAP2 and MAP1B (Microtubule-Associated Protein 1B), thus highlighting the importance of JNK1 in controlling the activity of microtubules and axonal elongation. In addition, MAP2 phosphorylation plays an important role in defining cell architecture (85). However, JNK1 could participate in other functions, such as controlling synaptic plasticity, through the transcription factors ATF-2 and c-Jun (85,96). Moreover, differential effects in chromatin modification exist between JNKs. Thus, JNK2 and JNK3 specifically phosphorylate serine 10 of histone H3 *in vitro*, an event that is associated with the relaxation of chromatin and active gene transcription (99).

7.2. Functional interactions between JNK pathway and JIP proteins

Cells ensure specificity and efficiency of a signalling cascade through the interaction of a variety of anchoring proteins that, despite the lack of enzyme activity, are added to various components of the same signalling pathway forming functional modules (107-110).

The JNK signalling pathway interacts with specific anchoring proteins, known as JIPs (c-Jun–N-terminal-kinase-interacting-proteins), forming a JNK signalling functional module. JIPs facilitate the activation of JNK in addition to different components of the MAPK cascade, as MKK7 and some MAP3K as MLK3 or DLK, forming a multi-enzyme complex.(109,110) JIP family consists of several members, JIP1, JIP2, JIP3 and JIP4 (8, 90).

JIP1 has been the best characterized. The gene encoding this protein, *jip1*, is expressed ubiquitously (111-113). JIP1 can interact with several MAP3K proteins (MEKK3, MLK3 and DLK), but only with one MAP2K (MKK7). Furthermore, JIP1 interacts with JNK isoforms, having a higher affinity for JNK1 isoform (109-113). Mice carrying a targeted deletion of the JNK-binding domain of JIP-1 have been found to be less vulnerable to KA-induced cell death in the hippocampus (113). It has been described that other kinases can phosphorylate JIP1 and change its affinity to the MAPKs (114). Specifically, JIP1 can bind to AKT promoting its activation and suppressing JNK signaling in neurons through the inhibition of the interaction between JNK and JIP1. However, exposure of these cells to an excitotoxic stress promotes the AKT release from JIP1, thereby increasing the interaction between JNK and JIP1, and triggering JNK activation through JIP1-JNK module (112-116).

JIP2 and JIP3 protein are expressed in neurons and neuroendocrine cells (113). JIP2 interacts specifically with MKK7 (109-115). JIP3 interacts with MEKK1, ASK1 and MLK3, and also with MAP2Ks, MKK4 and MKK7. However, it has been shown that JIP3 has a higher affinity for the JNK3 isoform than either JNK1 or JNK2 (112-114). A recent study reported an important role of JIP3 in seizures because the underexpression of JIP3 results in an anticonvulsive effect after KA administration (117,118).

The JIP4 protein is the most recently identified member of the JIP family (119). It interacts with MEKK3, MKK4 and MAPKs (JNK and p38a) (117-120).

7.3. Involvement of JNK in neurodegenerative mechanisms

Activation of the JNK pathway in the nervous system has been associated with neuronal cell death processes, either occurring naturally during embryonic development or arising in different brain pathologies (30). It has been proposed that the duration of JNK activation can determine the type of response. Thus, sustained JNK activation is related to apoptosis, whereas transient activation is related with cell survival and proliferation processes (121-128).

The two major pathways that initiate the apoptotic process are classified into the extrinsic pathway, activated by death receptors, and the intrinsic pathway, initiated by mitochondrial events. JNK appears to have a central role in both pathways, since it activates apoptotic signalling through upregulation of proapoptotic genes, and it is able to directly modulate mitochondrial activity through phosphorylation of both anti- and pro-apoptotic proteins (121,123).

Some authors support that upon activation of JNK, it translocates to the mitochondria, inducing the release of cytochrome c, which, in combination with Apaf-1, activates caspase-9 (122). However, the specific mechanism by which JNK mediates the release of cytochrome c is not clearly established. It has also been shown that JNK induces breakage of Bid (a pro-apoptotic member of the Bcl-2 family), or the phosphorylation of Bax (member of the Bcl-2 gene family) and Bad (Bcl-2-associated death promoter) (122).

In addition to the above described mechanisms, JNK can regulate the stability and transcriptional activity of p53, a key protein that mediates the regulation of the proapoptotic genes, apoptosis induced by DNA damage and oxidative stress (122-126).This indicates that JNK may cause both proapoptotic and antiapoptotic responses, even in the same cell type.

In particular, the JNK kinases play an important role in cerebral ischemia-induced excitotoxicity, epilepsy and in neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) (33,48,123,125).

It is well-known that AD is primarily characterized by the formation of neurofibrillary tangles consisting of tau protein hyperphosphorylation, and by the presence of β-amyloid (βA) plaques formed from the abnormal proteolysis of APP peptide (122-128). Recent studies suggest that the JNK pathway may play an important role in neuronal death and synaptic loss associated with AD (129-134). Furthermore, it has been described in neuronal cell cultures that βA-induced neurotoxicity is mediated through an increase in JNK activity, leading to neuronal death (130). Stimulation with βA and APP proteolysis requires a subsequent activation of c-Jun, caspase-8 and the expression of FasL (131). Similarly, it has been demonstrated that phosphorylation of JNK is related with hyperphosphorylation of Tau in neurodegeneration (129-132).

Likewise, it has been reported that the JNK signalling pathway probably playsa crucial role in PD (134-140). The main cause of this neurodegenerative disease is an increase of oxidative stress that causes the selective death of dopaminergic neurons of the substantia nigra (135-141). The degeneration of the nigrostriatal pathway produces clinical alterations such as akinesia, tremor, rigidity and postural disorders. Different studies showed that JNK is activated both in cultured of dopaminergic neurons subject to neurotoxicity by MPP^+ and following administration of MPTP (1-methyl-4-phenyl-1, 2,3,6 tetrahydropyridine) to nigrostriatal level (30).

It has also been demonstrated that there is JNK activation in experimental models of HD (30). This pathology is characterized by the selective loss of striatal neurons, mainly caused by a mutation in the *htt* gene (huntingtin) (30). *In vitro* studies, both in hippocampal neurons and striatal neurons treated with the neurotoxin 3-nitropropionic acid (3NP), evidenced that activation of JNK and c-Jun is associated with neuronal degeneration (30). However, there are currently no studies that describe the inhibitory effect of the JNK pathway in animal models of the disease (132-133,142).

The neuronal loss induced in a KA epileptic model could be mediated by the JNK activation and nuclear translocation induced by c-Jun phosphorylation, hence allowing the formation of the AP-1 complex which, in turn, induces transcription of proapoptotic factors. Different findings indicate that JNK-AP1 complex increases the expression of proapoptotic genes such as *tnf*, *fasl* and *bak* (Bcl-2 homologous antagonist/killer) (121-124).

The differential role of JNK isoforms in neurodegeneration and the specific mechanisms that regulate each isoform are little known. Analysis of genetically modified animals, as is the case of knockout mice for specific isoforms, provides the first evidence to understand the role of each isoform (142-145).

7.4. Specific isoform knock-out models of the JNK pathway

In the last decade, the use of murine genetic models has provided a major advance in the study of differential functions of each isoform of JNK

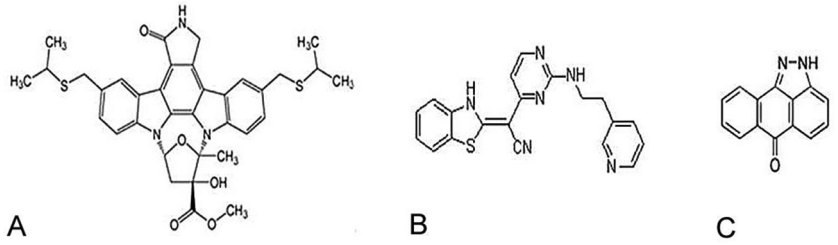

Figure 4. Molecular structure of JNK inhibitors: A. CEP-1347; B. AS601245; C. SP600125.

in vivo (143-146). Specific deletions of *jnk1*, *jnk2* and *jnk3* genes produce viable animals with normal development (143-147). The double knockout *jnk1/jnk2* is lethal, since the neural tube cannot be properly closed due to a deficiency in apoptosis, a crucial event in development process. However, the double knockout *jnk2/jnk3* and *jnk1/jnk3* are viable, thus demonstrating that both isoforms, JNK1 and JNK2, are essential in the regulation of apoptosis during embryonic brain development (141-150).

Some authors have described compensatory mechanisms in the expression of JNK isoforms in mice deficient in one of them. Thus, it has been shown that there is an increase in JNK1 levels in brain of *jnk2*$^{-/-}$ mice and an increase in JNK2 levels in *jnk3*$^{-/-}$ mice (104). Different studies with *jnk1*$^{-/-}$ and *jnk2*$^{-/-}$ mice showed that JNK1 has a central role in obesity and insulin resistance, while JNK2 could be involved in diabetes and in resistance to the neurotoxin MPTP (148,150). Furthermore, as mentioned above, both isoforms are involved in the regulation of brain development (148).

Interestingly, mice deficient in *jnk3* gene are less susceptible to excitotoxic stimuli, and have a reduced neuronal death in specific areas of the hippocampus after the administration of the neurotoxin KA. In these mice, a significant reduction of phosphorylation of c-Jun and of AP-1 activity was detected (144,145). Also, *jnk3*$^{-/-}$ mice exhibit reduced neuronal death in ischemic processes as well as in an experimental model of Parkinson's disease, established with the use of MPTP neurotoxin (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and 6-hydroxydopamine (150). Several *in vitro* studies show that cortical and hippocampal neurons deficient in JNK3 are resistant to βA toxicity and to oxygen and glucose deprivation (129-132). In addition, *in vivo* studies show that JNK3-deficient mice exhibit neuroprotection and decrease of seizures after KA administration, as well as resistance to apoptosis induced by hypoxia-ischemia (143,151).

It has been established that knockout mouse model for *c-jun* in which this gene is replaced with the mutant gene *junAA,* is viable. *JunAA* differs from the *wild-t*ype at serine 63 and 73, which are replaced by alanine. This knockout shows neuroprotection in different areas of the hippocampus after KA injections, suggesting that c-Jun protein can be the key substrate for JNK3 in the KA model (151). Nonetheless, the function of the individual JNK isoforms in the regulation of apoptotic pathways remains elusive.

Further knowledge of JNK physiological functions would be obtained with the deletion of JNK activators MKK4 and MKK7. However, mice deficient for MKK4 or MKK7 and double knockout *mkk4/mkk7* are unviable due to defects in hepatocyte proliferation (151). Moreover, since we have seen that the response of KA injections is different in neuronal and glial cells, it would be useful to study what happens when JNK pathway activity is specifically silenced in neurons or glial cells in the KA experimental model. This will be achieved by using conditional knockouts for *mkk4 and mkk7* genes using the Cre-loxP-System under specific neural promoters (GFAP and CAMKII).

Our findings will enable us to find therapeutic targets that can be used to silence JNK activity and therefore control the evolution of epileptogensis disease.

7.5. Inhibition of JNK pathway

Pharmacological inhibition of JNK is a well-known strategy for the protection of neuronal death. Several molecules have shown to be able to inhibit this pathway. Among them, the most important are CEP-1347, a specific inhibitor of MLK family; SP600125 and AS601245, both selective inhibitors of JNK activity, and D-JNKI a permeable peptide inhibitor of JNK that prevents phosphorylation of c-Jun (Figure 4) (135-141). The inhibitor CEP-1347 acts on MLKs kinases that activate the JNK pathway by competing for the ATP binding site (136). This compound, although it has low selectivity for JNK, shows neuroprotective effects in experimental mice models of PD, treated with MPP^+ (1-methyl-4-phenylpyridinium) in nigrostriatal neurons and prevents the toxicity of βA in neurons.(135). Although CEP-1347 is safe and well tolerated in patients with PD, is not effective in the treatment of this neurodegenerative disease. Further studies are needed to elucidate there mechanism of action (135,136).

SP600125 is a reversible inhibitor that competes for the ATP binding site with high selectivity for the three isoforms of JNK. The neuroprotective effect of SP600125 has been demonstrated in experimental models of Parkinson's disease, in ischemic processes and neurotoxic mechanisms of Alzheimer's disease (137,148). However, high doses of the drug may inhibit other kinases such as MKK3, MKK4, MKK6, and MKK7 (3,5,81).

The AS601245 inhibitor has been developed with a similar mechanism of action as SP600125, having a higher affinity toward the JNK3 isoform that JNK1 and JNK2 (138,139). It has been shown that AS601245 prevents neuronal death in cerebral ischemia models, and it also has an anti-inflammatory effect in models of rheumatoid arthritis (138,139). However, this drug does not provide sufficient efficiency to develop clinical trials intended for use in neurodegenerative diseases.

Moreover, a different strategy to inhibit the JNK pathway is the use of peptides that recognize the substrate binding domain or regulatory proteins thereof, such as JIPs. In this line, the D-JNKI peptide has been developed. It does not inhibit the enzymatic activity of JNK, but blocks the binding to its substrates (140). This peptide prevents phosphorylation of c-Jun, and thus exerts a neuroprotective effect in different models of excitotoxicity *in vitro*; it also protects from apoptotic cell death in experimental models of ischemia (140).

7.6. JNK3 as possible therapeutic target in neurodegenerative diseases

The specific JNK3 expression in brain makes it possible to consider this isoform as a potential therapeutic target for neurodegenerative diseases. As described above, *jnk3*$^{-/-}$ mice are viable and show protection against various apoptotic stimuli such as βA toxicity, oxygen and glucose deprivation, MPTP neurotoxin and KA, used as a model of mesial MTLE-HS. This data demonstrates the important role that JNK3 plays in neuronal death and explain why there is now currently interest in studying the involvement of this specific isoform in the development of neurological diseases (149-151). However, the high homology between JNK isoforms hinders the development of antibodies and selective inhibitors for each isoform. Thus, it is still unexplained why JNK3 plays a stronger role than the other two isoforms against brain damage, and whether it is the only JNK isoform with a neuroprotective role (105,106,130).

8. CONCLUSION

The KA experimental model shares similarities with the MTLE-HS epilepsy diseases, regarding the initial brain assaults, behavioral abnormalities associated to seizures and neuropathology. The use of this experimental animal model is a valuable tool to understand the mechanisms underlying epileptogenesis, specifically to study the potential pathways involved in the process of neuronal loss that occur in neurodegenerative processes. It is important to note that this model induces extensive neuronal damage in hippocampal structures, mainly a loss of pyramidal cells in the CA1 and CA3 regions. Considering that JNK pathway is activated in neuronal death, we focused on the analyses of JNK protein in mice KA experimental model, using different knock-outs for JNK (*jnk1*$^{-/-}$, *jnk2*$^{-/-}$ *and jnk3*$^{-/-}$). Previous data supported that *jnk3*$^{-/-}$ mice were protected from excitotoxicity induced with KA, and showed a decrease in seizures caused by this substance. Given this assumption, future studies should be performed treating this *jnk3*$^{-/-}$ knockout mice with KA. This will allow the evaluation of the potential genes involved in neuronal cell death/protection and will provide more tools to design effective drugs for neuronal protection. In addition, we suggest that JNK pathway inhibitors may constitute new adjuvant drugs for preventing seizure-induced neuronal death and may be used as potential therapeutic strategy for epileptogenesis.

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