



# Activation of the Extrinsic and Intrinsic Apoptotic Pathways in Cerebellum of Kindled Rats

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Published online: 6 May 2019

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## Abstract

The purpose of this study is to determine the activation of the extrinsic and intrinsic apoptotic pathways in the cerebellum of rats exposed to amygdaloid electrical kindling. Western blot analyses were carried out for caspase-8 and caspase-9, Bid, Bax, and Bcl-2 in the cerebellum and immunohistochemistry of Bid, Bax, cytochrome C, and VDAC (voltage-dependent anion channels) in the cerebellar cortex of Wistar male rats with 0, 15, and 45 kindling stimulations. In the experimental group of 45 stimuli, we observed an increase in protein activation of caspase-9 and truncated Bid and Bax, in addition to a decrease in expression of pro-caspase-8 and the anti-apoptotic protein Bcl-2, determined by Western blot. Moreover, we observed a cytosolic immunopositivity for cytochrome C and a mitochondrial immunolocalization for truncated Bid and Bax in the group of 45 stimuli. In this work, we found an increase of caspase-8, a cysteine-protease that can activate caspase-3 triggering extrinsic apoptosis by signaling of death receptors. However, it also can activate the intrinsic pathway releasing Bid, which performs mitochondrial translocation of Bax, inactivating Bcl-2 and allowing the release of cytochrome C through the opening of the mitochondrial permeability transition pore, promoting the activation of caspase-9 which activates caspase-3, the main executor caspase of apoptosis. Therefore, it is concluded that there is an activation of the intrinsic and extrinsic apoptotic pathways in the cerebellum of rats exposed to the kindling model. Apoptosis signaling pathways can be analyzed as an important developing object of research about the epileptic activity.

**Keywords** Epilepsy · Kindling · Cerebellum · Intrinsic apoptosis · Extrinsic apoptosis

## Highlights

- Presence of extrinsic and intrinsic apoptosis in the cerebellum after chronic epileptic activity.
- Increased expression of Bax and tBid and decreased expression of Bcl-2 in cells of the cerebellar layer after chronic epileptic seizures.
- Increased levels of initiator caspases of the extrinsic (caspase-8) and intrinsic (caspase-9) pathways in the cells of the cerebellar layer after chronic epileptic seizures.
- Presence of Bax and Bid in the mitochondria of cerebellar cells of rats exposed to generalized tonic-clonic seizures.
- Absence of cytochrome C in the mitochondria of cerebellar cells of rats exposed to generalized tonic-clonic seizures.

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## Introduction

Epilepsy has been described as a neurological disorder characterized by a series of neurophysiological and neurochemical changes that cause repeated seizures [1]. The complex physiopathology of epilepsy has allowed the design of multiple experimental models for its study [2, 3]. The kindling model has been referred to as a progressive development of electroencephalographic and behavioral seizures produced by repeated low-intensity electrical stimulation to discrete forebrain structures resulting in secondary generalized seizures [4]. The advantage of kindling is the control on the seizure frequency and the progression of seizure severity [5]. Other advantages include EEG registration that provides the morphologic changes similar to those present in human patients with secondary generalized partial seizures and the control of possible collateral effects of chemical models during the different states of kindling [6]. Using this model, it was demonstrated that there is apoptotic neuronal death in the cerebellum after the recurrence of epileptic seizures [7]. Although the amygdala or hippocampus are usually the epileptic focus, loss of Purkinje cells has been widely cited as a consequence of recurrent and prolonged seizures and patterns of cerebellar atrophy has been described in chronic epileptic patients [8, 9]. A clinical study has demonstrated that after an epileptic state, a generalized loss of Purkinje cells is observed [10]. Imaging studies have also revealed a generalized loss of cerebellar gray matter [11, 12]. In animal models, for example, Mongolian gerbils susceptible to seizures have decreased Purkinje cell densities [13, 14]. It is thought that degeneration can be excitotoxic induced by seizures because chronic intraventricular injections of glutamate and aspartate result in the loss of Purkinje cells [15]. However, the mechanisms by which the death of cerebellum cells occurs has not been determined in its entirety. The initiation of apoptosis occurs mainly for two different pathways: the intrinsic pathway and the extrinsic pathway [16, 17]. This can result in the activation of anti-apoptotic proteins of the Bcl-2 family to decrease the apoptotic effect [18, 19]. However, pro-apoptotic proteins (Bid, Bax) can also be activated to trigger a signaling cascade mediated by caspases and pro-apoptotic factors released from the mitochondrial inter-membrane space, amplifying the signal of death [20–23]. A reduction of Bcl-2 protein and the increase of mRNA for Bax protein in the hippocampus of mice after the systemic injection of kainic acid as model of epilepsy have been reported [24]. There was a significant positive correlation between serum levels of Fas (ligand of Fas death receptor) and Bcl-2 and they both were significantly increased in patients with uncontrolled epilepsy [25]. Therefore, the aim of this study was to determine the activation of apoptosis (extrinsic and intrinsic) in the cerebellum of kindled rats.

## Method

### Animal Conditions

Ten male Wistar rats weighing 250 to 290 g for each group (control, 15 and 45 stimuli) were used. The handling of rats was performed according to institutional and national guidelines (NOM-062-ZOO-1999) and the international principles of the Council for International Organizations of Medical Sciences. The rats were individually housed in transparent cages, which allowed them to move freely over a corncob bedding, and they were maintained at  $23 \pm 1$  °C under a 12-h light-dark cycle (lights on at 07:00 AM) with free access to food and water.

### Stereotaxic Surgery

Control rats were not operated; for the experimental groups, the rats were anesthetized intraperitoneally with 30 mg/kg of Zoletil 50 V® (Virbac Carros, France) and placed in a stereotaxic device (David Kopf) to carry out the implantation of electrodes for stimulation and recording in the basolateral nucleus of amygdala (previous coordinates of 6.2 mm, side of 5 mm and 1.5 mm in height), using the interaural line as reference in accordance with stereotaxic atlas of Paxinos and Watson [26]. Another electrode was placed in the cortex which indicated to register the propagation of electroencephalographic activity. Each electrode was made by an (0.005-in. diameter) isolated stainless steel and Teflon-coated, excepting for the ends. A screw implanted in the skull served as a reference source. The electrodes were attached to a mini-connector and fixed in the skull with acrylic dental, and the skin was sutured around the mini-connector.

### Kindling Model

After 10 days of postoperative recovery, the rats were placed in a silenced chamber (22.5 cm × 30 cm × 30 cm). The connector joined the flexible cables which connect to the rat with the Stimulator S88 Grass (GrassV® Instrument Company Model S88 Stimulator, USA) and a digital polygraph EBNeuro. The conditions to use the polygraph were 50  $\mu$ V of amplification and a filter between 0.3 and 30 Hz. The rats were stimulated every day with a frequency of 60 Hz, pulses of duration at 1.0 ms, and an intensity of 400  $\mu$ A [4]. The following parameters were measured: the duration of the afterdischarge registered in the amygdala and the behavior in accordance with the parameters described by Racine et al. [27]: stage 1: clonus of the facial muscles, one or both eyes closed; stage 2: oscillatory movements of the head; stage 3: myoclonic forelimbs in movement; stage 4: both extremities myoclonic movements; stage 5: generalized tonic-clonic seizure [27].

## Immunohistochemistry

The immunohistochemical analysis of the cerebellum was performed according to the study of Rubio et al. [5]. Five rats from each group (control, 15 and 45 stimuli) were sacrificed with sodium pentobarbital (63 mg) and transcardially perfused first with PBS (1×, pH 7.4) then fixed with paraformaldehyde dissolved in PBS (pH 7.2). Brains were extracted and gradually dehydrated with alcohol and xylol for the paraffin-embedded. Cerebellar coronal slices of 5 μm of thickness were obtained and mounted on silane-coated slides. Dewaxing and hydration were subsequently done in PBS followed by saturation with hydrogen peroxide. Non-specific sites were blocked with bovine serum albumin free of IgG1 (Sigma, St. Louis MO, USA). After washing each of the slides for VDAC immunohistochemistry, a drop of anti-VDAC antibody was added (goat monoclonal anti-VDAC antibody, Santa Cruz Biotechnology, USA) and diluted 1:500 overnight at 4 °C. Subsequently, tissues were washed and incubated with secondary antibody conjugated to fluorescein for 30 min. For double labeling of tBid, Bax, and cytochrome C, two compatible primary antibodies were consecutively applied overnight at 4 °C. The following primary antibodies were applied: mouse-monoclonal tBid antibody (1:100, Sigma, USA), rabbit-polyclonal Bcl2 antibody (1:100, Santa Cruz Biotechnology, USA), and mouse-monoclonal Bax antibody (1:100, Santa Cruz Biotechnology, USA). Other histological sections were used to determine the cytoarchitecture of the cerebellar cortex using double labeling with calbindin (1:100, Santa Cruz Biotechnology, USA) and glial fibrillary acid protein (GFAP) (1:100 DakoCytomation, USA). Subsequently, tissues were washed and incubated in secondary antibody conjugated to fluorescein for 30 min. Finally, they were placed on a mounting medium for fluorescence with a 4',6-diamidino-2-phenylindole (DAPI) staining and a mounting medium (Fluoroshield F6057 in blue, Sigma, USA). The slides were analyzed with an image program adapted to a microscope Olympus (1X81F3) and the immunopositivity was quantified through optical density analysis with the software Image-Pro Plus Version 7.0. The sections were obtained with an objective 40× in three fields of cerebellar Crus 1 folia for each animal.

## Western Blot

Five rats from each group (control, 15 and 45 stimuli) were sacrificed by decapitation to perform Western blot procedures. The levels of caspase-8 and 9, Bid, Bax, and Bcl-2 proteins expression were evaluated by Western blot analysis. Samples containing equal amounts of protein (50 μg) were mixed with an equal volume of sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.02% bromophenol blue, and 10% 2-mercaptoethanol) and boiled during 5 min. The samples were cooled on ice for 5 min, centrifuged for a short time, and

exposed to 10 to 12% SDS polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to a nitrocellulose membrane for 2 h at 70 V with 25 mM Tris-HCl, pH 8.0, 195 mM glycine, and 10% methanol. The membrane was blocked with 5% light milk in PBS for 1 h. Antibody for caspase-8 and caspase-9, Bid, Bax, and Bcl-2 (Santa Cruz Biotechnology, Ca) was added to the membrane for 24 h at 4 °C. After three consecutive washes in PBS, the membrane was incubated with a secondary antibodies IgG-horseradish peroxidase complex (Santa Cruz Biotechnology) during 1 h at room temperature, followed by three washes with PBS. Then, chemiluminescence was visualized using the enhanced chemiluminescence kit (Santa Cruz Biotechnology). The blot was exposed to Kodak XAR-5-ray film (Sigma Chemical Co.) for ± 1 min and then revealed. A similar procedure was used for β-actin, (Santa Cruz Biotechnology). The band intensities were quantified in a Molecular Dynamics (Durham, NC) computing densitometer using ImageQuant software version 3.2.2.

## Statistical Analysis

Statistical analyses were conducted using SPSS software (version 20). Data are expressed as the mean-standard error of the mean (SEM). Densitometry analyses were analyzed by one-way (ANOVA) followed by Tukey's test. In all cases, significance was considered when  $*p < 0.05$ .

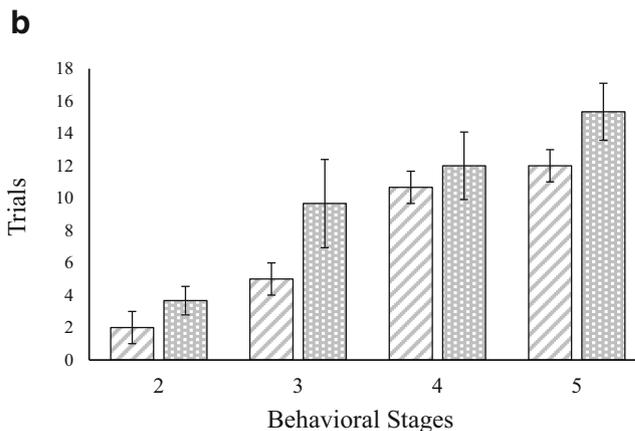
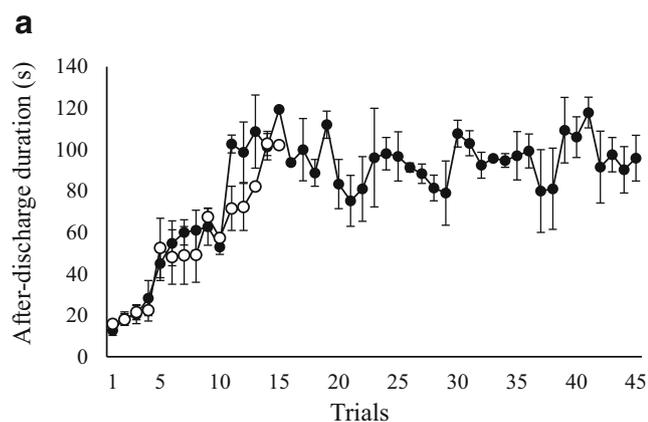
## Results

### Behavioral Activity Records

In both groups that received stimuli, the behavioral state presented was equivalent to generalized seizures, in accordance with the parameters described by Racine et al. [27]. We found that the duration of the afterdischarge in amygdala and cerebral cortex after generalized seizures is similar to the previous reports (Fig. 1).

### Immunohistochemistry

After double labeling with calbindin and GFAP, it was possible to determine the immunolocalization of Purkinje cells and surrounding glia respectively, allowing the boundaries of each cerebellar stratum to be very well defined (Fig. 2). We found a significantly increased immunolocalization for tBid that is an activated form of the pro-apoptotic protein involved in the activation of the intrinsic apoptotic pathway and VDAC (a subunit of the mitochondrial permeability pore) as the stimulation increases (Fig. 3 A and A'), suggesting an activation of the apoptotic cascade ( $f = 11.299$ ;  $df = 2.24$ ;  $p = 0.00$ ). A similar case has been observed for Bax that is the most important pro-apoptotic protein and VDAC, where both experimental



**Fig. 1** (a) Amygdaloid AD durations (s) for the 15 stimuli group (white circles) and 45 stimuli group (black circles). Values are expressed as means ± S.E.M. We determined that there is no statistical difference between groups. (b) Number of trials required to reach each kindling

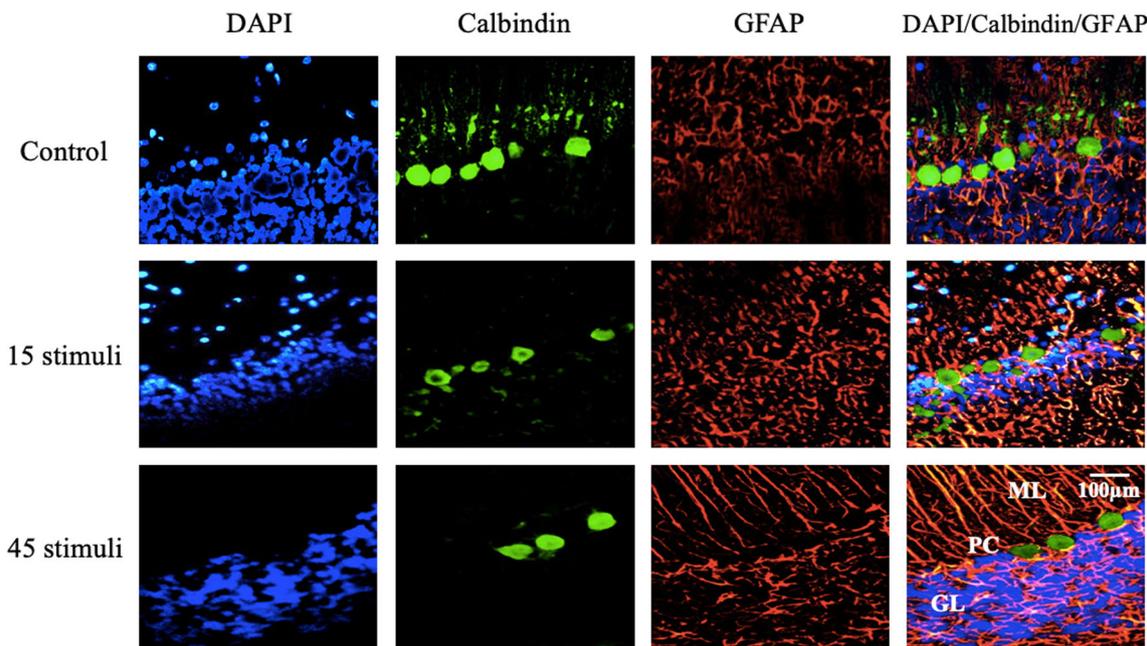
stage according to the Racine’s scale (1972) for the 15 stimuli group (diagonal bars) and 45 stimuli group (dotted bars). Values are expressed as means ± S.E.M. Between groups there are no significant differences

groups (15 and 45 stimuli) showed an increased immunolocalization compared with the control group (Fig. 4 A and A’), suggesting an activation of its apoptotic function ( $f=22.207$ ;  $df=2.24$ ;  $p=0.000$ ). Immunopositivity was localized in PC and furthermore in the cells of the granular layer of the cerebellar Crus 1 folia. Serapide et al. and Cicirata et al. showed that fibers coming from the pontine nuclei and from inferior olive establish a somatotopic projection to crus 1; for this reason, we perform this study in this cerebellar area [28, 29]. For cytochrome C that is the mean pro-apoptotic factor released from the inter-membrane mitochondria space and VDAC (Fig. 5 A and A’), we found a significantly decreased immunolocalization

in the group with 45 stimuli compared with the control group ( $f=9.364$ ;  $df=2.24$ ;  $p=0.001$ ), suggesting a triggered pro-apoptotic function of cytochrome C into the cytosol allowing an interaction with other factors involved. Immunopositivity was observed in the granular layer of the cerebellar cortex.

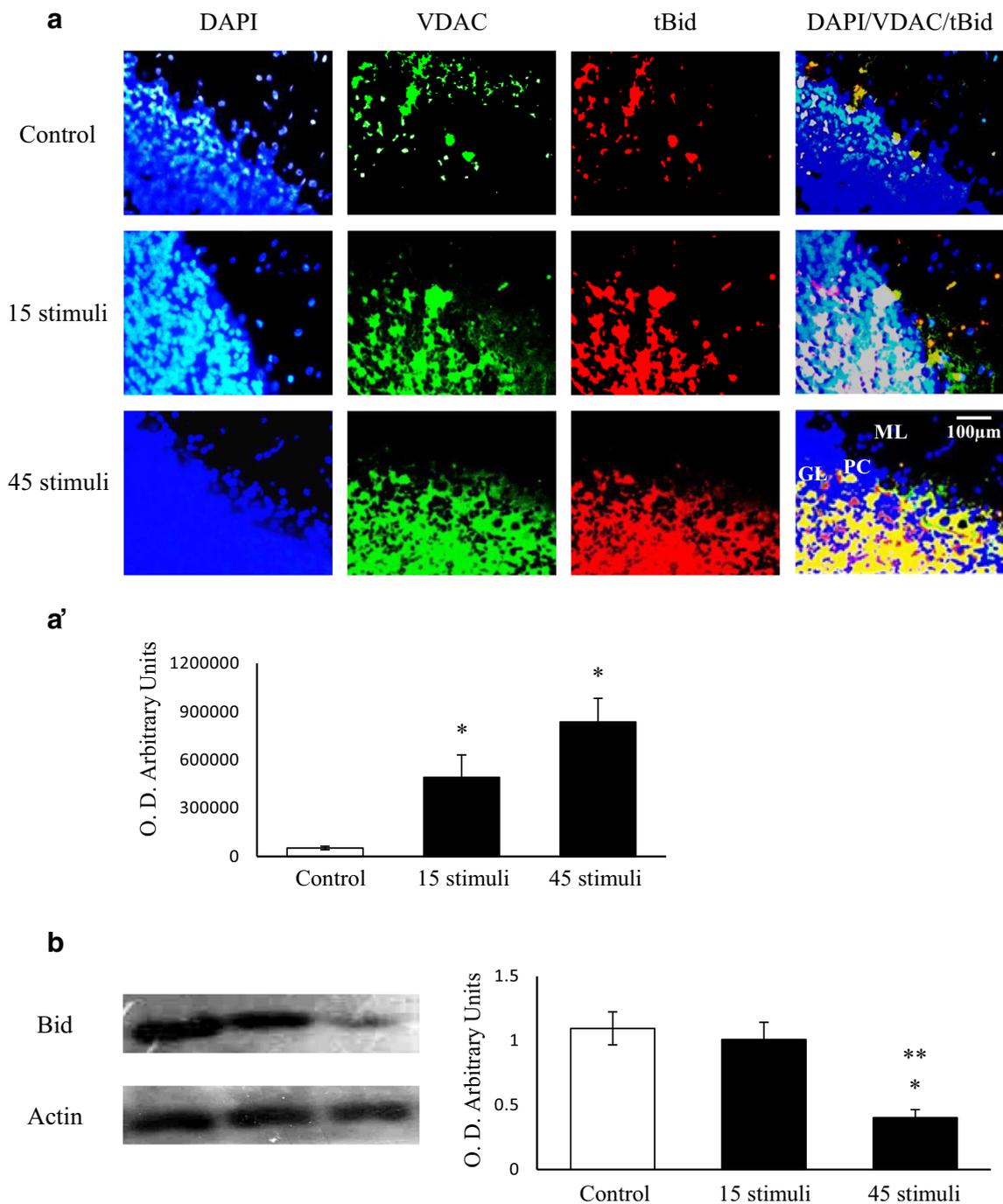
**Western Blot**

In the analysis done with Western blot, we observed a significant decrease of protein levels for pro-caspase-8 (Fig. 6), a complete form of caspase-8, the initiating caspase of extrinsic pathway, suggesting that the active form is expressed in the group with



**Fig. 2** Photomicrographs processed with immunohistochemistry assay (40×, scale bar 100 µm); the first column shows the DAPI in blue. The second column shows GFAP in red. The third column shows calbindin in

green and the last column shows the overlap of DAPI, GFAP, and calbindin. Molecular layer (ML); Purkinje cells (PC); granular layer (GL), after 0, 15, and 45 stimuli

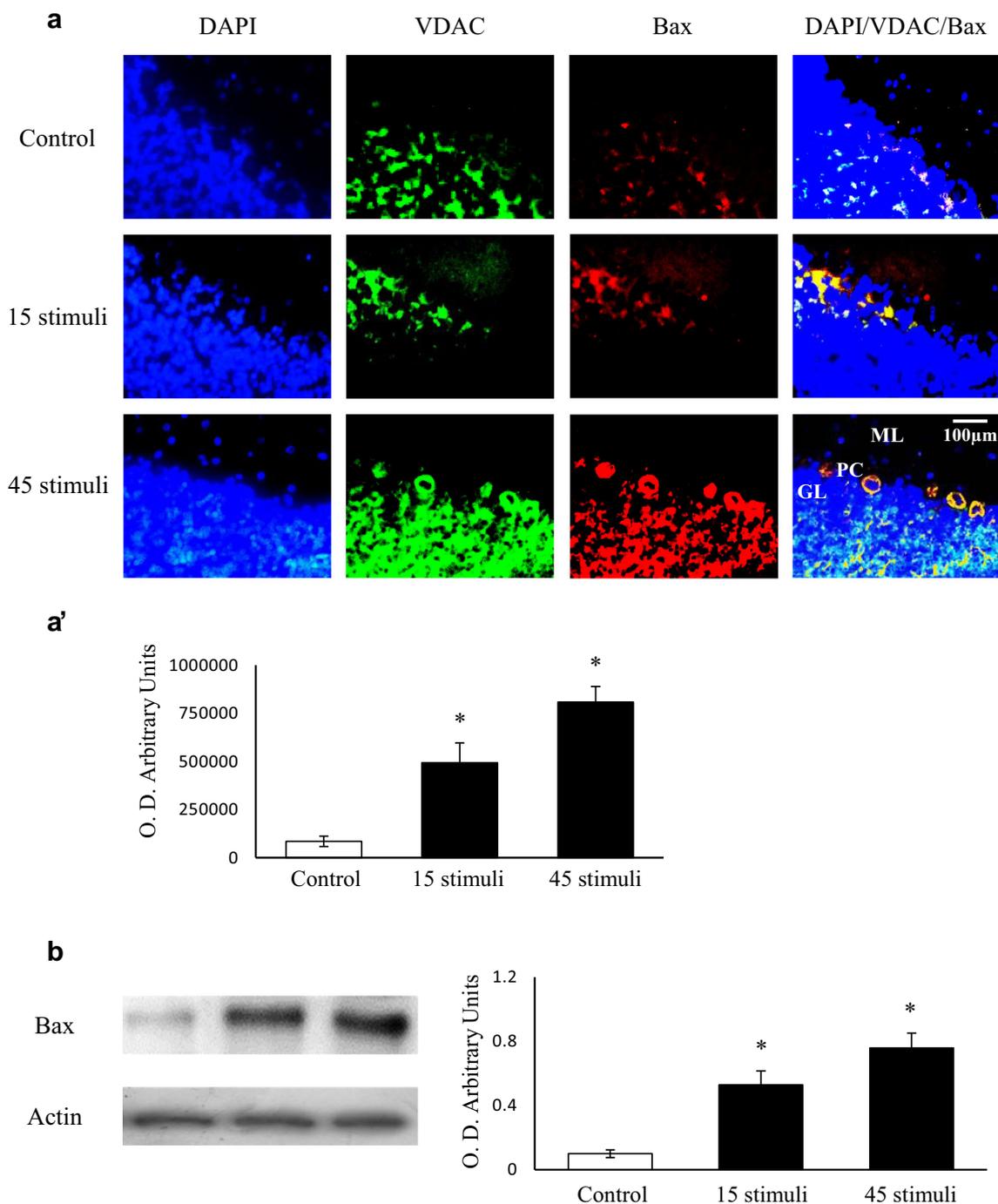


**Fig. 3** (A) Photomicrographs processed with immunohistochemistry assay (40 $\times$ , scale bar 100  $\mu$ m); the first column shows the DAPI in blue. The second column shows VDAC in green. The third column shows Bid in red and the last column shows the overlap of DAPI, VDAC, and Bid. Molecular layer (ML); Purkinje cells (PC); granular layer (GL). (A') Optical density for immunopositivity for VDAC

colocalizing with Bid. We observe that there is a significant increase in rats that received 15 and 45 stimuli ( $*p < 0.05$ , compared with control). (B) A representative Western blot of Bid and  $\beta$ -actin, and levels of expression after 0, 15, and 45 stimuli ( $*p < 0.05$ , compared with control;  $**p < 0.05$ , compared with 15 stimuli)

45 stimuli; a significantly decreased expression of Bcl-2 (Fig. 7 A), the mean anti-apoptotic protein of the Bcl-2 family, suggesting an inhibition of its function also in the group with 45 stimuli; and a decrease in the expression of total Bid (Fig. 3 B), the complete form of the pro-apoptotic protein involved in activation

of the intrinsic apoptotic pathway in the group with the highest number of stimuli, suggesting that the active form is expressed (pro-caspase-8:  $f = 17.094$ ;  $df = 2.6$ ;  $p = 0.003$ ; Bcl-2:  $f = 76.906$ ;  $df = 2.6$ ;  $p = 0.00$ ; total Bid:  $f = 11.270$ ;  $df = 2.6$ ;  $p = 0.009$ ). While for Bax, we observed a significant increase in both



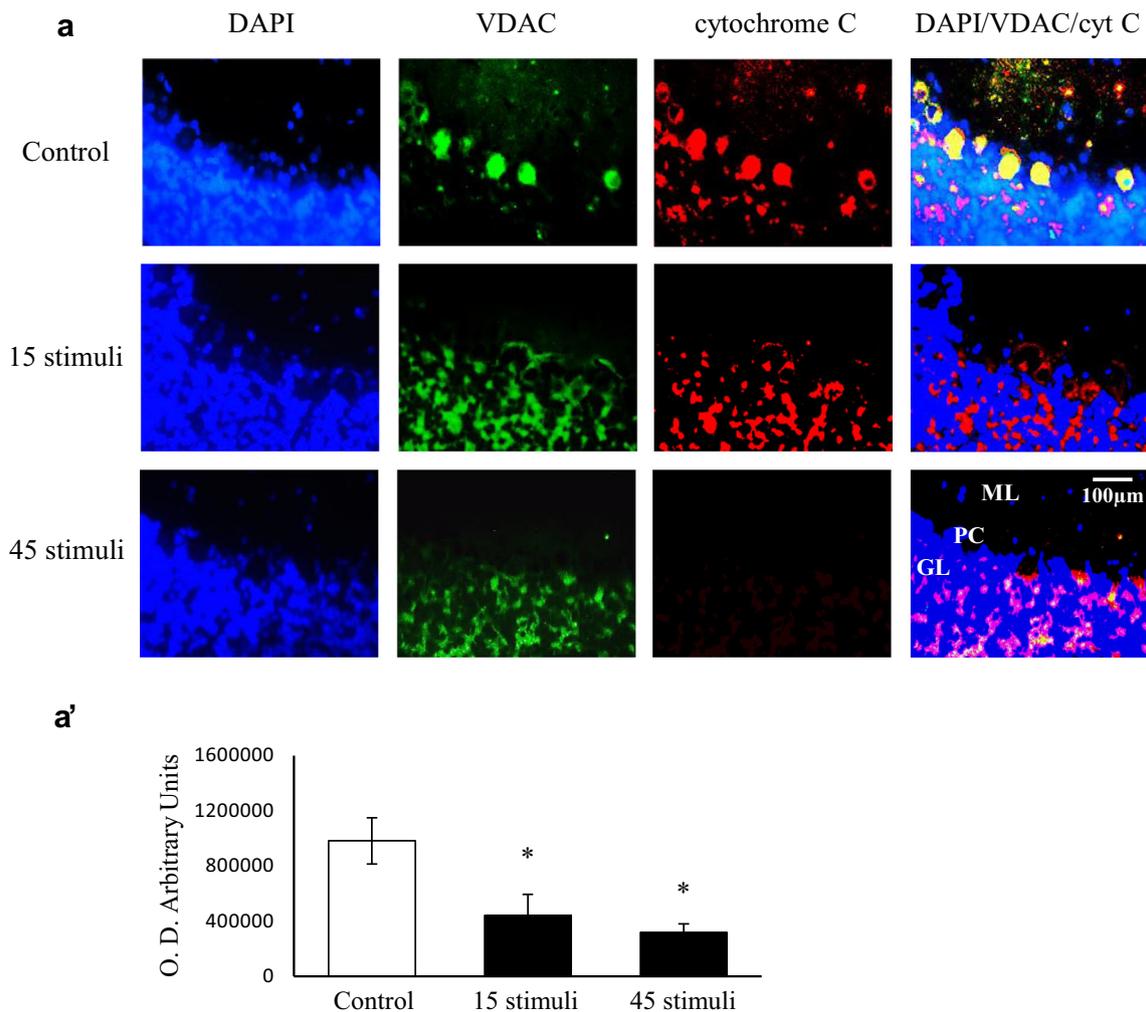
**Fig. 4** (a) Photomicrographs processed with immunohistochemistry assay (40×, scale bar 100 µm); the first column shows the DAPI in blue. The second column shows VDAC in green. The third column shows Bax in red and the last column shows the overlap of DAPI, VDAC, and Bax. Molecular layer (ML); Purkinje cells (PC); granular

layer (GL). (A') Optical density for immunopositivity for VDAC colocalizing with Bax. We observe that there is a significant increase in rats that received 15 and 45 stimuli (\* $p < 0.05$ , compared with control). (b) A representative Western blot of Bax and  $\beta$ -actin, and levels of expression after 0, 15, and 45 stimuli (\* $p < 0.05$ , compared with control)

experimental groups compared with the control group (Fig. 4 B), and for caspase-9 (Fig. 7 B), the initiating caspase of the intrinsic pathway, we observed a significant increase in both experimental groups (Bax:  $f = 20.409$ ;  $df = 2.6$ ;  $p = 0.002$ ; caspase-9:  $f = 26.863$ ;  $df = 2.6$ ;  $p = 0.001$ ). These results correlate with the results obtained through immunohistochemistry.

### Discussion

It has been described with various models of epilepsy that repeated or prolonged seizures induce a cellular degeneration causing cell death of neurons in the hippocampus, frontal cortex, cerebellum, and other regions of the limbic system [30].

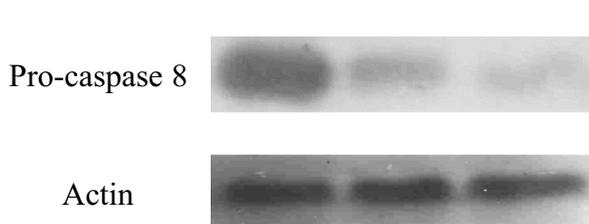


**Fig. 5** (a) Photomicrographs processed with immunohistochemistry assay (40 $\times$ , scale bar 100  $\mu$ m); the first column shows DAPI in blue. The second column shows VDAC in green. The third column shows cytochrome C (cyt C) in red and the last column shows the overlap of DAPI, VDAC, and cyt C. Molecular layer (ML); Purkinje cells (PC);

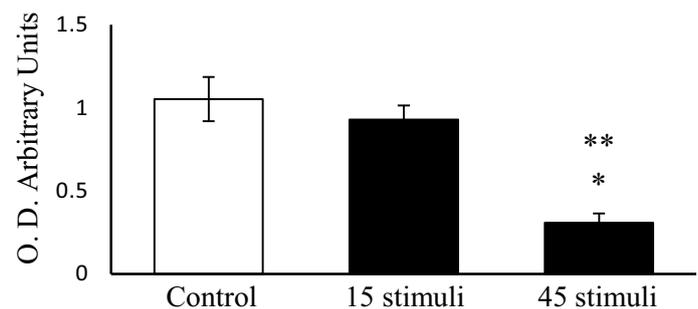
granular layer (GL). (a') Optical density for immunopositivity for VDAC colocalizing with cytochrome C. We observe that there is a significant decrease in rats that received 15 and 45 stimuli ( $*p < 0.05$ , compared with control)

The molecular mechanisms by which seizures induce neuronal death in cerebellum are unknown; however, we reported the presence of apoptosis in cerebellum after a chronic state of epilepsy [7]. One of the primary events in apoptosis induced

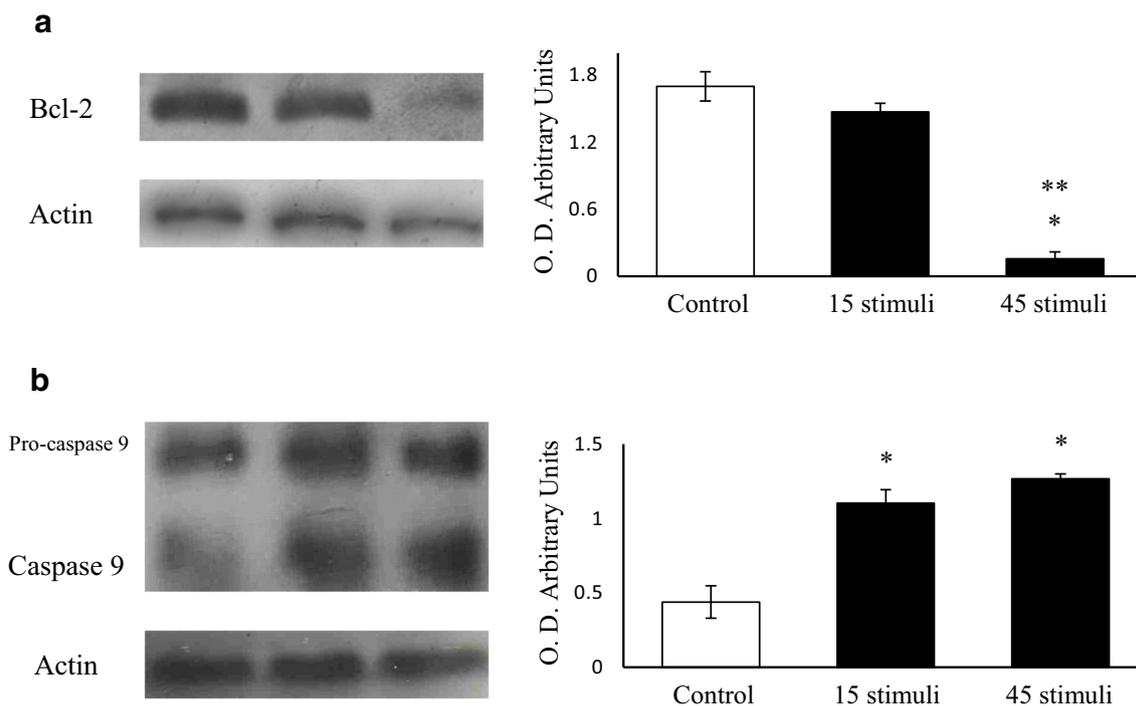
by seizures in the hippocampus is the excessive release of glutamate with consequent intracellular calcium ( $\text{Ca}^{2+}$ ) overload [31]. In the cerebellum, the PCs are highly susceptible to glutamate-mediated excitotoxicity, due to the excessive release



**Fig. 6** A representative Western blot of pro-caspase-8 and  $\beta$ -actin and levels of expression after 0, 15, and 45 stimuli. We observe a significant decrease in the expression of pro-caspase-8 as the stimulation increases



( $*p < 0.05$ , compared with control;  $**p < 0.05$ , compared with 15 stimuli), suggesting an expression of its active form (caspase-8)



**Fig. 7** (a) A representative Western blot of Bcl-2 and  $\beta$ -actin and levels of expression after 0, 15, and 45 stimuli. We observe a significant decrease in the expression of Bcl-2 in rats that received 45 stimuli ( $*p < 0.05$ , compared with control;  $**p < 0.05$ , compared with 15 stimuli), showing the inhibition of its anti-apoptotic function. (b) A

representative Western blot of caspase-9 and  $\beta$ -actin and levels of expression after 0, 15, and 45 stimuli. We observe a significant increase in the expression of caspase-9 as the stimulation increases ( $*p < 0.05$ , compared with control), for both groups that received stimuli

of glutamate from climbing fibers (arising from the inferior olive) and numerous parallel fibers (axons of granule cells). Glutamate excitotoxicity occurs when the increased  $\text{Ca}^{2+}$  influx and the release of  $\text{Ca}^{2+}$  from intracellular stores lead to the activation of  $\text{Ca}^{2+}$ -dependent enzymes, inducing degradative and apoptotic cell death pathways [32]. The extrinsic pathway of apoptosis is initiated via death receptors, with the binding of the ligand  $\text{TNF}\alpha$ , apoptosis-inducing ligand (TRAIL), and Fas ligand to its receptors of the TNF family (TNFR1, Fas, DR4 (TRAIL receptor 1), and DR5 (TRAIL receptor 2)), generating cytosolic release of cyt C and activation of caspase-9 [33, 34]. It is demonstrated that this family of receptors is associated with the maintenance and progression of temporal lobe epilepsy [35]. The cell death signaling for TNFR1 involved the recruitment of TNFR-associated death domain protein (TRADD) which binds Fas-associated death domain protein (FADD) and activates caspases such as caspase-8 and caspase-10 [36]. Meanwhile, Fas activation mediates the binding of TRADD, recruitment, and activity of caspase-10 which activate caspase-3 [37]. An overexpression of TNFR1-TRADD and formation of TRADD-FADD complexes in samples from hippocampus of patients with temporary intractable lobe epilepsy has been reported [38]. Moreover, it has been demonstrated that seizures that impair the hippocampus in rat mediate the formation of TNFR1 signaling, involving TRADD and FADD that might activate pro-

caspase-8 [39, 40]. In addition, Henshall et al. [39] demonstrated that seizures evoked by intra-amygdalar administration of kainic acid (KA) induce the expression of Fas, FADD, and activation of caspase-8 with cleavage of Bid to a truncated form (tBid) [41]. Inhibition of caspase-8 in vivo is neuroprotective against seizure damage; it might mitigate mitochondrial dysfunction through inhibition of Bid and cytosolic cyt C, inhibiting the activation of caspase-9, caspase-3, and DNA fragmentation [40, 42]. Thus, the extrinsic pathway contributes to the pathophysiology of seizure-induced neuronal death. On the other hand, in the intrinsic pathway of apoptosis, the family of Bcl-2 proteins has an important signaling role. Studies have confirmed that after seizures, tBid and its full-length form are localized in mitochondrial membrane, where the pro-apoptotic function of Bax is translocated and stimulated [43–45]. The activation of the intrinsic pathway can be dependent on the calcium current in mitochondria, that increases due to overactivation of Glutamate receptors after seizures. Additionally, the calcineurin (calcium dependent phosphatase) increase dephosphorylates and activates the pro-apoptotic protein Bad [46, 47]. It has been reported that under apoptotic stress and status epilepticus, Bad is released from 14 to 3-3 protein to replace Bax from the Bax – Bcl-xl complex; Bax translocates the mitochondria and promotes the release of cyt C, formation of apoptosomes, and activation of caspase-3 [48–51]. After i.p. administration of KA, nuclear DNA

fragmentation, downregulation of Bcl-2 protein expression, and upregulation of Bax mRNA expression in hippocampal and neocortex cells of mice have been reported, but the results are inconsistent [52–54]. Several anti-apoptotic proteins of Bcl-2 family may protect cell death after seizures as showed on animal models. Also, on patients with temporal lobe epilepsy, higher levels of anti-apoptotic proteins such as Bcl-2, Bcl-xl and Bcl-w were observed [55–57]. In our study, we showed alterations in the expression of members of the Bcl-2 family (decrease in Bcl-2, increase in levels on mitochondrial Bax, and post-transductional Bid processing), suggesting the activation of the intrinsic apoptotic pathway. The results obtained in the present study correlate with the previous reports of our group, with similar methodology that described the presence of apoptosis in the cerebellum of rats with generalized seizures and an increased signaling of Wnt/ $\beta$ -catenin pathway [7]. Furthermore, the overexpression of c-myc protein was demonstrated, which induces the expression of pro-apoptotic proteins such as Bax [58], Bim [59], Noxa [60], and Puma [61] as well as the transcription of fas ligand, Fas, and TNFR1 [62–64]. Also, it has been reported that activation of  $\beta$ -catenin induces mitochondrial apoptosis through downregulation of the anti-apoptotic protein Bcl-2, which leads to a loss of mitochondrial membrane potential with the release of cyt C and increased expression of caspase-9 and caspase-3 [65]. The current data and our previous work establish that seizures produced by the kindling model can activate the intrinsic and extrinsic pathways that involve cytochrome C and caspases, and the activation of pro-apoptotic and an inhibition of anti-apoptotic mechanisms, which provide a more complete understanding of the mechanisms by which epileptic seizures damage the cerebellum. However, additional studies are required to address the specific involvement of interneurons, glia, and Bergmann glia of the cerebellum on intrinsic and extrinsic death pathways, caused by epileptic activity.

## Conclusion

We conclude that the intrinsic and extrinsic apoptotic pathways are involved in the physiopathology of seizures in the cerebellum of rats with generalized seizures induced by the amygdaloid kindling model. Apoptosis signaling pathways appear to contribute to the neuronal loss that follows seizures and may also be engaged in the cerebellum of kindling rats.

**Acknowledgments** We thank the Armstrong Foundation for the undergraduate scholarship to César Mendoza and Emmanuel González.

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

## References

1. Sirven JI. Epilepsy: a spectrum disorder. *CSH Perspect Med.* 2015;5(9):1–16.
2. Ostergard T, Sweet J, Kusyk D, Herring E, Miller J. Animal models of post-traumatic epilepsy. *J Neurosci Meth.* 2016;272:50–5.
3. Rubio C, Rubio-Osornio M, Renata-Márquez S, López M, Custodio V, et al. In vivo experimental models of epilepsy. *Cent Nerv Syst Agents Med.* 2010;10:298–309.
4. Goddard GV, McIntyre DC, Leech CK. A permanent change in the brain function resulting from daily electrical stimulation. *Exp Neurol.* 1969;25:295–330.
5. Gortera JA, Van EA, Lopes da Silva FH. Which insights have we gained from the kindling and post-status epilepticus models? *J Neurosci Meth.* 2016;260:96–108.
6. Morimoto K, Fahnstock M, Racine RJ. Kindling and status epilepticus models of epilepsy, rewiring the brain. *Prog Neurobiol.* 2004;73:1–60.
7. Rubio C, Rosiles-Abonce A, Trejo-Solis C, Rubio-Osornio M, Mendoza C, et al. Increase signaling of Wnt/ $\beta$ -catenin pathway and presence of apoptosis in cerebellum of kindled rats. *CNS Neurol Disord Drug Targets.* 2017;16:772–80.
8. Crooks R, Mitchell T, Thom M. Patterns of cerebellar atrophy in patients with chronic epilepsy: a quantitative neuropathological study. *Epilepsy Res.* 2000;41:63–73.
9. Hermann BP, Bayless K, Hansen R, Parrish J, Seidenberg M. Cerebellar atrophy in temporal lobe epilepsy. *Epilepsy Behav.* 2005;7:279–87.
10. Fujikawa DG, Itabashi HH, Wu A, Shinmei SS. Status epilepticus-induced neuronal loss in humans without systemic complications or epilepsy. *Epilepsia.* 2000;41:981–91.
11. Oyegbile TO, Bayless K, Dabbs K, Jones J, Rutecki P, et al. The nature and extent of cerebellar atrophy in chronic temporal lobe epilepsy. *Epilepsia.* 2011;52:698–706.
12. Marcián V, Marecek R, Táková E, Pail M, Bareš M, et al. Morphological changes of cerebellar substructures in temporal lobe epilepsy: a complex phenomenon, not mere atrophy. *Seizure.* 2018;54:51–7.
13. Dam M, Bolwig T, Hertz M, Bajorec J, Lomax P, Dam AM. Does seizure activity produce Purkinje cell loss? *Epilepsia.* 1984;25:747–51.
14. Dam M. Quantitative neuropathology in epilepsy. *Acta Neurol Scand S1992;137:51–54.*
15. Sloviter RS, Dempster DW. “Epileptic” brain damage is replicated qualitatively in the rat hippocampus by central injection of glutamate or aspartate but not by GABA or acetylcholine. *Brain Res Bull.* 1985;15:39–60.
16. Ameisen J. On the origin, evolution, and nature of programmed cell death: a timeline of four billion years. *Cell Death Differ.* 2002;9:367–93.
17. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer.* 1972;26:239–57.
18. Dejean LM, Martinez-Caballero S, Manon S, Kinnally KW. Regulation of the mitochondrial apoptosis-induced channel, MAC, by BCL-2 family proteins. *Biochim Biophys.* 2006;1762:191–201.
19. Duprez L, Wirawan E, Vanden T, Vandenabeele P. Major cell death pathway at a glance. *Microbes Infect.* 2009;11:1050–62.
20. Coutlas L, Strasser A. The role of the Bcl-2 protein family in cancer. *Semin Cancer Biol.* 2003;13:115–23.
21. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell.* 1998;94:481–90.

22. Yoshida H, Kong YY, Yoshida R, Elia AJ, Hakem A, Hakem R, et al. Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell*. 1998;94:739–50.
23. Kiuda K, Haydar TF, Kuan CY, Gu Y, Taya C, et al. Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell*. 1998;94:235–337.
24. Simonato M, Hosford DA, Labiner DM, Shin C, Mansbach HH, McNamara JO. Differential expression of immediate early genes in the hippocampus in the kindling model of epilepsy. *Brain Res Mol Brain Res*. 1991;11(2):115–24.
25. El-Hodhod MA, Tomoum HY, Abd AA, Samaan SM, Samaan SM. Serum Fas and Bcl-2 in patients with epilepsy. *Acta Neurol Scand*. 2006;113:315–21.
26. Paxinos G, Watson CH. The rat brain in stereotaxic coordinates. New York: Academic Press; 1982.
27. Racine R. Modification of seizure activity by electric stimulation II: motor seizure. *Electroencephalogr Clin Neurophysiol*. 1972;32:218–27.
28. Serapide MF, Panto MR, Parenti R, Zappalá A, Cicirata F. Multiple zonal projections of the basilar pontine nuclei to the cerebellar cortex of the rat. *J Comp Neurol*. 2001;430:471–84.
29. Cicirata F, Zappala A, Serapide MF, Parenti R, Panto MR, et al. Different pontine projections to the two sides of the cerebellum. *Brain Res Rev*. 2005;49:280–94.
30. Henshall DC, Simon RP. Epilepsy and apoptosis pathways. *J Cereb Blood Flow Metab*. 2005;25(12):1557–72.
31. Fujikawa DG. Prolonged seizures and cellular injury: understanding the connection. *Epilepsy Behav*. 2005;7:S3–11.
32. Trump BF, Berezsky IK. Calcium-mediated cell injury and cell death. *FASEB J*. 1995;9(2):219–28.
33. Meldrum BS, Bruton CJ. Epilepsy. In: Greenfield's neuropathology, Eds. Adams JH, Duchon LW. New York: Oxford University Press 1992:1246–1283.
34. Ashkenazi A, Dixit V. Apoptosis control by death and decoy receptors. *Curr Opin Cell Biol*. 1999;11:255–60.
35. Ananias M, D'Souza-Li L. Apoptosis through death receptors in temporal lobe epilepsy-associated hippocampal sclerosis. *Mediat Inflamm*. 2016:1–12.
36. Strasser A, O'Connor L, Dixit VM. Apoptosis signaling. *Annu Rev Biochem*. 2000;69:217–45.
37. Micheau O, Tschopp J. Induction of TNF receptor mediated apoptosis via two sequential signaling complexes. *Cell*. 2003;114:181–90.
38. Yamamoto A, Schindler CK, Murphy BM, Bellver-Estelles C, So NK, et al. Evidence of tumor necrosis factor receptor 1 signaling in human temporal lobe epilepsy. *Exp Neurol*. 2006;202(2):410–20.
39. Henshall DC, Araki T, Schindler CK, Shinoda S, Lan J-Q, Simon RP. Expression of death-associated protein kinase and recruitment to the tumor necrosis factor signaling pathway following brief seizures. *J Neurochem*. 2003;86:1260–70.
40. Shinoda S, Skradski SL, Araki T, Schindler CK, Meller R, Lan JQ, et al. Formation of a tumor necrosis factor receptor 1 molecular scaffolding complex and activation of apoptosis signal-regulating kinase 1 during seizure-induced neuronal death. *Eur J Neurosci*. 2003;17:2065–76.
41. Henshall DC, Bonislawski DP, Skradski SL, Araki T, Lan JQ, Schindler CK, et al. Formation of the Apaf-1/cytochrome c complex precedes activation of caspase-9 during seizure-induced neuronal death. *Cell Death Differ*. 2001b;8:1169–81.
42. Henshall DC, Bonislawski DP, Skradski SL, Meller R, Lan J-Q, et al. Cleavage of Bid may amplify caspase-8-induced neuronal death following focally evoked limbic seizures. *Neurobiol Dis*. 2001a;8:568–5680.
43. Engel T, Caballero-Caballero A, Schindler CK, Plesnila N, Strasser A, Prehn JHM, et al. BH3-only protein bid is dispensable for seizure-induced neuronal death and the associated nuclear accumulation of apoptosis-inducing factor. *J Neurochem*. 2010;115(1):92–101.
44. Murphy B, Dunleavy M, Shinoda S, Schindler C, Meller R, Bellver-Estelles C, et al. Bcl-w protects hippocampus during experimental status epilepticus. *Am J Pathol*. 2007;171(4):1258–68.
45. Schindler CK, Shinoda S, Simon RP, Henshall DC. Subcellular distribution of Bcl-2 family proteins and 14-3-3 within the rat hippocampus during seizure induced neuronal death in the rat. *Neurosci Lett*. 2004;356:163–6.
46. Meldrum BS. Excitatory amino acids in epilepsy and potential novel therapies. *Epilepsy Res*. 1992;12(2):189–96.
47. Wang HG, Pathan N, Ethell IM, Krajewski S, Shibasaki F, et al. Ca<sup>2+</sup>-induced apoptosis through calcineurin dephosphorylation of BAD. *Science*. 1999;284(5412):339–43.
48. Datta SR, Ranger AM, Lin MZ, Sturgill JF, Ma YC, Cowan CW, et al. Survival factor-mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis. *Dev Cell*. 2002;3(5):631–43.
49. Kelekar A, Chang BS, Harlan JE, Fesik SW, Thompson CB. Bad is a BH3 domain-containing protein that forms an inactivating dimer with Bcl-XL. *Mol Cell Biol*. 1997;17(12):7040–6.
50. Meller R, Schindler CK, Chu XP, Xiong ZG, Cameron JA, Simon RP, et al. Seizure-like activity leads to the release of BAD from 14-3-3 protein and cell death in hippocampal neurons in vitro. *Cell Death Differ*. 2003;10(5):539–47.
51. Henshall DC, Araki T, Schindler CK, Lan JQ, Tiekoter KL, et al. Activation of Bcl-2-associated death protein and counter-response of Akt within cell populations during seizure-induced neuronal death. *Mol Cell Biol J Neurosci*. 2002;22(19):8458–65.
52. Gillardon F, Wickert H, Zimmermann M. Up-regulation of bax and down-regulation of bcl-2 is associated with kainate-induced apoptosis in mouse brain. *Neurosci Lett*. 1995;192(2):85–8.
53. Lopez E, Pozas E, Rivera R, Ferrer I. Bcl-2 and Bax expression following methylazoxymethanol acetate-induced apoptosis in the external granule cell layer of the developing rat cerebellum. *Dev Brain Res*. 1999;112(1):149–53.
54. Akcali K, Sahiner M, Sahiner T. The role of Bcl-2 family of genes during kindling. *Epilepsia*. 2005;46(2):217–23.
55. Lawrence MS, Ho DY, Sun GH, Steinberg GK, Sapolsky RM. Overexpression of Bcl-2 with herpes simplex virus vectors protects CNS neurons against neurological insults in vitro and in vivo. *J Neurosci*. 1996;16(2):486–96.
56. Ju KL, Manley NC, Sapolsky RM. Anti-apoptotic therapy with a Tat fusion protein protects against excitotoxic insults in vitro and in vivo. *Exp Neurol*. 2008;210(2):602–7.
57. Engel T, Henshall DC. Apoptosis, Bcl-2 family proteins and caspases: the ABCs of seizure-damage and epileptogenesis? *Int J Physiol Pathophysiol Pharmacol*. 2009;1(2):97–115.
58. Mitchell KO, Ricci MS, Miyashita T, Dicker DT, Jin Z, Reed JC, et al. Bax is a transcriptional target and mediator of c-myc-induced apoptosis. *Cancer Res*. 2000;60(22):6318–25.
59. Egle A, Harris AW, Bouillet P, Cory S. Bim is a suppressor of Myc-induced mouse B cell leukemia. *Proc Natl Acad Sci U S A*. 2004;101(16):6164–9.
60. Nikiforov MA, Riblett M, Tang WH, Gratchouk V, Zhuang D, Fernandez Y, et al. Tumor cell-selective regulation of NOXA by c-MYC in response to proteasome inhibition. *Proc Natl Acad Sci U S A*. 2007;104(49):19488–93.
61. Jeffers JR, Parganas E, Lee Y, Yang C, Wang JL, Brennan J, et al. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell*. 2003;4(4):321–8.

62. Amanullah A, Liebermann D, Hoffman B. Deregulated c-Myc prematurely recruits both type I and II CD95/Fas apoptotic pathways associated with terminal myeloid differentiation. *Oncogene*. 2002;21(10):1600–10.
63. Brunner T, Kasibhatla S, Pinkoski M, Fruttschi C, Yoo NJ, et al. Expression of Fas ligand in activated T cells is regulated by c-Myc. *J Biol Chem*. 2000;275(13):9767–72.
64. Kasibhatla S, Beere HM, Brunner T, Echeverri F, Green DR. A 'non-canonical' DNA-binding element mediates the response of the Fas-ligand promoter to c-Myc. *Curr Biol*. 2000;10(19):1205–8.
65. Ming M, Wang S, Wu W, Senyuk V, Le Beau MM, et al. Activation of Wnt/ $\beta$ -catenin protein signaling induces mitochondria-mediated apoptosis in hematopoietic progenitor cells. *J Biol Chem*. 2012;287(27):22683–90.

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