Dantrolene protects neurons against kainic acid induced apoptosis *in vitro* and *in vivo*

B. O. Popescu ^{a, b, *}, M. Oprica ^c, Maria Sajin ^d, Cristina L. Stanciu ^b, O. Bajenaru ^a, Andreea Predescu ^a, Cristina Vidulescu ^{b, e}, L. M. Popescu ^{b, e}

^a Department of Neurology, University Hospital, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania

^b "Victor Babes" National Institute of Pathology, Bucharest, Romania

^c Neurotec, Section of Experimental Geriatrics, KFC, NOVUM, Karolinska Institutet, Stockholm, Sweden

^d Department of Pathology, University Hospital, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania

^e Division of Cellular and Molecular Medicine, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania

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Abstract

Apoptotic cell death induced by kainic acid (KA) in cultures of rat cerebellar granule cells (CGC) and in different brain regions of Wistar rat pups on postnatal day 21 (P21) was studied. *In vitro*, KA (100-500 μ M) induced a concentration-dependent loss of cell viability in MTT assay and cell death had apoptotic morphology as studied by chromatin staining with propidium iodide (PI). *In vivo*, twenty-four hours after induction of status epilepticus (SE) by an intraperitoneal KA injection (5 mg/kg) we quantified apoptotic cells in hippocampus (CA1 and CA3), parietal cortex and cerebellum using PI staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique. We report that dantrolene, a specific ryanodine receptor antagonist, was able to significantly reduce the apoptotic cell death in CGC cultures and in hippocampal CA1 and parietal cortex regions. Our finding can be valuable for neuroprotective therapy strategies in patients with repeated generalized seizures or status epilepticus.

Keywords: apoptosis • epilepsy • kainic acid • dantrolene • ryanodine receptors • cerebellar granule cells

Introduction

Glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system (CNS)

and it plays crucial roles in normal CNS function (for review, see [1]). Three classes of glutamate ionotropic receptors located on postsynaptic membranes transduce glutamate signals: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, N-methyl-D-aspartate (NMDA) receptors and kainate receptors. Besides its neuro-

* Correspondence to: Bogdan O. POPESCU, MD, PhD
Department of Neurology, University Hospital, "Carol Davila"
University of Medicine and Pharmacy, 169, Splaiul Independentei,
Bucharest 5, Romania, Tel +40744.35.34.33, Fax +4021.312.81.02,
E-mail: Bogdan.Popescu@univermed-cdgm.ro

transmitter role, glutamate in excess leads to sustained high intracellular calcium and neuronal death by excitotoxicity. Whilst many studies focused on NMDA receptors involvement in neuronal death and on NMDA receptors antagonists potential for neuroprotection (for review see [2]), far less assessed the mechanisms of neuronal apoptosis triggered by non-NMDA glutamate receptors. Still, there are studies suggesting that AMPA and kainate receptors might be important for neuronal loss in both acute and chronic neurological diseases. It has been shown that kainic acid (KA) induces apoptosis in cerebellar granule cell (CGC) neurons [3], non-NMDA receptors antagonists are neuroprotective for cerebral ischemia [4] and cholinergic neurons affected in Alzheimer's disease are vulnerable to AMPA/kainate mediated toxicity [5].

KA was extensively used for its epileptogenic properties in a rat model of mesial temporal lobe epilepsy (MTLE, [6]). In animal models for MTLE, significant neurodegeneration occurs in the hippocampus, mainly after status epilepticus [7]. When inducing status epilepticus with KA, it is not clarified to what extent neuronal damage is due to actions of KA *per se* or is secondary to seizure-induced release of excitatory amino acids (EAA). However, it was shown that seizure-induced neuronal injury is developmentally regulated, being dependent on expression and distribution of glutamate receptors at different developmental stages [8–10]. Moreover, it seems that neuronal loss induced by KA is biphasic, dying neurons being found at 24 hours but also weeks after KA treatment [11]. It is also well established that humans experiencing recurrent epileptic seizures show biochemical [12, 13], neuropsychological [14] and neuroimaging [15, 16] evidence of neuronal injury. Hence, beside the use of anticonvulsants, it is important to identify drugs with neuroprotective and antiepileptogenic properties.

Previous reports showed that seizure-induced neuronal loss can be due to both necrosis and apoptosis [17, 18]. However, in KA-triggered seizures in a rat model, several caspases are activated at early time-points within hippocampus [19, 20] and pro-apoptotic protein Bax accumulates at mitochondrial membranes [21]. Apoptosis triggered by KA in cerebellar granule cells *in vitro* associates with elevated intracellular calcium concentration ($[Ca^{2+}]_i$, [22]) and regulation of L-type channel calcium flux protects against KA excitotoxicity [23]. Moreover, inositol 1,4,5-triphosphate

(IP₃) receptors, which are able to release calcium from the endoplasmic reticulum (ER), were highly expressed in rat hippocampal neurons undergoing apoptosis after KA administration [24].

Here we have used both *in vitro* and *in vivo* experimental models to study neuronal death triggered by KA. We addressed the role of calcium release from internal stores during KA-induced cell death by using dantrolene, a specific ryanodine receptor antagonist [25]. Our findings support the role of ER-released calcium in mediating neuronal apoptosis caused by excess EAA.

Materials and methods

Chemicals

Dantrolene sodium, propidium iodide (PI), kainic acid (KA), sodium pentobarbital and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) powder were purchased from Sigma (St. Louis, MO, USA).

Cerebellar granule cell cultures and treatments

Primary CGC cultures were prepared as previously described [26]. Cell culture supplies were purchased from Nunc A/S (Roskilde, Denmark) and foetal calf serum, Dulbecco's modified Eagle's medium (DMEM), L-glutamine, penicillin-streptomycin, phosphate buffered saline (PBS) and trypsin were from Sigma (St. Louis, MO, USA). In brief, cerebella from 5 day-old Wistar rat pups were minced and dissociated with 1500 UI/ml trypsin and DNase I (Boehringer Mannheim, Germany) 0.1 mg/ml and then DMEM was added. Individual cell suspension was obtained by mechanical trituration and cells were cultured in DMEM supplemented with 10% (v/v) heat inactivated calf serum, 1% (v/v) penicillin-streptomycin, 1% (v/v) glutamine, at 37°C in a humidified 5% CO₂ atmosphere. Cytosine-β-arabino-furanoside (10 μM, Sigma) was added to cultures after 24 h to limit glial proliferation. All the experiments were done 5 days after seeding the cultures. Cells were exposed to 100, 200 or 500 μM KA in culturing media for either 3 h or 24 h. KA was prepared as a stock of 500 mM in DMSO. The control cells were treated for the same period with a similar concentration of the

solvent. Dantrolene (10 μ M) was added in the same time with KA, as cotreatment or as sole treatment. We used a single concentration of dantrolene that was found efficient in both signalling [27] and cell death [28] studies.

Assessment of cell death

MTT assay

Cell viability was assessed by MTT assay that detects the cellular capacity to convert MTT tetrazolium salt to formazan [29]. MTT powder was dissolved in minimum essential medium (MEM) without phenol red (Sigma) at 0.3 mg/ml and added to the cells. After 1 h at 37°C, the medium was removed and the formazan crystals were dissolved in isopropanol. Aliquots were transferred to 96-well-plates and the optical density was read at 550 nm in a Bio-Rad PR 1100 Microplate Reader. Results were expressed as a percentage of viable cells compared with control (unexposed cells).

Chromatin staining

Nuclear morphology was assessed by propidium iodide staining as previously described [30]. At the indicated time-points after exposure, medium was removed and cells gently washed with 37°C PBS. Cells were subsequently fixed with 2% paraformaldehyde and 0.2% glutaraldehyde Sorensen solution, washed with PBS and stained with 50 μ g/ml PI in PBS for 10 min in the dark. A glycerol/PBS (1:1) solution was added to wells and cells were visualised in fluorescence microscopy, with a Nikon Eclipse TE 200 microscope. Cell counting was unbiased, and a minimum of 500 cells in at least 5 different fields was counted for each experimental situation.

Animals

Wistar rats pups (P 21) were provided by "Victor Babes" National Institute of Pathology Animal Department. The animal experiments were performed in agreement with the international ethical rules and regulations concerning animal experiments. Rat pups received two intraperitoneal injections at 1 h interval, being divided in 4 groups (each group, N=3): 1) saline + saline, 2) saline + KA, 3) dantrolene + KA, 4) dantrolene + saline. KA was used at a sublethal dose of 5 mg/kg, higher doses being associated with high mortality rates for rat pups of this age. Dantrolene was used at 10 mg/kg, dose that was shown to be protective in other studies, e.g. [31]. Each

injection volume was 0.1 ml. Rats were observed for behavioural evidence of seizures. All rats in groups 2 and 3 developed seizures starting 30 min after KA injection, with lethargy, rigid posture, then falling, tonic-clonic seizures, progressing to status epilepticus (SE). For rats in groups 1 and 4 no behavioural disturbance was noted.

Histology

Brain specimens were processed as previously described in [32]. Briefly, 24 h after the second injection the animals were anaesthetised with sodium pentobarbital (30 mg/kg) and then perfused intracardiac with PBS followed by glutaraldehyde 2.5% in Sørensen solution, brains were removed and fixed in 4% paraformaldehyde at 4°C for 5 days and then specimens were included in paraffin. Brain coronal 10 μ m thick sections were deparaffinized, washed in PBS, and then either stained with PI (50 μ g/ml) for 5 min in dark, or subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) technique, following the manufacturer instructions ("in situ cell death detection kit", Boehringer Mannheim, Germany) as described [33]. Tissue was visualised by fluorescence microscopy (Nikon Eclipse TE 200). Cell counting was unbiased, and cells in at least 5 different fields (20x objective) were counted for each experimental situation.

Statistical analysis

The statistical analysis of cell death assays and quantitative histology was performed by one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test, using Statistica 6 software. The results are expressed by the mean followed by the confidence interval (CI) and the degree of significance.

Results

Kainic acid triggers apoptosis in cerebellar granule cells

CGC cultures were exposed to three different concentrations of KA (100, 200 and 500 μ M) and cell viability was evaluated by MTT assay. At both 3 h

Fig. 1 MTT assay on cell viability. Cerebellar granule cells were exposed to 100, 200 or 500 μ M kainic acid (KA) treatment for 3h (**a**) or 24 h (**b**), in the absence or presence of 10 μ M dantrolene (Dan). CGC control cells were treated with solvent (DMSO) or with 10 μ M dantrolene. Bars indicate confidence intervals and stars mark the statistical significance ($n=3$, ‘*’ = $p<0.05$, ‘**’ = $p<0.01$, ‘***’ = $p<0.001$, ANOVA with Bonferroni post hoc test).

Fig. 2 Microscopy on cerebellar granule cells (CGC). **a**) Phase-contrast image of control (untreated) CGC culture. **b**) Fluorescence microscopy image of control CGC cells stained with propidium iodide (PI). **c**) Fluorescence microscopy image of PI-stained CGC cells treated with 200 μ M kainic acid (KA) for 24 h. Note condensed, bright nuclei, characteristic for apoptotic cells. **d**) Fluorescence microscopy image of PI-stained CGC cells treated with 200 μ M KA and 10 μ M dantrolene. Arrows indicate normal cells and arrowheads indicate apoptotic cells. Bar = 41.8 μ m (Ob 40 x).

Fig. 3 Quantification of condensed (apoptotic) nuclei in cerebellar granule cells (CGC) stained with propidium iodide. CGC were exposed to 100, 200 or 500 μ M kainic acid (KA) treatment for 3h (a) or 24 h (b), in the absence or presence of 10 μ M dantrolene (Dan). CGC control cells were treated with solvent (DMSO) or with 10 μ M dantrolene. Bars indicate confidence intervals and stars mark the statistical significance ($n=3$, $*$ = $p<0.05$, $**$ = $p<0.01$, $***$ = $p<0.001$, ANOVA with Bonferroni post hoc test).

and 24 h, MTT assay showed that KA induced a significant dose and time dependent loss of cell viability (Fig. 1). Considering that MTT assay estimates only the mitochondrial viability and is not able to detect the form of cell death, we performed chromatin staining with PI in control cells and cells treated with different concentrations of KA. The KA treated cells displayed characteristic apoptotic nuclear morphology (Fig. 2b, c) in a dose-dependent proportion (Fig. 3).

Dantrolene protects cerebellar granule cells against KA-triggered apoptosis

In order to verify whether Ca^{2+} released from ER is important in KA-triggered apoptosis, CGC cultures were treated with both KA (100, 200, 500 μ M) and dantrolene (10 μ M) and MTT assay and PI staining were performed. At 3 h time point, the cell viability measured by MTT assay was significantly increased in cells treated with 200 μ M KA + dantrolene (70.06%; 0.95 CI = 64.88-75.23%) as compared to cells treated with 200 μ M KA (58.27%; 0.95 CI = 51.70-64.83%; $p < 0.05$), and in cells treated with 500 μ M KA + dantrolene (54.40%; 0.95CI = 39.76-69.03%) as compared to cells treated with 500 μ M KA (40.68%; 0.95CI = 37.49-43.86%; $p < 0.01$), as shown in Fig. 1a. At 24 h time point, dantrolene significantly increased cell viability in cells treated with 200 μ M KA (60.53%; 0.95CI = 46.42-67.63%

as compared to 43.95%; 0.95CI = 37.01-50.88%; $p < 0.001$), as shown in Fig. 1b.

Further on, we investigated whether the rescuing effects of dantrolene were due to inhibiting apoptosis. As shown in Fig. 3a, dantrolene was able to significantly reduce the percentage of cells with condensed nuclei at 3 h time point from 21.63% (0.95CI = 15.47-27.80%) to 14.78% (0.95CI = 9.24-20.32%; $p < 0.05$) in 200 μ M KA treated cells and from 30.21% (0.95CI = 23.23-37.18%) to 22.49% (0.95CI = 16.69-28.29%; $p < 0.01$) in 500 μ M KA treated cells, respectively. At 24 h time point, dantrolene significantly reduced the percentage of cells with condensed nuclei from 33.87% (0.95CI = 20.49-47.25%) to 21.25% (0.95CI = 13.78-28.72%; $p < 0.01$) in 200 μ M KA treated cells and from 38.91% (0.95CI = 28.27-49.54%) to 28.86% (0.95CI = 23.19-34.53%; $p < 0.05$) in 500 μ M KA treated cells, respectively (Fig. 3b). No significant effects were seen for 100 μ M KA concentration.

We also noticed a significant decrease in MTT values at 24 h time point in cells treated with dantrolene alone as compared with control cells (Fig. 1b). This finding did not correlate with the percentage of apoptotic cells estimated by PI staining (Fig. 3b). Hence, this effect is probably due to metabolic causes, such as interference with succinate dehydrogenases, rather than to toxicity. Obviously, this effect could not be responsible for the rescuing effect of dantrolene, since it is decreasing the apparent cell viability.

Kainic acid generates DNA damage characteristic for apoptosis in various brain regions

Considering our findings in the cell culture experimental paradigm we wanted to further characterise cell death in various regions of rat brain after KA-induced status epilepticus (SE). We chose a morphological method (PI staining) and a biochemical method (TUNEL technique) to estimate the proportion of cells displaying nuclear hallmarks of apoptosis at 24 h after KA treatment (5 mg/kg). We

counted condensed nuclei and TUNEL-positive cells in hippocampus (CA1 and CA3 regions), parietal cortex and cerebellum. As shown in Fig. 4-6, we found both cells with condensed chromatin and TUNEL-positive cells in all explored brain regions. The highest proportion of apoptotic cells was found in the hippocampus (CA3) and the lowest in the cerebellum, by both PI-staining (Fig. 7a) and TUNEL technique (Fig. 7b). Animals treated with dantrolene alone didn't show any difference in condensed nuclei or TUNEL positive cells as compared to control animals (data not shown).

Fig. 4 Histology of CA1 hippocampal subregion in rat pups (P21). **a)** Hematoxylin-eosin staining of CA1 subregion in control brain. **b)** Fluorescence microscopy image of CA1 subregion in control brain stained with propidium iodide (PI). **c)** Fluorescence microscopy image of PI-stained CA1 subregion in rat pup treated with 5 mg/kg kainic acid (KA), 24 h after treatment. Note condensed nuclei in apoptotic cells. **d)** Fluorescence microscopy image of PI-stained CA1 subregion in rat pup treated with 5 mg/kg KA and 10 mg/kg dantrolene, 24 h after treatment. **e)** Fluorescence microscopy image of CA1 subregion in control brain stained with TUNEL method. **f)** Fluorescence microscopy image of TUNEL-stained CA1 subregion in rat pup treated with 5 mg/kg kainic acid (KA), 24 h after treatment. Note intense stained, bright green, positive TUNEL cells, undergoing apoptosis. **g)** Fluorescence microscopy image of TUNEL-stained CA1 subregion in rat pup treated with 5 mg/kg KA and 10 mg/kg dantrolene, 24 h after treatment. Arrows indicate normal cells and arrowheads indicate apoptotic cells. Bar = 41.8 μ m (Ob 40 x).

Dantrolene protects hippocampus and cerebral cortex against kainic acid induced apoptosis

We wanted to further confirm our findings in CGC paradigm by testing the effect of dantrolene *in vivo*, on rat pups subjected to KA-triggered SE. Considering that KA effects are seen in a short time after treatment, we administered dantrolene (10 mg/kg) 1 h prior to KA treatment, to enable an effective blockade of ryanodine receptors. The ani-

mals that received dantrolene and KA had a similar pattern of seizures as compared to animals treated with KA alone, and they also reached SE. At 24 h time point, animals treated with both dantrolene and KA showed significantly fewer cells with condensed nuclei as compared to animals treated with KA alone in two of the examined areas: CA1 region of the hippocampus (9.65%; 0.95CI = 6.88-12.42 as compared to 14.98%; 0.95CI = 12.36-17.60, respectively, $p < 0.01$) and in the parietal cortex (10.85%;

0.95CI = 4.83-16.88 as compared to 20.35%; 0.95CI = 15.85-24.85, respectively, $p < 0.001$), as shown in Fig. 4c, d, 5c, d and 7a. A similar result was obtained using the TUNEL technique, as shown in Fig. 4f, g, 5f, g and 7b. Animals treated with both dantrolene and KA had 1.72 (0.95CI = 1.29-2.14) TUNEL positive cells/1000 μm^2 in CA1 region, as compared to 2.43 (0.95CI = 1.79-3.08) TUNEL positive cells/1000 μm^2 in animals treated only with

KA ($p < 0.05$). Animals treated with both dantrolene and KA had 1.41 (0.95CI = 0.99-1.84) TUNEL positive cells/1000 μm^2 in the parietal cortex, as compared to 2.04 (0.95CI = 1.54-2.55) TUNEL positive cells/1000 μm^2 in animals treated only with KA ($p < 0.05$). No significant dantrolene protection occurred in the CA3 hippocampal region and in the cerebellum as assessed with either PI staining (Fig. 7a) or TUNEL technique (Fig. 7b).

Fig. 5 Histology of parietal cortex in rat pups (P21). **a)** Hematoxylin-eosin staining of parietal cortex in control brain. **b)** Fluorescence microscopy image of parietal cortex in control brain stained with propidium iodide (PI). **c)** Fluorescence microscopy image of PI-stained parietal cortex in rat pup treated with 5 mg/kg kainic acid (KA), 24 h after treatment. Note condensed nuclei in apoptotic cells. **d)** Fluorescence microscopy image of PI-stained parietal cortex in rat pup treated with 5 mg/kg KA and 10 mg/kg dantrolene, 24 h after treatment. **e)** Fluorescence microscopy image of parietal cortex in control brain stained with TUNEL method. **f)** Fluorescence microscopy image of TUNEL-stained parietal cortex in rat pup treated with 5 mg/kg kainic acid (KA), 24 h after treatment. Note intense stained, bright green, positive TUNEL cells, undergoing apoptosis. **g)** Fluorescence microscopy image of TUNEL-stained parietal cortex in rat pup treated with 5 mg/kg KA and 10 mg/kg dantrolene, 24 h after treatment. Arrows indicate normal cells and arrowheads indicate apoptotic cells. Bar = 41.8 μ m (Ob 40 x).

Discussion

It is well established that activation of ionotropic glutamate receptors is involved in excitotoxicity, which is responsible for neuronal loss in several acute and chronic neurologic disorders [34]. Cells treated with glutamate can undergo either necrosis or apoptosis, and this option is dependent on glutamate concentration and is settled by mitochondrial function [35].

Several mechanisms might be involved in KA-induced neurodegeneration [36, 37]. First, KA activates excitatory pathways, leading to release of important amounts of EAA, which can cause cell death through excitotoxic mechanisms. Second, KA triggers epileptic seizures that associate brain oedema and hypoxia, known to be responsible for neuronal injury. The third possibility is that KA causes apoptosis directly in cells where it binds kainate

receptors, through a mechanism similar to the one encountered in neuronal cell cultures.

It has been shown that KA can increase $[Ca^{2+}]_i$ in CGC [22]. Non-NMDA glutamate receptors are generally not permeable for Ca^{2+} ions. Still, their activation can lead to increased $[Ca^{2+}]_i$ by secondary activation of membrane voltage sensitive calcium channels. High $[Ca^{2+}]_i$ can trigger enzymes responsible for cell death, like phospholipases, nitric oxide synthase or calpains [38], and can determine the

mitochondrial permeability transition pore formation with release of molecules responsible for caspase activation, like cytochrome c or APAF-1 [39].

A recent report showed that KA-induced cell death in CGC is not blocked by caspase inhibition, even though cell death have apoptotic features [40]. However, activation of caspases is well documented in rat brain in KA-induced SE model [19, 20]. Interestingly, transgenic mice with increased superoxide dismutase activity are more

Fig. 6 Histology of cerebellum in rat pups (P21). **a)** Hematoxylin-eosin staining of cerebellum in control brain. **b)** Fluorescence microscopy image of cerebellum in control brain stained with propidium iodide (PI). **c)** Fluorescence microscopy image of PI-stained cerebellum in rat pup treated with 5 mg/kg kainic acid (KA), 24 h after treatment. Note few condensed nuclei in apoptotic cells. **d)** Fluorescence microscopy image of PI-stained cerebellum in rat pup treated with 5 mg/kg KA and 10 mg/kg dantrolene, 24 h after treatment. **e)** Fluorescence microscopy image of cerebellum in control brain stained with TUNEL method. **f)** Fluorescence microscopy image of TUNEL-stained cerebellum in rat pup treated with 5 mg/kg kainic acid (KA), 24 h after treatment. Note few intense stained, bright green, positive TUNEL cells, undergoing apoptosis. **g)** Fluorescence microscopy image of TUNEL-stained cerebellum in rat pup treated with 5 mg/kg KA and 10 mg/kg dantrolene, 24 h after treatment. Arrows indicate normal cells and arrowheads indicate apoptotic cells. Bar = 41.8 μ m (Ob 40 x).

resistant to kainate-induced hippocampal DNA damage [41].

Considering that $[Ca^{2+}]_i$ rises early in CGC treated with KA [22], we investigated whether calcium-induced calcium release (CICR) phenomenon, which implies mobilisation of Ca^{2+} from ER stores, plays a role in KA-triggered apoptosis. We used dantrolene, a specific ryanodine receptor antagonist, to inhibit the release of Ca^{2+} from the ER through stimulation of ryanodine

receptors. We noticed that upon treatment with high concentrations of KA (200 and 500 μ M), CGC viability decreases at an early time point (3 h). This early loss in cell viability was accompanied by increased nuclear condensation, a hallmark of nuclear apoptosis. Dantrolene was able to partially reverse both the decrease in cell viability and the nuclear condensation, probably by sequestering Ca^{2+} within ER and preventing further increase in $[Ca^{2+}]_i$. However, it was shown that

Fig. 7 Quantification of condensed (apoptotic) nuclei stained with propidium iodide (a) and of TUNEL-positive cells (b) in different brain regions in rat pups (P21) treated with saline (control), 5 mg/kg kainic acid (KA), or 5 mg/kg KA and 10 mg/kg dantrolene (Dan), 24 h after treatment. (N=3, ‘*’ = $p < 0.05$, ‘**’ = $p < 0.01$, ‘***’ = $p < 0.001$, ANOVA with Bonferroni post hoc test). CA1 = CA1 hippocampal subregion, CA3 = CA3 hippocampal subregion, Cortex = parietal cortex, Cerebel = cerebellum.

KA can activate the expression of IP₃ receptors in hippocampal cells undergoing apoptosis [24]. Hence, it is probable that in our model there is still a Ca²⁺ release from the ER, through IP₃ receptors. Further studies can elucidate this by the use of both IP₃ and ryanodine receptors antagonists.

At 24 h time point, dantrolene was able to improve cell viability only in cells treated with 200 μM KA, but not with 500 μM KA. For this late time point the PI staining revealed that dantrolene protected against nuclear condensation in both 200 and 500 μM KA treated cells. This difference can be due to the fact that cells committed to undergo apoptosis can instead end up by necrosis under various conditions, like caspase inhibition or ATP depletion [42]. Interestingly, it was shown that in KA-induced apoptosis in CGC neither caspases nor calpains play an essential role [40]. This is in agreement with the concept that there is caspase-independent cell death bearing apoptotic morphology [43]. Moreover, in neuronal-like cells treated with A 23187 calcium ionophore apoptotic morphology is induced with minimal activation of caspase-3 [44]. However, dantrolene significantly reduced both loss of cell viability and nuclear condensation in cells treated with KA, which suggest that the increase in [Ca²⁺]_i is a primary event in KA-induced cell death and control of ER Ca²⁺ stores can modulate KA-induced neuronal injury.

KA is commonly used in a rat model of MTLE [6] and triggers hippocampal neurodegeneration in adult rats, generating both necrosis and apoptosis [17]. SE induced by KA or other epileptogenic agents is also associated with cell loss in the hippocampus [7]. Neonatal rats seem to show resistance to glutamate toxicity [8] but there is a general agreement that SE triggered by KA [11] or pilocarpine [10] induce important acute neurodegeneration with apoptotic hallmarks, at least starting with the third week of life. It was also shown that SE experienced early in life has long term consequences, like delayed neuronal loss [11] and subsequent spontaneous epileptic seizures [10].

Considering all these data we chose to study P21 rat pups and investigate if dantrolene has any protective effect *in vivo* against KA induced neuronal injury. We observed that cells identified as apoptotic by PI staining and TUNEL method are not restricted to hippocampus, being also present in the cerebral cortex and cerebellum. The widespread distribution of brain lesions following systemic KA administration in rats was reported before [45]. Interestingly, certain brain regions were more inclined to be protected by dantrolene against KA-induced injury. In our study dantrolene protected the hippocampal CA1 region and the parietal cortex, but not the hippocampal CA3 region and the cerebel-

lum. Based on our current study we cannot conclude which are the mechanisms of this selectivity but it can be speculated that different ryanodine receptor distribution and density within different brain regions could be responsible. It is also possible that different brain cell populations possess distinct ways in regulating Ca^{2+} or distinct $[\text{Ca}^{2+}]_i$ thresholds for activating cell death pathways.

Our results are in agreement with a previous study [46], which also showed a protective effect of dantrolene in rats treated with KA. This previous report was based on cell counting and no apoptotic markers were used. Moreover, the observations were limited to the hippocampus, and no *in vitro* study was performed. To our knowledge, we report for the first time that dantrolene significantly decrease the KA induced apoptosis *in vitro* and *in vivo*. Dantrolene has the great advantage that has passed clinical trials, being already in clinical use for treatment of malignant hyperthermia, where it decreases body temperature. However, dantrolene ability to counteract hyperthermia would not affect its neuroprotective potential, being known that hypothermia is neuroprotective in several acute neurological diseases [47-50]. It was also reported that hypothermia protects from neuronal cell death induced by KA [51, 52]. Intracerebral [53] or intraperitoneal [54, 55] administration of KA in rats produced a biphasic effect on core temperature, an initial hypothermia followed by a longer hyperthermic effect. Since it has been shown that dantrolene can decrease rectal temperature in different experimental paradigms [56, 57] we cannot exclude the modulation of core and/or brain temperature as a potential mechanism that can account for at least some part of the neuroprotective effect of dantrolene against KA-induced neuronal death observed in our study.

In conclusion, our findings could be valuable for the development of neuroprotective and antiepileptogenic treatment strategies for patients with recurrent generalised seizures or SE.

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References

1. **Lipton S.A., Rosenberg P.A.**, Excitatory amino acids as a final common pathway for neurologic disorders, *N. Engl. J. Med.*, **330**: 613-622, 1994
2. **Kemp J.A., McKernan R.M.**, NMDA receptor pathways as drug targets, *Nat. Neurosci.*, **S**: 1039-1042, 2002
3. **Simonian N.A., Getz R.L., Leveque J.C., Konradi C., Coyle J.T.**, Kainic acid induces apoptosis in neurons, *Neuroscience*, **75**: 1047-1055, 1996
4. **Sheardown M., Nielsen E., Hansen A., Jacobsen P., Honore T.**, Dihydro-6-nitro-sulfamoyl-benzo(F)quinoline: a neuroprotectant for cerebral ischemia, *Science*, **247**: 571-573, 1990
5. **Weiss J., Yin H., Choi D.**, Basal forebrain cholinergic neurons are selectively vulnerable to AMPA/kainate receptor-mediated toxicity, *Neuroscience*, **60**: 659-664, 1994
6. **White H.S.**, Animal models of epileptogenesis, *Neurology*, **59**: S7-S14, 2002
7. **Holmes G.L.**, Seizure-induced neuronal injury, *Neurology*, **59**: S3-S6, 2002
8. **Marks J.D., Friedman J.E., Haddad G.G.**, Vulnerability of CA1 neurons to glutamate is developmentally regulated, *Dev. Brain Res.*, **97**: 194-206, 1996
9. **Liu Z., Stafstrom C.E., Sarkisian M., Tandon P., Yang Y., Hori A., Holmes G.L.**, Age-dependent effects of glutamate toxicity in the hippocampus, *Dev. Brain Res.*, **97**: 178-184, 1996
10. **Sankar R., Shin D.H., Liu H., Mazarati A., Pereira de Vasconcelos A., Wasterlain C.G.**, Patterns of status epilepticus-induced neuronal injury during development and long-term consequences, *J. Neurosci.*, **18**: 8382-8393, 1998
11. **Humphrey W.M., Dong H.X., Csernansky C.A., Csernansky J.G.**, Immediate and delayed hippocampal neuronal loss induced by kainic acid during early postnatal development in the rat, *Dev. Brain Res.*, **137**: 1-12, 2002
12. **Henshall D.C., Clark R.S., Adelson P.D., Chen M., Watkins S.C., Simon R.P.**, Alterations in bcl-2 and caspase gene family protein expression in human temporal lobe epilepsy, *Neurology*, **55**: 250-257, 2000
13. **Rabinowicz A.L., Correale B., Boutros R.B., Couldwell W.T., Henderson C.W., DeGiorgio C.M.**, Neuron-specific enolase is increased after single seizures during inpatient video/EEG monitoring, *Epilepsia*, **37**: 122-125, 1996

14. **Dodrill C.B.**, Progressive cognitive decline in adolescents and adults with epilepsy, *Prog. Brain Res.*, **135**: 397-408, 2002
15. **Kalviainen R., Salmenpera T., Partanen K., Vainio P., Riekkinen P., Pitkanen A.**, Recurrent seizures may cause hippocampal damage in temporal lobe epilepsy, *Neurology*, **50**: 1377-1382, 1998
16. **Tasch E., Cendes F., Li L.M., Dubeau F., Andermann F., Arnold D.L.**, Neuroimaging evidence of progressive neuronal loss and dysfunction in temporal lobe epilepsy, *Ann. Neurol.*, **45**: 568-576, 1999
17. **Sloviter R.S., Dean E., Sollas A.L., Goodman J.H.**, Apoptosis and necrosis induced in different hippocampal neuronal populations by repetitive perforant path stimulation in the rat, *J. Comp. Neurol.*, **366**: 516-533, 1996
18. **Bengzon J., Kokaia Z., Elmer E., Nanobashvili A., Kokaia M., Lindvall O.**, Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures, *Proc. Natl. Acad. Sci. USA*, **94**: 10432-10437, 1997
19. **Henshall D.C., Chen I., Simon R.P.**, Involvement of caspase-3 like protease in the mechanism of cell death following focally evoked limbic seizures, *J. Neurochem*, **74**: 1215-1223, 2000
20. **Henshall D.C., Bonislawski D.P., Skradski S.L., Araki T., Lan J.Q., Schindler C.K., Meller R., Simon R.P.**, Formation of Apaf-1/cytochrome c complex precedes activation of caspase-9 during seizure-induced neuronal death, *Cell Death Differ*, **8**: 1169-1181, 2001
21. **Henshall D.C., Araki T., Schindler C.K., Lan J.Q., Tiekoter K.L., Taki W., Simon R.P.**, Activation of Bcl-2-associated death protein and counter-response of Akt within cell populations during seizure-induced neuronal death, *J. Neurosci.*, **22**: 8458-8465, 2002
22. **Leski M.L., Valentine S.L., Coyle T.**, L-type voltage-gated calcium channels modulate kainic acid neurotoxicity in cerebellar granule cells, *Brain Res.*, **828**: 27-40, 1999
23. **Brines M.L., Ling Z., Broadus A.E.**, Parathyroid hormone-related protein protects against kainic acid excitotoxicity in rat cerebellar granule cells by regulating L-type channel calcium flux, *Neurosci. Lett.*, **274**: 13-16, 1999
24. **Blackshaw S., Sawa A., Sharp A.H., Ross C.A., Snyder S.H., Khan A.A.**, Type 3 inositol 1,4,5-triphosphate receptor modulates cell death, *FASEB J.*, **14**: 1375-1379, 2000
25. **Zhao F., Li P., Chen S.R.W., Louis C.F., Fruen B.R.**, Dantrolene inhibition of ryanodine receptor Ca^{2+} release channels, *J. Biol. Chem.*, **276**: 13810-13816, 2001
26. **Popescu A.T., Vidulescu C., Stanciu C.L., Popescu B.O., Popescu L.M.**, Selective protection by phosphatidic acid against staurosporine-induced apoptosis, *J. Cell. Mol. Med.*, **6**: 433-438, 2002
27. **Cedazo-Minguez A., Popescu B.O., Ankarcrona M., Nishimura T., Cowburn R.F.**, The presenilin 1 $\Delta E9$ mutation gives enhanced basal phospholipase C activity and a resultant increase in intracellular calcium concentrations, *J. Biol. Chem.*, **277**: 36646-36655, 2002
28. **Mattson M.P., Zhu H., Yu J., Kindy M.S.**, Presenilin-1 mutation increases neuronal vulnerability to focal ischemia and to hypoxia and glucose deprivation in cell culture: involvement of perturbed calcium homeostasis, *J. Neurosci.*, **20**: 1358-1364, 2000
29. **Mosmann T.**, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J. Immunol. Methods*, **65**: 55-63, 1983
30. **Popescu B.O., Popescu L.M.**, Neuronal apoptosis triggered by anti-Fas (CD95, APO-1) antibody is enhanced by dexamethasone, *J. Med. Biochem.*, **4**: 1-14, 2000
31. **Schneider I., Reverse D., Dewachter I., Ris L., Caluwaerts N., Kuiperi C., Gilis M., Geerts H., Kretzschmar H., Godaux E., Moechars D., Van Leuven F., Herms J.**, Mutant presenilins disturb neuronal calcium homeostasis in the brain of transgenic mice, decreasing the threshold for excitotoxicity and facilitating long-term potentiation, *J. Biol. Chem.*, **276**: 11539-11544, 2001
32. **Zagrean L., Moldovan M., Munteanu A.M., Spulber S., Voiculescu B.A., Popescu B.O.**, Early electrocortical changes consistent with ischemic preconditioning in rat, *J. Cell. Mol. Med.*, **4**: 215-223, 2000
33. **Nat R., Voiculescu B., Stanciu C., Vidulescu C., Cergan R., Badiu C., Popescu L.M.**, Apoptosis in human embryo development: 2. Cerebellum, *J. Cell. Mol. Med.*, **5**: 179-187, 2001
34. **Beal M.F.**, Mechanisms of excitotoxicity in neurological diseases, *FASEB J.*, **6**: 3338-3344, 1992
35. **Ankarcrona M., Dybukt J.M., Bonfoco E., Zhivotovsky B., Orrenius S., Lipton S.A., Nicotera P.**, Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function, *Neuron*, **15**: 961-973, 1995

36. **Malva J.O., Carvalho A.P., Carvalho C.M.,** Kainate receptors in hippocampal CA3 subregion: evidence for a role in regulating neurotransmitter release, *Neurochem. Int.*, **32**: 1-6, 1998
37. **Zagulska-Szymczak S., Filipkowski R.K., Kaczmarek L.,** Kainate-induced genes in the hippocampus: lessons from expression patterns, *Neurochem. Int.*, **38**: 485-501, 2001
38. **Nicotera P., Bellomo G., Orrenius S.,** The role of Ca^{2+} in cell killing, *Chem. Res. Toxicol.*, **3**: 484-494, 1990
39. **Friberg H., Wieloch T.,** Mitochondrial permeability transition in acute neurodegeneration, *Biochimie*, **84**: 241-250, 2002
40. **Verdaguer E., Garcia-Jorda E., Jimenez A., Stranges A., Sureda F.X., Canudas A.M., Escubedo E., Camarasa J., Pallas M., Camins A.,** Kainic acid-induced neuronal cell death in cerebellar granule cells is not prevented by caspase inhibitors, *Br. J. Pharmacol.*, **135**: 1297-1307, 2002
41. **Hirata H., Cadet J.L.,** Kainate-induced hippocampal DNA damage is attenuated in superoxide dismutase transgenic mice, *Mol. Brain Res.*, **48**: 145-148, 1997
42. **Nicotera P., Leist M., Fava E., Berliocchi L., Volbracht C.,** Energy requirement for caspase activation and neuronal cell death, *Brain Pathol.*, **10**: 276-282, 2000
43. **Nicotera P.,** Apoptosis and age-related disorders: role of caspase-dependent and caspase-independent pathways, *Toxicol. Lett.*, **127**: 189-195, 2002
44. **Popescu B.O., Cedazo-Minguez A., Popescu L.M., Winblad B., Cowburn R.F., Ankarcrona M.,** Caspase cleavage of exon 9 deleted presenilin-1 is an early event in apoptosis induced by calcium ionophore A 23187 in SH-S5Y neuroblastoma cells, *J. Neurosci. Res.*, **66**: 122-134, 2001
45. **Schwob J.E., Fuller T., Price J.L., Olney J.W.,** Widespread patterns of neuronal damage following systemic or intracerebral injections of kainic acid: a histological study, *Neuroscience*, **5**: 991-1014, 1980
46. **Berg M., Bruhn T., Fransen A., Schousboue A., Diemer N.H.,** Kainic acid-induced seizures and brain damage in the rat: role of calcium homeostasis, *J. Neurosci. Res.*, **40**: 641-646, 1995
47. **Clausen T., Bullock R.,** Medical treatment and neuroprotection in traumatic brain injury, *Curr. Pharm. Des.*, **7**: 1517-1532, 2001
48. **Rees K., Beranek-Stanley M., Burke M., Ebrahim S.,** Hypothermia to reduce neurological damage following coronary artery bypass surgery, *Cochrane Database Syst. Rev.*, **1**: CD002138, 2001
49. **Gunn A.J.,** Cerebral hypothermia for prevention of brain injury following perinatal asphyxia, *Curr. Opin. Pediatr.*, **12**: 111-115, 2000
50. **Thoresen M.,** Cooling the asphyxiated brain – ready for clinical trials?, *Eur. J. Pediatr.*, **158**: S5-S8, 1999
51. **Liu Z., Gatt A., Mikati M., Holmes G. L.,** Effect of temperature on kainic acid-induced seizures, *Brain Res.*, **631**: 51-58, 1993
52. **Maeda T., Hashizume K., Tanaka T.,** Effect of hypothermia on KA-induced limbic seizures: an electroencephalographic and ^{14}C -deoxyglucose autoradiographic study, *Brain Res.*, **818**: 228-235, 1999
53. **Turski L., Kleinrok Z.,** Effects of kainic acid on body temperature of rats: role of catecholaminergic and serotonergic systems, *Psychopharmacol.*, **71**: 35-39, 1980
54. **Oprica M., Forslin Aronsson Å., Post C., Eriksson C., Ahlenius S., Popescu L. M., Schultzberg M.,** Effects of α -MSH on kainic acid induced changes in core temperature in rats, *Peptides*, **23**: 143-149, 2002
55. **Ahlenius S., Oprica M., Eriksson C., Winblad B., Schultzberg M.,** Early hypothermic effect of kainic acid: unmasking by dizocilpine, *Neuropharmacol.*, **43**: 28-35, 2002
56. **Wu C.C., Yen M.H.,** Beneficial effects of dantrolene on lipopolysaccharide-induced haemodynamic alterations in rats and mortality in mice, *Eur. J. Pharmacol.*, **327**: 17-24, 1997
57. **Nisijima K., Yoshino T., Yui K., Katoh S.,** Potent serotonin (5-HT)(2A) receptor antagonists completely prevent the development of hyperthermia in an animal model of the 5-HT syndrome, *Brain Res.*, **890**: 23-31, 2001