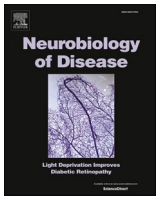
Neurobiology of Disease 157 (2021) 105441

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi



Increased ATP release and CD73-mediated adenosine A_{2A} receptor activation mediate convulsion-associated neuronal damage and hippocampal dysfunction

Elisabete Augusto [a,b], Francisco Q. Gonçalves [a], Joana E. Real [a], Henrique B. Silva [a], Daniela Pochmann [a], Tiago S. Silva [a], Marco Matos [a,b], Nélio Gonçalves [a], Ângelo R. Tomé [a,c], Jiang-Fan Chen [b,d], Paula M. Canas [a], Rodrigo A. Cunha [a,e,*]

[a] CNC-Center for Neuroscience and Cell Biology, University of Coimbra, Portugal
[b] Department of Neurology, Boston University School of Medicine, Boston, MA 02118, USA
[c] Department of Life Sciences, Faculty of Sciences and Technology, University of Coimbra, Portugal
[d] Molecular Neuropharmacology Lab, School of Optometry and Ophthalmology, Wenzhou Medical University, Wenzhou, China
[e] Faculty of Medicine, University of Coimbra, Portugal

ARTICLE INFO

Keywords:
ATP
P2 receptors
CD73
Ecto-nucleotidases
A_{2A} receptors
Epilepsy
Astrocytes
Memory

ABSTRACT

Extracellular ATP is a danger signal to the brain and contributes to neurodegeneration in animal models of Alzheimer's disease through its extracellular catabolism by CD73 to generate adenosine, bolstering the activation of adenosine A_{2A} receptors (A_{2A}R). Convulsive activity leads to increased ATP release, with the resulting morphological alterations being eliminated by A_{2A}R blockade. However, it is not known if upon convulsions there is a CD73-mediated coupling between ATP release and A_{2A}R overactivation, causing neurodegeneration. We now show that kainate-induced convulsions trigger a parallel increase of ATP release and of CD73 and A_{2A}R densities in synapses and astrocytes of the mouse hippocampus. Notably, the genetic deletion of CD73 attenuates neuronal degeneration but has no impact on astrocytic modifications in the hippocampus upon kainate-induced convulsions. Furthermore, kainate-induced convulsions cause a parallel deterioration of hippocampal long-term potentiation (LTP) and hippocampal-dependent memory performance, which is eliminated by knocking out CD73. This demonstrates the key role of the ATP release/CD73/A_{2A}R pathway to selectively control synaptic dysfunction and neurodegeneration following an acute brain insult, paving the way to consider CD73 as a new therapeutic target to prevent neuronal damage upon acute brain damage.

1. Introduction

Adenosine is a neuromodulator that mainly acts through inhibitory A_1 receptors to decrease basal excitatory synaptic transmission and through A_{2A} receptors (A_{2A}R) to bolster synaptic plasticity processes in different brain areas (Cunha, 2008). The differential engagement of A_{2A}R under condition of synaptic plasticity is ensured by a particular pool of adenosine formed by ecto-nucleotidases using adenine nucleotides as substrates (Augusto et al., 2013; Gonçalves et al., 2019; Rebola et al., 2008) coupled to particularly high release of ATP from nerve terminals upon more intense or greater frequency of stimulation (Cunha et al., 1996; Wieraszko et al., 1989). However, the overactivation of A_{2A}R is also concomitant with the onset of neurodegeneration (reviewed in Cunha, 2016). The overfunction of A_{2A}R in chronic brain diseases is associated with an upregulation of A_{2A}R (*e.g.* Espinosa et al., 2013; Kaster et al., 2015; Rebola et al., 2005; Temido-Ferreira et al., 2020), but also requires increased levels of adenosine to activate this increased number of A_{2A}R (Carmo et al., 2019; Gonçalves et al., 2019). Notably, in different brain diseases there is an increase of extracellular levels of ATP, which can act as prominent danger signal in the brain (reviewed in Rodrigues et al., 2015); furthermore, the blockade of ecto-5′-nucleotidase or CD73, the rate-limiting ecto-nucleotidase controlling ATP-derived adenosine formation (Cunha, 2001; Zimmermann et al., 2012), mimics the neuroprotection afforded by A_{2A}R in animal models of neurodegenerative disorders such as Alzheimer's (Gonçalves et al., 2019) or Parkinson's disease (Carmo et al., 2019). However, it is not

* Corresponding author at: CNC-Center for Neuroscience and Cell Biology, University of Coimbra, Portugal.
E-mail address: cunharod@gmail.com (R.A. Cunha).

https://doi.org/10.1016/j.nbd.2021.105441
Received 6 March 2021; Received in revised form 12 June 2021; Accepted 30 June 2021
Available online 3 July 2021

known if the inhibition of CD73 also prevents acutely induced neurodegeneration.

The exposure to the neurotoxin kainate triggers a rapid increase of excitability in limbic cortical circuits, followed by a re-organization of the afflicted neuronal circuits, which involves neurodegeneration and alteration ('activation') of glial cells (Nadler et al., 1980; Zaczek et al., 1980). Accordingly, kainate exposure is a useful model of acutely-induced neurotoxicity and is also a valuable model of temporal lobe epilepsy (*e.g.* Ben-Ari, 1985; Upadhya et al., 2019). We have previously used this model to show that it triggers a rapid upregulation of $A_{2A}R$ in excitatory synapses, which control kainate-induced remodeling of hippocampal circuits that precedes neurodegeneration accompanied by astrogliosis in the hippocampus (Canas et al., 2018). We now aim to further exploit this kainate model to test if the ATP release – CD73 – $A_{2A}R$ pathway is critical for the expression of convulsion-associated neurodegeneration.

2. Material and methods

2.1. Animals

CD73-KO mice from a C57bl\6 background, were generated and crossbred as previously described (Augusto et al., 2013; Gonçalves et al., 2019) and male KO (36 animals) and wild type littermates (41 animals), as well as wild type mice (23 animals; purchased from Charles River, Barcelona, Spain) at 8–12 weeks were used in the experiments. Animals were housed in groups of 3–4 *per* cage in a temperature-controlled room (22 ± 1 °C), with free access to food and water, and with a 12 h light/12 h dark cycle (lights on at 7:00 am). All studies were conducted in accordance with the principles and procedures outlined as "3Rs" in the

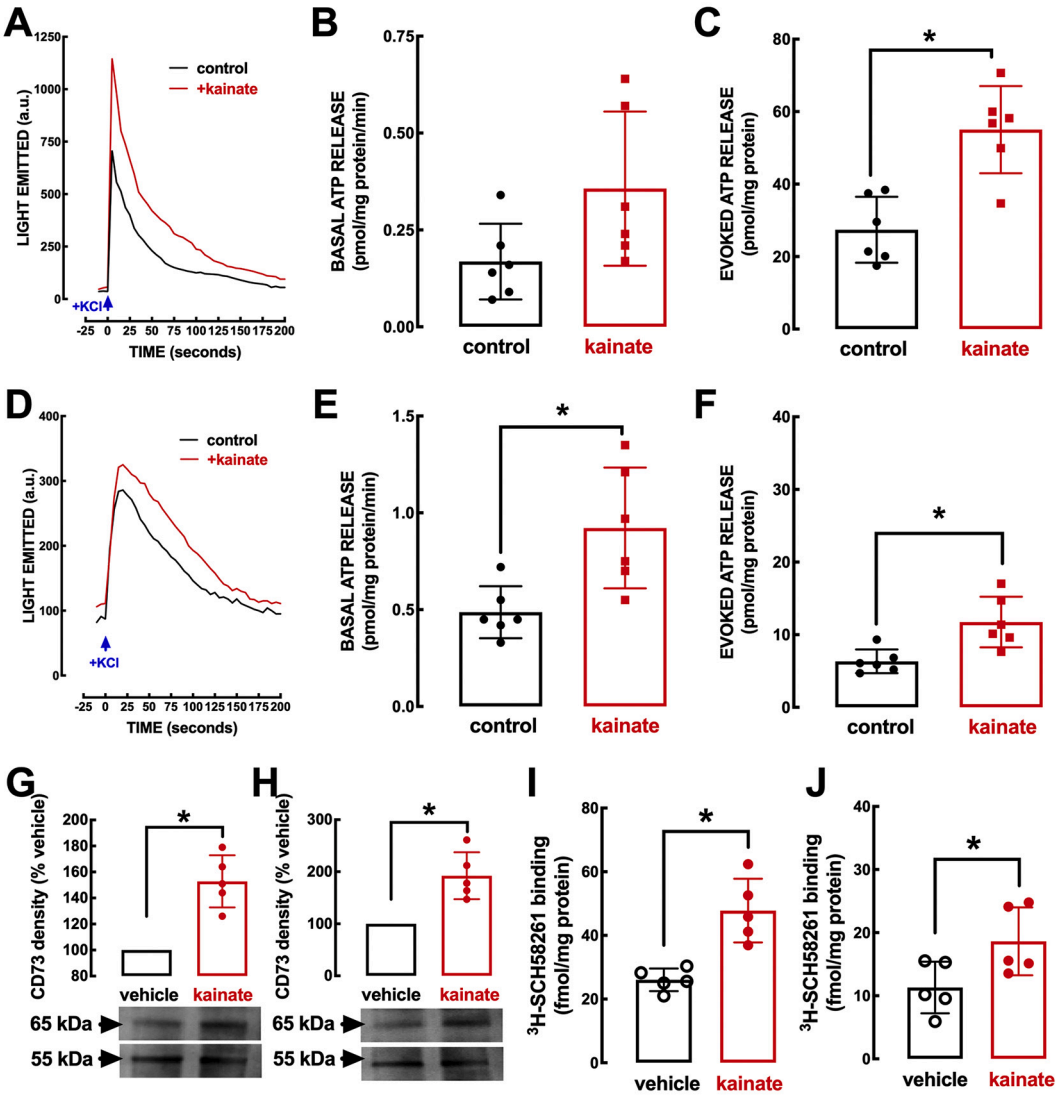


Fig. 1. Kainate-induced convulsions triggered an increased ATP release and increased density of CD73 and of adenosine A_{2A} receptors in hippocampal nerve terminals and in gliosomes. (A) Time course of ATP outflow from synaptosomes of control (vehicle-treated, black) and from synaptosomes prepared 24 h after kainate subcutaneous administration (red), which displayed a similar basal release of ATP (B), but a higher KCl (20 mM)-evoked release (C). (D) Time course of ATP outflow from hippocampal gliosomes of control and kainate-treated mice, showing an increased basal outflow (E) and K^+-evoked outflow of ATP in gliosomes from kainate-treated compared to control mice (F). The density of CD73 was increased in both hippocampal synaptosomes (G, upper blot) and gliosomes (H, upper blot) collected 24 h after kainate administration (red) compared to control mice (black), with a similar immunoreactivity of β3-tubulin (G, lower blot) and α-tubulin (H, lower blot). The density of $A_{2A}R$, assessed as the specific binding of the selective $A_{2A}R$ antagonist ^{3}H-SCH58261 (2 nM), was also higher in both hippocampal synaptosomes (I) and gliosomes (J) collected 24 h after kainate administration (red) compared to control mice (black). Data are mean ± standard deviations of 5–6 experiments (number of different animals tested). * $p < 0.05$ using an unpaired Student's *t*-test with Welsh correction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

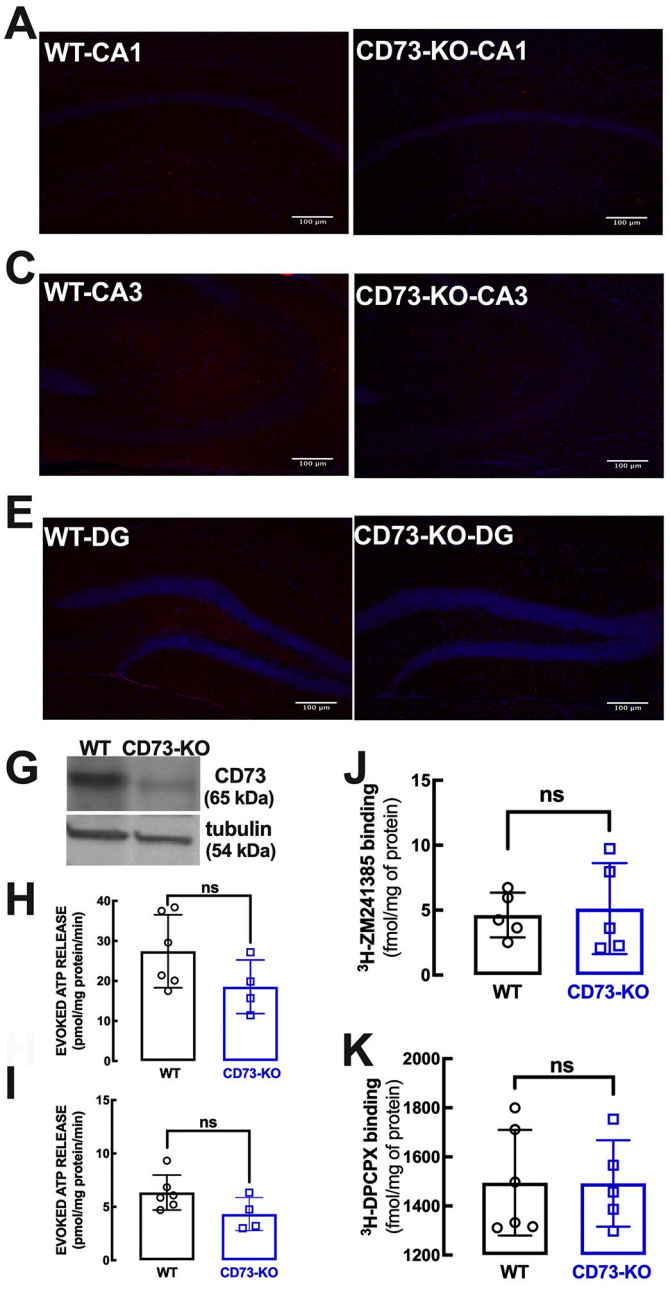
EU guidelines (210/63) and were approved by the Animal Care Committee of the Center for Neuroscience and Cell Biology (ORBEA 78/2013) and by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

2.2. Kainate model of convulsions

In some experiments, kainate was directly delivered to the hippocampus as previously described (Gouder et al., 2004), by stereotaxic injection of 50 nL of a 20 mM solution of kainic acid (KA) in 0.9% NaCl (*i.e.*, 1 nmol of KA) into the right dorsal hippocampus [coordinates with bregma as reference: anteroposterior (AP) -2.0, mediolateral (ML) -1.5, dorsoventral (DV) -1.8 mm] using a stainless steel cannula connected to a micro-syringe (Hamilton), under general anesthesia using vaporized isoflurane supplemented with oxygen. Each injection was performed over 1 min. At the end of the injection, the cannula was left in place for an additional period of 8–10 min to limit reflux along the cannula track. Control animals received vehicle, phosphate-buffered saline (PBS: 137 mM NaCl, 2.1 mM KCl, 1.8 mM KH_2PO_4 and 10 mM Na_2HPO_4, pH 7.4).

In other experiments, mice were injected subcutaneously (sc) with either PBS or kainate (35 mg/kg), after being handled daily for 3 days, as previously described (Canas et al., 2018), between 8 AM and 10 AM. A total of 4 WT mice died after the subcutaneous administration of kainate, which represents a lethality of 8%, below our expected average lethality of *circa* 15% in previous experiments.

The neurochemical characterization of the impact of the subcutaneous kainate administration on the release of ATP and densities of CD73 and $A_{2A}R$ (data in Fig. 1) involved the comparison of naïve wild type mice half of them challenged with kainate (n = 5–6/group). The exploration of alterations of the purinergic system upon genetic CD73 deletion (data in Fig. 2) involved the comparison of CD73-KO mice and their WT littermates, none of them challenged with kainate (n = 5–6/group). The histochemical characterization of the impact of the intrahippocampal kainate administration (data in Figs. 2 and 3) was



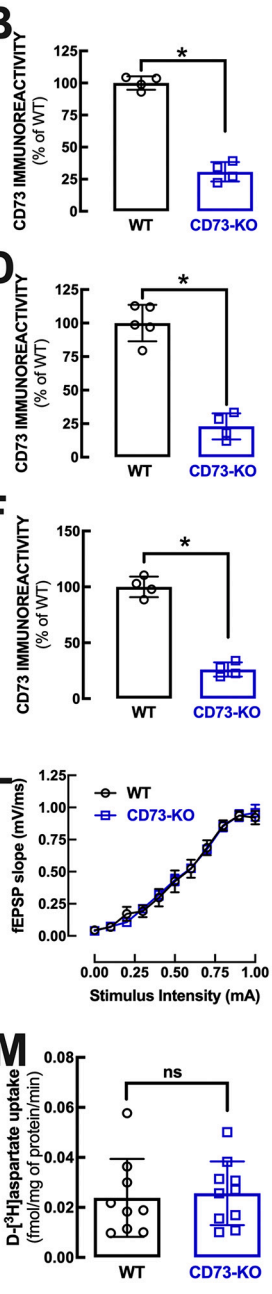


Fig. 2. CD73 knockout (KO) mice display a selective reduction of CD73 immunoreactivity in the hippocampus, without alterations of ATP release or density of adenosine receptors or function of synapses or astrocytes in the hippocampus. (A-F) Immunohistochemical staining of CD73 in hippocampal section revealed a decrease of CD73 immunoreactivity in the CA1 area (A, B), CA3 area (C,D) and dentate gyrus (E,F) of CD73-KO mice compared to their wild type (WT) littermates. Western blot analysis confirmed the decrease of CD73 immunoreactivity in total hippocampal membranes of CD73-KO mice compared to their WT littermates (G). The KCl (20 mM)-evoked release of ATP from hippocampal synaptosomes (H) or gliosomes (I) was similar in WT (black) and CD73-KO mice (blue). (J) The density of A_{2A} receptors, assessed as the specific binding of ^{3}H-ZM241385 (3 nM), and the density of A_1 receptors (K), assessed as the specific binding of ^{3}H-DPCPX (2 nM), was similar in total hippocampal membranes of WT (black) and CD73-KO mice (blue). (L) Synaptic function, evaluated as input-output curves of synaptic transmission in Schaffer fibers-CA1 pyramid synapses of hippocampal slices, and astrocytic function (M), evaluated as the uptake of ^{3}H-D-aspartate (50 nM) in hippocampal gliosomes were not significantly different between WT (black) and CD73-KO mice (blue). Data are mean ± standard deviations of 4–10 experiments (number of different animals tested). * $p < 0.05$ using an unpaired Student's *t*-test with Welsh correction; ns: non-significant. Scale bars in each photograph are 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

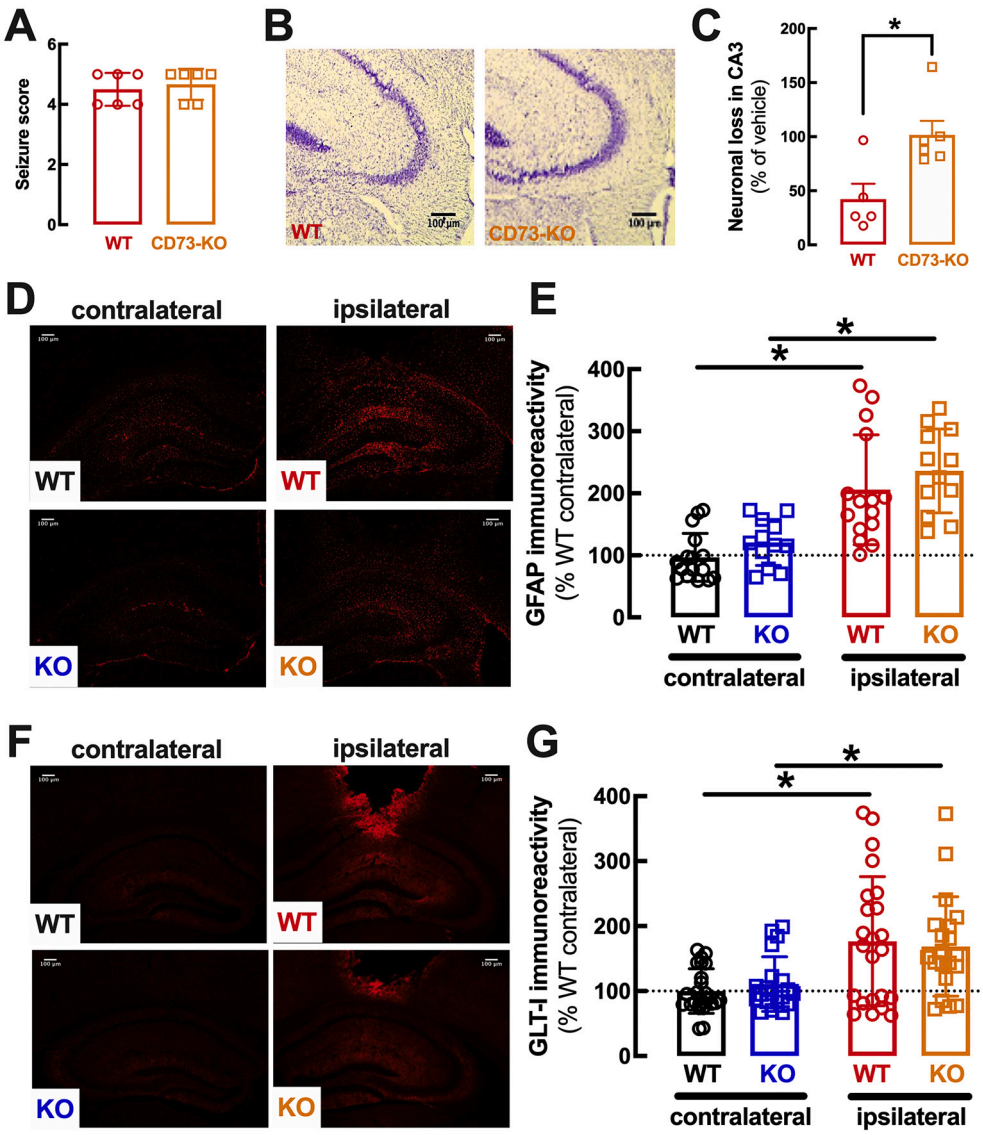


Fig. 3. The genetic deletion of CD73 did not affect the severity of kainate-induced convulsions but selectively eliminated kainate-induced hippocampal neurodegeneration without altering astrogliosis. (A) The subcutaneous administration of kainate triggered a similar pattern of convulsions in CD73-KO mice and in their wild type (WT) littermates. (B,C) However, the hippocampal neuronal degeneration, assessed as the dispersion of pyramidal cell layer of the CA3 region with Nissl staining, was more evident in WT than in CD73-KO mice after intra-hippocampal administration of kainate. Data are mean ± SEM of 6 experiments (number of different animals tested). * $p < 0.05$ using a Student's *t*-test with Welsh correction for comparison between two groups. The immunoreactivity of GFAP (D,E) and glutamate transporter type I (GLT-I) (F, G) induced by kainate was similar in the hippocampus of WT mice and their CD73-KO littermates after intra-hippocampal administration of kainate. Data are mean ± SEM of 12–23 sections from 6 mice per group. * $p < 0.05$ using a Newman-Keuls *post hoc* test after a two-way ANOVA. Scale bars in each photograph are 100 μm.

carried out in 4 groups of mice: CD73-KO mice treated with vehicle, CD73-KO mice exposed to kainate, WT littermates treated with vehicle, WT littermates exposed to kainate (n = 6/group). Likewise, 4 similar groups were used to test the electrophysiological and behavioral impact of the subcutaneous administration of kainate (n = 9–10/group; data in Figs. 3 and 4).

2.3. Scoring of behavioral convulsions

After the subcutaneous injections of kainate, the animals were placed in individual cages kept at room temperature. They were monitored continuously for 3 h to score the kainate-induced behavioral convulsions according to a previously established six-point seizure scale (Schauwecker and Steward, 1997) adapted from a five-point scale for rats (Racine, 1972), using the following criteria: stage 1: immobility; stage 2: forelimb and/or tail extension, rigid posture; stage 3: repetitive movements, head bobbing; stage 4: rearing and falling; stage 5: continuous rearing and falling; stage 6: severe tonic-clonic seizures. The scoring was made by direct visual inspection by two independent researchers blind to the group of animals tested.

2.4. Histological analysis

Twenty-four hours after the administration of kainate, mice were anesthetized with avertin (2,2,2-tribromoethanol) and brain tissue was fixed by transcardiac perfusion with 4% paraformaldehyde in PBS, post-fixed overnight in PBS with 4% paraformaldehyde and cryopreserved in PBS containing 25% sucrose. Frozen brains were sectioned (30 μm coronal slices) with a Leica CM3050S cryostat (Leica Microsystems). Two of every six hippocampal sections (*i.e.*, two sections separated by 150 μm from each other) were mounted on slides, allowed to dry at room temperature and stained with cresyl violet staining of Nissl bodies: this semi-quantitative assay, which we previously validated by comparison with other cell death assays (Canas et al., 2018), allows estimating neuronal cell loss. Briefly, sections were rehydrated through 100% and 95% alcohol for 1 min each and then with distilled water. Sections were incubated for 10 min in pre-warmed (40 °C) cresyl violet solution (IHCWorld) and then rinsed quickly in distilled water. After dehydration in 95% alcohol for 2 min, and twice more in 100% alcohol for 5 min each, the sections were cleared twice with xylene for 5 min each and mounted with DPX mounting medium (Sigma). Sections were then examined under a fluorescence Nikon eclipse E600 microscope, with SPOT software 4.7 (Diagnostic instruments, Inc.). We analyzed the CA3 region since the CA1 region often displayed an altered morphology due

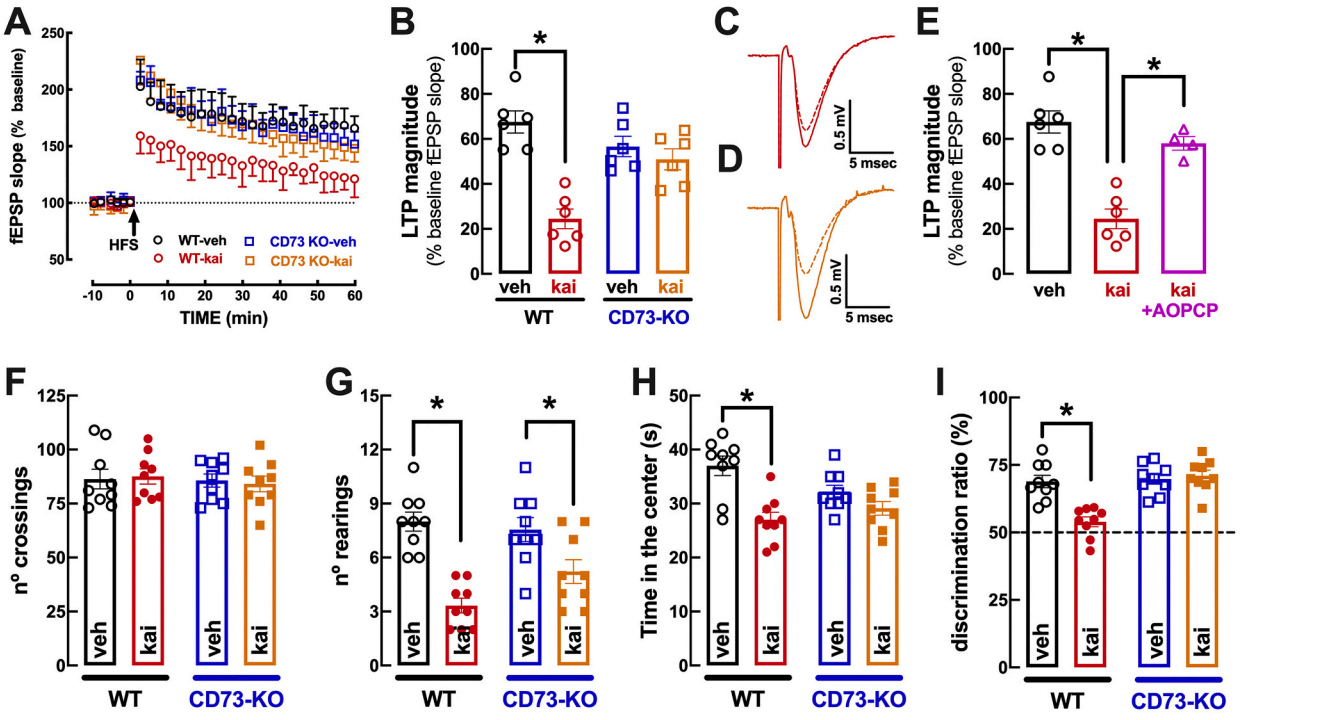


Fig. 4. The genetic deletion of CD73 eliminates the kainate-induced depression of hippocampal synaptic plasticity and of hippocampal-dependent memory without affecting spontaneous locomotion or anxiety. (A,B) The long-term potentiation (LTP), quantified as the high-frequency stimulation (HFS)-induced enhancement of the slope of field excitatory postsynaptic potentials (fEPSP) recorded in Schaffer fibers-CA1 pyramid synapses, was reduced in hippocampal slices collected 24 h after the subcutaneous administration of kainate (35 mg/kg) in wild type (WT) mice (black and red symbols), but not in CD73 knockout (KO) mice (blue and orange symbols). (C,D) Representative fEPSP traces before and 60 min after HFS recorded in slices from kainate-treated WT mice (C, red) and kainate-treated CD73-KO mice (D, orange). (E) The CD73 inhibitor α,β-methylene ADP (AOPCP, 100 μM) applied directly to hippocampal slices, reverted the reduction of LTP magnitude present in kainate-treated WT mice. Data are mean ± SEM of 4–6 experiments (number of different animals tested). (F—I) Behavioral analysis of vehicle-treated (black, blue) and kainate-treated (red, orange) WT (black, red) and CD73-KO mice (blue, orange) show that subcutaneous kainate administration did not modify spontaneous locomotion in an open field test (F), but decreased number of rearing events (G), and the time spent in the center of the open field test (H), which were not consistently affected by the genetic deletion of CD73. Kainate deteriorated hippocampal-dependent memory performance in WT mice and this effect was eliminated in CD73-KO mice (I), assessed by the object displacement test. Data are mean ± SEM of 9 mice/group. * $p < 0.05$ using a Newman-Keuls *post hoc* test after a Two-way ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to the local injections. The quantification performed by two independent subjects blind to the groups of animals tested, as previously described (Cho et al., 2006); the scoring was semi-quantitative ranging from 0, no observable damage; +1, < 10%; +2, 11–25%; +3, 26–50%; +4, 51–75%; +5, 76–90%; +6 > 91% of observable cell loss.

For immunohistochemistry, four of every six hippocampal sections (*i.e.*, four sections separated by 150 μm from each other) were used (Canas et al., 2018). After rinsing for 5 min with PBS at room temperature, the sections were permeabilized and blocked with PBS containing 0.2% Triton X-100 and 5% donkey serum for 1 h, incubated in the presence of the rabbit anti-murine CD73 antibody (1:500; kindly provided by J. Sévigny and produced as in Fausther et al., 2012), mouse anti-GFAP-Cy3 antibody (1:200; Sigma; Matos et al., 2012) or mouse anti-GLT-I/EAAT2 (1:300; Millipore; Matos et al., 2012) overnight at room temperature, rinsed three times for 10 min in PBS, and then incubated with donkey anti-mouse and/or donkey anti-rabbit secondary antibodies conjugated with a fluorophore (Alexa Fluor 488 or Alexa Fluor 555, 1:200, Invitrogen) for 2 h at room temperature. After rinsing three times for 10 min in PBS, the sections were mounted on slides and allowed to dry. Vectashield mounting medium with DAPI (Vector Laboratories) was applied as well as the cover glass and sections were examined as described above.

2.5. Preparation of synaptosomes and gliosomes

Twenty-four hours after the subcutaneous administration of kainate, mice were anesthetized with avertin (2,2,2-tribromoethanol) and killed by decapitation for isolation of both hippocampi. Mouse hippocampal synaptosomes (enriched synaptic fraction) and gliosomes (enriched astrocytic fraction) were obtained using a discontinuous Percoll (Sigma-Aldrich, Munich, Germany) gradient (2%, 6%, 15%, and 23%, *v*/v, of Percoll in a medium containing 0.32 M sucrose and 1 mM EDTA, pH 7.4), as previously described (Matos et al., 2012). The use of these fractions requires their careful resuspension yielding a suspension rather than a solution, which imposes a period not longer than 2 h to use these suspensions as a near homogenous distribution of the particles.

2.6. ATP release

The release of ATP was measured on-line using the luciferin-luciferase assay (Carmo et al., 2019; Gonçalves et al., 2019). Briefly, a suspension containing synaptosomes or gliosomes, an ATP assay mix (with luciferin and luciferase; from Sigma) and Krebs-HEPES solution was equilibrated at 25 °C for 10 min to ensure the functional recovery of the preparations. The suspension was then transferred to a white 96-well plate and measurements were performed in a luminometer (Victor3, Perkin Elmer). After 60 s to measure basal ATP outflow, the evoked release of ATP was triggered with 20 mM of KCl (isomolar substitution of NaCl in the Krebs-HEPES solution), a well-established neurochemical strategy to trigger optimal signal-to-noise calcium-dependent vesicular release without damaging these artificial structures (Raiteri et al., 2007; Stigliani et al., 2006). The evoked release of ATP was calculated by integration of the area of the peak upon subtraction of the estimated basal ATP outflow. We always confirmed the integrity of the

preparations by quantifying the amount of lactate dehydrogenase and of glutamine in the incubation medium after the assays, as previously described (Carmo et al., 2019; Gonçalves et al., 2019).

2.7. Western blot analysis

Western blot analysis was carried out by SDS-PAGE using synaptosomal or gliosomal membranes to evaluate CD73 levels using a rabbit polyclonal anti-CD73 antibody (1:300, Santa-Cruz) as previously described (Gonçalves et al., 2019). Membranes were re-probed for β3-tubulin (1:1000; Abcam) or α-tubulin (1:1000; Abcam) as a loading control. The selectivity of the other used anti-CD73 antibody (1:500) was also assessed by Western blot analysis comparing total hippocampal membranes of CD73-KO mice and of their WT littermates.

2.8. Receptor binding assay

The density of $A_{2A}R$ in synaptosomes and gliosomes was estimated by radioligand binding assays using a supra-maximal concentration of ^{3}H-SCH58261 (2 nM; offered by E.Ongini, Schering-Plough, Italy), as previously described (Kaster et al., 2015; Rebola et al., 2005). The density of A_1R and $A_{2A}R$ in total hippocampal membranes, prepared as previously described (Rebola et al., 2003, 2005) was assessed using 2 nM of the selective A_1R antagonist, ^{3}H-DPCPX (Perkin Elmer) and 3 nM of the selective $A_{2A}R$ antagonist, ^{3}H-ZM241385 (Perkin Elmer). The binding reactions were carried out at 37 °C with 0.1–0.2 mg of protein and were performed for 1 h in 50 mM Tris and 10 mM $MgCl_2$ with 4 U/mL of adenosine deaminase (Sigma) for ^{3}H-ZM241385 and 2 h in 50 mM Tris and 2 mM $MgCl_2$ with 4 U/mL of adenosine deaminase for ^{3}H-DPCPX binding. Specific binding was always determined by subtraction of non-specific binding, measured using 3 μM XAC (Tocris).

2.9. D-^{3}H-aspartate uptake

The uptake of the non-metabolizable glutamate analogue D-^{3}H-aspartate, a validated readout of the activity of glutamate transporters (Anderson and Swanson, 2000), was carried out as previously described (Matos et al., 2012). Briefly, the gliosomes were diluted in NMG buffer (Krebs buffer constituted by 132 NaCl, 4 KCl, 1.2 Na_2HPO_4, 1.4 $MgCl_2$, 6 glucose, 10 HEPES, and 1 $CaCl_2$, pH 7.4, where NaCl is replaced by *N*-methylglucamine-NMG) and equilibrated at 37 °C for 10 min. Triplicates (150 μL) of each fraction were added to 150 μL of Krebs or NMG medium containing a final concentration of 50 nM D—^{3}H aspartate (11.3 ci/mmol; PerkinElmer). The mixtures were incubated for 10 min at 37 °C and the reaction terminated by rapid vacuum filtration over Whatman GF/C filters (GE Healthcare) and further washed 3 times with ice cold NMG buffer. The filters were dried overnight and the radioactivity was measured after adding 5 mL of scintillation liquid (Perkin Elmer). The specific uptake of D-^{3}H-aspartate was calculated by subtraction from the total uptake of the non-specific uptake measured in a Na^+-free medium (NMG).

2.10. Behavioral analysis

Behavioral analysis was performed one day (22–26 h) after the subcutaneous administration of kainate, between 9 AM and 2 PM, under low-intensity light (12 lx) in a sound-attenuated room, where mice had been habituated for at least 1 h before the beginning of the tests. Behavior was video-monitored with the ANY Maze® video tracking system (Stoelting Co., Wood Dale, IL, USA) and analyzed by a researcher blind to the group of mice tested. The apparatuses were cleaned with 10% ethanol in-between animal testing to avoid odor cues. Locomotion and exploratory behavior were monitored using an open-field apparatus with a gridded floor to count the total number of crossings and the number of crossings in the central (more aversive) region of the field as an estimate of anxiety, as previously detailed (Gonçalves et al., 2015). Using the same open-field boxes (habituation), hippocampal-dependent memory was next evaluated using the object displacement test (Kaster et al., 2015; Silva et al., 2013), where mice first explored the box with two similar objects (training session) and explored it again 2 h later, now with one object displaced (test session). The ratio of the time interacting with each object allowed defining a discrimination index as a measure of memory (Gonçalves et al., 2019; Kaster et al., 2015).

2.11. Electrophysiological recordings

After the behavioral evaluation, mice were anesthetized under halothane atmosphere, decapitated and the brain was rapidly removed and submerged in ice-cold aCSF solution (in mM: 124.0 NaCl, 4.0 KCl, 1.25 Na_2HPO_4, 26.0 $NaHCO_3$, 2.0 $CaCl_2$, 1.2 $MgCl_2$, 10.0 glucose; osmolality: 290–310 mOsmol/kg) gassed with a 95% O_2 and 5% CO_2 mixture. Recordings of excitatory synaptic transmission and plasticity were performed in superfused slices (400 μm thick) of the dorsal hippocampus, as previously described (Gonçalves et al., 2019; Kaster et al., 2015). Briefly, Schaffer fibers were stimulated every 15 s to evoked field excitatory postsynaptic potentials (fEPSPs) recorded in the CA1 *stratum radiatum* to measure the fEPSP slope. The intensity of stimulation was chosen between 40 and 50% of maximal fEPSP response, determined based on input/output curves in which the fEPSP slope was plotted *versus* stimulus intensity. LTP was induced with a high-frequency train (100 Hz for 1 s) and was quantified as the percentage changed between the fEPSP slopes 60 min after and 10 min before the train. When the effect of α,β-methylene ADP (AOPCP, from Sigma) was tested, it was added through the superfusion solution for at least 20 min before LTP induction and the effect of AOPCP was assessed by comparing LTP magnitude in the absence and presence of the drug in experiments carried out in different slices from the same animal.

2.12. Statistical analysis

In all experimental procedures, four or more animals were used for each parameter analyzed and the individual sample size (n = number of animals) was specified for each experiment. All data were presented as mean ± SEM, and significance was considered at $p < 0.05$ using either a one-sample *t*-test to assess the effect of any individual drug or treatment in electrophysiological experiments, a two-tailed Student's *t*-test with Welsh correction for comparison between two groups and one-way ANOVA (followed by a Bonferroni's *post hoc* test) or two-way ANOVA (followed by a Newman-Keuls *post hoc* test) for comparison of multiple groups.

3. Results

3.1. Kainate treatment increases ATP release

Extracellular ATP can act as a danger signal in the brain (reviewed in Rodrigues et al., 2015) and its extracellular levels are augmented upon seizure-like activity (reviewed in Beamer et al., 2019). We now used synaptosomes and gliosomes to disentangle the alteration of ATP release from nerve terminals and from astrocytes, respectively, after the subcutaneous administration of kainate (Fig. 1A,D). The basal outflow of ATP from hippocampal synaptosomes was similar between kainate-treated and vehicle-treated mice (0.357 ± 0.081 and 0.168 ± 0.040 pmol/mg protein, respectively, $n = 6$–7; $p = 0.064$) (Fig. 1A,B), whereas the evoked ATP release, triggered by a chemically-induced depolarization using a moderate (20 mM) increase of extracellular K^+ (*e.g.* Blaustein, 1975; McMahon and Nicholls, 1990), was significantly larger in kainate-treated than in vehicle-treated mice (55.05 ± 4.91 and 27.42 ± 3.72 pmol/mg protein, respectively, $n = 6$–7; $p = 0.001$) (Fig. 1A,C). In gliosomes of vehicle-treated mice, the basal outflow of ATP was significantly larger (0.487 ± 0.055 pmol/mg protein, n = 6; $p < 0.001$), whereas the K^+-evoked ATP outflow from gliosomes was significantly

lower (6.34 ± 0.67 pmol/mg protein, n = 6; p < 0.001) than in synaptosomes of vehicle-treated mice. The basal outflow of ATP from gliosomes from kainate-treated mice was larger than vehicle-treated mice (0.922 ± 0.127 pmol/mg protein, n = 6; $p = 0.017$) (Fig. 1D,E), the same occurring for the evoked ATP release (11.75 ± 1.42 pmol/mg protein, n = 6–9; $p = 0.011$) (Fig. 1D,F). Thus, the depolarization of nerve terminals seems to be a major contributor to the increase of extracellular ATP after kainate-induced seizures.

3.2. Kainate treatment increases CD73 and A_{2A} receptor density

In addition to the long-term effects of ATP on P2X7R to alter microglia phenotype and trigger neuroinflammation (*e.g.* Bianco et al., 2005) and on P2X4R to control NMDA receptors (Bertin et al., 2021), extracellular ATP is also a source of adenosine, upon CD73-mediated formation of ATP-derived adenosine, to activate adenosine A_{2A}R that are both necessary and sufficient to disrupt synaptic plasticity and hippocampal-dependent function (reviewed in Agostinho et al., 2020). To test how this ATP-CD73-A_{2A}R pathway is affected by seizures, we quantified the alteration of the densities of CD73 and A_{2A}R in hippocampal synaptosomes and gliosomes after the subcutaneous administration of kainate. As shown in Fig. 1G,H, the density of CD73 was higher both in synaptosomes (52.8 ± 8.97% higher, $n = 5$–6; $p = 0.004$) and gliosomes (92.4 ± 20.2% higher, n = 5; $p = 0.010$) from kainate-treated than vehicle-treated mice. Moreover, the density of A_{2A}R was also higher both in synaptosomes (83.4 ± 9.32% higher, $n = 5$; $p = 0.006$) and gliosomes (64.8 ± 13.4% higher, n = 6–9; $p = 0.044$) from kainate-treated than vehicle-treated mice (Fig. 1I,J). Thus, there is a parallel increase of ATP release, CD73 and A_{2A}R densities both in synaptosomes and gliosomes upon kainate-induced seizure activity.

3.3. Impact of the genetic deletion of CD73 on the purinergic system in the hippocampus

To test whether the alteration of ATP-derived adenosine can cause alterations of hippocampal morphology and function, triggered by the exposure to kainate, we resorted to a genetic deletion of CD73, which ensures the formation of extracellular adenosine from released adenine nucleotides (Augusto et al., 2013; Cunha et al., 1992; Gonçalves et al., 2019; Kulesskaya et al., 2013; Lovatt et al., 2012). This first requires testing if the hippocampal purinergic system is significantly affected upon genetic deletion of CD73. As shown in Figs. 2A-F, there is a significant reduction of CD73 immunoreactivity in the hippocampus of CD73 knockout (KO) mice compared to wild type mice in all three main hippocampal areas ($p < 0.001$ for each area). This was confirmed by the observed decrease by over 90% (90.5 ± 5.67%, $n = 4$) of CD73 immunoreactivity in total hippocampal membranes from CD73-KO mice compared to WT mice as assessed by Western blot quantification (Fig. 2G). The residual labeling with the anti-CD73 antibody that was observed in hippocampal sections from CD73-KO mice shows that the antibody only has a good selectivity (but not absolute specificity), since the genotyping of CD73-KO mice confirmed the complete elimination of CD73 mRNA, ensuring that CD73-KO mice are indeed a full KO of CD73.

The evoked release of ATP from synaptosomes was not significantly different in CD73-KO mice compared to WT mice (27.42 ± 3.72 and 18.54 ± 3.35 pmol/mg protein, respectively, n = 4–6, $p = 0.115$), the same occurring for the evoked ATP outflow from gliosomes (6.34 ± 0.67 and 4.33 ± 0.77 pmol/mg protein, respectively, n = 4–6, $p = 0.091$) (Fig. 2H,I). There was also no evident alteration in CD73-KO mice of the density of the main adenosine receptors in the hippocampus (Fredholm et al., 2005), with similar densities of binding of the selective A_{2A}R antagonist ^{3}H-ZM241385 in total membranes (4.62 ± 0.77 and 5.13 ± 1.57 fmol/mg protein, $n = 5$, $p = 0.782$) (Fig. 2J) and of the selective A_1R antagonist ^{3}H-DPCPX in total membranes (1495 ± 88 and 1492 ± 79 fmol/mg protein, n = 5–6, $p = 0.982$) (Fig. 2K). Finally, to gain an overall insight on putative alteration of the function of synapses and astrocytes, we compared excitatory synaptic transmission and the uptake of aspartate as surrogate markers. As shown in Fig. 2L, input-output curves were nearly superimposable in slices from CD73-KO and WT mice and there was no alteration of the uptake of ^{3}H-aspartate in gliosomes (0.0257 ± 0.0040 in CD73-KO mice and 0.0238 ± 0.0052 fmol/mg protein/min in WT littermates, $n = 8$–9, $p = 0.779$) (Fig. 2M). Thus, the genetic deletion of CD73 effectively reduces CD73 density in the hippocampus, without major alterations of ATP release or adenosine receptors or major functional changes in excitatory synapses or astrocytes.

3.4. ATP-derived adenosine critically controls kainate-induced neuronal degeneration, but not astrocytic alterations in the hippocampus

The intensity of convulsions triggered by the subcutaneous administration of kainate was similar in WT and CD73-KO mice ($p = 0.600$; $n = 6$ per group; Fig. 3A). This argues for a lack of a major effect of the genetic deletion of CD73 on the formation of extracellular adenosine associated with the activation of inhibitory A_1 adenosine receptors known to curtail seizure duration (reviewed in Tescarollo et al., 2020) or on the amount of extracellular ATP known to aggravate seizures through P2X7 receptor activation (reviewed in Engel et al., 2016).

However, in contrast to the degeneration of neurons prominently observed in the CA3 region of the hippocampus of WT mice 24 h after the intrahippocampal administration of kainate (Fig. 3B,C), there was a higher preservation of Nissl body stained with cresyl violet in the hippocampus of CD73-KO mice (Fig. 3B,C). Notably, this preservation of kainate-induced neurodegeneration upon genetic deletion of CD73 is not accompanied by preservation of kainate-induced alterations of astrocytes. In fact, the increase of hippocampal GFAP immunoreactivity observed 24 h after kainate exposure was similar between WT and CD73-KO mice ($p = 0.320$; n = 6 per group in duplicate; Fig. 3D,E). Likewise, the increase in hippocampal immunoreactivity of the glutamate transporters type 1 (GLT-1, a predominant astrocytic protein; *e.g.* Berger et al., 2005) observed 24 h after kainate exposure was also similar in WT and CD73-KO mice ($p = 0.766$; n = 6 per group in duplicate; Fig. 3F,G).

3.5. ATP-derived adenosine critically controls kainate-induced deterioration of hippocampal synaptic plasticity and spatial memory

The particular ability of CD73 genetic deletion to affect neurons rather than astrocytes after kainate exposure was further confirmed by its ability to eliminate the deleterious effects of kainate exposure on hippocampal synaptic plasticity. As shown in Fig. 4A-B, one day after the subcutaneous administration of kainate to induce convulsions in WT mice, there was a decrease in the magnitude of long-term potentiation (LTP) in Schaffer fiber-CA1 pyramid synapses (24.5 ± 4.39% over baseline, n = 6) compared to slices obtained from WT mice treated with vehicle (67.5 ± 4.92% over baseline, n = 6; $p < 0.001$, Tukey's test after two-way ANOVA). This contrasts with CD73-KO mice, where LTP magnitude in slices from vehicle-treated mice (56.6 ± 4.54% over baseline, n = 6) was similar ($p = 0.821$, Tukey's test after two-way ANOVA) to that observed in slices from kainate-treated mice (50.9 ± 4.63% over baseline, n = 6) (Fig. 4A,B). Moreover, the decrease of LTP magnitude caused by kainate exposure in WT mice was reverted upon acute blockade of CD73 through superfusion of slices with 100 μM α,β-methylene ADP (58.0 ± 2.98% over baseline, $n = 4$; $p < 0.001$ *vs.* LTP magnitude in untreated slices from WT mice exposed to kainate *in vivo*) (Fig. 4C-E).

Since synaptic plasticity is regarded as the neurophysiological trait of memory (*e.g.* Martin et al., 2000; Lynch, 2004), we next investigated if kainate caused a hippocampal-dependent memory deterioration that could be prevented by CD73 genetic deletion. The spontaneous locomotion assessed as the number of crossings in an open field was similar between WT mice (86.33 ± 4.55, $n = 9$) and CD73-KO mice (85.67 ± 3.03, n = 9) and was not significantly modified by kainate, either in WT

mice (87.56 ± 3.52, n = 9; $p = 0.995$ *vs.* vehicle) or CD73-KO mice (84.11 ± 3.59, n = 9; $p = 0.991$ *vs.* vehicle) (Fig. 4F). Analysis with a two-way ANOVA of the number of rearing events in the open field, revealed an effect of kainate ($F_{1,32} = 49.45$, $p < 0.001$) but not of genotype ($F_{1,32} = 2.107$, $p = 0.219$) or their interaction ($F_{1,32} = 5.492$, $p = 0.052$) (Fig. 4G). Analysis with a two-way ANOVA of anxiety, assessed as the time in the more aversive central area of the open field arena, revealed an effect of kainate ($F_{1,32} = 35.28$, $p < 0.001$) but not of genotype ($F_{1,32} = 1.463$, $p = 0.357$) and an interaction kainate x genotype ($F_{1,32} = 9.745$, $p = 0.022$); however, a post-hoc Tukey's test indicated a difference between WT mice treated with vehicle and with kainate ($p < 0.001$), whereas there was no difference of anxiety between vehicle- and kainate-treated mice ($p = 0.423$) (Fig. 4H).

This profile enabled the evaluation of spatial memory performance in the object displacement test, carried out in the same arena as the open field (habituation). In the training phase of the object displacement test, there was no difference in the total time exploring both objects between the different groups (vehicle-treated WT mice: 34.11 ± 2.245 s; kainate-treated WT mice: 28.11 ± 1.263 s; vehicle-treated CD73-KO mice: 32.67 ± 2.128 s; kainate-treated CD73-KO mice: 28.89 ± 1.033 s; n = 9 for each). In the test phase, a two-way ANOVA analysis of the discrimination of the displaced object revealed an effect of kainate ($F_{1,32} = 14.48$, $p = 0.002$) and an interaction kainate x genotype ($F_{1,32} = 20.53$, $p < 0.001$); a post-hoc Tukey's test indicated a difference between WT mice treated with vehicle and with kainate ($p < 0.001$), whereas there was no difference of spatial memory performance either between vehicle-treated WT and CD73-KO mice ($p = 0.990$) or between vehicle-treated and kainate-treated CD73-KO mice ($p = 0.967$) (Fig. 4I).

4. Discussion

The present study provides evidence for the upregulation and key role in the control of neurodegeneration of a pathway constituted by increased ATP release, CD73-mediated formation of extracellular adenosine from this released ATP and activation of adenosine A_{2A} receptors ($A_{2A}R$). This provides a synthesis into a single framework of two established mechanisms associated with neurodegeneration, namely ATP as a danger signal in the brain (reviewed in Rodrigues et al., 2015) and the overactivation of $A_{2A}R$, which genetic and pharmacological blockade affords a robust neuroprotection (reviewed in Cunha, 2016). Ecto-5′-nucleotidase or CD73 emerges as a key link between these mechanisms, indicating that ATP released as a danger signal is a prominent source of adenosine responsible for the overactivation of $A_{2A}R$. The association between CD73 and $A_{2A}R$ has a rather robust molecular support (Augusto et al., 2013) and was previously shown to have functional relevance to control synaptic plasticity in physiological conditions (Gonçalves et al., 2019; Rebola et al., 2008) as well as in morpho-functional alterations in animal models of neurodegenerative disorders such as Alzheimer's (Gonçalves et al., 2019) or Parkinson's disease (Carmo et al., 2019). Using the kainate model of acutely-induced neurodegeneration, we now showed that the up-regulation of this CD73 pathway mediating $A_{2A}R$ overactivation upon bolstered release of ATP is actually of critical importance for the early onset of acutely-induced neurodegeneration.

Our sub-cellular fractionation allowed gaining additional insights on the predominant compartments involved in this CD73-mediated ATP-derived $A_{2A}R$ overactivation. Thus, we observed that a convulsive period induced a parallel up-regulation of ATP release and of the densities of CD73 and $A_{2A}R$ in nerve terminals (see also Bonan et al., 2000; Canas et al., 2018; Rebola et al., 2003, 2005) as well as in astrocytes (see also Barros-Barbosa et al., 2016). However, the amplitude of the changes of ATP release and of $A_{2A}R$ density was higher in nerve terminals than in gliosomes. Accordingly, the genetic deletion of CD73 mostly affected neurodegeneration rather than morpho-functional alterations in astrocytes. Altogether, these observations prompt the conclusion that the kainate-induced upregulation of CD73-mediated ATP-derived $A_{2A}R$ activation is most relevant to control neuronal rather than astrocytic function, at least for the immediate alterations occurring after a convulsive period. Indeed, kainate administration causes an early (within 6 h) $A_{2A}R$-mediated hyperexcitability of glutamatergic synapses in the hippocampus that leads to their subsequent failure, decreasing synaptic plasticity and causing synaptotoxicity, which pre-dates glial alterations (Canas et al., 2018). Accordingly, we now report that the genetic elimination of CD73 prevents alterations of synaptic plasticity in hippocampal synapses, which allows a preservation of hippocampal-dependent memory performance. Furthermore, the acute *in vitro* inhibition of CD73 after kainate-induced convulsions was sufficient to revert the alterations of hippocampal synaptic plasticity, which mimics the effect of $A_{2A}R$ antagonists (see Canas et al., 2018). This further argues for the early engagement of a constitutive increase of the CD73-mediated ATP-derived $A_{2A}R$ activation pathway after an acute deleterious challenge such as kainate-induced convulsions, to sustain the evolving neurodegeneration well-described for this kainate model of temporal lobe epilepsy (see Sperk et al., 1983). The present observation that this increased CD73-mediated ATP-derived $A_{2A}R$ activation pathway mostly controls neuronal rather than astrocytic morphological or functional alterations at the early onset of neurodegeneration further argues for a prominent role of synaptic dysfunction in processes of activity-dependent neurodegeneration, as has been proposed in different neuropsychiatric conditions (see Duman and Aghajanian, 2012; Lepeta et al., 2016; Selkoe, 2002; Schirinzi et al., 2016).

The tight and prominent association of released ATP and $A_{2A}R$ overactivation can be somewhat surprising since extracellular ATP can directly signal through a broad family of P2 receptors (Burnstock et al., 2011). In fact, the inhibition of the extracellular conversion of ATP into adenosine would be expected to increase the extracellular levels of adenine nucleotides apart from decreasing the extracellular levels of adenosine. However, a putative impact of altered P2 receptor-mediated effects on convulsions and neurodegeneration upon knock out of CD73 seems unlikely. In fact, P2 receptor activation is proposed to be pro-convulsive (Engel et al., 2016), which would imply an aggravation of convulsions and a worsening of neurodegeneration following convulsions. In contrast, knocking out CD73 failed of alter the profile of behavioral convulsions and instead largely eliminated neurodegeneration upon convulsions. This argues for a key role of ATP-derived $A_{2A}R$ activation and excludes a direct action of ATP on P2 receptors. However, there is previous robust evidence that antagonists of P2X7 receptors or the down-regulation of this receptor can decrease the neurodegeneration resulting from convulsive activity in animal models (Jimenez-Mateos et al., 2015; Jimenez-Pacheco et al., 2016). This apparent paradox is probably a consequence of a crosstalk between P2X7 receptors and $A_{2A}R$, which was recently unveiled in a rodent model of restraint stress (Dias et al., 2021). Additionally, the presently observed prominent ATP-derived $A_{2A}R$-mediated control of neurodegeneration does not exclude an ability of P2 receptors to control glial function upon convulsions, as elegantly shown by others (Alves et al., 2020; Avignone et al., 2008). In fact, microglia 'activation' only occurs in an evident manner later than synaptotoxicity in this kainate model of induced neurodegeneration (Canas et al., 2018) and we observed that CD73 controlled synaptic dysfunction and neuronal damage rather than astrocytic alterations. Thus, the main role of CD73-mediated ATP-derived $A_{2A}R$-mediated control of neurodegeneration does not exclude other roles of extracellular ATP acting through P2 receptors, namely in the control of later adaptive features involving astrocytes or microglia, in light of the spatio-temporal interplay between the different arms of purinergic signaling to control brain function (reviewed in Agostinho et al., 2020).

A major limitation of the present study is the lack of a direct electrophysiological characterization of the pattern of seizure activity, which is directly associated with neurodegeneration. We only accessed the pattern of behavioral convulsions, which proved to be an ancillary readout supporting the association of CD73 and $A_{2A}R$ activities. In fact,

the knockout of CD73 displayed a pattern superimposable to that of knocking out $A_{2A}R$, both decreasing neurodegeneration without affecting behavioral convulsions. The observed lack of impact of knocking out CD73 on behavioral convulsions also indicates a lack of involvement of inhibitory A_1 adenosine receptors upon CD73 knockout in the control of convulsions and neurodegeneration. In fact, based on the ability of A_1 receptor actvation to terminate seizures (reviewed in Tescarollo et al., 2020), one would expect that CD73-KO mice would develop longer periods of seizures that would translate into an aggravated profile of behavioral convulsions as well as a more extensive neurodegeneration, precisely the opposite of what was observed.

The second major limitation of our study derives from the use of only male mice. Previous studies have noted the impact of sex on the development of epilepsy in different animal models (reviewed in Scharfman and MacLusky, 2014). This led us to optimize the different protocols of kainate-induced seizures in male rodents, to decrease the variability of the measures and the number of animals used, as strictly imposed by the regular audits of our animal house. The cost of this option is that the validity of the findings can only be ascertained for a subgroup of animals (males) and further studies will have to be carried out to extend the presently drawn conclusion to females.

5. Conclusion

The present study provides the first direct demonstration of the importance of the ATP release-CD73-$A_{2A}R$ activation pathway to control early neurodegenerative features following a kainate-induced convulsive episode. This prompts this pathway as a critical player linking a deleterious challenge to the brain with the onset of neurodegeneration, which is prominently associated with a dysfunction of synaptic plasticity.

Author contribution

RAC, JFC and EA designed research; EA, FQG, JER, HBS, DP, TSS, MM, NG and ART performed experiments and analyzed data; all authors commented on the manuscript text.

Declaration of Competing Interest

RAC is a scientific consultant for the Institute for Scientific Information on Coffee. All other authors declare no conflict of interests.

Acknowledgements

Supported by La Caixa Foundation (LCF/PR/HP17/52190001), Centro 2020 (CENTRO-01-0145-FEDER-000008:BrainHealth 2020 and CENTRO-01-0246-FEDER-000010) and FCT (POCI-01-0145-FEDER-03127 and UIDB/04539/2020).

References

Agostinho, P., Madeira, D., Dias, L., Simões, A.P., Cunha, R.A., Canas, P.M., 2020. Purinergic signaling orchestrating neuron-glia communication. Pharmacol. Res. 162, 105253.

Alves, M., Smith, J., Engel, T., 2020. Differential expression of the metabotropic P2Y receptor family in the cortex following status epilepticus and neuroprotection via P2Y1 antagonism in mice. Front. Pharmacol. 10, 1558.

Anderson, C.M., Swanson, R.A., 2000. Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia 32, 1–14.

Augusto, E., Matos, M., Sévigny, J., El-Tayeb, A., Bynoe, M.S., Müller, C.E., Cunha, R.A., Chen, J.F., 2013. Ecto-5′-nucleotidase (CD73)-mediated formation of adenosine is critical for the striatal adenosine A_{2A} receptor functions. J. Neurosci. 33, 11390–11399.

Avignone, E., Ulmann, L., Levavasseur, F., Rassendren, F., Audinat, E., 2008. Status epilepticus induces a particular microglial activation state characterized by enhanced purinergic signaling. J. Neurosci. 28, 9133–9144.

Barros-Barbosa, A.R., Ferreirinha, F., Oliveira, Â., Mendes, M., Lobo, M.G., Santos, A., Rangel, R., Pelletier, J., Sévigny, J., Cordeiro, J.M., Correia-de-Sá, P., 2016. Adenosine A_{2A} receptor and ecto-5′-nucleotidase/CD73 are upregulated in hippocampal astrocytes of human patients with mesial temporal lobe epilepsy (MTLE). Purinergic Signal. 12, 719–734.

Beamer, E., Conte, G., Engel, T., 2019. ATP release during seizures - a critical evaluation of the evidence. Brain Res. Bull. 151, 65–73.

Ben-Ari, Y., 1985. Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. Neuroscience 14, 375–403.

Berger, U.V., DeSilva, T.M., Chen, W., Rosenberg, P.A., 2005. Cellular and subcellular mRNA localization of glutamate transporter isoforms GLT1a and GLT1b in rat brain by in situ hybridization. J. Comp. Neurol. 492, 78–89.

Bertin, E., Deluc, T., Pilch, K.S., Martinez, A., Pougnet, J.T., Doudnikoff, E., Allain, A.E., Bergmann, P., Russeau, M., Toulmé, E., Bezard, E., Koch-Nolte, F., Séguéla, P., Lévi, S., Bontempi, B., Georges, F., Bertrand, S.S., Nicole, O., Boué-Grabot, E., 2021. Increased surface P2X4 receptor regulates anxiety and memory in P2X4 internalization-defective knock-in mice. Mol. Psychiatry 26, 629–644.

Bianco, F., Pravettoni, E., Colombo, A., Schenk, U., Möller, T., Matteoli, M., Verderio, C., 2005. Astrocyte-derived ATP induces vesicle shedding and IL-1 beta release from microglia. J. Immunol. 174, 7268–7277.

Blaustein, M.P., 1975. Effects of potassium, veratridine, and scorpion venom on calcium accumulation and transmitter release by nerve terminals in vitro. J. Physiol. 247, 617–655.

Bonan, C.D., Walz, R., Pereira, G.S., Worm, P.V., Battastini, A.M., Cavalheiro, E.A., Izquierdo, I., Sarkis, J.J., 2000. Changes in synaptosomal ectonucleotidase activities in two rat models of temporal lobe epilepsy. Epilepsy Res. 39, 229–238.

Burnstock, G., Krügel, U., Abbracchio, M.P., Illes, P., 2011. Purinergic signalling: from normal behaviour to pathological brain function. Prog. Neurobiol. 95, 229–274.

Canas, P.M., Porciúncula, L.O., Simões, A.P., Augusto, E., Silva, H.B., Machado, N.J., Gonçalves, N., Alfaro, T.M., Gonçalves, F.Q., Araújo, I.M., Real, J.I., Coelho, J.E., Andrade, G.M., Almeida, R.D., Chen, J.F., Köfalvi, A., Agostinho, P., Cunha, R.A., 2018. Neuronal adenosine A_{2A} receptors are critical mediators of neurodegeneration triggered by convulsions. eNeuro 5. ENEURO.0385-18.2018.

Carmo, M., Gonçalves, F.Q., Canas, P.M., Oses, J.P., Fernandes, F.D., Duarte, F.V., Palmeira, C.M., Tomé, A.R., Agostinho, P., Andrade, G.M., Cunha, R.A., 2019. Enhanced ATP release and CD73-mediated adenosine formation sustain adenosine A_{2A} receptor over-activation in a rat model of Parkinson's disease. Br. J. Pharmacol. 176, 3666–3680.

Cho, I.H., Kim, S.W., Kim, J.B., Kim, T.K., Lee, K.W., Han, P.L., Lee, J.K., 2006. Ethyl pyruvate attenuates kainic acid-induced neuronal cell death in the mouse hippocampus. J. Neurosci. Res. 84, 1505–1511.

Cunha, R.A., 2001. Regulation of the ecto-nucleotidase pathway in rat hippocampal nerve terminals. Neurochem. Res. 26, 979–991.

Cunha, R.A., 2008. Different cellular sources and different roles of adenosine: A_1 receptor-mediated inhibition through astrocytic-driven volume transmission and synapse-restricted A_{2A} receptor-mediated facilitation of plasticity. Neurochem. Int. 52, 65–72.

Cunha, R.A., 2016. How does adenosine control neuronal dysfunction and neurodegeneration? J. Neurochem. 139, 1019–1055.

Cunha, R.A., Sebastião, A.M., Ribeiro, J.A., 1992. Ecto-5′-nucleotidase is associated with cholinergic nerve terminals in the hippocampus but not in the cerebral cortex of the rat. J. Neurochem. 59, 657–666.

Cunha, R.A., Vizi, E.S., Ribeiro, J.A., Sebastião, A.M., 1996. Preferential release of ATP and its extracellular catabolism as a source of adenosine upon high- but not low-frequency stimulation of rat hippocampal slices. J. Neurochem. 67, 2180–2187.

Dias, L., Lopes, C.R., Gonçalves, F.Q., Nunes, A., Pochmann, D., Machado, N.J., Tomé, A. R., Agostinho, P., Cunha, R.A., 2021. Crosstalk between ATP-P2X7 and adenosine A_{2A} receptors controlling neuroinflammation in rats subject to repeated restraint stress. Front. Cell. Neurosci. 15, 639322.

Duman, R.S., Aghajanian, G.K., 2012. Synaptic dysfunction in depression: potential therapeutic targets. Science 338, 68–72.

Engel, T., Alves, M., Sheedy, C., Henshall, D.C., 2016. ATPergic signalling during seizures and epilepsy. Neuropharmacology 104, 140–153.

Espinosa, J., Rocha, A., Nunes, F., Costa, M.S., Schein, V., Kazlauckas, V., Kalinine, E., Souza, D.O., Cunha, R.A., Porciúncula, L.O., 2013. Caffeine consumption prevents memory impairment, neuronal damage, and adenosine A_{2A} receptors upregulation in the hippocampus of a rat model of sporadic dementia. J. Alzheimers Dis. 34, 509–518.

Fausther, M., Lecka, J., Soliman, E., Kauffenstein, G., Pelletier, J., Sheung, N., Dranoff, J. A., Sévigny, J., 2012. Coexpression of ecto-5′-nucleotidase/CD73 with specific NTPDases differentially regulates adenosine formation in the rat liver. Am. J. Phys. 302, G447–G459.

Fredholm, B.B., Chen, J.F., Cunha, R.A., Svenningsson, P., Vaugeois, J.M., 2005. Adenosine and brain function. Int. Rev. Neurobiol. 63, 191–270.

Gonçalves, F.Q., Lopes, J.P., Silva, H.B., Lemos, C., Silva, A.C., Gonçalves, N., Tomé, Â. R., Ferreira, S.G., Canas, P.M., Rial, D., Agostinho, P., Cunha, R.A., 2019. Synaptic and memory dysfunction in a β-amyloid model of early Alzheimer's disease depends on increased formation of ATP-derived extracellular adenosine. Neurobiol. Dis. 132, 104570.

Gonçalves, F.Q., Pires, J., Pliássova, A., Beleza, R., Marques, J.M., Rodrigues, R.J., Canas, P.M., Cunha, R.A., Rial, D., 2015. Adenosine A_{2B} receptors control A_1 receptor-mediated inhibition of synaptic transmission in the mouse hippocampus. Eur. J. Neurosci. 41, 876–886.

Gouder, N., Scheurer, L., Fritschy, J.M., Boison, D., 2004. Overexpression of adenosine kinase in epileptic hippocampus contributes to epileptogenesis. J. Neurosci. 24, 692–701.

Jimenez-Mateos, E.M., Arribas-Blazquez, M., Sanz-Rodriguez, A., Concannon, C., Olivos-Ore, L.A., Reschke, C.R., Mooney, C.M., Mooney, C., Lugara, E., Morgan, J.,

Langa, E., Jimenez-Pacheco, A., Silva, L.F., Mesuret, G., Boison, D., Miras-Portugal, M.T., Letavic, M., Artalejo, A.R., Bhattacharya, A., Diaz-Hernandez, M., Henshall, D.C., Engel, T., 2015. microRNA targeting of the P2X7 purinoceptor opposes a contralateral epileptogenic focus in the hippocampus. Sci. Rep. 5, 17486.

Jimenez-Pacheco, A., Diaz-Hernandez, M., Arribas-Blázquez, M., Sanz-Rodriguez, A., Olivos-Oré, L.A., Artalejo, A.R., Alves, M., Letavic, M., Miras-Portugal, M.T., Conroy, R.M., Delanty, N., Farrell, M.A., O'Brien, D.F., Bhattacharya, A., Engel, T., Henshall, D.C., 2016. Transient P2X7 receptor antagonism produces lasting reductions in spontaneous seizures and gliosis in experimental temporal lobe epilepsy. J. Neurosci. 36, 5920–5932.

Kaster, M.P., Machado, N.J., Silva, H.B., Nunes, A., Ardais, A.P., Santana, M., Baqi, Y., Müller, C.E., Rodrigues, A.L., Porciúncula, L.O., Chen, J.F., Tomé, Â.R., Agostinho, P., Canas, P.M., Cunha, R.A., 2015. Caffeine acts through neuronal adenosine A_{2A} receptors to prevent mood and memory dysfunction triggered by chronic stress. Proc. Natl. Acad. Sci. U. S. A. 112, 7833–7838.

Kulesskaya, N., Võikar, V., Peltola, M., Yegutkin, G.G., Salmi, M., Jalkanen, S., Rauvala, H., 2013. CD73 is a major regulator of adenosinergic signalling in mouse brain. PLoS One 8, e66896.

Lepeta, K., Lourenco, M.V., Schweitzer, B.C., Martino Adami, P.V., Banerjee, P., Catuara-Solarz, S., de La Fuente, Revenga M., Guillem, A.M., Haidar, M., Ijomone, O.M., Nadorp, B., Qi, L., Perera, N.D., Refsgaard, L.K., Reid, K.M., Sabbar, M., Sahoo, A., Schaefer, N., Sheean, R.K., Suska, A., Verma, R., Vicidomini, C., Wright, D., Zhang, X.D., Seidenbecher, C., 2016. Synaptopathies: synaptic dysfunction in neurological disorders - a review from students to students. J. Neurochem. 138, 785–805.

Lovatt, D., Xu, Q., Liu, W., Takano, T., Smith, N.A., Schnermann, J., Tieu, K., Nedergaard, M., 2012. Neuronal adenosine release, and not astrocytic ATP release, mediates feedback inhibition of excitatory activity. Proc. Natl. Acad. Sci. U. S. A. 109, 6265–6270.

Lynch, M.A., 2004. Long-term potentiation and memory. Physiol. Rev. 84, 87–136.

Martin, S.J., Grimwood, P.D., Morris, R.G., 2000. Synaptic plasticity and memory: an evaluation of the hypothesis. Annu. Rev. Neurosci. 23, 649–711.

Matos, M., Augusto, E., Santos-Rodrigues, A.D., Schwarzschild, M.A., Chen, J.F., Cunha, R.A., Agostinho, P., 2012. Adenosine A_{2A} receptors modulate glutamate uptake in cultured astrocytes and gliosomes. Glia 60, 702–716.

McMahon, H.T., Nicholls, D.G., 1990. The relationship between cytoplasmic free Ca^{2+} and the release of glutamate from synaptosomes. Biochem. Soc. Trans. 18, 375–377.

Nadler, J.V., Perry, B.W., Gentry, C., Cotman, C.W., 1980. Degeneration of hippocampal CA3 pyramidal cells induced by intraventricular kainic acid. J. Comp. Neurol. 192, 333–359.

Racine, R.J., 1972. Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr. Clin. Neurophysiol. 32, 281–294.

Raiteri, L., Zappettini, S., Milanese, M., Fedele, E., Raiteri, M., Bonanno, G., 2007. Mechanisms of glutamate release elicited in rat cerebrocortical nerve endings by 'pathologically' elevated extraterminal K^+ concentrations. J. Neurochem. 103, 952–961.

Rebola, N., Coelho, J.E., Costenla, A.R., Lopes, L.V., Parada, A., Oliveira, C.R., Soares-da-Silva, P., de Mendonça, A., Cunha, R.A., 2003. Decrease of adenosine A_1 receptor density and of adenosine neuromodulation in the hippocampus of kindled rats. Eur. J. Neurosci. 18, 820–828.

Rebola, N., Porciúncula, L.O., Lopes, L.V., Oliveira, C.R., Soares-da-Silva, P., Cunha, R.A., 2005. Long-term effect of convulsive behavior on the density of adenosine A_1 and A_{2A} receptors in the rat cerebral cortex. Epilepsia 46 (Suppl. 5), 159–165.

Rebola, N., Luján, R., Cunha, R.A., Mulle, C., 2008. Adenosine A_{2A} receptors are essential for long-term potentiation of NMDA-EPSCs at hippocampal mossy fiber synapses. Neuron 57, 121–134.

Rodrigues, R.J., Tomé, A.R., Cunha, R.A., 2015. ATP as a multi-target danger signal in the brain. Front. Neurosci. 9, 148.

Scharfman, H.E., MacLusky, N.J., 2014. Sex differences in the neurobiology of epilepsy: a preclinical perspective. Neurobiol. Dis. 72, 180–192.

Schauwecker, P.E., Steward, O., 1997. Genetic determinants of susceptibility to excitotoxic cell death: implications for gene targeting approaches. Proc. Natl. Acad. Sci. U. S. A. 94, 4103–4108.

Schirinzi, T., Madeo, G., Martella, G., Maltese, M., Picconi, B., Calabresi, P., Pisani, A., 2016. Early synaptic dysfunction in Parkinson's disease: insights from animal models. Mov. Disord. 31, 802–813.

Selkoe, D.J., 2002. Alzheimer's disease is a synaptic failure. Science 298, 789–791.

Silva, C.G., Métin, C., Fazeli, W., Machado, N.J., Darmopil, S., Launay, P.S., Ghestem, A., Nesa, M.P., Bassot, E., Szabó, E., Baqi, Y., Müller, C.E., Tomé, A.R., Ivanov, A., Isbrandt, D., Zilberter, Y., Cunha, R.A., Esclapez, M., Bernard, C., 2013. Adenosine receptor antagonists including caffeine alter fetal brain development in mice. Sci. Transl. Med. 5, 197ra104.

Sperk, G., Lassmann, H., Baran, H., Kish, S.J., Seitelberger, F., Hornykiewicz, O., 1983. Kainic acid induced seizures: neurochemical and histopathological changes. Neuroscience 10, 1301–1315.

Stigliani, S., Zappettini, S., Raiteri, L., Passalacqua, M., Melloni, E., Venturi, C., Tacchetti, C., Diaspro, A., Usai, C., Bonanno, G., 2006. Glia re-sealed particles freshly prepared from adult rat brain are competent for exocytotic release of glutamate. J. Neurochem. 96, 656–668.

Temido-Ferreira, M., Ferreira, D.G., Batalha, V.L., Marques-Morgado, I., Coelho, J.E., Pereira, P., Gomes, R., Pinto, A., Carvalho, S., Canas, P.M., Cuvelier, L., Buée-Scherrer, V., Faivre, E., Baqi, Y., Müller, C.E., Pimentel, J., Schiffmann, S.N., Buée, L., Bader, M., Outeiro, T.F., Blum, D., Cunha, R.A., Marie, H., Pousinha, P.A., Lopes, L.V., 2020. Age-related shift in LTD is dependent on neuronal adenosine A_{2A} receptors interplay with mGluR5 and NMDA receptors. Mol. Psychiatry 25, 1876–1900.

Tescarollo, F.C., Rombo, D.M., DeLiberto, L.K., Fedele, D.E., Alharfoush, E., Tomé, Â.R., Cunha, R.A., Sebastião, A.M., Boison, D., 2020. Role of adenosine in epilepsy and seizures. J. Caffeine Adenosine Res. 10, 45–60.

Upadhya, D., Kodali, M., Gitai, D., Castro, O.W., Zanirati, G., Upadhya, R., Attaluri, S., Mitra, E., Shuai, B., Hattiangady, B., Shetty, A.K., 2019. A model of chronic temporal lobe epilepsy presenting constantly rhythmic and robust spontaneous seizures, co-morbidities and hippocampal neuropathology. Aging Dis. 10, 915–936.

Wieraszko, A., Goldsmith, G., Seyfried, T.N., 1989. Stimulation-dependent release of adenosine triphosphate from hippocampal slices. Brain Res. 485, 244–250.

Zaczek, R., Simonton, S., Coyle, J.T., 1980. Local and distant neuronal degeneration following intrastriatal injection of kainic acid. J. Neuropathol. Exp. Neurol. 39, 245–264.

Zimmermann, H., Zebisch, M., Strater, N., 2012. Cellular function and molecular structure of ecto-nucleotidases. Purinergic Signal. 8, 437–502.