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Corresponding Author	Family Name	**Bustamante**
	Particle	
	Given Name	**J.**
	Suffix	
	Division	Laboratory of Free Radical Biology, School of Pharmacy and Biochemistry
	Organization	University of Buenos Aires
	Address	Junin 956, C1113AAD, Buenos Aires, Argentina
	Email	juanitab@ffyb.uba.ar
Author	Family Name	**Lores-Arnaiz**
	Particle	
	Given Name	**S.**
	Suffix	
	Division	Laboratory of Free Radical Biology, School of Pharmacy and Biochemistry
	Organization	University of Buenos Aires
	Address	Junin 956, C1113AAD, Buenos Aires, Argentina
	Email	
Author	Family Name	**Tallis**
	Particle	
	Given Name	**S.**
	Suffix	
	Division	Laboratory of Portal Hypertension, School of Pharmacy and Biochemistry
	Organization	University of Buenos Aires
	Address	Junin 956, C1113AAD, Buenos Aires, Argentina
	Email	
Author	Family Name	**Roselló**
	Particle	
	Given Name	**D. M.**
	Suffix	
	Division	Laboratory of Portal Hypertension, School of Pharmacy and Biochemistry
	Organization	University of Buenos Aires
	Address	Junin 956, C1113AAD, Buenos Aires, Argentina
	Email	
Author	Family Name	**Lago**
	Particle	
	Given Name	**N.**
	Suffix	

	Division	Pathology Centre, Pathology Department, School of Medicine
	Organization	University of Buenos Aires
	Address	J Uriburu 950, C1114AAD, Buenos Aires, Argentina
	Email	
Author	Family Name	**Lemberg**
	Particle	
	Given Name	**A.**
	Suffix	
	Division	Laboratory of Portal Hypertension, School of Pharmacy and Biochemistry
	Organization	University of Buenos Aires
	Address	Junin 956, C1113AAD, Buenos Aires, Argentina
	Email	
Author	Family Name	**Boveris**
	Particle	
	Given Name	**A.**
	Suffix	
	Division	Laboratory of Free Radical Biology, School of Pharmacy and Biochemistry
	Organization	University of Buenos Aires
	Address	Junin 956, C1113AAD, Buenos Aires, Argentina
	Email	
Author	Family Name	**Perazzo**
	Particle	
	Given Name	**J. C.**
	Suffix	
	Division	Laboratory of Portal Hypertension, School of Pharmacy and Biochemistry
	Organization	University of Buenos Aires
	Address	Junin 956, C1113AAD, Buenos Aires, Argentina
	Email	

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Abstract

In this study, we describe the presence of apoptosis, associated with a mitochondrial dysfunction in the hippocampus of animals in an experimental model defined as minimal hepatic encephalopathy (MHE). This experimental model was studied after 10 days of induced portal vein calibrated stricture, leading to portal hypertension and to a moderate hyperammonemia, without the presence of other evident central nervous system changes. The molecular mechanisms here proposed indicate the presence of apoptotic intrinsic pathways that point to hippocampal mitochondria as an important mediator of apoptosis in this experimental model. In this model of MHE, the presence of DNA fragmentation is documented by 2.3-times increased number of TUNEL-positive cells. These findings together with a higher ratio of the Bcl-2 family members Bax/Bcl-xL in the outer mitochondrial membrane of the MHE animals together with 11% of cytochrome c release indicate the presence of apoptosis in this experimental model. A detailed analysis of the hippocampal mitochondrial physiology was performed after mitochondrial isolation. The determination of the respiratory rate in the presence of malate plus glutamate and ADP showed a 45% decrease in respiratory control in MHE animals as compared with the sham group. A marked decrease of cytochrome oxidase (complex IV of the electron transport chain) was also observed, showing 46% less activity in hippocampal mitochondria from MHE animals. In addition, mitochondria from these animals showed less ability to maintain membrane potential ($\Delta \Psi_m$) which was 13% lower than the sham group. Light scattering experiments showed that mitochondria from MHE animals were more sensitive to swell in the presence of increased calcium concentrations as compared with the sham group. In addition, in vitro studies performed in mitochondria from

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Mitochondrial dysfunction as a mediator of hippocampal apoptosis in a model of hepatic encephalopathy

J. Bustamante · S. Lores-Arnaiz · S. Tallis · D. M. Roselló · N. Lago · A. Lemberg · A. Boveris · J. C. Perazzo

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Abstract In this study, we describe the presence of apoptosis, associated with a mitochondrial dysfunction in the hippocampus of animals in an experimental model defined as minimal hepatic encephalopathy (MHE). This experimental model was studied after 10 days of induced portal vein calibrated stricture, leading to portal hypertension and to a moderate hyperammonemia, without the presence of other evident central nervous system changes. The molecular mechanisms here proposed indicate the presence of apoptotic intrinsic pathways that point to hippocampal mitochondria as an important mediator of apoptosis in this experimental model. In this model of MHE, the presence of DNA fragmentation is documented by 2.3-times increased number of TUNEL-positive cells. These findings together with a higher ratio of the Bcl-2 family members Bax/Bcl-xL in the outer mitochondrial membrane of the MHE animals together with 11% of cytochrome c release indicate the presence of apoptosis in this experimental model. A detailed analysis of the hippocampal mitochondrial physiology was performed after mitochondrial isolation. The determination of the respiratory rate in the presence of malate plus glutamate and ADP showed a 45% decrease in respiratory control in MHE animals as compared with the sham group. A marked decrease of cytochrome oxidase (complex IV of the electron transport chain) was also observed, showing 46% less activity in hippocampal mitochondria from MHE animals. In addition, mitochondria from these animals showed less ability to maintain membrane potential ($\Delta\Psi_m$) which was 13% lower than the sham group. Light scattering experiments showed that mitochondria from MHE animals were more sensitive to swell in the presence of increased calcium concentrations as compared with the sham group. In addition, in vitro studies performed in mitochondria from sham animals showed that mitochondrial permeability transition (MPT) could be a mitochondrial mediator of the apoptotic signaling in the presence of NH_4^+ and calcium.

Keywords Hepatic encephalopathy · Hippocampal apoptosis · Hippocampal mitochondrial dysfunction

Abbreviations

ADP	Adenosine diphosphate
Ca^{2+}	Calcium
CsA	Cyclosporine A
DiOC6	3,3′dihexyloxocarbocyanine iodide
FCCP	Carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone
FL-1	Green fluorescence
HE	Hepatic encephalopathy
H_2O_2	Hydrogen peroxide
MHE	Minimal hepatic encephalopathy
MSH	Mannitol–sacarose–Hepes
MPT	Mitochondrial permeability transition
NH_4^+	Ammonia
NO	Nitric oxide

J. Bustamante (✉) · S. Lores-Arnaiz · A. Boveris
Laboratory of Free Radical Biology, School of Pharmacy and Biochemistry, University of Buenos Aires, Junin 956, C1113AAD Buenos Aires, Argentina
e-mail: juanitab@ffyb.uba.ar

S. Tallis · D. M. Roselló · A. Lemberg · J. C. Perazzo
Laboratory of Portal Hypertension, School of Pharmacy and Biochemistry, University of Buenos Aires, Junin 956, C1113AAD Buenos Aires, Argentina

N. Lago
Pathology Centre, Pathology Department, School of Medicine, University of Buenos Aires, J Uriburu 950, C1114AAD Buenos Aires, Argentina

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L-NNA	*N*-nitro-L-arginine
OTC	Ornitine transcarbamilase
PVS	Portal vein stricture
RCR	Respiratory control rate

Introduction

Previous studies have described that after portal vein stricture, rats developed moderated hyperammonemia, prehepatic portal hypertension (PPH) associated with morphological and functional damage of hippocampal mitochondria [1–3]. The main reason of increased ammonium is a reduced capacity of hepatic synthesis of urea and glutamate by which the normal liver removes ammonia (NH_4^+) from the portal blood [4]. Hepatic encephalopathy (HE) is a severe complication of acute and chronic liver disease with symptoms and signs ranging from subtle mental disturbances to coma. Although the responsible mechanisms of HE remain elusive, and the molecular mechanisms present in the different models of NH_4^+ toxicity are not clear enough, the neurotoxin NH_4^+ is generally considered as the main molecule involved in the HE pathogenesis [5–7]. Although the developing brain is more susceptible to the deleterious effects of NH_4^+ toxicity, we can not discard that the magnitude and duration of the increased plasma NH_4^+ levels in adult brain are an important and dangerous condition that can be associated with neuronal or astrocytic cell death.

In neurons and astrocytes, as in almost all mammalian cells, two distinct but ultimately converging signaling events termed extrinsic and intrinsic pathways mediate the molecular mechanisms of apoptosis [8]. Intrinsic signals such as neuronal growth factor deprivation, stress, or the presence of cytotoxic drugs or metabolites can initiate the apoptotic pathways. These stimuli can activate different cell receptors inducing different pathways such as alteration in calcium homeostasis which, latter on, interact with the pro-apoptotic members of the Bcl-2 family, leading to alteration of the pro and anti apoptotic members content in the cell membranes changing the cytosolic Bax pool [9] that immediately associate to the outer mitochondrial membrane. The presence of this pro-apoptotic member at the surface of the outer mitochondrial membrane alters mitochondrial physiology inducing increased membrane permeability; in fact mitochondrial permeability transition (MPT) has been closely associated with different signaling pathways of cell death [10].

This study was carried out in an experimental model of portal calibrated stricture operation, called Minimal Hepatic Encephalopathy (MHE), in which the animals were sacrificed 10 days after surgery. The purpose of this study was to evaluate if in this experimental model the increased portal blood pressure and the slight increase in plasma ammonia levels could be associated with the presence of apoptotic signaling and if mitochondrial dysfunction could be a mediator of this cell death pathway in hippocampal cells.

Materials and methods

Materials

Ethylenediaminetetraacetic acid, proteinase K, and Folin reagent were obtained from Sigma Chemical Co. (Saint Louis, MO, USA). Other reagents were of analytical grade. Antibodies, against Bax (N-20) sc-493, Bcl-xL (S-18) sc-634, and cytochrome c (H-104 sc7159) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Terminal deoxynucleotidyl transferase (TUNEL enzyme) and Nucleotide mix, containing fluorescein-dUTP and dNTP (TUNEL label) were obtained from Boehringer Mannheim Biochemical (Indianapolis IN, USA).

Animal model

Male Wistar Kyoto rats (240–270 g) housed in a humidity and temperature-controlled environment with an automatic 12:12-h light–dark cycle and fed standard rat chow and tap water ad libitum were divided at random into two groups of 12 rats each: SHAM and MHE. MHE rat model was induced by performing a calibrated stenosis of the portal vein [1]. In brief, rats were anaesthetized with ether; a midline abdominal incision was made, a 20-gauge blunt-end needle was placed alongside the portal vein, and a 3–0 silk ligature was placed around the vein and snugly tied. The needle was subsequently removed to yield a calibrated stenosis of the portal vein. Sham-operated rats underwent identical procedure except for the portal vein, which was exposed but not stenosed. The stricture of portal vein diminished the lumen vein in around the 66% of the vessel area under these experimental conditions [11].

Animal handling was in accordance with the American Physiological Society "Guiding Principles in the Care and Use of Animals" and with the 6344/96 regulation of Argentine National Drug Food and Medical Technology Administration (ANMAT). The animal room facilities were provided by the Animal House of the School of Pharmacy and Biochemistry.

Plasma pressure and plasma ammonia determination

10 days after portal vein stenosis, rats were anaesthetized with sodium pentobarbital (40 mg/Kg, i.p). To measure

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portal pressure, a midline abdominal incision was made, the spleen was exposed, and a needle was placed in the splenic pulp, fixed with cyanoacrilate glue, and connected to a pressure transducer through a polyethylene cannula (PE 50) filled with heparinized saline solution (25 U/ml). The needle was connected to a Statham Gould P23ID pressure transducer (Statham, Hato Rey, Puerto Rico) coupled to a Grass 79D polygraph (Grass Instrument, Quincy, MA, USA) for the measurement of portal pressure. Plasma ammonia concentrations were determined using "Ammoniac Enzymatique U.V. kits", Biomerieux (France).

Terminal dUTP nick end labeling (TUNEL)

10 days after surgery, rats were deeply anaesthetized with sodium pentobarbital (i.p); brains were quickly removed, washed in physiological solution, and the hippocampus was dissected according to the surgical procedure previously described [12]. Hippocampal tissue was fixed in 3% glutaraldehyde in cacodylate buffer 0.1 M (pH 7.4) and paraffin-embedded. Nuclear DNA fragmentation was determined by the in situ nick end labeling (TUNEL) assay in thin hippocampal tissue sections (40 μm) by a modification of the initially described method [13]. Dewaxed and rehydrated samples were washed with distilled water and PBS twice and incubated with proteinase K (20 μg/ml in Tris–HCl 10 mM pH 7.4) during 30 min at 37°C. After rinsed twice with PBS, ice maintained samples were permeabilized with 0.1% Triton X-100, in 0.1% sodium citrate during 2 min, and incubated with 100 μl of a reaction mixture [10 μl of TDT buffer 10×, 300 mM Tris, pH 7.2 with sodium cacodylate 1.4 M, 10 mM $CoCl_2$, 5 μl distilled H_2O, 5 μl of TUNEL enzyme (Terminal deoxynucleotidyl transferase), and 55 μl of TUNEL label (fluorescein-dUTP and dNTP)] during 60 min at 37°C. Negative controls for TUNEL staining were performed by omitting terminal deoxynucleotidyl-transferase from the labeling mixture and the primary antibody, respectively, which resulted in no specific labeling.

Number of TUNEL-positive fluorescent foci were analyzed and counted, within the whole hippocampal sections. Four animals per group and at least five sections from each individual, from the two groups of animals (sham and MHE), were analyzed for quantification.

Isolation of rat hippocampal mitochondrial fraction

Brains from the two groups of animals ($n = 12$ each) were extracted, and the hippocampal region (100-150 mg each) was dissected as described. Tissues were suspended in MSTE (0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA, and 10 mM Tris–HCl, pH 7.2), homogenized 1:5 (w/v) in the presence of complete protease inhibitors (1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.4 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin), and centrifuged at 600 and 8000*g* for 10 min. The resulting pellet was washed and resuspended in the same buffer, and contained both synaptic and non-synaptic mitochondria, corresponding to hippocampal mitochondria from neurons and glia. These mitochondria were able to carry out oxidative phosphorylation. Submitochondrial membranes were obtained from mitochondria after three cycles of freezing and thawing and homogenizing the suspension by passing it through a 15/10 hypodermic needle [3]. Protein content was assayed by using Folin phenol reagent, and bovine serum albumin was used as standard. All isolation procedures were performed at 0–4°C.

Mitochondrial respiration

Oxygen consumption of the total isolated hippocampal mitochondria was measured with a high-resolution respirometer (Oroboros Oxygraph, Paar KG, Graz, Austria). Hippocampal mitochondria (0.5–1 mg protein/ml) were incubated in a reaction medium consisting of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl, 5 mM malate plus glutamate, 5 mM KH_2PO_4, 4 mM $MgCl_2$ (pH 7.4), and 0.2% bovine serum albumin at 30°C. State 3 was set by the addition of 1 mM ADP, and the respiratory control ratio (RCR) was calculated from the ratio of the states 3/4 respiratory rates [14, 15]. The mitochondrial fraction obtained from the brain hippocampal tissue showed a respiratory control rate (RCR) between 4.0 and 7.0 ($n = 5$) determined with malate plus glutamate as substrates [3].

Evaluation of mitochondrial respiratory complexes I–III, II–III, and IV

NADH-cytochrome c reductase activity (complex I–III) was measured in hippocampal submitochondrial membranes by following spectrophotometrically the reduction of cytochrome c at 550 nm ($\varepsilon = 19.6\ mM^{-1}\ cm^{-1}$) in a reaction medium containing 100 mM phosphate buffer (pH 7.4), 0.2 mM NADH, 0.1 mM cytochrome c, and 0.5 mM KCN at 30°C. Enzyme activity was expressed in nmoles cytochrome c reduced per min per milligram of protein. Succinate cytochrome c reductase activity (complex II + III) was similarly determined and expressed, except that NADH was substituted by 20 mM succinate. Cytochrome oxidase activity (complex IV) was assayed spectrophotometrically at 550 nm by following the rate of oxidation of 50 μM ferrocytochrome c [16]. The activity was expressed as nmoles cytochrome c oxidized per min per milligram of protein.

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Western blotting and chemiluminescence

Bax, Bcl-xL association to the mitochondrial fractions and cytochrome c content were determined in hippocampal mitochondrial fractions as described: equal total protein amount of purified hippocampal mitochondria from sham and MHE animals was separated by SDS-PAGE (12%), blotted into a nitrocellulose membrane (Bio-Rad, München, Germany), and probed against rabbit polyclonal Bax antibodies (dilution 1:500), against rabbit polyclonal Bcl-xL antibodies (dilution 1:500), and against rabbit polyclonal cytochrome c antibodies (dilution 1:500). Then, the nitrocellulose membrane was incubated with a secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (dilution 1:5000), followed by development of chemiluminescence with the ECL reagent for 2–4 min [17]. Relative concentration was assessed by densitometric analysis of digitized autographic images, using the NIH Image J Program. The ratio Bax/Bcl-xL was calculated, and cytochrome c content remaining in the mitochondrial fractions of sham and MHE was also indicated.

Determination of MPT by transmembrane potential and mitochondrial swelling analysis

The two main characteristics of this mitochondrial condition (MPT), loss of transmembrane potential, and swelling were evaluated. For estimation of the mitochondrial membrane potential, isolated rat hippocampal mitochondria (25 µg/ml) from sham and MHE groups of animals were incubated at 37°C for 20 min in MSH buffer supplemented with 5 mM malate, 5 mM glutamate, and 1 mM phosphate in the presence of 30 nM of the potentiometric probe DiOC6 [18, 19]. The fluorescence changes were determined by cytometric measurement of FL-1 DiOC6 fluorescence. Samples were protected from light until acquired by the cytometer. Fresh mitochondria were prepared for each experiment, and were used within 4 h. Auto fluorescence of the selected mitochondrial population, without probe, was measured, and the protonophore FCCP (0.5 µM) was used as a depolarizing agent (positive control). A common marker, indicating the relative fluorescence intensity of the mitochondrial population, was used to quantify the resulting changes in membrane potential from three different experiments.

Swelling was evaluated in fresh isolated rat hippocampal mitochondria from sham and MHE groups of animals (250 µg/ml) after incubation in MSH buffer with 5 mM malate, 5 mM glutamate, 1 mM phosphate, and 2 mM $MgCl_2$. Hippocampus mitochondrial swelling was monitored as a decrease in absorbance at 540 nm ($\Delta A_{540\ nm}$/min mg protein) at 30°C after addition of 100 µM [Ca^{2+}]. Swelling was obtained by drawing a tangent to the plot of absorbance/time at its steepest point [9, 18]. Maximal mitochondrial swelling was achieved after adding the 20-residue channel-forming peptide alamethicin [19–21].

Effect of NH_4^+ on MPT

The effect of ammonia in vitro was studied in isolated mitochondria from sham rats. As described before, MPT was studied by swelling and loss of transmembrane potential after ammonia and calcium pretreatment. $\Delta\Psi_m$ was evaluated in three different conditions: 1 mM NH_4Cl, 50 μM Ca^{2+}, and 50 μM Ca^{2+} and 1 mM NH_4Cl. Mitochondria (25 µg/ml) were incubated in MSH buffer supplemented with 5 mM malate, 5 mM glutamate, and 1 mM phosphate. After 2 min preincubation in the described conditions (1 mM NH_4Cl, 50 μM Ca^{2+}, and 50 μM Ca^{2+} and 1 mM NH_4Cl), mitochondria were loaded with 30 nM DiOC6, and $\Delta\Psi_m$ was determined as described in the preceding section. Hippocampus mitochondrial swelling was monitored as a decrease in absorbance at 540 nm ($\Delta A_{540\ nm}$/min mg protein) at 30°C as described before. Pretreatment with 1 μM CsA before Ca^{2+} and NH_4Cl was used to verify the involvement of MPT [21, 22].

Statistics

Values in Tables and figures are mean values ± SEM. The Western blotting experiments were typical results of three different experiments. At least three independent experiments for each experimental condition of MPT and mitochondrial membrane potential determination were performed. Results were compared using an unpaired Student *t*-test.

Results

Portal blood pressure was significantly increased in the MHE group (12 ± 1 mm Hg) after 10 days of portal vein stricture as compared with values in the sham group (9.0 ± 1.5 mm Hg). Similarly, plasma ammonia concentration in MHE rats was 2.5 times increased being 55.6 ± 7.5 µM as compared with the sham group (22 ± 1 µM).

Moderated hyperammonemia induces apoptosis in hippocampal tissue

The presence of apoptosis was investigated through the presence of nuclear DNA fragmentation in hippocampal tissue sections (40 µm), from MHE 10 days after operation. Hippocampal apoptosis was visualized by the terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP

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nick end labeling assay as described previously. As demonstrated in Fig. 1a, in the sham-operated group of animals almost none TUNEL-positive focus were observed in the hippocampus. In contrast (Fig. 1b), clear green fluorescent foci indicate the presence of apoptosis in hippocampal tissue. The analysis for quantification was performed by counting the number of total fluorescent foci (TUNEL-positive cells) in the whole hippocampal sections from MHE and sham animals (Fig. 1c.)

Protein expression of the pro-apoptotic Bax, the antiapoptotic Bcl-xL, and cytochrome c in hippocampal mitochondria from MHE and sham animals

An important intrinsic apoptotic signal consisted in the migration of the pro-apoptotic protein Bax from the cytosol to the outer mitochondrial membrane alterating the ratio between others antiapoptotic members of the Bcl-2 family, such as Bcl-xL. The presence of Bax association to the mitochondrial membranes and the Bcl-xL mitochondrial content was analyzed by western blot assays in the hippocampal mitochondrial fraction from MHE and sham animals. The results showed a clear increase in a 23 kDa protein reacting with antibodies directed against Bax and no change in a protein of less than 30 kDa that reacts with antibodies directed against the Bcl-2 family member Bcl-xL (Fig. 2a). Quantification was performed by densitometric analysis as the ratio of Bax/Bcl-xL being 4.3 times higher in MHE animals as compared with the sham group (Fig. 2b). In addition, the amount of cytochrome c content that remained in the mitochondrial fraction was evaluated in MHE and in sham animals, being only 15% of total cytochrome c released from mitochondria in MHE animals as compared with the sham group (Fig. 2c).

Respiratory function

Hippocampal mitochondrial oxygen uptake in states 4 and 3 was evaluated in isolated mitochondria from sham and MHE animals, and the results are shown in Table 1. A 45% decrease in state 3 respiratory rate was observed in MHE animals, as compared with the sham group of animals. A 40% decrease in the RCR was observed in hippocampal mitochondria from MHE animals as compared with that obtained in the sham group (Table 1), meanwhile no changes were observed in state 4.

Activity of the respiratory complex I–III and II–III

The analysis of the different components of the electron transport chain is described in Table 2. Hippocampal mitochondria from MHE animals showed 21% increased activity of respiratory complex I–III, similar activities of complex II–III and a marked decrease of 46% in Complex IV activity, as compared with sham animals.

MPT in hippocampal mitochondria from MHE animals

Loss of $\Delta\Psi_m$ and the presence of swelling were observed in hippocampal mitochondria from MHE animals. DiOC6 fluorescence histograms obtained in a typical experiment showed a clear $\Delta\Psi_m$ decrease in mitochondria from MHE animals (Fig. 3a). The quantification analysis of these

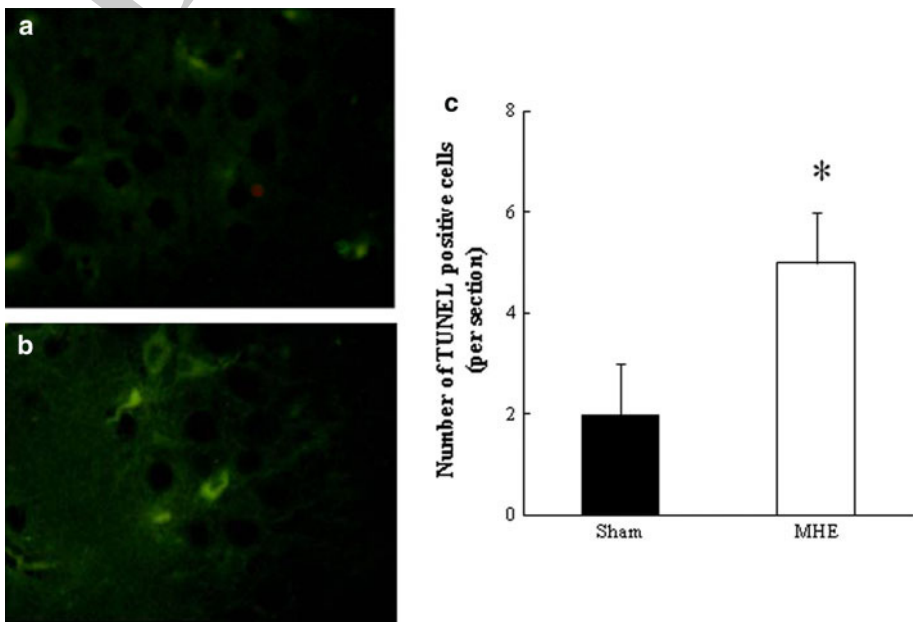

Fig. 1 **a–c** Terminal dUTP nick end labeling (TUNEL). **a** (20×), Hippocampal tissue from sham-operated rats without portal stenosis, showing very few TUNEL-positive cells. **b** (20×) Hippocampal tissue from MHE animals showing TUNEL-positive cells. **c** Quantification of the terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling assay. The number of total TUNEL-positive cells was performed by counting the total brightly *green* foci per section of the whole hippocampus (*$P < 0.05$) significantly different from control

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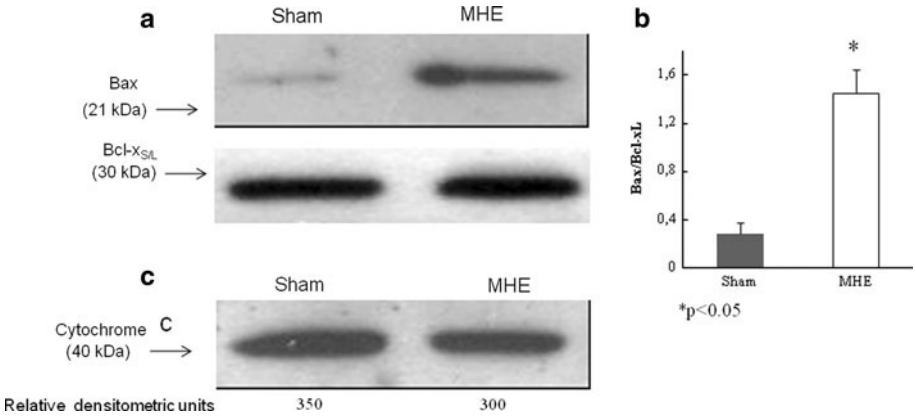

Fig. 2 **a–c** Hippocampal mitochondrial Bax association and Bax/Bcl-xL ratio in a model of MHE. Detection of Bax, Bcl-xL, and cytochrome c protein expression in hippocampal mitochondrial fraction was performed by Western blot assay. Equal amounts of protein were loaded for the assay. **a** Bands of Bax and Bcl-xL protein expression in Sham and MHE. **b** Ratio Bax/Bcl-xL. **c** Cytochrome c content in the mitochondrial membranes. Relative densitometric quantification of protein bands indicate significantly different from sham group (*$P < 0.01$)

Table 1 Respiratory rates of isolated, hippocampal mitochondria of sham and MHE animals

Oxygen uptake (ng-atom O/min mg protein)			
Condition	State 4	State 3	RC
Sham	6.1 ± 0.9	42 ± 2	6.9 ± 0.8
MHE	5.5 ± 0.8	21 ± 1*	3.8 ± 0.3*

* $P < 0.05$, significantly different from control

Table 2 Activity of respiratory complexes I–III, II–III, and IV from isolated hippocampal mitochondria from sham and MHE animals

	Complex I–III	Complex II–III (nmol cyt c/min mg protein)	Complex IV
Sham	160 ± 4	27 ± 3	71 ± 8
MHE	193 ± 2*	22 ± 2	38 ± 2*

* $P < 0.05$, significantly different from control

results showed a 13% less $\Delta\Psi_m$ in isolated hippocampal mitochondria from MHE animals as compared with sham rats (Fig. 3b). An important decrease in absorbance at 540 nm, in a typical swelling assay in the presence of 50 μM [Ca^{2+}] (which is not able to induce MPT in isolated control mitochondria from sham animals), was observed in hippocampal mitochondria from MHE animals, indicating a mitochondrial incapacity to tolerate an increase in calcium concentration in these animals, being more prone to induce swelling as compared with mitochondria from the sham group (Fig. 3c), Mitochondrial samples after alamethicin treatment showed a maximal decrease in absorbance (data not shown). The quantification analysis showed a 40% increase in mitochondrial swelling in MHE animals as compared with the sham group of animals (Fig. 3d).

In vitro effects of NH_4^+ on Ca^{2+}-induced MPT

The induction of MPT after NH_4^+ and Ca^{2+} incubation was analyzed in vitro in isolated hippocampal mitochondria from sham rats through studies of $\Delta\Psi_m$ and swelling. The $\Delta\Psi_m$ was evaluated in mitochondria from sham animals (Fig. 4) after 50 μM Ca^{2+}, 1 mM NH_4Cl, 50 μM Ca^{2+} and 1 mM NH_4Cl, and after CsA pretreatment; typical DiOC6 fluorescence histograms were obtained for the different conditions (Fig. 4a). Quantification of $\Delta\Psi_m$ results (Fig. 4b) showed 17% depolarization after 50 μM Ca^{2+} incubation and 19% depolarization after 1 mM NH_4Cl, indicating that 1 mM NH_4Cl has a similar ability to induce hippocampal mitochondria depolarization as 50 μM Ca^{2+}. However, when mitochondria were exposed simultaneously to agents, 50 μM Ca^{2+} and 1 mM NH_4Cl, a higher level of depolarization was observed (40%). Mitochondria incubated with 1 μM of the immunosuppressant CsA were able to maintain a good level of mitochondrial polarization. Mitochondrial treatment with the depolarizing agent, FCCP, showed 70% depolarization, as expected (Fig. 4a and b).

A typical swelling in vitro experiment was also performed with isolated mitochondria from sham rats, in the presence of 50 μM Ca^{2+}, 1 mM NH_4Cl, and 50 μM Ca^{2+} and 1 mM NH_4Cl as well as CsA pre-treatment before 50 μM Ca^{2+} and 1 mM NH_4Cl, as shown in Fig. 5a. Results quantification (Fig. 5b) showed a $\Delta A_{540\ nm}$ of 0.035 ± 0.005 for untreated mitochondrial samples, meanwhile after 50 μM Ca^{2+} and 1 mM NH_4Cl, a higher decrease in $\Delta A_{540\ nm}$ (0.080 ± 0.007) was observed,

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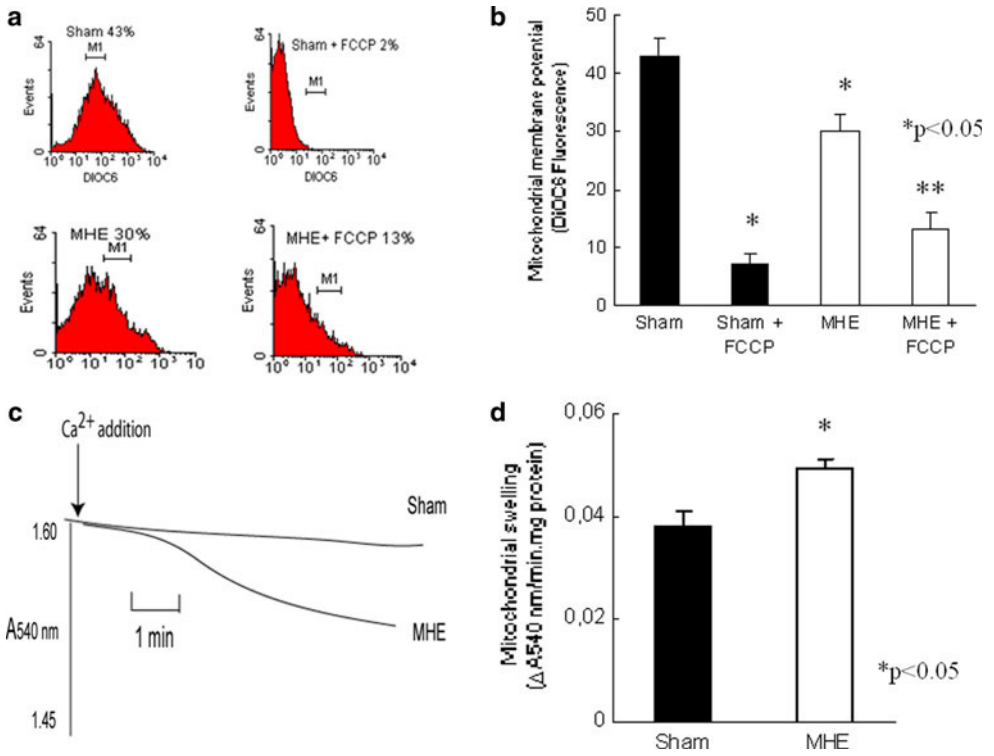

Fig. 3 **a–d** Spontaneous permeability transition evaluated by transmembrane potential and mitochondrial swelling. Isolated mitochondria (25 μg/ml) were loaded with 20 nM of the potentiometric probe DiOC6. Cytometric measurement of the FL-1 green fluorescence was expressed as histograms. **a** Transmembrane potential of Sham, MHE, Sham and FCCP, and MHE and FCCP. The inset auto fluorescence (unloaded mitochondria) was performed for loading control. **b** Quantification of the DiOC6 fluorescence histograms in three different experiments, ($*P < 0.05$) significantly different from Sham samples. **c** Typical swelling spectrophotometric assay of decrease in ΔAbs at 540 nm/min mg protein for Sham and MHE-isolated mitochondria. **d** Quantification of three different swelling experiments, ($*P < 0.05$) significantly different from Sham samples; ($**P < 0.05$) significantly different from MHE samples

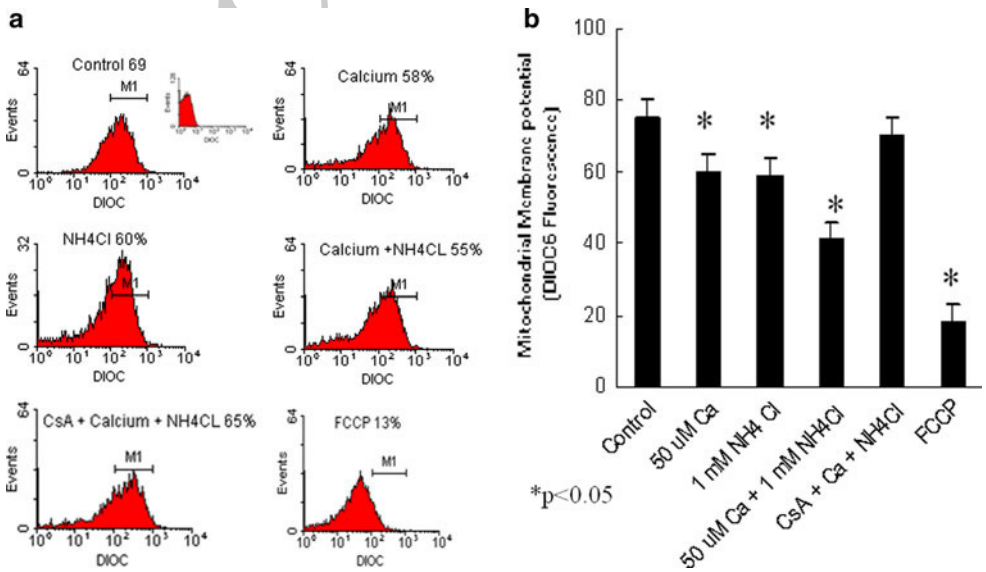

Fig. 4 **a–b** In vitro analysis of MPT: ammonium and calcium effect on mitochondrial transmembrane potential. MPT was evaluated in vitro by $\Delta\Psi_m$ in isolated mitochondria (25 μg/ml) from Sham animals. **a** Histograms of relative fluorescence intensity were registered after no addition: control, 50 μM Ca^{2+}, 1 mM NH_4Cl, 50 μM Ca^{2+} and 1 mM NH_4Cl, CsA pretreatment before 50 μM Ca^{2+} and 1 mM NH_4Cl, and FCCP as a positive control. **b** Quantification of the DiOC6 fluorescence histograms obtained in three different experiments. $*P < 0.05$ significantly different from sham animals

indicating that both agents, Ca^{2+} and NH_4Cl together, present stronger ability to induce mitochondrial swelling. Again, in these swelling studies, CsA pretreatment showed a significant difference indicating that this drug was able to protect against swelling induced by the presence of Ca^{2+} and NH_4Cl (Fig. 5b).

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Discussion

The main purpose of this study was to investigate if in the experimental model of MHE used in this study, 10 days after portal calibrated stricture, the presence of cell death via apoptosis, could be observed in hippocampal tissue and which could be the molecular mechanism involved. Mitochondria have been increasingly implicated as sensors and executioners of this process, determining the mode of cell death chosen by the different hippocampal cell populations. The two molecular mechanisms of cell death, apoptosis and necrosis, mainly depend on cellular energetic metabolism [23]. In this model, 2.5 times increased plasma ammonia levels were observed, resulting in a moderate hyperammonemia associated with a marked increase in portal blood pressure. Several studies point the NH_4^+ increase as the main cause responsible for the brain observed changes in different models of HE [24].

In this study, the results showed the presence of TUNEL-positive cells in the hippocampal tissue indicating the occurrence of apoptosis, in association with a clear mitochondrial dysfunction. In fact, mitochondrial respiration in the presence of malate plus glutamate clearly showed that the increase in plasma NH_4^+ levels could be one of the factors that definitely interferes with the electron transport system, leading to an impairment of the electron transport chain with a decrease in ADP dependent oxygen uptake and an important reduction (46%) in the activity of the last component of the respiratory chain, the cytochrome oxidase.

The BCL-2 family proteins are able to alterate the mitochondrial permeability by its association with different outer mitochondrial membrane proteins. t-Bid, a pro-apoptotic member, is able to close the porine VDAC, which may account for the VDAC inhibition observed during apoptosis [25]. Meanwhile, Bax association to the outer mitochondrial membrane results in the acceleration of the opening of the MPT pore, inducing the release of several proteins from the inter-membrane space, such as cytochrome c, AIF and Diabolo, to the cytosol [9]. Our results describing an increased mitochondrial Bax association and an increased Bax/Bcl-xL ratio in the mitochondrial membranes of MHE animals as compared with the sham group suggest that ammonia could be closely involved in the signaling pathways of apoptosis. These facts are in agreement with the observations that Bax is required for apoptosis and that Bax deletion permanently rescues developing cells from target-dependent cell death in different tissues [22, 26, 27]. In addition, Bax-ability to induce conformational changes, oligomerize with other Bcl-2 members, and form pores in the outer mitochondrial membrane inducing permeability changes and transmembrane potential ($\Delta\Psi_m$) collapse, has been observed in many apoptotic models [18, 28–31]. In fact, in this study the $\Delta\Psi_m$ collapse observed in MHE hippocampal mitochondria could be also related to the effect that ammonia and calcium exert on mitochondrial physiology. It is possible that ammonia and cytosolic calcium alterations themselves or by means of inducing intra and extra-cellular signals could alter the association of a set of different proteins with the mitochondria, which have the ability to modulate mitochondrial permeability leading to the induction of intrinsic pathways of apoptosis.

It is well known that the amount of cytochrome c release from mitochondria can vary depending on the

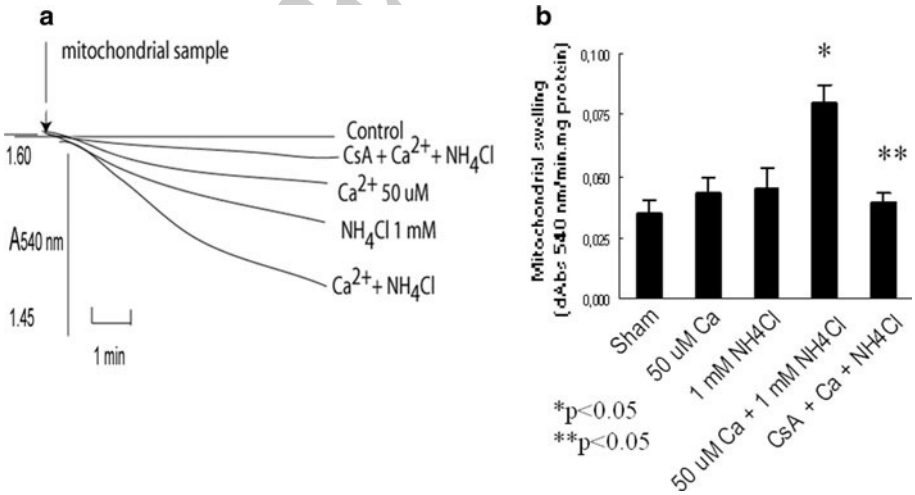

Fig. 5 **a–b** In vitro analysis of MPT: ammonium and calcium effect on mitochondrial swelling. MPT was also evaluated in vitro after ammonium and calcium mitochondrial exposure by the induction of swelling. Mitochondria from Sham animals were exposed to 50 μM Ca^{2+}, 1 mM NH_4Cl, to both 50 μM Ca^{2+} and 1 mM NH_4Cl, and 3 min of CsA pretreatment before 50 μM Ca^{2+} and 1 mM NH_4Cl. **a** The decrease in ΔAbs at 540 nm/min mg protein was registered. **b** Quantification of the amount of mitochondrial swelling was performed from three different experiments. *$P < 0.05$ significantly different from sham animals

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mitochondrial conditions present at the moment of the induction of apoptosis; in fact other important proteins, such as AIF/Diabolo, play also important roles amplifying the degradation cascade during apoptosis. Different studies have suggested that MPT could be the cause of the energetic failure present in HE [32]. In addition, it is well known that MPT occurs with a decreased $\Delta\Psi_m$ [33], an increased mitochondrial swelling, and a release of protein factors from the intermembrane space to the cytosol [8, 24]. This process is triggered by an increase in matrix Ca^{2+}, prooxidants, and other unknown mitochondrial conditions. This mega channel appears to be non specific and permeable to solutes under 1.5 kDa [34]. In this study, only 15% of total cytochrome c was released from mitochondria of MHE animals as compared with the sham group, indicating that an important amount of cytochrome c is still inside the organelle.

Our studies on MPT indicate that mitochondria from MHE animals were more susceptible to swell in the presence of 50 μM calcium soon after their isolation, being not able to maintain their proton gradient across its inner membrane, as compared with the mitochondria from sham animals. This mitochondrial swelling tendency was correlated with a clear decrease in mitochondrial polarization as part of the mitochondrial dysfunction observed in this model.

Our in vitro studies on MPT showed that increases in ammonia levels alone were not able to induce a marked swelling and depolarization, as observed previously by other study [35] in which addition of ammonia ions at neurotoxic concentrations alone were not able to induce a decrease in light scattering. However, in this study, when 1 mM ammonia and 50 μM calcium were added together, a marked increase in swelling and depolarization was evident.

The role of ammonia in the inhibition of the tricarboxylic acid cycle (TCA) [36] and in the activation of glutamate (NMDA) [37] receptor, at the moment, do not completely explain the complex effects of ammonia and calcium in brain ATP metabolism. Thus, a minor disturbance of cellular ammonia and Ca^{2+} homeostasis can complicate mitochondrial function, being important factors disturbing the brain energetic metabolism during HE. Further studies should be performed to clarify the close relationship between these two molecules and their effects on brain mitochondrial dysfunction.

The data presented in this study strongly suggest that the observed alterations in brain hippocampal mitochondrial physiology of MHE animals could be closely associated with the increased ammonia levels causing alterations in energy metabolism and inducing apoptotic intrinsic signaling pathways in this tissue.

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References

1. Vorobioff J, Bredfelt JE, Groszmann RJ (1983) Hyperdynamic circulation in portal hypertensive rat model: a primary factor for maintenance of chronic portal hypertension. Am J Physiol 244:G52–G57
2. Perazzo JC, Lores Arnaiz S, Fernández M, Lago N, Lemberg A (2006) Prehepatic portal hypertension and mitochondrial dysfunction in brain hippocampus. In: Häussinger D, Schliess H (eds) Hepatic encephalopathy and nitrogen metabolism, vol 14. Springer, Dordrecht, pp 191–201
3. Lores Arnaiz S, Perazzo JC, Prestifilipo JC, Lago N, Dámico G, Czerniczyniec A, Bustamante J, Boveris A, Lemberg A (2005) Hippocampal mitochondrial dysfunction with decreased mtNOS activity in prehepatic portal hypertensive rats. Neurochem Int 47:362–368
4. Clemmesen JO, Larsen FS, Kondrup J, Hansen BA, Ott P (1999) Cerebral herniation in acute liver failure is correlated with arterial ammonia concentration. Hepatology 29:648–853
5. Butterworth RF (2003) Pathogenesis of hepatic encephalopathy: new insights from neuroimaging and molecular studies. J Hepatol 39:278–285
6. Felipo V, Butterworth RF (2002) Neurobiology of ammonia. Prog Neurobiol 67:259–279
7. Kosenko E, Kaminsky Y, Lobata O, Muravyov N, Kaminsky A, Hermenegildo D, Felipo V (1998) Nitroarginine an inhibitor of nitric oxide synthase, prevents changes in superoxide radical and antioxidant enzymes induced by ammonemia intoxication. Metab Brain Dis 13:29–41
8. Kroemer G, Zamzami N, Susin SA (1997) Mitochondrial control of apoptosis. Immunol Today 18:44–51
9. Gogvadze V, Robertson JD, Zhivotovsky B, Orrenius S (2001) Cytochrome c release occurs via Ca^{2+} dependent and Ca^{2+} independent mechanisms that are regulated by Bax. Proc Natl Acad Sci USA 99(3):1259–1263
10. Bernardi P, Scorrano L, Colonia R, Petronilli V, Di Lisa F (1999) Mitochondria and cell death. Mechanistic aspects and methodological issues. Eur J Biochem 264:687–701
11. Butterworth R, Noremberg M, Felipo V, Ferenci P, Albrecht J, Blei A (2009) Experimental models of hepatic encephalopathy: ISHEN guidelines. Liver Int 1478:783–788
12. Navarro A, López-Cerero JM, Sandez MJ, Sánchez-Pino MJ, Gomes C, Cadenas E, Boberis A (2008) Hippocampal mitochondrial dysfunction in rat aging. Am J Physiol Regul Integr Comp Physiol 294(2):R501–R509
13. Wijsman JH, Jonker RR, Keijzer R, Van De Velde CJH, Cornelisse CJ, Van Dierendock JH (1993) A new method to detect apoptosis in paraffin sections. In situ end-labeling of fragmented DNA. J Histochem Cytochem 41:7–12
14. Estabrook RW (1967) Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. Methods Enzymol 10:41–47
15. Chance B, Williams GR (1956) The respiratory chain and oxidative phosphorylation. Adv Enzymol 17:65–134
16. Yonetani T (1967) Cytochrome oxidase: beef heart. Methods Enzymol 10:332–335
17. Bustamante J, Czerniczyniec A, Cymering C, Lores Arnaiz S (2008) Age related changes from youth to adulthood in rat brain

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cortex: nitric oxide synthase and mitochondrial respiratory function. Neurochem Res 33(7):1216–1223
18. Mattiasson G, Friberg H, Hansson M, Elmér E, Wieloch T (2003) Flow cytometric analysis of mitochondria from CA1 and CA3 regions of rat hippocampus reveals differences in permeability transition pore activation. J Neurochem 87:532–544
19. Czerniczyniec A, Bustamante J, Lores Arnaiz S (2006) Modulation of brain mitochondrial function by deprenyl. Neurochem Int 48:235–241
20. Bustamante J, Caldas Lopez E, Garcia M, Di Libero E, Alvarez E, Hajos S (2004) Disruption of mitochondrial membrane potential during apoptosis induced by PSC 833 and CsA in multidrug-resistant lymphoid leukemia. TAAP 199:44–51
21. Cafiso DS (1994) Alamethicin a peptide model for voltage gaiting and protein-membrane interactions. Ann Rev Biophys Biomol Struct 23:141–165
22. Hansson MJ, Persson T, Friberg H, Keep MF, Rees A, Wieloch T, Elmér E (2003) Powerful cyclosporine inhibition of calcium-induced permeability transition in brain mitochondria. Brain Res 960:99–111
23. Scorticati C, Prestifilipo JP, Murer G, Lemberg A, Perazzo JC (2001) Cambios funcionales en el sistema nervioso central de ratas hipertensas portales prehepáticas. Medicina 61:673–675
24. Tsujimoto Y (1997) Apoptosis and necrosis: intracellular ATP levels as a determinant for cell death modes. Cell Death Differ 4:429–434
25. Rostovtseva T, Antonsson B, Suzuki M, Youle RJ, Colombini M, Bezrukov SM (2004) Bid, but not Bax, regulates VDAC channels. J Biol Chem 279:13575–13583
26. Bustamante J, Nutt L, Orrenius S, Gogvadze V (2005) Arsenic stimulates release of cytochrome c from isolated mitochondria via induction of mitochondrial permeability transition. TAAP 207(Suppl 2):110–116
27. Woong S, Winseck A, Vinsant S, Park O, Kim H, Oppenheim RW (2004) Programmed cell death of adult-generated hippocampal neurons is mediated by the proapoptotic gene Bax. J Neurosci 24:11205–11213
28. Deckwerth TL, Elliot JL, Knudson CM, Johnson EM Jr, Snider WD, Korsmeyer SJ (1996) Bax is required for neuronal death after trophic factor deprivation and during development. Neuron 17:401–411
29. Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B, Orrenius S, Lipton SA, Nicotera PL (1995) Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron 15:961–973
30. Martinou JC, Green DR (2001) Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol 2:63–67
31. Green DR, Kroemer G (2004) The pathophysiology of mitochondrial cell death. Science 305:626–629
32. Norenberg MDJ, Jayakumar AR, Rama Rao KV, Panikar KS (2007) New concept in the mechanism of ammonia-induced astrocyte swelling. Metab Brain Dis 22:219–234
33. Zoratti M, Szabo I (1994) Electrophysiology of the inner mitochondrial membrane. J Bioenerg Biomembr 26:543–553
34. Zoratti M, Szabo I (1995) The mitochondrial permeability transition. Biochim Biophys Acta 1241:139–176
35. Ziemiska E, Dolinska M, Lazarewicz JW, Albrecht J (2000) Induction of permeability transition and swelling of rat brain mitochondria by glutamine. Neurotoxicology 21(3):295–300
36. Lay JCK, Cooper AJL (1986) Brain α-ketoglutarate dehydrogenase complex: kinetic properties, regional distribution and effects of inhibitors. J Neurochem 47:1376–1386
37. Kosenko E, Kaminsky Y, Grau E, Miñana MD, Marcaida G, Grisolia S, Felipo V (1994) Brain ATP depletion induced by acute ammonia intoxication in rats is mediated by activation of the NMDA receptor and Na+, K+-ATPase. J Neurochem 63:2172–2178

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