

Maternal seizures can affect the brain developing of offspring

Ana Carolina Cossa¹ · Daiana Correia Lima¹ · Tiago Gurgel do Vale² ·
Anna Karynna Alves de Alencar Rocha¹ · Maria da Graça Naffah-Mazzacoratti^{1,3} ·
Maria José da Silva Fernandes¹ · Debora Amado¹

Received: 17 November 2015 / Accepted: 11 April 2016 / Published online: 16 April 2016
© Springer Science+Business Media New York 2016

Abstract To elucidate the impact of maternal seizures in the developing rat brain, pregnant Wistar rats were subjected to the pilocarpine-induced seizures and pups from different litters were studied at different ages. In the first 24 h of life, blood glucose and blood gases were analyzed. ¹⁴C-leucine [¹⁴C-Leu] incorporation was used to analyze protein synthesis at PN1, and Western Blot method was used to analyze protein levels of Bax, Bcl-2 and Poly(ADP-ribose) polymerase-1 (PARP-1) in the hippocampus (PN3-PN21). During the first 22 days of postnatal life, body weight gain, length, skull measures, tooth eruption, eye opening and righting reflex have been assessed. Pups from naive mothers were used as controls. Experimental pups showed a compensated metabolic acidosis and hyperglycemia. At PN1, the [¹⁴C-Leu] incorporation into different studied areas of experimental pups was lower than in the control pups. During development, the protein levels of Bax, Bcl-2 and PARP-1 in the hippocampus of experimental pups were altered when compared with control pups. A decreased level of pro- and anti-apoptotic proteins was verified in the early postnatal age (PN3), and an increased level of pro-apoptotic proteins concomitant with a reduced level of anti-apoptotic protein was observed at the later stages of the development (PN21). Experimental pups had a delay in

postnatal growth and development beyond disturb in protein synthesis and some protein expression during development. These changes can be result from hormonal alterations linked to stress and/or hypoxic events caused by maternal epileptic seizures during pregnancy.

Keywords Epilepsy · Pilocarpine · Pregnancy · Development · Offspring

Introduction

Using antiepileptic drugs (AEDs) during pregnancy may cause teratogenic effect on the offspring and lead to neuropsychological disorders later in life (Meador et al. 2006). On the other hand, seizures themselves present risk to the fetus. Generalized tonic-clonic seizures (GTCSs) can cause maternal and fetal hypoxia and acidosis (Yerby 2000). Fetal intracranial hemorrhages (Minkoff et al. 1985), miscarriages, and stillbirths are reported after a single GTCS.

Previous studies confirm that seizures during pregnancy affect the development of the offspring's brain. Changes were observed in interneurons expressing calcium-binding proteins in the hippocampus of developing rats, fact that indicates imbalance in the excitation and inhibition mechanisms (Vale et al. 2010). Moreover, the increased neuroglobin expression in the cerebellum of pups reflected low O₂ rates that possibly result from the response to maternal seizures (Lima et al. 2011). The authors also showed the presence of ischemic infarct in the placenta of epileptic female rats and it suggests that seizures caused hypoxic–ischemic (HI) insults in their pups (Lima et al. 2011).

Cerebral ischemia triggers several changes linked to functional disturbances, thus leading to the loss of brain structural

✉ Ana Carolina Cossa
carol_cossa@hotmail.com

¹ Departamento de Neurologia e Neurocirurgia - Disciplina de Neurologia Experimental, Universidade Federal de São Paulo, UNIFESP, Rua Pedro de Toledo, 669, 2º Andar, São Paulo, SP, Brasil

² Centro Universitário São Camilo, Av. Nazaré, 1501 São Paulo, SP, Brasil

³ Departamento de Bioquímica, Universidade Federal de São Paulo, UNIFESP - Rua 3 de maio, 100 São Paulo, BR, Brasil

integrity (Krause et al. 1988). Among the notably disturbed functions, protein synthesis is of particular interest.

Protein synthesis suppression is a common reactive cell response to severe forms of stress, including thermal, physical and metabolic stress or viral infection (Clemens et al. 2000). Many studies have shown that three minutes of ischemia are enough to stop protein synthesis during reperfusion (Nowak et al. 1985). Protein synthesis inhibition is considered to be an early indicator of subsequent neuronal cell death (Hossmann et al. 1992), which is an important feature observed after HI injury. Ischemia-induced cell death may occur via apoptosis or necrosis (Northington et al. 2001).

An important protein family involved in apoptosis is the B-cell lymphoma-2 (Bcl-2), which regulates caspase activation by regulating the mitochondrial outer membrane permeability (MOMP) (Autret and Martin 2009). This family encodes specific proteins which regulate programmed cell death in different physiological and pathological condition and can be divided into two categories according to their effects on apoptosis. The products of one group of genes, which include Bcl-2, Bcl-w and Bcl-xL, promote cell survival. On the other hand, Bax, a member of the pro-apoptotic bcl-2 family, accelerates apoptotic cell death, as well as Bak, Bim and Bad proteins (White 1996). The balance between pro- and anti-apoptotic bcl-2 family members is important for neural survival during development (Korsmeyer 1999).

Additional studies argue that the bcl-2 family may also play a previously unsuspected role as mitochondrial morphogenesis regulators. This scenario is quite apart from this family's role in apoptosis (Autret and Martin 2009), and it includes a crucial role in neurons (Autret and Martin 2010).

Another protein involved in the mitochondrial function is the Poly (ADP-ribose) polymerase-1 (PARP-1), which plays also a number of other possible roles, including gene expression regulation and amplification, cell differentiation, cell division, malignant transformation, DNA replication and cell death (Herceg and Wang 2001). PARP-1 is potently activated by DNA strand nicks and breaks and facilitates DNA repair (Lautier et al. 1993; Smulson et al. 2000).

The present study evaluated the impact of maternal seizures during the intrauterine life, without drug administration, on the brain of rat pups, at different ages after birth, by investigating metric parameters, fetal distress, protein synthesis rates as well as the Bax, Bcl-2 and PARP-1 expressions in the hippocampus.

Material and methods

The experiments were performed with ethical approval from the Institutional Ethics Committee of the Universidade Federal de São Paulo (UNIFESP) on the registration number n°2018/07. All efforts were made to minimize animal

suffering according to the International Ethical Guidelines for Biomedical Research (CIOMS/OMS 1985).

Prepartum period

Adult female and male Wistar rats weighing 200–250 g were housed in groups of five under environmentally controlled conditions (12/12 h light/dark cycle, and constant temperature of 20°–22 °C) with free access to food and water. *Status Epilepticus (SE)* was induced in female rats by administration of 320–350 mg/kg (i.p.) of pilocarpine (PILO; Sigma, St. Louis, MO), a muscarinic agonist (Cavalheiro et al. 1987). Scopolamine methyl nitrate (Sigma, 1 mg/kg s.c.) was injected 30 min before pilocarpine to prevent peripheral cholinergic effects. Treatment with pilocarpine was performed during the estrus phase of the estrous cycle due to a higher incidence of animals with spontaneous recurrent seizures and a lower mortality compared to rats injected on other phases of estrous cycle (Amado and Cavalheiro 1998; Valente et al. 2002). Following four hours from the *SE* onset, rats received thiopental (30 mg/kg, i.p.) to block seizures. Rats subjected to *SE* received special care including hydration and a fractionated diet. Animals that survived *SE* were continuously monitored by video until the appearance of the first spontaneous seizure, which defines the onset of the chronic phase. A blinded investigator visually scored the frequency of behavioral seizures characterized by clonic/tonic/tonic-clonic movements of the forelimbs culminating with rearing and falling (grade IV–V from Racine scale) (Racine 1972) from videotapes obtained during recording sessions.

Females were placed in cages with males during the period of estrus. Vaginal contents were analyzed daily and the presence of sperm was used as a marker for pregnancy onset. During pregnancy, epileptic and non epileptic females were housed in separate cages (transparent acrylic cylinder) and the epileptic rats were video-monitored 24 h/day to determine seizure frequency and duration.

Postpartum period

In most cases, epileptic females do not take care of their pups and usually present an aggressive behavior towards them (Vale 2007). Thus, it was necessary to exchange the litters of these epileptic animals by control animals (cross-fostering), which, in turn, nested and fed their offspring. Thus, immediately after birth, the litters were subjected to cross-fostering with untreated control rats from a standard colony delivered in the same day (Vale et al. 2010; Lima et al. 2010). The control group was subjected to the same mating procedure, and pups born from non-epileptic rats (control) were compared to those born from epileptic rats (experimental).

Immediately postpartum (P0), 20 animals from 7 litters of control group and 7 animals from 4 litters of experimental

group were randomly chosen and sacrificed. Mixed arteriovenous blood sample was obtained by decapitation and use of a capillary sampling tube. Glucose and blood gases analyze was made with Rapid Lab 1200 Blood Gas Analyzer (Siemens).

Some metric parameters were evaluated throughout the offspring development (PN1–PN21): weight, length and cranial measures divided in anteroposterior and laterolateral distances. A total of 46 animals from 8 litters of control group and 46 animals from 7 litters of experimental group were measured every 2 days. The onset of eye opening and the first dental eruption were also observed in 5 control litters and in 6 experimental litters. The appearance of the righting reflex was also evaluated daily.

The profile of protein level was analyzed in pups at PN1 ($n = 5$, 01 pup per litter). This period was chosen because it allows studying events very close to the period of intrauterine seizures.

The analysis of Bax, Bcl-2 and PARP-1 was performed using Western blotting. Samples of hippocampus of control and experimental pups at PN3, PN7, PN14 and PN21 with the same sex (male) were used ($n = 4$ per group, 1 pup per litter).

¹⁴C-L-leucine [¹⁴C-Leu] incorporation to study protein synthesis

At PN1, five pups from epileptic rats were weighed and injected (s.c.) with ¹⁴C-Leu at a dose of 3 μ Ci/10 g (50–60 mCi/mmol; Amersham Pharmacia Biotech) (Tuor et al. 1999). Five pups of control rats treated with saline instead of pilocarpine were injected with equal dose of ¹⁴C-Leu, and 90 min after the injection of the tracer, the animals were killed by decapitation. Brains were rapidly removed, frozen and cut into 20 μ m coronal sections in a cryostat (Leica), wherein for each four slices collected for the autoradiograph, one was collected for Nissl Staining, and five slices were discarded. This cycle was repeated until the end of the brain. Sections were autoradiographed on Amersham Biomax MR film along with calibrated ¹⁴C standards (GE against the sections). The autoradiographs were then digitized and analyzed by densitometry with an image-processing system (Biocom, Les Ulis, France). The *Densitag* Program was used to calibrate the gray levels with eight ¹⁴C standards (30.0–862.0 nCi/g), and then proceeded to read the regions of interest. Regions of interest were chosen according to their implication with the circuitry of limbic seizures in the pilocarpine model: entorhinal cortex, posterior hypothalamus, hippocampal formation (CA1, CA3, hilus and dentate gyrus), thalamus and piriform cortex. The optical density from each region was the ratio of two measurements in four sections, and the average used in statistical analysis. The localization of specific nuclei was assessed on adjacent sections stained with cresil violet (Nissl Staining) and was made with the aid of the Neonatal Rat Brain Atlas

(PN1, Coronal Plates and Coronal Fig. 1 through 30) (Ramachandra and Subramanian 2011).

Western blot analysis

Protein expression of Bax, Bcl-2 and PARP-1 were analyzed in the hippocampus of pups at PN3, PN7, PN14 and PN21 ($n = 4$ per age group, 1 per litter) by using Western blot. Tissues were dissected and stored at -80 °C until assay. Samples were homogenized in lysis buffer with protease inhibitor cocktails (0.1 M NaCl, 0.01 M Tris-HCl pH 7.6, 0.001 M EDTA pH 8.0, 1 % NP-40, 10 % glycerol, 10 μ M PMSF, 1 mM sodium metavanadate, 0.05 M NaF, 2 nM okadaic acid). Protein content was determined using the Lowry method (Lowry et al. 1951). Samples (25–40 μ g) were mixed with Laemmli buffer containing 0.125 M Tris (pH 6.8), 20 % glycerol, 10 % beta-mercaptoethanol, 4 % SDS and 0.002 % bromophenol blue, and heated at 95 °C for five min. Protein was loaded on a 15 % SDS-PAGE gel to Bax and Bcl-2 or on a 10 % SDS-PAGE to PARP-1 separated by electrophoresis using a Bioed system with molecular weight standards (Rainbow-GE). Protein transfer to PVDF (Millipore) (Bax and Bcl-2) or nitrocellulose membranes (GE) (PARP-1) was carried out in a transfer system (Biorad) using 25 mM Tris, 192 mM glycine, 20 % (v/v) methanol, pH 8.3. Membranes were washed with 0.1 M Tris-Tween 20, blocked with 0.1 M Tris containing 5 % skimmed milk (PARP-1) or 5 % fetal bovine serum (Bax and Bcl-2), and then incubated with the primary antibodies Bax, Bcl-2 and PARP-1 at 4 °C overnight (1:1000 dilution, Calbiochem). After rinsing, the membranes were incubated with the corresponding secondary antibody (goat anti-rabbit IgG, Calbiochem) at a dilution of 1:8000 in 0.1 M Tris containing 2 % fetal bovine serum for 60 min. After washing them twice with 0.1 M Tris, membranes were ready for the blocking stage to re-probing with the monoclonal anti- β -actin immunoglobulin (Sigma-Aldrich, 1:2000) used as internal control of the reaction.

After rinsing, blots were incubated for 90 min in streptavidin-horseradish peroxidase (Vector Laboratories, Burlingame, CA), then with a primary antibody anti- β -actin peroxidase (1:3000, Sigma) used as internal control of the reaction, and revealed with 3,3-diaminobenzidine (DAB, Sigma). The rate between optical density of Bax, Bcl-2, and PARP-1 over β -actin bands is presented as means \pm standard deviation (SD).

Statistical analysis

The results were analyzed with the following tests, as appropriate: analysis of variance (ANOVA) followed by Tukey test, Student's t test and χ^2 test.

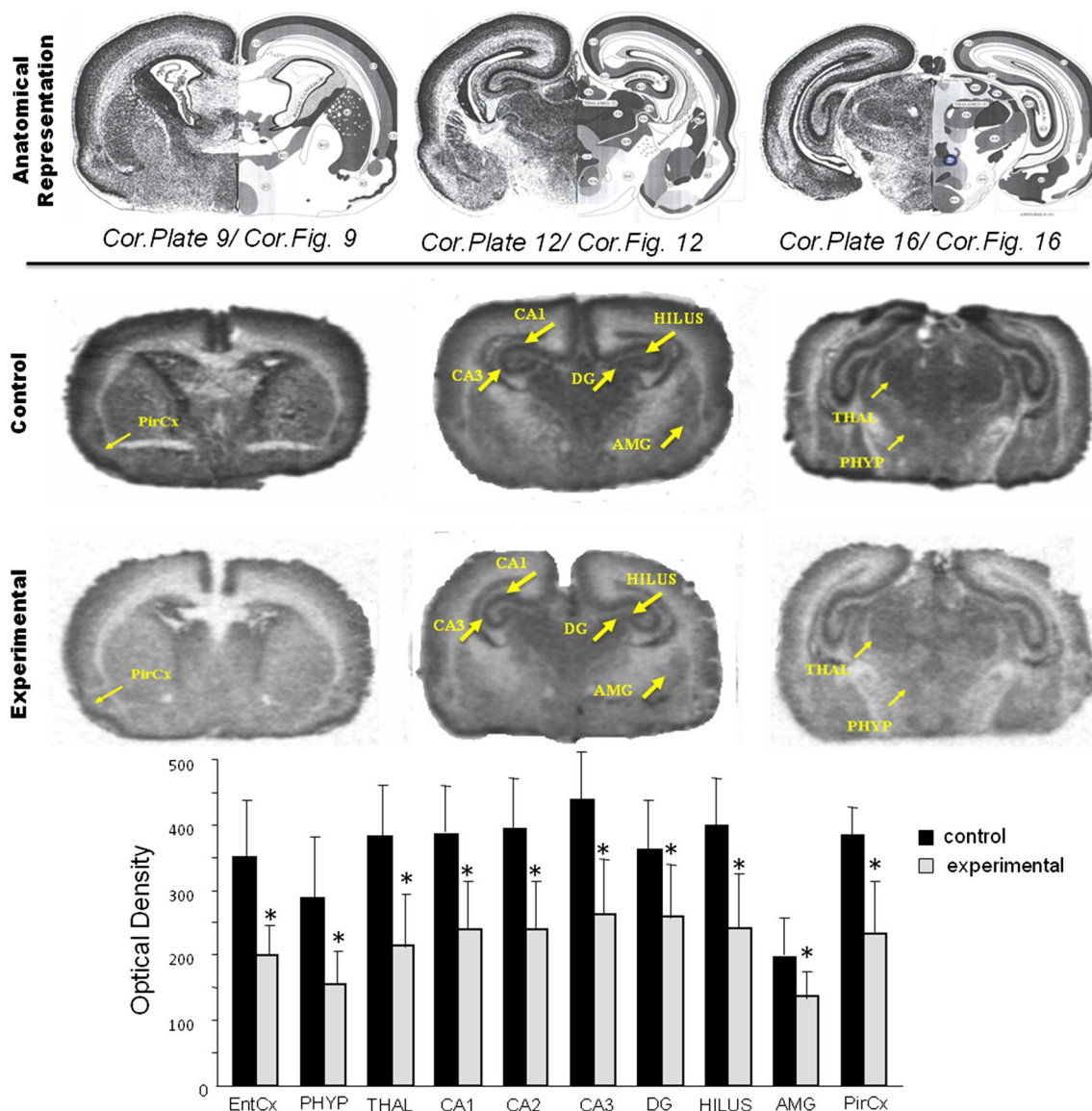


Fig. 1 Protein synthesis in brain of rat pups after maternal seizures. ¹⁴C-Leu autoradiographs of brain sections of rats at first postnatal day (PN1). Note the low grain density in the hippocampal formation (CA1, CA3, HILUS and dentate gyrus (DG), piriform cortex (PirCx), amygdala (AMG), posterior hypothalamus (PHYP) and thalamus (THAL) of experimental pups compared to controls (arrows), indicating low protein synthesis. **On the bottom** - Optical density (OD) values (means

± standard deviation, SD) of brain areas of experimental and control pups studied at PN1. Data shows a significant decrease in OD of all studied brain areas of experimental offspring compared to control (* *p* < 0.05, Student's *t* test). Coronal Plate (Cor. Plate) and Coronal Figure (Cor. Fig.) at top panel were used to represent the anatomy of regions of interest (Ramachandra and Subramanian 2011); EntCx- Entohinal Cortex

Results

Prepartum period

We observed that epileptic rats had a reduction of fertility in 35 % whereas only 13 of the 20 rats that were mated became pregnant. These data are consistent with previous data already published by our group. It was observed that epileptic rats have a reduction of the fertility in 34.4 % of the animals, i.e. in a group of 15 epileptic rats only 10 become pregnant during period of 3 weeks of exposure to male in contra-position to

100 % of the non-epileptic animals that became pregnant at the same period (Amado and Cavalheiro 1998). Our data are also consistent with other studies (Vale et al. 2010) which showed that pregnancy lasted 21.3 ± 0.25 days in the control group and 21.3 ± 0.18 days in the experimental group (no difference between groups). We did not observe any difference in relation to weight gain during the gestation period between the groups, which is in agreement with previous data (Vale 2007).

Seizure frequency was monitored over entire pregnancy as well as before this period. Epileptic rats exhibited generalized

seizures classified according to Racine's scale as grade IV or V. No tonic seizures were detected.

The frequency of spontaneous seizures was recorded in 13 female rats. Prior to pregnancy, mean seizure frequency was 15 ± 4.5 per week. During pregnancy, all rats presented at least five seizures, wherein the distribution along the three weeks declined significantly when compared to prepregnancy period. In the first week there were 3 ± 4 seizures/week ($p < 0,05$ vs the prepregnancy rate; ANOVA and Tukey's test) followed by 3 ± 2 ($p < 0,05$) and 4 ± 3 ($p < 0,05$) seizures/week at the second and third weeks chronologically. The mean duration of seizures was 39 ± 14 s, which lasted from 16 to 82 s. Results are presented as means \pm standard deviation, SD.

Postpartum period

Our results are similar with previous results (Lima et al. 2010) where no significant difference was observed in the number of live born pups per litter. In the control group ($n = 12$) there were 11 ± 0.7 pups/litter, and in the experimental group ($n = 11$) there were 9 ± 0.6 pups/litter (Student's t test; $p > 0,05$).

At PN1, it was observed a significant decrease at experimental group ($n = 20$) when compared with control ($n = 20$) in length ($50,75 \pm 0,5$ mm; $p < 0,01$; ANOVA and Tukey post hoc), weight ($6,12 \pm 0,2$ g; $p < 0,01$), and anteroposterior distances ($17,06 \pm 0,1$ mm; $p < 0,001$) (Table 1). The experimental pups ($n = 48$) also showed a decrease in weight compared with control pups ($n = 46$) at PN3 and PN7 (Table 1). The length was also decreased in experimental pups at PN3, PN7 and PN14 (Table 1). Except at PN1, the cranial measures were not significantly different at all periods analyzed.

All control and experimental pups presented the eye opening at PN14. This procedure was analyzed in 5 offsprings per group. The tooth eruption was considered positive when the bone structure was observed. At PN11, all the control pups presented superior tooth eruption while the experimental group presented it at PN12 (no statistical difference, χ^2 test). The righting reflex was positive at PN4 for the control pups

and at PN5 for experimental pups (no statistical difference, χ^2 test).

Blood gases and blood glucose

At experimental group, we observed a discrete hyperoxia with a bicarbonate decrease (Student's t test; $p < 0,05$), featuring a metabolic acidosis. Glycemia was highly increased at experimental group (Student's t test; $p < 0,001$) (Table 2).

Autoradiographic analysis

The experimental pups studied at Autoradiographic analysis were born from 05 epileptic dams that had $4,66 \pm 4,5$ seizures at the first week, $2 \pm 1,73$ at the second and $3 \pm 2,64$ at the third gestational week (mean \pm SD). Optical densities (O.D.) analysis of ^{14}C -Leu incorporation revealed lower grain density in brain sections of experimental pups compared to control pups (Student's t test; $p < 0,05$) (Fig 1A). The reduction in ^{14}C -Leu incorporation induced by seizures during pregnancy ranged from 37 to 46 % and was observed in distinct cortical, hippocampal, thalamic-hypothalamic and amygdaline regions from experimental pups compared to pups from control group (Fig 1).

Analysis of bax, bcl-2 and PARP-1 by Western blot

Semi-quantitative analyses of Bax (21 KDa), Bcl-2 (24 KDa) and PARP-1 (85KDa) proteins were performed according to previous descriptions (Merry and Korsmeyer 1997).

The levels of Bax, Bcl-2 and PARP-1 proteins assayed in the hippocampus at different ages are shown in Fig. 2. A significant decrease of Bax and Bcl-2 protein level was observed at experimental pups at PN3 (Table 3); although at PN14 they showed a significant increase in the levels of Bax and Bcl-2 proteins when compared to control pups (Fig 2a and b, Table 3). At PN21, the level of Bax protein remained increased while Bcl-2 was significantly decreased (Fig 2a and b, Table 3). The level of PARP-1 protein was significantly

Table 1 Developmental metric parameters: weight, length, and cranial measures

	WEIGHT (g)		LENGTH (mm)		AP (mm)		LL (mm)	
	CONT	EXP	CONT	EXP	CONT	EXP	CONT	EXP
PN1	6,42 \pm 0,08	6,12 \pm 0,17*	53,35 \pm 0,24	50,75 \pm 0,46**	17,15 \pm 0,13	17,06 \pm 0,11**	9,75 \pm 0,09	8,93 \pm 0,14**
PN3	8,68 \pm 0,12	7,73 \pm 0,16**	63,19 \pm 0,34	58,87 \pm 0,45**	19,9 \pm 0,16	19,8 \pm 0,13	10,52 \pm 0,07	10,33 \pm 0,11
PN7	14,60 \pm 0,24	13,12 \pm 0,28*	75,97 \pm 0,56	70,62 \pm 0,57**	24,86 \pm 0,19	24,23 \pm 0,17	12,94 \pm 0,09	12,64 \pm 0,09
PN14	26,83 \pm 0,42	26,27 \pm 0,31	104,45 \pm 0,83	99,46 \pm 0,52**	32,56 \pm 0,14	32,10 \pm 0,15	15,71 \pm 0,08	15,51 \pm 0,09
PN21	38,01 \pm 0,46	38,39 \pm 0,53	122,17 \pm 0,68	119,74 \pm 0,67	36,80 \pm 0,26	36,04 \pm 0,28	17,91 \pm 0,39	17,46 \pm 0,19

AP Anteroposterior distance; LL: Laterolateral distance; CONT: control rats; EXP: Experimental rats. Results are presented as mean \pm standard deviation (SD). Statistical significances are represented at * $p < 0,01$ and ** $p < 0,001$ (ANOVA and Tukey)

Table 2 Physiological parameters in pups in the birth day (PN0). Blood gas and glucose were measured in control (20 pups from 7 litters) and in experimental pups (7 pups from 3 litters)

	CONTROL	EXPERIMENTAL
pH	7,4 ± 0,02	7,48 ± 0,06
pCO ₂ (mmHg)	37,5 ± 2,5	24,2 ± 6,3
pO ₂ (mmHg)	65,0 ± 8,3	113,5 ± 15,0*
SatO ₂ (%)	80,9 ± 3,7	90,5 ± 7,5
HCO ₃ (mmol/L)	22,4 ± 1,2	15,5 ± 2,2*
Glycemia (mg/dL)	80,6 ± 4,3	206,7 ± 1,4**

All values are mean ± standard deviation (SD). Statistical significances were presented at * $p < 0,05$; ** $p < 0,001$ (Student's t test)

increased in almost all phases of the development studied, i.e., PN7, PN14 and PN21 in experimental compared to control pups (Fig 2c, Table 3), indicating a possible occurrence of DNA damage in experimental pups. The samples at a given stage showed in Fig. 2 were cut out from the same gel. All assays were performed with 4 animals per age group, 1 per litter.

Discussion

Data in the literature show that women with epilepsy have increased fetal risk in comparison to that of the general

population (Yerby 2000; Hvas et al. 2000). Even with the reduction at seizure frequency observed during pregnancy, likely due to changes in hormonal status (Amado and Cavaleiro 1998), the present study shows that animals born from epileptic rats presented reduced weight, length and anteroposterior cranial measures at birth when they were compared to control rats. At this time, metabolic changes and disturbances were also observed in the protein synthesis rates in the offspring hippocampus, probably due to the occurrence of seizures during pregnancy, which, in turn, could modify the expression of important proteins. The level of anti-apoptotic proteins was reduced at PN3 and it was followed by pro-apoptotic proteins level increase at later development stages (PN14–PN21).

Protein synthesis is a high-energy expenditure process accounting for the consumption of 18–26 % cellular energy reserves (Hawkins 1991). Studies on a variety of HI experimental models have shown that energy and/or energy substrates reduction may cause protein synthesis inhibition in the brain (Albrecht and Smiatek 1975; Nowak et al. 1985). By considering that seizures are high energy expenditure processes, the occurrence of seizures during pregnancy may cause ischemia and it results in the "freezing" effect on the protein synthesis in the fetus' brain due to low ATP levels. According to previous results, several changes may be related to fetal ischemia caused by seizures: the presence of ischemic infarct in the placenta of epileptic female rats, changes in the development of hippocampal interneurons that express

Fig. 2 Representative Western blots of Bax (a); Bcl-2 (b); and PARP-1 (c) proteins in the hippocampus of rat offspring at postnatal day 3, 7, 14 and 21. Blots were normalized to β -actin to control for equal protein loading between lanes. Data are representative of four rats/group/age. Densitometric analysis of Western blots is shown as normalized proteins expression, represented as mean ± standard deviation (SD). Statistical significance are presented at * $p \leq 0.05$ (Student's t test) compared to control

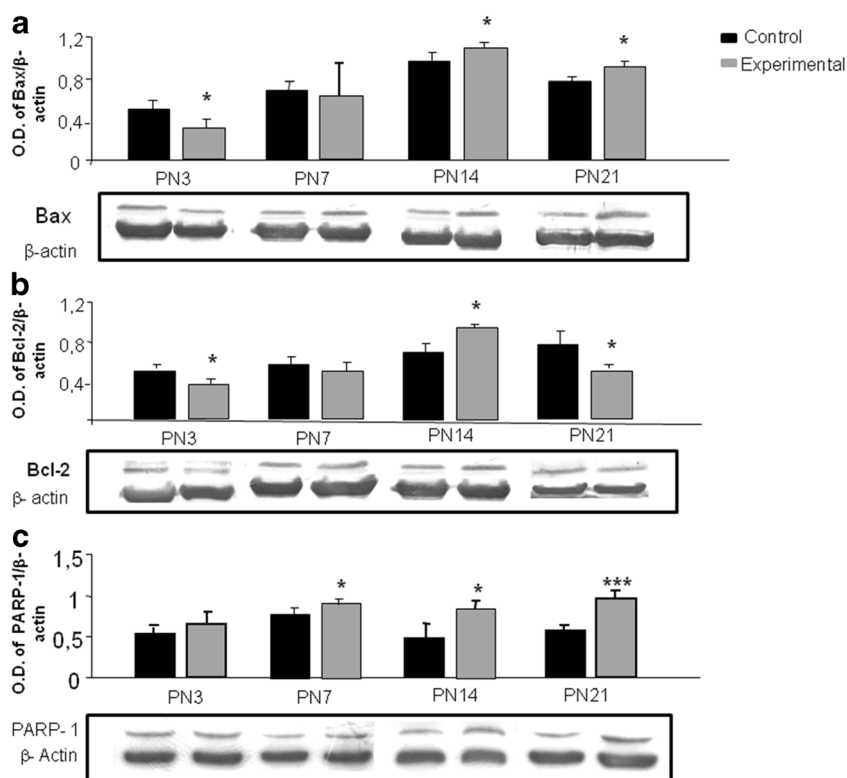


Table 3 Bax, Bcl-2, and PARP-1 proteins in rat hippocampus aged 3, 7, 14 and 21 days. Values represent densitometric analysis of protein bands normalized with β -actin in Western blots (four rats/group/age)

	BCL-2		BAX		PARP	
	CONT	EXP	CONT	EXP	CONT	EXP
PN3	0,55 \pm 0,03	0,46 \pm 0,08*	0,52 \pm 0,07	0,35 \pm 0,08*	0,55 \pm 0,08	0,65 \pm 0,14
PN7	0,58 \pm 0,09	0,53 \pm 0,07	0,67 \pm 0,06	0,63 \pm 0,30	0,73 \pm 0,06	0,84 \pm 0,04*
PN14	0,69 \pm 0,12	0,92 \pm 0,03*	0,96 \pm 0,08	1,09 \pm 0,04*	0,49 \pm 0,15	0,74 \pm 0,07*
PN21	0,76 \pm 0,15	0,54 \pm 0,05*	0,78 \pm 0,04	0,87 \pm 0,04*	0,57 \pm 0,06	0,93 \pm 0,09**

Data are represented as mean \pm standard deviation (SD). Statistical significance are presented at * $p \leq 0.05$, ** $p < 0.001$

calcium-binding proteins, which may reflect an imbalance in the mechanisms of excitation and inhibition, and an increased expression of neuroglobin observed in the cerebellum of pups, reflecting low O_2 rates (Vale et al. 2010; Lima et al. 2011).

It was observed that the offspring from epileptic rats exhibited lower optical densities of ^{14}C -Leu incorporation in numerous brain areas at birth when they were compared to the offspring from normal rats. Reduction in ^{14}C -Leu incorporation ranging from 37 to 46 % was mainly observed in the piriform and entorhinal cortex, amygdala, thalamus, as well as in the hypothalamus and hippocampal formation. The data in the present study indicate that seizures during pregnancy may alter protein synthesis in the offspring brain, and its consequences still need to be elucidated. The similarity in seizure frequency among the three gestational weeks allows thinking that the herein observed impairment may have resulted from the entire pregnancy period. However, despite this similarity, it is also possible that the different damages are related to the gestational period in which the seizures occurred. This will be the subject of further studies.

In addition to present a reduced protein synthesis, pups of epileptic rats also presented a decreased weight, length and anteroposterior cranial extent, which is associated with intrauterine growth delay. These events could be caused by changes in uterine blood flow during the seizures, since there is an increase in cerebral blood flow when these events take place and a redistribution of this flow consequently. Furthermore, the increase in hypothalamic-pituitary-adrenal (HPA) axis hormones related to maternal seizure stress may decrease utero-placental perfusion and, as a result, alter fetal growth (Cosmi et al. 1990). Previous studies have shown the presence of ischemic infarcts at the placenta of epileptic female rats and it meets the placental low flow, thus suggesting that maternal seizures may cause HI insults in the offspring (Lima et al. 2010).

The metabolic changes observed at the first 24 postnatal hours may also result from insults during intrauterine life. Blood gas analysis showed hyperoxia and low bicarbonate in pups from epileptic rats (Table 2). These findings are related to compensatory metabolic acidosis with respiratory alkalosis, which may result from fetal distress. Hyperglycemia in the

first 24 h was another finding to meet fetal distress in offspring from epileptic rats. Prolonged-birth newborns that suffered perinatal hypoxia showed initial hyperglycemia resulting from the increased catecholamines and cortisol release (Lagercrantz and Bistoletti 1977; Procianoy and Silveira 2001).

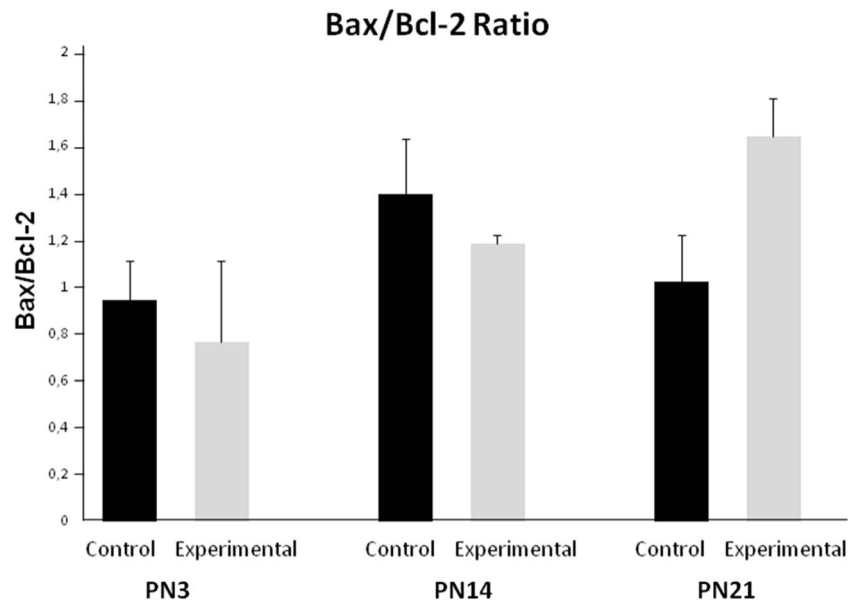
Therefore, literature findings (Challis et al. 2000; Austin et al. 2005) allowed thinking that hormonal changes due to stress throughout the gestational period, along with the hypoxic environment - both arising from maternal seizures - are responsible for the fetal development alterations, such as fetal suffering and intrauterine growth delay right after birth.

During their development (PN3-PN21), the experimental pups showed weight curve below the control up to PN7 and lower length curve up to PN14 (Table 1). These results show postnatal growth delay followed by recovery during the nutritional rehabilitation process, when the weight gain is faster than the length gain.

In addition to the physical changes seen in the first days after birth, pups from epileptic rats exhibited Bax and Bcl-2 protein level reduction at PN3 and it was followed by increase in these markers and in PARP-1 at PN14 when they were compared to the normal offspring. Bax and PARP-1 levels remained higher at PN21, whereas Bcl-2 levels were lower than those found in the control rats.

Low Bax and Bcl-2 protein levels in the early developmental stages may have resulted from the intrauterine seizures. This imbalance between pro- and anti-apoptotic protein expressions may affect the neuronal survival. As the ratio of Bcl-2 to Bax appears to determine the susceptibility to apoptotic stimuli (Oltvai et al. 1993; Yin et al. 1994), the low Bax/Bcl-2 ratio (lower than 1) observed in the hippocampus of pups at PN3 suggests that repair mechanisms may be activated in these regions to prevent cell death (Fig 3). On the other hand, it was possible to observe increased Bax and Bcl-2 protein levels at PN14, and it was followed by Bcl-2 decrease at PN21. These results could indicate the presence of caspase-dependent apoptosis, since the activation of either Bax or Bak is associated with changes in their conformation. These changes take place to induce permeabilization in the outer mitochondrial membrane (Autret and Martin 2009). This

Fig. 3 Ratio between O.D. of Bax and Bcl-2 proteins of experimental and control groups at postnatal day 3, 14 and 21. At PN7, no significant difference of Bax and Bcl-2 proteins levels were observed at this age between the groups and therefore it was not included



hypothesis is corroborated by the increased Bax/Bcl-2 ratio at PN21 (Fig 3).

However, although Bcl-2 proteins have always been linked to apoptotic signaling, some recent studies show that these proteins may modulate biochemical pathways other than mitochondria-regulated apoptosis, such as the mitochondrial remodeling regulation through fusion and fission (Autret and Martin 2009; Autret and Martin 2010). These mitochondrial dynamics are crucial to allow accumulating these organelles in subcellular regions that require high metabolic activity. Therefore, they are tightly implicated in the disease pathogenesis. Thus, despite the occurrence of apoptotic cell death, the disturbed balance of interactions within the Bcl-2 family could generate important impact upon the mitochondrial network connectivity.

Under cell-stress conditions, the mitochondria release death factors, such as the cytochrome c and the apoptosis-inducing factor (AIF) (Pospisilik et al. 2007). The mitochondrial-associated AIF release and the translocation to the nucleus is the commitment point for parthanatos, which is a caspase-independent cell death. Besides the fact that PARP-1 plays a pivotal role in multiple neurologic diseases by mediating parthanatos (Wang et al. 2009), it is likely that PARP-1 participates in cell death by multiple mechanisms (Ha and Snyder 2000).

PARP-1 is known as key regulator of cell survival and cell death and it is activated in response to noxious stimuli such as free radicals, hydrogen peroxide, hydroxyl radical, and peroxynitrite, which trigger DNA strand nicks and breaks. Thus, when there is mild DNA damage, the PARP-1 activity increases, as it was observed in the experimental offspring at PN7, PN14 and PN21, in comparison to the control levels.

Despite the changes observed at the Bcl-2, Bax and PARP-1 expression levels, no other signs were observed to prove cell death occurrence. Previous analysis showed no significant neuronal loss in the hippocampus of the offspring from epileptic rats at PN6 (Vale et al. 2010). However, these rats showed change in specific neuronal subpopulations and it suggests abnormal development or differentiation of these neurons (Vale et al. 2010). Therefore, the altered PARP-1 expression observed in the current study could be a way to repair the impairment caused by the insults of the epileptic mother, since many studies indicate that this protein is an important molecule in response to DNA damage (Herceg and Wang 2001). In addition, Bax may be also stimulated by the signal of DNA fragmentation (Lee et al. 2008).

The harmful effects caused by the insults from intrauterine life were verified by the alterations found right after birth. These changes kept on inducing disturbances at the cellular machinery after 21 days, which, in turn, could cause long-lasting consequences. Previous studies performed by our group indicate that the exposure to maternal seizures within the uterus may cause behavioral effects on the offspring during their adult life (Lima et al. 2010). These studies evaluated 3-month-old pups and showed that pups from epileptic mothers presented significant deficits in some behavioral tests, such as the open field test, which showed increased immobility duration; and the rotarod test, which found significant motor deficits (Lima et al. 2010).

Therefore, the present data provides new evidence that the intrauterine exposure to maternal seizures may have consequences for the fetal brain. These consequences may be linked to the occurrence of ischemia and/or maternal stress and it results in fetal suffering, intrauterine growth delay, altered

protein synthesis and changes in the expression of apoptotic proteins, which, in turn, may cause long-term disturbances in the brain function. These findings are particularly interesting to pregnant women who are afraid to take anticonvulsant medications, since they show the important harmful effects of the seizures per se.

Acknowledgments The authors are grateful for Hilda da Silva Reis for technical assistance, and Lara Ribeiro Silva for assistance with autoradiography. The study was supported by grants from FAPESP, CAPES, CNPq, PRONEX, CInAPCe, and FAPESP/CNPq/MCT-Instituto Nacional de Neurosciência Translacional.

Compliance with ethical standards

Ethical approval We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Conflict of interest statement None of the authors has any conflict of interest to disclose

References

- Albrecht J, Smiatek M (1975) Effect of hypoxia, ischemia and carbon monoxide intoxication on in vivo protein synthesis in neuron and glia cell enriched fractions from rat brain. *Acta Neuropath (Berl)* 31: 257–262
- Amado D, Cavalheiro EA (1998) Hormonal and gestational parameters in female rats submitted to the pilocarpine model of epilepsy. *Epilepsy Res* 32:266–274
- Austin MP, Leader LR, Reilly N (2005) Prenatal stress, the hypothalamic-pituitary-adrenal axis, and fetal and infant neurobehavior. *Early Hum Dev* 81:917–926
- Autret A, Martin SJ (2009) Emerging role for members of the bcl-2 family in mitochondrial morphogenesis. *Mol Cell* 36:355–363
- Autret A, Martin SJ (2010) Bcl-2 family proteins and mitochondrial fission/fusion dynamics. *Cell Mol Life Sci* 67:1599–1606
- Cavalheiro EA, Silva DF, Turski WA, Calderazzo-Filho LS, Bortolotto ZA, Turski L (1987) The susceptibility of rats to pilocarpine-induced seizures is age-dependent. *Dev Brain Res* 37:43–58
- Challis JRG, Matthews SG, Gibb W (2000) Endocrine and paracrine regulation of birth at term and preterm. *Endocr Rev* 21:514–555
- CIOMS/OMS (1985) Council for International Organizations of Medical Services. WHO Distribution and sales service, 1211 Geneva 27, Switzerland, International Guiding Principles for Biomedical Research Involving Animals.
- Clemens MJ, Bushell M, Jeffrey IW, Pain VM, Morley SJ (2000) Translation initiation factor modifications and the regulation of protein synthesis in apoptotic cells. *Cell Death Differ* 7(7):603–615
- Cosmi EV, Luzzi G, Gori F, Chiodi A (1990) Response of utero-placental fetal blood flow to stress situation and drugs. *Eur J Obstet Gynecol Reprod Biol* 36:239–245
- Ha HC, Snyder SH (2000) Poly (ADP-ribose) polymerase-1 in the nervous system. *Neurobiol Dis* 7:225–239
- Hawkins AJS (1991) Protein turnover: a functional appraisal. *Funct Ecol* 5:222–233
- Herceg Z, Wang ZQ (2001) Functions of poly (ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. *Mutat Res* 477:97–110
- Hossmann K-A, Widmann R, Wiessner C, Dux E, Djuricic B, Röhn G (1992) Protein synthesis after global cerebral ischemia and selective vulnerability. In *J pharmacology of cerebral ischemia*, edited by Krieglstein. H. Oberpichler-Schwenk, Wissenschaftliche Verlagsgesellschaft, Stuttgart, p. 289
- Hvas C, Henriksen T, Ostergaard J, Dam M (2000) Epilepsy and pregnancy: effect of antiepileptic drugs and lifestyle on birthweight. *Br J Obstet Gynaecol* 107:896–902
- Korsmeyer SJ (1999) BCL-2 gene family and the regulation of programmed cell. *Death Cancer Res* 59:1693s–1700s
- Krause GS, Whitte BC, Aust SD, Nayini NR, Kumar KL (1988) Brain cell death following ischemia and reperfusion: a proposed biochemical consequence. *Crit Care Med* 16:714–726
- Lagercrantz H, Bistoletti P (1977) Catecholamine release in the newborn infant at birth. *Pediatr Res* 11:889–893
- Lautier D, Lagueux J, Thibodeau J, Menard L, Poirier GG (1993) Molecular and biochemical features of poly (ADP-ribose) metabolism. *Mol Cell Biochem* 122:171–193
- Lee HY, Naha N, Kim JH, Jo MJ, Min KS, Seong HH, et al. (2008) Age- and area-dependent distinct effects of ethanol on bax and bcl-2 expression in prenatal rat brain. *J Microbiol Biotechnol* 18(9):1590–1598
- Lima DC, Vale TG, Arganãraz GA, Varella PP, Frussa-Filho R, Cavalheiro EA, et al. (2010) Behavioral evaluation of adult rats exposed in utero to maternal epileptic seizures. *Epilepsy Behav* 18:45–49
- Lima DC, Cossa AC, Perosa SR, Oliveira EM, Junior JAS, Fernandes MJS, et al. (2011) Neuroglobin is up regulated in cerebellum of pups exposed to maternal epileptic seizures. *Int J Dev Neurosci* 29(8): 891–897
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275
- Meador KJ, Baker GA, Finnell RH, Kalayjian LA, Liporace JD, Loring DW, et al. (2006) In utero antiepileptic drug exposure: fetal death and malformations. *Neurology* 67:407–412
- Merry DE, Korsmeyer SJ (1997) Bcl-2 gene family in the nervous system. *Annu Rev Neurosci* 20:245–267
- Minkoff H, Schaffer R, Delke I, Grunevaum A (1985) Diagnosis of intracranial hemorrhage in utero after a maternal seizure. *Obstet Gynecol* 65:225–245
- Northington FJ, Ferriero DM, Graham EM, Traystman RJ, Martin LJ (2001) Early neurodegeneration after hypoxia–ischemia in neonatal rat is necrosis while delayed neuronal death is apoptosis. *Neurobiol Dis* 8:207–219
- Nowak JTS, Fried RL, Lust D, Passonneau JV (1985) Changes in brain energy metabolism and protein synthesis following transient bilateral ischemia in the gerbil. *J Neurochem* 44:487–494
- Oltvai ZN, Millman CL, Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homology, bax, that accelerates programmed cell death. *Cell* 74:609–619
- Pospisilik JA, Knauf C, Joza N, Benit P, Orthofer M, Cani PD, et al. (2007) Targeted deletion of AIF decreases mitochondrial oxidative phosphorylation and protects from obesity and diabetes. *Cell* 131: 476–491
- Procianoy RS, Silveira RC (2001) Hypoxic-ischemic syndrome. *J Pediatr* 77:S63–S70
- Racine R (1972) Modification of seizure activity by electrical stimulation II. Motor seizure. *Electroencephalogr Clin Neurophysiol* 32:281–294
- Ramachandra R, Subramanian T (2011) Atlas of the neonatal rat brain, 1st edn. CRC Press, Taylor & Francis Group, New York
- Smulson ME, Simbulan-Rosenthal CM, Boulares AH, Yakovlev A, Stoica B, Iyer S, et al. (2000) Roles of poly(ADP-ribosylation) and

- PARP in apoptosis, DNA repair, genomic stability and functions of p53 and E2F-1. *Adv Enzym Regul* 40:183–215
- Tuor UI, Manley JJ, Fyfe CA, Bascaramurty S (1999) Dexamethasone effects on cerebral protein synthesis prior to and following hypoxia-ischemia in immature rat. *Brain Res Bull* 48(1):61–64
- Vale, TG (2007) Efeito de crises epilépticas maternas sobre o desenvolvimento da prole. PhD Thesis. Universidade Federal de São Paulo, Departamento de Neurologia e Neurocirurgia, Unpublished results.
- Vale TG, Silva AV, Lima DC, Lima E, Torres LB, Cossa AC, et al. (2010) Seizures during pregnancy modify the development of hippocampal interneurons of the offspring. *Epilepsy Behav* 19:20–25
- Valente SG, Naffah-Mazzacoratti MG, Pereira M, Silva I, Santos NF, Baracat EC, Cavalheiro EA, Amado D (2002) Castration in female rats modifies the development of pilocarpine model of epilepsy. *Epilepsy Res* 49:181–188
- Wang Y, Dawson VL, Dawson TM (2009) Poly ADP-ribose signals to mitochondrial AIF: a key event in parthanatos. *Exp Neurol* 218(2): 193–202
- White E (1996) Life, death and the pursuit of apoptosis. *Genes Dev* 10:1–15
- Yerby MS (2000) Quality of life, epilepsy advances, and the evolving role of anticonvulsants in women with epilepsy. *Neurology* 55:21–31
- Yin XM, Oltvai ZN, Korsmeyer SJ (1994) BH1 and BH2 domains of bcl-2 are required for inhibition of apoptosis and heterodimerization with bax. *Nature* 369:321–323