Neuroscience 180 (2011) 191–200

NUCLEOSIDE TRIPHOSPHATE DIPHOSPHOHYDROLASES ROLE IN THE PATHOPHYSIOLOGY OF COGNITIVE IMPAIRMENT INDUCED BY SEIZURE IN EARLY AGE

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Abstract—Studies have shown that seizures in young animals lead to later cognitive deficits. There is evidence that long-term potentiation (LTP) and long-term depression (LTD) might contribute to the neural basis for learning and memory mechanism and might be modulated by ATP and/or its dephosphorylated product adenosine produced by a cascade of cell-surface transmembrane enzymes, such as E-NTPDases (ecto-nucleoside triphosphate diphosphohydrolases) and ecto-5′-nucleotidase. Thus, we have investigated if hippocampal ecto-nucleotidase activities are altered at different time periods after one episode of seizure induced by kainic acid (KA) in 7 days old rats. We also have evaluated if 90 day-old rats previously submitted to seizure induced by KA at 7 days of age presented cognitive impairment in Y-maze behavior task. Our results have shown memory impairment of adult rats (Postnatal day 90) previously submitted to one single seizure episode in neonatal period (Postnatal day 7), which is accompanied by an increased ATP hydrolysis in hippocampal synaptosomes. The metabolism of ATP evaluated by HPLC confirmed that ATP hydrolysis was faster in adult rats treated with KA in neonatal period than in controls. Surprisingly, the mRNA and protein levels as seen by PCR and Western blot, respectively, were not altered by the KA administration in early age. Since we have found an augmented hydrolysis of ATP and this nucleotide seems to be important to LTP induction, we could assume that impairment of memory and learning observed in adult rats which have experienced a convulsive episode in postnatal period may be a consequence of the increased ATP hydrolysis. These findings correlate the purinergic signaling to the cognitive deficits induced by neonatal seizures and contribute to a better understanding about the mechanisms of seizure-induced memory dysfunction.

Key words: ATP, memory, early seizure, adenosine, NTPDases, 5′-nucleotidase.

Seizures occur more frequently in the neonatal period and early childhood than any other time in life (Holmes, 1997). Furthermore, there is general agreement that epilepsy in childhood carries a significant risk for a variety of problems involving cognition and behavior. To support this idea, it has been shown that prolonged or frequent seizures in young animals lead to later cognitive deficits, which can often be subtle (Holmes, 2004; Cilio et al., 2003; Huang et al., 2002). In fact, one single seizure induced by Kainic acid (KA) in rats at 7 days of age was able to induce impairment on spatial memory in radial and water mazes (Sayin et al., 2004) and Y-maze task in adulthood (Cognato et al., 2010). Despite several studies on this matter, the mechanism responsible for these seizure-related cognitive deficits in the developing brain is not clearly defined.

There is several evidence that long-term potentiation (LTP) and long-term depression (LTD) contribute to the neural basis for learning and memory mechanism (see Cooke and Bliss, 2006). These synaptic plasticity phenomena may be modulated by ATP or adenosine. Application of extracellular ATP in CA1 neurons of hippocampus was able to induce LTP after 10 min (Fujii, 2004). Meanwhile, adenosine attenuates both LTP and LTD through adenosine A_1 receptors in the same hippocampus CA1 neurons (De Mendonça and Ribeiro, 1997). Furthermore, the neuromodulatory effects of this nucleoside remain persistent after the LTP and LTD (De Mendonça et al., 2002).

ATP may be hydrolyzed to its respective nucleoside adenosine by a cascade of cell-surface-bound enzymes, named ecto-nucleotidases. In this group of enzymes, it has been proposed that NTPDases and ecto-5′-nucleotidase constitute an enzymatic cascade able to promote the hydrolysis of triphosphate and diphosphate nucleotides to the respective nucleosides. In rats, this hydrolysis can occur in the central nervous system (CNS) (Battastini et al., 1991;

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Abbreviations: ADP, adenosine di-phosphate; AMP, adenosine monophosphate; ATP, adenosine tri-phosphate; CNS, central nervous system; GPI, Glycosylphosphatidylinositol; ITI, inter-trial interval; KA, kainic acid; LTD, long-term depression; LTP, long-term potentiation; NTPDases, nucleoside triphosphate diphosphohydrolases; Pi, inorganic phosphate; PN, postnatal day.

0306-4522/11 $ - see front matter
doi:10.1016/j.neuroscience.2011.01.065

Cruz Portela et al., 2002) by the action of the same set of enzymes. Eight different enzymes are described as members of the NTPDase family (Robson et al., 2006; Zimmermann, 2006). NTPDase1, -2, -3 and -8 are ecto-enzymes (E-NTPDases), with their catalytic site facing to extracellular space. On the other hand, the enzymes classified as NTPDase4, -5, -6, and -7 show intracellular localization. Among them, NTPDase5 and -6 could present secreted forms (Robson et al., 2006). Ecto-5′-nucleotidase is a Glycosylphosphatidylinositol-anchored enzyme which was also described in rat CNS (Sadasivudu et al., 1980; Braun et al., 1994). This ecto-enzyme was identified on mossy fibers that sprout after seizures in kainate-treated and kindled rats (Schoen et al., 1999). Over the years, our laboratory has studied the involvement of nucleotidases in epilepsy. A significant increase in nucleotide hydrolysis has been demonstrated in hippocampal synaptosomes of rats submitted to pilocarpine and KA models of epilepsy (Bonan et al., 2000b) as well as kindling induced by Pentylenetetrazole (PTZ) (Bonan et al., 2000a). However, the increase of adenosine tri-phosphate (ATP), adenosine di-phosphate (ADP), and adenosine mono-phosphate (AMP) hydrolysis showed in adult animals was not observed in young rats (Cognato et al., 2005). The enhancement of nucleotide hydrolysis has been also observed in blood serum (Bruno et al., 2003) and cerebrospinal fluid (Oses et al., 2007) of rats submitted to administration of PTZ.

Thus, in the present work, we have investigated if hippocampal ecto-nucleotidase activities are altered at different time periods after one episode of seizure induced by KA in 7 day-old rats. The times analyzed correspond to the maturation period of the brain (7, 14, and 21 days of age) and in the adult brain (90 days of age). We also have evaluated if 90 day-old rats previously submitted to seizure induced by KA at 7 days of age presented cognitive impairment in Y-maze behavior task.

EXPERIMENTAL PROCEDURES

Reagents

Nucleotides (ATP, ADP, and AMP), Percoll, Trizma base, Malachite Green Base, Coomassie Brilliant Blue G, EDTA, HEPES, and Kainic Acid were purchased from Sigma, St. Louis, MO, USA. Trizol reagent, SuperScript™ III First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) kit, and Taq DNA polymerase were purchased from Invitrogen, USA. All other reagents were of analytical grade.

Animals

Male Wistar rats (postnatal day 7; PN7) were used in the initial phase of this study. Animals had access to food and water *ad libitum* and were housed in plastic cages with lights on from 7:00 to 19:00 at room temperature of 23±1 °C. To avoid interlitter variations, different treatment groups (saline and KA) as well as the age of analyses of each group (PN7, PN14, PN21, and PN90) were represented in each litter. During the induction of seizure, all pups were transiently separated from their mothers. Procedures for the care and use of animals were adopted according to the regulations of Brazilian College of Animal Experimentation (COBEA), based on the Guide for the Care and Use of Laboratory Animals National Research Council.

Induction of seizures

Seizures were induced by the systemic administration of KA on postnatal day 7 (PN7). KA was dissolved in saline and administered i.p. (1 ml/kg) to male rats at a dosage of 2 mg/kg. According to previous studies, this dosage reliably induces electrographic and behavior seizures (Stafstrom et al., 1992; Lynch et al., 2000). Male littermates, injected with 0.9% (wt/vol) NaCl, were considered the control group. Seizures were observed and their characteristics noted over a 3 h period. After that, animals were returned to their dams and were allowed to reach the following ages, in which nucleotide hydrolysis was analyzed: 14 days old (postnatal day 14; PN14), 21 days old (postnatal day 21; PN21), and 90 days old (postnatal day 90; PN90). Nucleotide hydrolysis of PN7 animals was analyzed 12 h after the injection of KA. Adult animals (PN90) were also evaluated in behavioral tests.

Behavioral tasks

In order to verify if KA seizures induced at PN7 could lead to later cognitive impairment, we have performed behavioral tasks in adult rats (PN80–PN90) previously submitted to seizures in early childhood. Open-field task was applied on PN80. On the next day, rats were submitted to elevated plus maze task. Finally, on PN83, rats performed a memory task in a Y-maze apparatus with an inter-trial interval (ITI) of 2 min. 1 week later, rats (PN90) were submitted to a Y-maze task with 2 h of ITI. All behavioral experiments were performed between 9:00 AM and 4:00 PM (light phase).

Open-field and elevated plus maze tasks

Locomotor activity and exploratory behavior were monitored by using an open-field apparatus which consisted in a 50×50 cm^2 arena, divided into four squares of 25 cm. The proposal of this test was to determine whether the control and experimental groups differed in baseline locomotor activity. The exploratory behavior of the rats was evaluated by counting the total number of line crossings over a period of 5 min without prior habituation to the arena. In parallel, the number of rearings was also counted.

Evaluation of the anxiety was also tested by using the elevated plus maze. The apparatus consisted of two open arms 50×10 cm^2 at right angles to two covered (closed) arms, 50×40×10 cm^3. The maze was elevated to a height of 50 cm above the ground, forming an aversive stimulus to animals in the open arms. The animal was placed in the center of the maze with its head facing a closed arm. Rats were tested for 5 min. Entry into a particular arm was defined as the placement of all four feet into the arm. The relative time spent in open versus close arms is a measure of anxiety with anxious rats preferring closed arms.

Y-maze task

The Y-maze is a simple two-trial recognition test for measuring spatial recognition memory in rodents. Y-maze apparatus consisted of three arms (10 cm of width, 50 cm of length, and 20 cm of height) with an angle of 120° between each arm. The three identical arms were randomly designated: start arm, in which the rat started to explore (always open), novel arm, which was blocked during the first trial, but open during the second trial, and other arm (always open). The maze was placed in a separate room with a red light. The floor of the maze was covered with sawdust, which was mixed after each individual trial in order to eliminate olfactory stimuli. Visual cues were placed next to the walls of the maze. The Y-maze test consisted of two trials separated by an ITI to assess response to novelty (2 min ITI) and spatial recognition memory (2 h ITI). The first trial (training) had 5 min duration and allowed the rat to explore only two arms (start arm and other arm) of the maze, with the third arm (novel arm) blocked. For the second trial (after ITI), the rat was placed back in

the maze in the same starting arm, with free access to all three arms for 5 min. The number of entries and time spent in each arm were analyzed. Data were expressed as percentage of total time spent in arms as well as percentage of total numbers of entries in each arm with SEM of each group.

Subcellular fraction

Animals previously submitted to KA-seizures at 7 days of age were euthanized by decapitation at different ages (PN7, PN14, PN21, and PN90). Their brains were removed and placed in ice-cold isolation medium (320 mM sucrose, 5 mM HEPES, pH 7.5, and 0.1 mM EDTA) and were cut longitudinally. Total hippocampi of both hemispheres were immediately dissected on ice. The total hippocampi were gently homogenized in five volumes of ice-cold isolation medium with a motor-driven Teflon-glass homogenizer. The synaptosomes were isolated as previously described (Nagy and Delgado-Escueta, 1984). Briefly, 0.5 ml of crude mitochondrial fraction was mixed with 4.0 ml of an 8.5% Percoll solution and layered onto an isoosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were washed twice at 15,000×g for 20 min with the same ice-cold medium to remove the contaminating Percoll and the synaptosome pellet was resuspended to a final protein concentration of approximately 0.5 mg/ml. The material was prepared fresh daily and maintained at 0–4 °C throughout preparation.

Enzyme assays

The reaction medium used to assay the ATP and ADP hydrolysis was essentially as described previously (Battastini et al., 1991). The reaction medium contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 ml. The synaptosome preparation (10–20 μg protein) was added to the reaction mixture and preincubated for 10 min at 37 °C. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1.0 mM and the reaction was stopped by the addition of 200 μl 10% trichloroacetic acid. The released inorganic phosphate (Pi) was measured as previously described (Chan et al., 1986). The reaction medium used to assay the 5′-nucleotidase activity (AMP hydrolysis) contained 10 mM $MgCl_2$, 0.1 M Tris–HCl, pH 7.0, and 0.15 M sucrose in a final volume of 200 μl (Heymann et al., 1984). The synaptosome preparation (10–20 μg protein) was preincubated for 10 min at 37 °C. The reaction was initiated by the addition of AMP to a final concentration of 1.0 mM and was stopped by the addition of 200 μl 10% trichloroacetic acid; the released inorganic phosphate (Pi) was measured as previously described (Chan et al., 1986). In all enzyme assays, incubation times and protein concentration were chosen in order to ensure the linearity of the reactions (Battastini et al., 1991; Heymann et al., 1984). Controls with the addition of the enzyme preparation after addition of trichloroacetic acid were used to correct nonenzymatic hydrolysis of the substrates. All samples were run in triplicate.

Protein determination

Protein was measured by the Coomassie Blue method, using bovine serum albumin as standard (Bradford, 1976).

Analysis of extracellular ATP metabolism by high performance liquid chromatography (HPLC)

Synaptosomal samples were obtained as described before. The reaction medium contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, and 45 mM Tris–HCl buffer (pH 8.0) in a final volume of 200 μl. The synaptosome preparation (10–20 μg protein) was added to the reaction mixture and preincubated for 10 min at 37 °C. To start the reaction, ATP was added to the medium in a final concentration of 0.1 mM at 37 °C. Aliquots of the sample were collected at different incubation times (0–120 min), with the reaction being stopped on ice. All samples were centrifuged 14,000×g for 15 min. Aliquots of 40 μl were applied to a reversed-phase HPLC system using a 25 cm C_{18} Shimadzu column (Shimadzu, Japan) at 260 nm with a mobile phase containing 60 mM KH_2PO_4, 5 mM tetrabutylammonium chloride, pH 6.0, in 30% methanol according to a method previously described (Voelter et al., 1980). The peaks of purines (ATP, ADP, AMP, and adenosine) were identified by their retention times and quantified by comparison with standards. The results are expressed as nmoles of the different compounds per mg of protein for each different incubation time. All incubations were carried out in triplicate and the controls to correct nonenzymatic hydrolysis of nucleotides were performed by measuring the peaks present into the same reaction medium without synaptosomes. The control for intrinsic synaptosomal purines was performed by incubation of the preparation without the substrate under the same conditions.

Analysis of gene expression by semi-quantitative RT-PCR

The expression analysis of NTPDase1–3 was carried out by a semi-quantitative RT-PCR assay. Rats at PN90 (saline $n=4$, KA $n=5$) were euthanized by decapitation, the brains were removed and placed in ice-cold RNAse free water. Hippocampus of both hemispheres was dissected and immediately frozen with liquid nitrogen for storage in −80 °C freezer. The total RNA extraction was performed using Trizol reagent in accordance with the manufacturer instructions. The cDNA species were synthesized with SuperScript SuperScript™ III First-Strand Synthesis System for RT-PCR from 1 μg of total RNA and oligo (dT) primer in accordance with the suppliers. RT reactions were performed for 50 min at 50 °C. cDNA (1 μl) was used as a template for PCR with specific primers for NTPDase1, -2, and -3. Rat DNA sequences encoding NTPDase1 (NM_022587.1), NTPDase2 (O35795), NTPDase3 (NM_178106) were retrieved from GenBank database and aligned using ClustalX program. Regions with low scores of similarity among sequences were used to construct specific primers using Oligos 9.6 program. Each primer was blasted against rat genome in order to confirm its specificity. The strategy adopted to design the primers does not allow cross-amplification. Specific primers were also constructed to β-actin (NM_031144). β-actin-PCR was performed as a control for cDNA synthesis. PCR reactions had a volume of 25 μl using a concentration of 0.4 μM of each primer indicated below, 200 μM dNTP, 2 mM $MgCl_2$, and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer.

Conditions for all PCRs were as follow: Initial 1 min denaturation step at 94 °C, 1 min annealing step (NTPDase1 and -3: 65 °C; NTPDase2: 66 °C; β-actin: 58.5 °C), 1 min extension step at 72 °C for 35 cycles and a final 10 min extension at 72 °C. The PCR products were: NTPDase1—543 bp; NTPDase2—331 bp; NTPDase3—267 bp, β-actin—210 bp. PCR products were submitted to electrophoresis using a 1% agarose gel. Bands intensities were analyzed by Kodak 1D v.3.5.4 software. The following set of primers were used: RnNTPDase1F: 5′-GAT CAT CAC TGG GCA GGA GGA AGG-3′ and RnNTPDase1R 5′-AAG ACA CCG TTG AAG GCA CAC TGG-3′; RnNTPDase2F: 5′-GCT GGG TGG GCC GGT GGA TAC G-3′ and RnNTPDase2R 5′-ATT GAA GGC CCG GGG ACG CTG AC-3′; RnNTPDase3F: 5′-CGG GAT CCT TGC TGT GCG TGG CAT TTC TT-3′ and RnNTPDase3R 5′-TCT AGA GGT GCT CTG GCA GGA ATC AGT-3′; and for β-actin: RnβactinF 5′-TAT GCC AAC ACA GTG CTG TCT GG-3′ and RnβactinR 5′-TAC TCC TGC TTC CTG ATC CAC AT-3′.

The relative abundance of each mRNA versus β-actin was determined by densitometry using the freeware ImageJ 1.37 for Windows. Each experiment was repeated four times using RNA

isolated from independent extractions. The expression analysis was performed in replicate and representative findings were shown.

Western blotting analysis

After all behavioral tests, the rats were killed by decapitation for the preparation of Percoll-purified hippocampal synaptosomes as previously described (e.g. Rebola et al., 2005). Briefly, the hippocampi from rats were homogenized at 4 °C in sucrose solution (0.32 M) containing 50 mM Tris–HCl, 2 mM EGTA, and 1 mM dithiothreitol, pH 7.6. The resulting homogenates were centrifuged at 3000×g for 10 min at 4 °C, the supernatants collected and centrifuged at 14,000×g for 20 min at 4 °C. The pellets were resuspended in 1 ml of a 45% (v/v) Percoll solution made up in a Krebs solution (composition 140 mM NaCl, 5 mM KCl, 25 mM HEPES, 1 mM EDTA, 10 mM glucose, pH 7.4). After centrifugation at 14,000×g for 2 min at 4 °C, the top layer was removed (synaptosomal fraction), and washed in 2 ml Krebs solution. This mixture was centrifuged at 14,000×g for 2 min at 4 °C. The pellet corresponded to the nerve terminal membranes and was immediately homogenized in 5% SDS with a protease inhibitor cocktail (Sigma, São Paulo/Brazil) and frozen at −70 °C. After defrost, the protein content was determined by Bicinchoninic acid assay (Smith et al., 1985) using bovine serum albumin (BSA) as standard (Pierce, São Paulo/Brazil). Samples extracts were diluted to a final protein concentration of 2 μg/μl in SDS-PAGE buffer under nonreducing conditions. 50μg of the samples and prestained molecular weight standards (Bio-Rad, São Paulo/Brazil) were separated by SDS-PAGE (7.5% with 4% concentrating gel). After electro-transfer, the membranes were blocked with Tris–buffered saline 0.1% Tween-20 (TBS-T) containing 3% BSA for 1 h. The membranes were then incubated overnight at 4 °C with rabbit anti rat-NTPDase1 (rN1-6$_L$(I_4,I_5); dilution 1:3000; Vorhoff et al., 2005), rabbit anti-rat NTPDase2 (BZ3-4F; dilution 1:500; Kishore et al., 2005), and rabbit anti-rat NTPDase3 (rN3-1$_L$(I_4I_5); rN3-2$_L$(I_4I_5); rN3-3$_L$(I_4I_5); dilution 1:500; Kittel et al., 2007). After primary antibodies incubation, membranes were washed and incubated with rabbit-peroxidase conjugated secondary antibodies (dilution 1:10,000 from Abcam) for 1 h at room temperature and developed with chemiluminescence ECL kit (Amersham, São Paulo/Brazil). The autoradiographic films were scanned and densitometric analyses were performed by densitometry using the freeware ImageJ 1.37 for Windows. The membranes were then re-probed and tested for β-actin (1:10,000 dilution, from Abcam) immunoreactivity to confirm that similar amounts of protein were applied to the gels.

Statistical analysis

Data are mean±SD of n animals in all experiments except for behavioral experiments, when data were represented by mean±SEM. Significance ($P<0.05$) was assessed by Student's *t*-test or by a two-way ANOVA to compare data experimental groups.

RESULTS

Behavioral changes after KA injection

Status epilepticus (SE) was observed in all KA-treated animals. PN7 rats initially became immobile with loss of limb tone and ataxia. Then, they exhibited intermittent hyperactivity with rhythmic "bicycling" movements of all extremities, opisthotonic arching, and tonic limb extension.

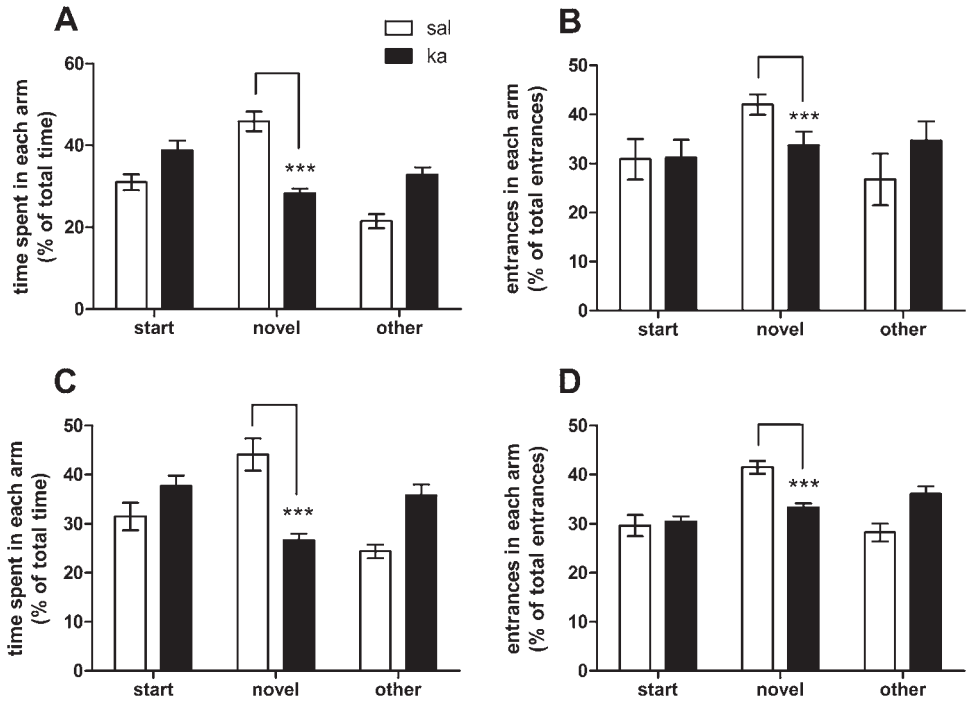

Fig. 1. Rats submitted to a kainate-induced convulsive period early in childhood display selective memory impairment in adulthood. Pups with 7 d of age received an i.p. injection of either saline (open bars) or kainate (2 mg/kg, black bars), which caused a period of convulsions. Rats were then behaviorally analyzed at postnatal day 83–90 (PN83–90). Data represent the percentage time spent in each arm (A with 2 min ITI; C with 2 h ITI) and the percentage of total entrances (B with 2 min ITI; D with 2 h ITI). Bars represent the mean±SEM of eight (saline) and nine (KA) animals. Novel arm was analyzed individually using the Student's *t*-test with *** $P<0.005$.

Y-maze task

Initially, rats previously submitted to KA-seizures at PN7 were allowed to reach adulthood and the response to novelty (at PN93) and spatial memory (at PN100) were tested in Y-maze task (Fig. 1). A decrease ($P<0.0001$) in the time spent in the novel arm (28.3±1.1) was observed in rats submitted to KA seizures at PN7 when compared to control group (45.9±2.4; Fig. 1A) after the ITI of 2 min KA-treated rats also presented a reduced number of entrances in novel arm (33.6±0.9) when compared to control group (42.0±0.7; $P<0.0001$; Fig. 1B) after the ITI of 2 min. Spatial memory (2 h ITI) of early seizures rats also revealed impairment in Y-maze test. Time spent in novel arm was reduced for rats submitted to KA seizures at neonatal period (26.6±1.3) when compared with control groups that did not receive KA (44.1±3.3; $P<0.0001$; Fig. 1C) after ITI of 2 h. Finally, KA-treated rats also presented a diminished number of entrances in novel arm (33.3±0.7) when compared to control animals (41.5±1.3; $P<0.0001$; Fig. 1D). It is important to mention that no alterations in locomotor activity (Fig. 2A) and anxiety profile (Fig. 2B, C) were observed in animals submitted to KA seizures in neonatal period.

Nucleotide hydrolysis

The extracellular hydrolysis of ATP, ADP, and AMP was evaluated at different time points in hippocampal synaptosomes after one single seizure episode induced by KA in PN7 rat pups.

ATP hydrolysis

12 h after the seizure (PN7), ATP hydrolysis from KA group (92.7±2.6 nmol Pi. $min^{-1}.mg^{-1}$ protein) was not significantly different in relation to control group (88.2±6.6 nmol Pi. $min^{-1}.mg^{-1}$ protein; $P>0.05$; Fig. 3A. ATP hydrolysis from KA groups at PN14 (113±15.6 nmol Pi. $min^{-1}.mg^{-1}$ protein) and PN21 (129±33 nmol Pi. $min^{-1}.mg^{-1}$ protein) also were not significantly different when compared to the respective control groups (PN14—112±17 nmol Pi. $min^{-1}.mg^{-1}$ protein and PN21—102±17 nmol Pi. $min^{-1}.mg^{-1}$ protein; $P>0.05$; Fig. 3A). However, ATP hydrolysis in PN90 rats previously submitted to a convulsive episode at PN7 was increased (234±45 nmol Pi. $min^{-1}.mg^{-1}$ protein) when compared to the respective control group (160±34 nmol Pi. $min^{-1}.mg^{-1}$ protein; $P<0.05$; Fig. 3A). Our results also indicate an enhancement of ATP hydrolysis from PN7 (88±7 nmol Pi. $min^{-1}.mg^{-1}$ protein) to PN90 (160±34 nmol Pi. $min^{-1}.mg^{-1}$ protein; $P>0.05$; Fig. 3A) in saline-treated animals throughout aging.

ADP hydrolysis

ADP hydrolysis from PN7 rats formerly treated with KA (43.4±5.9 nmol Pi. $min^{-1}.mg^{-1}$ protein) was not significantly different in relation to control group (36.6±1.1 nmol Pi. $min^{-1}.mg^{-1}$ protein; $P>0.05$; Fig. 3B). Likewise, PN14 rats submitted to KA at PN7 presented an ADP hydrolysis (45.4±3.0 nmol Pi. $min^{-1}.mg^{-1}$ protein) statistically similar to the control group (50.4±4.9 nmol Pi. $min^{-1}.mg^{-1}$ protein; $P>0.05$; Fig. 3B). The ADP hydrolysis from KA group at PN21 (71.3±7.3 nmol Pi. $min^{-1}.mg^{-1}$ protein) was also not significantly different from ADP hydrolysis in the respective control groups (64±23 nmol Pi. $min^{-1}.mg^{-1}$ protein; $P>0.05$; Fig. 3B). PN90 rats previously submitted to a convulsive episode at PN7 showed an ADP hydrolysis (68.5±7.4) statistically similar to the one observed in the respective control group (56.5±9.5 nmol Pi. $min^{-1}.mg^{-1}$ protein; $P<0.05$; Fig. 3B). An enhancement in ADP hydrolysis was observed throughout aging in saline-treated animals from PN7 (36.6±1.1 nmol Pi. $min^{-1}.mg^{-1}$ protein) to PN21 (71.3±7.3 nmol Pi. $min^{-1}.mg^{-1}$ protein) and PN90 (56.5±9.5 nmol Pi. $min^{-1}.mg^{-1}$ protein; $P>0.05$; Fig. 3B).

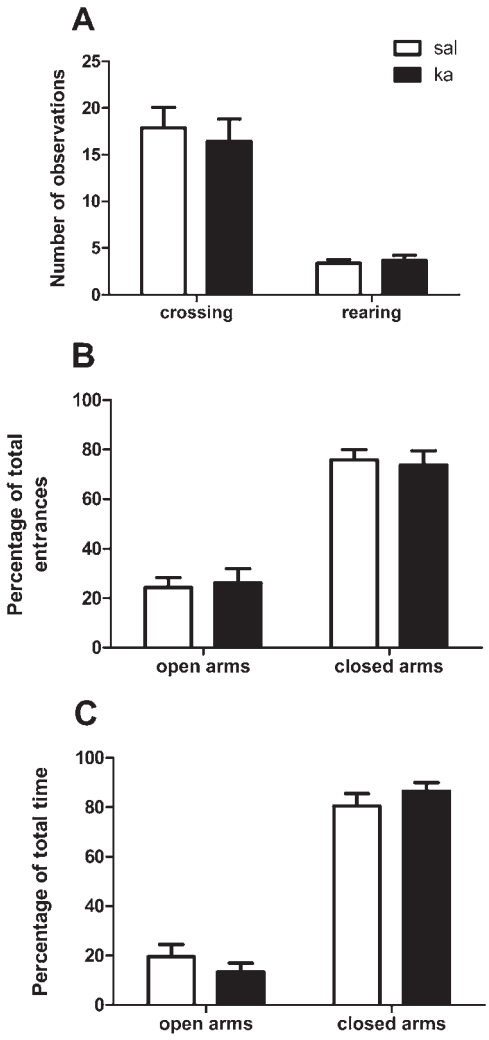

Fig. 2. Rats submitted to kainate-induced convulsive period early in childhood display no alterations in locomotor and anxiety behaviors. Locomotion was evaluated in an open field arena (A) by counting the number of crossings and rearings of the rats during 5 min. The degree of anxiety was determined by their preference for the closed and open arms of an elevated-plus maze (B, C). Data represent the means±SEM of eight (saline) and nine (KA) animals.

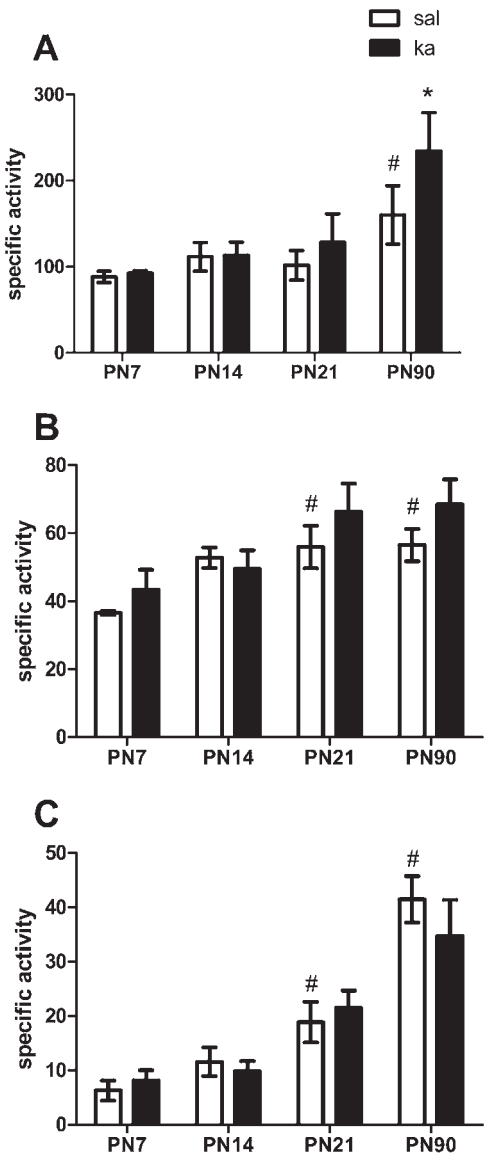

Fig. 3. Nucleotide hydrolysis of hippocampal synaptosomes of rats at PN90 previously submitted to early seizure at PN7. Specific enzyme activities were expressed as nmol Pi. $min^{-1}.mg^{-1}$ protein. Data represent the mean±SD of ATP (A), ADP (B), and AMP (C) hydrolysis of at least five animals per group. * Indicates difference between control (white bars) and KA (black bars)-treated groups at the same age. # indicates difference of control groups when compared to PN7 controls ($P<0.05$: two-way ANOVA followed by Tukey's posttest).

AMP hydrolysis

AMP hydrolysis from KA group at PN7 (8.2±1.8 nmol Pi. $min^{-1}.mg^{-1}$ protein) was not significantly different in relation to control group (5.3±0.7 nmol Pi. $min^{-1}.mg^{-1}$ protein; $P>0.05$; Fig. 3C). The AMP hydrolysis of KA groups at PN14 (8.3±1.4 nmol Pi. $min^{-1}.mg^{-1}$ protein) and PN21 (21.5±3.2 nmol Pi. $min^{-1}.mg^{-1}$ protein) were also not significantly different when compared to respective the respective control groups (PN14—8.7±1.5 nmol Pi. $min^{-1}.mg^{-1}$ protein and PN21—18.9±3.7 nmol Pi. $min^{-1}.mg^{-1}$ protein; $P>0.05$; Fig. 3C). Likewise, AMP hydrolysis at PN90 of rats previously treated with KA at PN7 (34.7±6.6 nmol Pi. $min^{-1}.mg^{-1}$ protein) was statistically similar to the respective control group (38.4±7.9 nmol Pi. $min^{-1}.mg^{-1}$ protein; $P<0.05$; Fig. 3C). Our results also have shown an increase in AMP hydrolysis from PN7 (5.25±0.7 nmol Pi. $min^{-1}.mg^{-1}$ protein) to PN21 (21.48±3.2 nmol Pi. $min^{-1}.mg^{-1}$ protein) and PN90 (38.35±7.9 nmol Pi. $min^{-1}.mg^{-1}$ protein; $P>0.05$; Fig. 3C).

Extracellular ATP metabolism by high performance liquid chromatography (HPLC)

As seen in Fig. 4, the control group hydrolyzed extracellular ATP in a slower fashion then PN90 rats submitted to one single seizure in early age. This pattern of hydrolysis was clearly evident from the steady accumulation of AMP (from 10 to 60 min of incubation) and adenosine (from 60 to 120 min of incubation) in the KA-treated group. The final levels of adenosine were 7.8 times higher in KA-treated group.

Expression of NTPDases at PN90

Since only ATP hydrolysis at PN90 rats was altered by the previous convulsive episode at PN7, we have evaluated the relative gene expression pattern and immunoreactivity of ecto-nucleotidases involved in ATP hydrolysis in brain: NTPDase1, NTPDase2, and NTPDase3. In the matter of gene relative expression, NTPDase1 at PN90 rats previously submitted to a single seizure in neonatal period (71±7% of relative expression) was not significantly different from NTPDase1 relative expression observed in control group (72±5% of relative expression; $P>0.05$; Fig. 5A). NTPDase2 relative expression of PN90 KA group (88±6% of relative expression) also was not significantly different from NTPDase2 relative expression in control group (79±6% of relative expression; $P>0.05$; Fig. 5A). Likewise, the relative expression of NTPDase3 of PN90 rats treated with KA at PN7 (49±9% of relative expression) was statistically similar to the one found in the control group (53±7% of relative expression; $P>0.05$; Fig. 5A). In agreement, the immunoreactivity of NTPDases as assessed by Western blot were also similar between PN90 KA group (NTPDase1, 66±6%; NTPDase2, 85±3%; and NTPDase3, 68±4% of immunoreactivity) and control group (NTPDase1, 62±4%; NTPDase2, 73±7%; and NTPDase3, 79±6% of immunoreactivity; $P>0.05$; Fig. 5B).

DISCUSSION

The present study has shown memory impairment of adult rats (PN90) previously submitted to one single seizure episode in neonatal period (PN7), which is accompanied by an increased ATP hydrolysis in hippocampal synaptosomes but no changes in ADP and AMP hydrolysis. Furthermore, the present results have shown that the relative expression and protein amount of NTPDases responsible for ATP hydrolysis was not altered by the KA administration in early age.

Previous studies had already reported that a single administration of KA at PN5–PN10 can lead to spatial

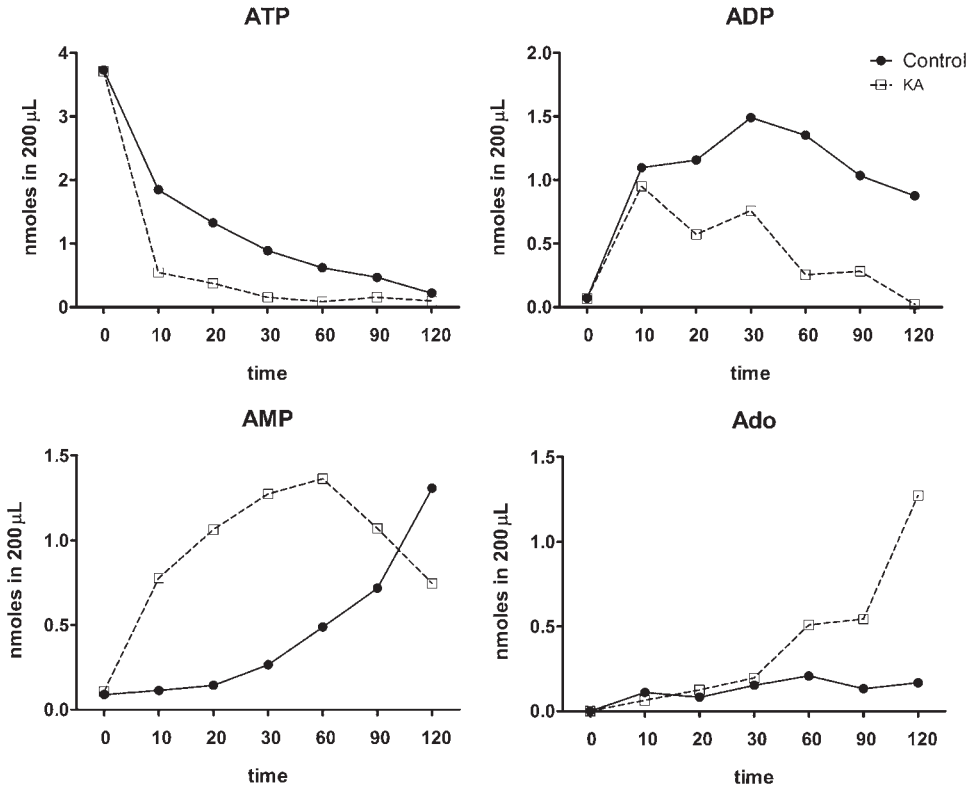

Fig. 4. Metabolism of ATP and product formation in synaptosomes of hippocampus from control rats and KA-treated PN90 rats. Synaptosomes were incubated with 0.1 mM ATP. ATP, ADP, AMP, and adenosine (ADO) contents in saline (black circles) and kainate (white squares) groups are presented as the mean (nmol)±SEM. Data represents a typical result of three independent experiments.

memory impairment later in life (Lynch et al., 2000; Sayin et al., 2004; Cornejo et al., 2007; Cognato et al., 2010). Similarly, induction of convulsive activity with different agents in immature rodents also triggers visuo-spatial memory dysfunction later in life (De Rogalski-Landrot et al., 2001; Lee et al., 2001; Huang et al., 2002; Cilio et al., 2003; Bo et al., 2004). Some of these studies confirmed that KA-induced neonatal convulsions-associated memory deficits are present at PN90 and later (Lynch et al., 2000; Sayin et al., 2004). However, other studies reported memory deficits early in adulthood (PN60) albeit they were rather marginal and only observed in some tasks (Cornejo et al., 2007). The present results have shown that the spatial memory deficits were observed at PN90 and corroborate with the data reported in literature. Furthermore, there were no changes in exploratory and locomotor activities (see also Lynch et al., 2000; Sayin et al., 2004) as well as no modification of anxiety-like behavior in the elevated-plus maze test, which could influence the performance in the Y-maze task. The findings related to anxiety are in disagreement with previous report (Sayin et al., 2004) which has shown a greater degree of anxiety of adult rats treated with KA in early age. This discrepancy might be related to the use of different rat strains (which were not specified in Sayin et al., 2004).

Once we have verified the cognitive deficits in adulthood induced by one single seizure episode in neonatal period and since ATP and adenosine are involved in the modulation of plastic phenomena (LTP and LTD) involved in learning and memory mechanisms, we have tested if enzymes responsible for the availability of ATP and adenosine in the synaptic cleft are changed by previous KA treatment. The time points chosen for the analysis correspond to the maturation periods of the brain (PN7, PN14, and PN21) and the adult age which presented the cognitive deficits after the neonatal seizure (PN90). Our results have demonstrated that there were no significantly changes in nucleotide hydrolysis at earlier time points after the seizure induction (PN7, PN14, and PN21). In fact, several studies have discussed that immature brain is less vulnerable to morphological and physiological alterations after status epilepticus when compared to mature brain (Haut et al., 2004; Cilio et al., 2003; Haas et al., 2001), although changes in behavior and brain connectivity are detected in adulthood (Holmes, 2004). The unaffected hydrolysis of nucleotides observed in the present study is in agreement with a previous study which revealed that ATP, ADP, and AMP hydrolysis were not changed in young rats exposed to pilocarpine model of epilepsy (Cognato et al., 2005). Our results also demonstrate that the ATP, ADP, and AMP hydrolysis observed in the control group have increased from PN7 to PN90. These data are in agreement with a previous study which has demonstrated that ATP and ADP hydrolysis increased significantly from birth until the second postnatal week in synaptosomal preparations of rat cerebral cortex (Müller et al., 1990). In addition, it is

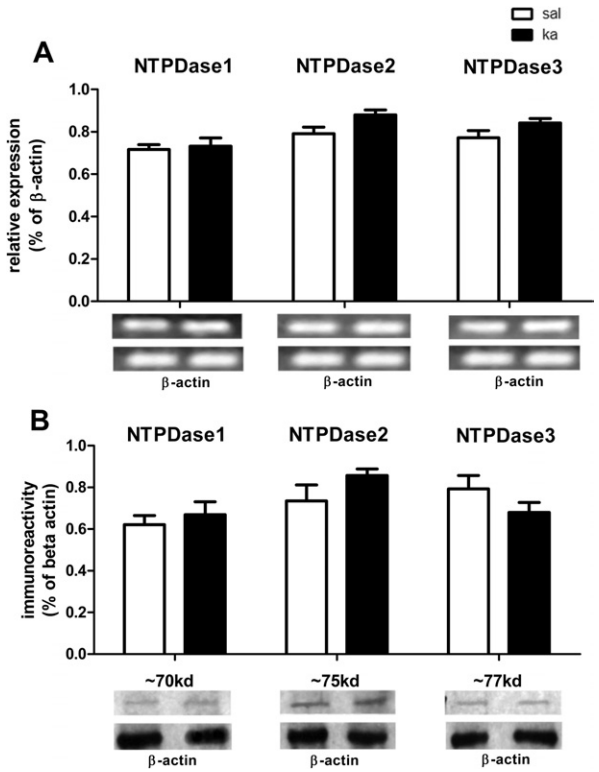

Fig. 5. Expression of hippocampal ecto-nucleotidases of rats at PN90 previously submitted to early seizure at PN7. Data represent the relative mRNA expression (A) and the Western blot analysis (B) of NTPDase1, NTPDase2, and NTPDase3 with the respective representative images (below). At least three (A) and four (B) independent experiments were performed, with entirely consistent results. There are no significant differences between control (white bars) and kainate (black bars)-treated groups using Student's *t*-test ($P>0.05$).

possible that ecto-nucletidases may be related to the process of adhesion and recognition between cells during development (Braun et al., 2003). Furthermore, the ecto-5′-nucleotidase is expressed at the surface of developing nervous cells and is regarded as a marker of neural development (Braun and Zimmermann, 1998). Also, it was reported that this enzyme activity increased in aging rat brain (Fuchs, 1991). Our results are in agreement with this study, since we have observed an increase in AMP hydrolysis from PN7 until PN90.

The results also have shown an increased ATP hydrolysis in hippocampal synaptosomes of PN90 rats, which were formerly submitted to KA seizure at PN7. We have also observed that adult animals treated with KA in early age revealed a faster ATP metabolism than PN90 control rats. Previous studies have already observed an enhancement in ecto-nucleotidase activities from CNS after induction of different models of epilepsy (Bonan et al., 2000a,b; Cognato et al., 2005, 2007; Oses et al., 2007). However, this is the first study demonstrating an increased nucleotide hydrolysis following a long period after the induction of one single seizure in early age. Despite short convulsive periods early in life not led to evident morphological modifications (Stafstrom, 2002; Holmes, 2005), it has been reported that the neural activity during development not only determinates the organization of neural circuits but may also influence the capacity for circuit plasticity later in life (Abraham and Bear, 1996; Feldman and Knudsen, 1998). Once ATP seems to be related to LTP and synaptic plasticity induction (Wieraszko, 1996; Fujii, 2004), the increased hydrolysis of this nucleotide observed in memory impaired adult rats could be one of several brain mechanisms altered by early seizure. In fact, Lynch and co-workers (2000) have observed that PN90 rats, formerly submitted to KA-induced seizures in early age (PN7), presented impairment in hippocampal LTP induction. Since we have found an augmented hydrolysis of ATP and this nucleotide seems to be important to LTP induction, we could assume that impairment of memory and learning observed in adult rats which have experienced a convulsive episode in postnatal period may be a consequence of the increased ATP hydrolysis. Interestingly, it reported a decrease in nucleotide hydrolysis of hippocampal synaptosomes of rats immediately euthanized after the training session of the inhibitory avoidance task (Bonan et al., 1998). This finding assumes that the presence of ATP in the synaptic cleft may be required for the mechanisms of memory retention and highlights the opposite situation of our data, in which ATP hydrolysis is enhanced and the memory impairment is observed.

We also have observed that enhanced ATP hydrolysis was not accompanied by change in gene expression or immunoreactivity of the NTPDase1–3, suggesting that the effects observed were not due to an increased synthesis of these proteins. Previous study has also reported that nucleotide hydrolysis changed by seizures was not accompanied by an increase in relative expression in ecto-nucleotidases. Oses and co-workers (2007) have found an increased AMP and GMP hydrolysis in hippocampal slices of rats submitted to kindling by pentylenetetrazole at 10 days after the last injection with no significant changes in the relative expression of 5′-nucleotidase (Oses et al., 2007). A possible explanation for the enhanced nucleotide hydrolysis accompanied by no changes in gene expression or immunoreactivity of the NTPDase1-3 observed in our data could be related to posttranslational events. According to analysis performed in NetPhosk, a kinase-specific prediction of protein phosphorylation sites tool (http://www.cbs.dtu.dk/), ecto-nucleotidase sequences present potential PKC phosphorylation sites. Protein phosphorylation is considered a key mechanism in processes underlying the induction of long-lasting changes in synaptic activity, including learning and memory formation (Kandel and Schwartz, 1982; Neary et al., 1981). The presence of neuronal ecto-protein kinase (Ehrlich et al., 1986) is able to regulate several neuronal functions, using extracellular ATP secreted from synaptic vesicles as a substrate in the ecto-protein phosphorylation (Ehrlich et al., 1990). Indeed, there is evidence that ecto-apyrase (now called NTPDase1) could be a phosphoprotein, and that such phosphorylation could affect its enzymatic activity (Wink et al., 2000).

CONCLUSION

The present study shows that one single seizure episode early in life caused a selective impairment of memory performance later in adulthood. This cognitive impairment was accompanied by an enhancement of extracellular ATP hydrolysis in hippocampal tissue. Since ATP and adenosine seems to be related to LTP, LTD, and synaptic plasticity induction, the control of nucleotide levels promoted by NTPDases could be one of the neurochemical mechanisms involved in the pathophysiology of adult cognitive deficit induced by early KA exposure. We also observed that there were no changes in gene expression as well as immunoreactivity of enzymes responsible for ATP hydrolysis. These findings correlate the purinergic signaling to the cognitive deficits induced by neonatal seizures and contribute to a better understanding about the mechanisms of seizure-induced memory dysfunction.

Acknowledgments—This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), FINEP research grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" 01.06.0842-00. J.S. was also the recipient of a Junior 2 scholarship from the Fonds de Recherche en Santé du Québec.

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(Accepted 31 January 2011)
(Available online 16 February 2011)