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Ontogenetic profile of ectonucleotidase activities from brain synaptosomes of pilocarpine-treated rats

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Abstract

Adenosine, a well-known neuromodulator, can act as an endogenous anticonvulsant via the activation of adenosine A_1 receptors. This adenine nucleoside can be produced in the synaptic cleft by the ectonucleotidase cascade, which includes the nucleoside triphosphate diphosphohydrolase (NTPDase) family and ecto-5′-nucleotidase. It has been previously reported that ectonucleotidase activities are increased in female adult rats submitted to the pilocarpine model of epilepsy. Several studies have suggested that the immature brain is less vulnerable to morphologic and physiologic alterations after status epilepticus (SE). Here, we evaluate the ectonucleotidase activities of synaptosomes from the hippocampus and cerebral cortex of male and female rats at different ages (7–9, 14–16 and 27–30-day old) submitted to the pilocarpine model of epilepsy. Our results show that ATP and ADP hydrolysis in the hippocampus and cerebral cortex were not altered by the pilocarpine treatment in female and male rats at 7–9, 14–16 and 27–30 days. There were no changes in AMP hydrolysis in female and male rats submitted to the model at different ages, but a significant increase in AMP hydrolysis (71%) was observed in synaptosomes from the cerebral cortex of male rats at 27–30 days. Pilocarpine-treated male rats (60–70-day old) presented an enhancement in ectonucleotidase activities in the synaptosomes of the cerebral cortex (33, 40 and 64% for ATP, ADP and AMP hydrolysis, respectively) and hippocampus (55, 98 and 101% for ATP, ADP and AMP hydrolysis, respectively). These findings highlight differences between the purinergic system of young and adult rats submitted to the pilocarpine model of epilepsy.

Keywords: Ectonucleotidases; Adenosine; Epilepsy; Pilocarpine model; Young rats

1. Introduction

Studies have suggested that the immature brain is less vulnerable to morphologic and physiologic alterations after status epilepticus (SE) (Haut et al., 2004; Cilio et al., 2003; Haas et al., 2001; Stafstrom et al., 1993; Sperber et al., 1991). There is a different sensitivity of developing rats to the pilocarpine model of temporal lobe epilepsy when compared to adult rats (Cavalheiro et al., 1987). Indeed, a previous study demonstrated that none of a group of 7- to 17-day-old rats developed spontaneous recurrent seizures after pilocarpine-induced SE in adulthood (Priel et al., 1996) and the susceptibility to spontaneous recurrent seizures in the chronic period of pilocarpine model increases with age (Priel et al., 1996).

Adenosine is a ubiquitous homeostatic substance and its role as a neuromodulator is extensively reported (Ribeiro et al., 2003). There is evidence that adenosine can act as an endogenous anticonvulsant (During and Spencer, 1992; Dragunow, 1988) probably by the activation of adenosine A_1 receptors, which lead to an inhibition in neurotransmitter release (Brundege and Dunwiddie, 1997). It is known that the expression of adenosine A_1 receptors and their coupling with G-proteins and other signaling molecules are similar in young and adult rats (Rivkees, 1995). Several studies have reported an anticonvulsant action of adenosine and its analogues in adult rats submitted to different animal models of epilepsy (Aden et al., 2004; Avsar and Empson, 2004; Boison et al., 2002; Khan et al., 2000; Turski et al., 1985).

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In addition to bi-directional transport, extracellular adenosine may be produced through ATP catabolism mediated by ectonucleotidases (Dunwiddie and Masino, 2001) such as nucleoside triphosphate diphosphohydrolases (NTPDases) and ecto-5′-nucleotidase. It has been shown that hydrolysis of ATP, ADP and AMP increases with age (Fuchs, 1991; Müller et al., 1990); our laboratory has shown increased ectonucleotidase activities in adult rats using several animal models of epilepsy, including the pilocarpine model (Bonan et al., 2000).

In order to identify neurochemical differences between immature and mature brains in pilocarpine-treated rats and bearing in mind that the ectonucleotidases are involved in the plasticity promoted by epilepsy, we investigated ATP, ADP and AMP hydrolysis in the synaptosomes of the cerebral cortex and the hippocampus of female and male rats at different ages following induction of the pilocarpine model.

2. Material and methods

2.1. Reagents

Nucleotides (ATP, ADP and AMP), Percoll, Trizma base, Malachite Green Base, Coomassie Brilliant Blue G, EDTA, HEPES, pilocarpine hydrochloride and methylscopolamine nitrate were purchased from Sigma, St. Louis, MO, USA. All other reagents were of analytical grade.

2.2. Animals

Female and male rats of different ages (7–9, 14–16 and 27–30 days) were used throughout 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 a temperature of 23 ± 1 °C. To avoid interlitter variations in susceptibility to seizures, different treatment groups (saline and pilocarpine) were represented in each litter. During the induction of the epilepsy model, the pilocarpine and saline-treated rats were transiently separated from their mothers. We also used male adult rats (60–70 days) to compare the ectonucleotidase activities in mature and immature brains. Previous studies from our laboratory submitted female adult Wistar rats to the pilocarpine model of epilepsy (Bonan et al., 2000). 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).

2.3. Pilocarpine model

The pilocarpine model has been previously described (Cavalheiro et al., 1991; Mello et al., 1993). A total of 130 animals were injected with pilocarpine and monitored behaviorally for at least 2 h. Only animals that evolved to SE, characterized by generalized motor seizures, were used. In brief, 30 min after subcutaneous pretreatment with 1 mg/kg scopolamine methylnitrate (to minimize peripheral cholinergic effects), a single dose of pilocarpine or saline (control group) was injected intraperitoneally (1 ml/kg). Pilocarpine doses were chosen according to the age of the animal (7–9-day old: 380 mg/kg; 14–16-day old: 250 mg/kg; 27–70-day old: 350 mg/kg of pilocarpine diluted in 0.9% saline solution), as previously described (Cavalheiro et al., 1987). After pilocarpine injection, the animal became hypoactive; generalized convulsions and limbic SE usually occurred 40–80 min after injection (Cavalheiro et al., 1991; Mello et al., 1993). The animals were sacrificed at 7 days after the induction of pilocarpine treatment. Mortality reached 18, 16, 40 and 44% for female and male rats at 7–9, 14–16, 27–30 and 60–70-day old, respectively.

2.4. Animal preparation and subcellular fraction

Animals were sacrificed by decapitation and 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 and total cerebral cortex of both hemispheres were immediately dissected on ice. In pilot experiments, we determined that the vehicle (saline) or scopolamine injections did not alter enzyme activities. Therefore, a group of saline-treated rats were used as a control and the subcellular fractionation and enzyme assays were carried out simultaneously with the pilocarpine-treated groups. The total hippocampi and cerebral cortex were gently homogenized in 5 and 10 volumes, respectively, 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 $15{,}000 \times g$ 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.

2.5. 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 μl. 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 phosphoate (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 phosphoate (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). Other conditions, such as medium reaction, pH and cation concentrations were used to assure the optimal enzyme activities (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 non-enzymatic hydrolysis of the substrates. All samples were run in triplicate.

2.6. Protein determination

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

2.7. Statistical analysis

The data obtained are represented as mean ± S.D. Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a Duncan multiple range test, considering $P < 0.05$ as significant.

3. Results

3.1. Behavioral changes

Animals of all ages presented akinesia, ataxic lurching, masticatory automatisms and head tremor 5–10 min after the pilocarpine treatment. This behavior progressed to seizures with rearing, forelimb clonus, salivation, intense masticatory

movements and falling (data not shown). These behavior alterations are similar to those observed by Cavalheiro et al., 1987.

3.2. Ectonucleotidase activities of female rats

3.2.1. Pilocarpine model

We evaluated the ontogenetic profile of ectonucleotidase activities in the hippocampal and cerebral cortical synaptosomes of female pups at 7–9, 14–16 and 27–30-day old after submission to the pilocarpine model of epilepsy. At all ages analyzed and in both brain regions, no significant difference in ATP, ADP and AMP hydrolysis was observed between pilocarpine-treated rats and their respective control groups (Figs. 1 and 2).

3.2.2. Ontogenetic profile

The ontogenetic profile, however, presented an increase in synaptosomal ATP and AMP hydrolysis in the cerebral cortex and hippocampus of saline-treated female rats at 14–16 days when compared to other ages. Synaptosomes from the cerebral cortex of saline-treated female rats at 14–16 days showed a significant enhancement in ATP hydrolysis (43 and 54%, respectively), when compared to female rats at 7–9 and 27–30 days (Fig. 1A). AMP hydrolysis in the cerebral cortical synaptosomes of 14–16-day old female rats (Fig. 1C) was significantly increased (115%) in relation to the 7–9-day old group. In hippocampal synaptosomes, a similar enzyme ontogenetic profile to that of saline-treated female rats was observed. ATP hydrolysis was significantly increased at 14–16 days (50%; Fig. 2A) in relation to rats at 27–30 days. AMP hydrolysis in 14–16 and 27–30-day old saline-treated rats was significantly increased (188 and 127%, respectively) in relation to control rats at 7–9 days (Fig. 2C). There were no significant changes in ADP hydrolysis in cerebral cortical and hippocampal synaptosomes of saline-treated female rats at different ages (Figs. 1B and 2B).

3.3. Ectonucleotidase activities of male rats

3.3.1. Pilocarpine model

The ontogenetic profile of ectonucleotidase activities in the synaptosomes of the hippocampus and the cerebral cortex of saline and pilocarpine-treated male rats at different ages (7–9, 14–16, 27–30 and 60–70 days) was also investigated. Our results demonstrated a significant increase in ATP, ADP and AMP hydrolysis in synaptosomes of the cerebral cortex (33, 40 and 64%, respectively) and the hippocampus (55, 98 and 101%, respectively) of pilocarpine-treated male rats (60–70-day old) when compared to saline-treated rats. No differences were observed between saline and pilocarpine-treated young rats (7–9, 14–16 and 27–30-day old), however, AMP hydrolysis in the cerebral cortical synaptosomes of pilocarpine-treated male rats at 27–30 days was increased when compared to the respective control group (71%; Fig. 3C).

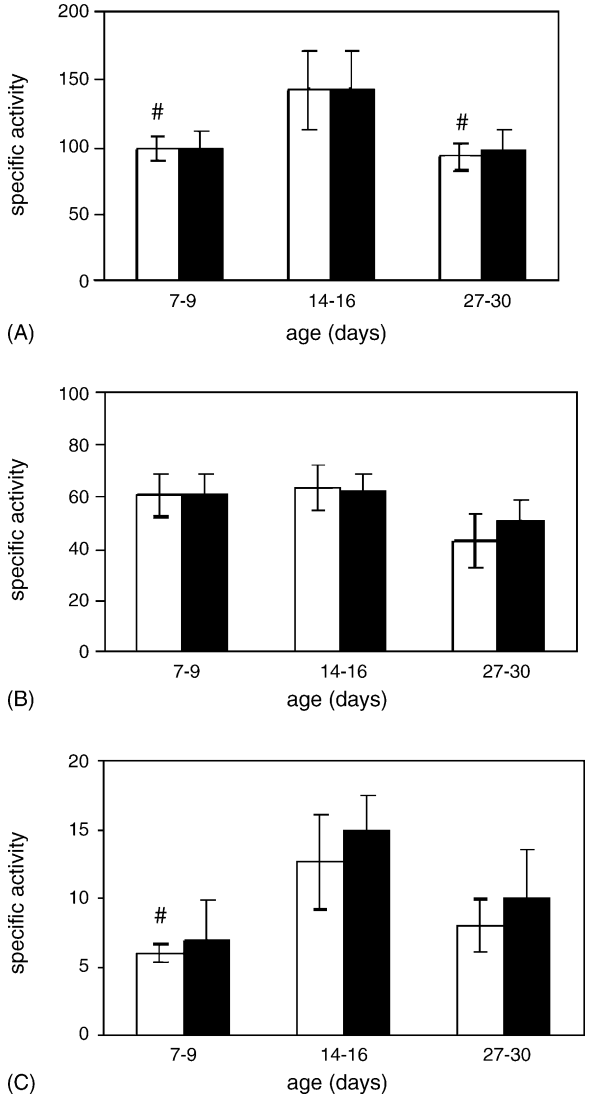

Fig. 1. Influence of the pilocarpine model of epilepsy on (A) ATP, (B) ADP and (C) AMP hydrolysis in cerebral cortical synaptosomes of female rats at 7–9 (n saline = 6 /n pilocarpine = 8), 14–16 (n saline = 6/n pilocarpine = 9) and 27–30-day old (n saline = 6/n pilocarpine = 9). Specific enzyme activities were expressed as nmol Pi min^{-1} mg^{-1} protein. # Indicates difference from control group at 14–16 days ($P < 0.05$: one-way ANOVA followed by Duncan multiple range test).

3.3.2. Ontogenetic profile

The ectonucleotidases from the cerebral cortical and hippocampal synaptosomes of saline-treated male rats at different ages were also compared. Cerebral cortex synaptosomes of control rats at 14–16-day old presented a significant enhancement in ATP (42%), ADP (35%) and AMP (141%) hydrolysis when compared to control rats at 7–9-day old (Fig. 3). With regard to ATP and ADP hydrolysis in cerebral cortical synaptosomes, the control enzyme activities of 14-day old rats were significantly increased (62 and 86%, respectively) when compared to adult control rats (60–70-day old) (Fig. 3A and B). AMP hydrolysis, however, was similar in 14–16 and 60-day-old rats (Fig. 3C). In hippocampal synaptosomes, ATP hydrolysis in 14–16-day-old rats was significantly increased (56%) when compared to 27–30-day-old rats (Fig. 4A). In

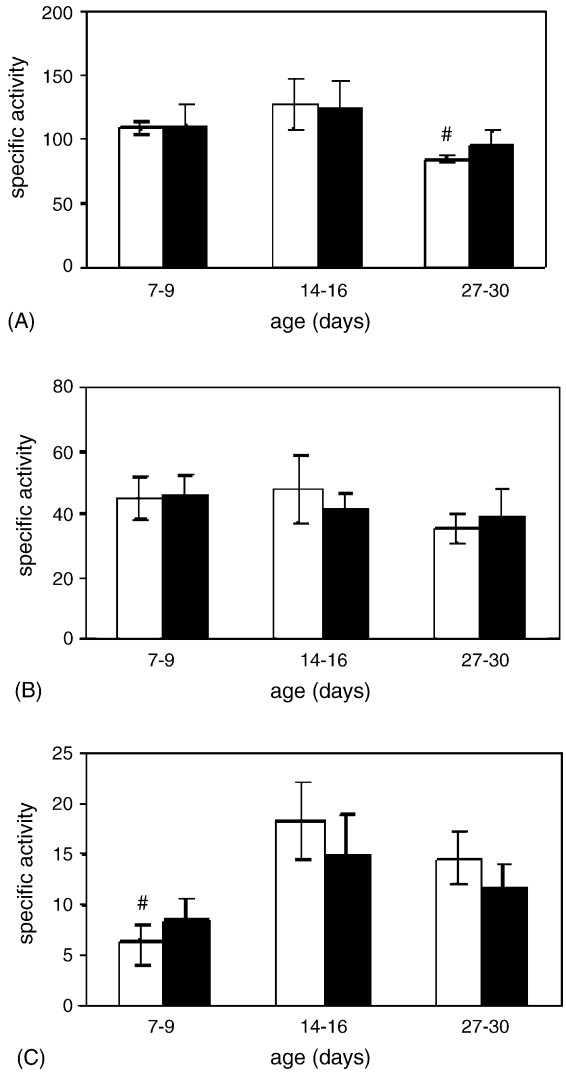

Fig. 2. Influence of the pilocarpine model of epilepsy on (A) ATP, (B) ADP and (C) AMP hydrolysis in hippocampal synaptosomes of female rats at 7–9 (*n* saline = 6/*n* pilocarpine = 8), 14–16 (*n* saline = 6/*n* pilocarpine = 9) and 27–30-day old (*n* saline = 6/*n* pilocarpine = 9). Specific enzyme activities were expressed as nmol Pi min^{-1} mg^{-1} protein. # Indicates difference from control group at 14–16 days ($P < 0.05$: one-way ANOVA followed by Duncan multiple range test).

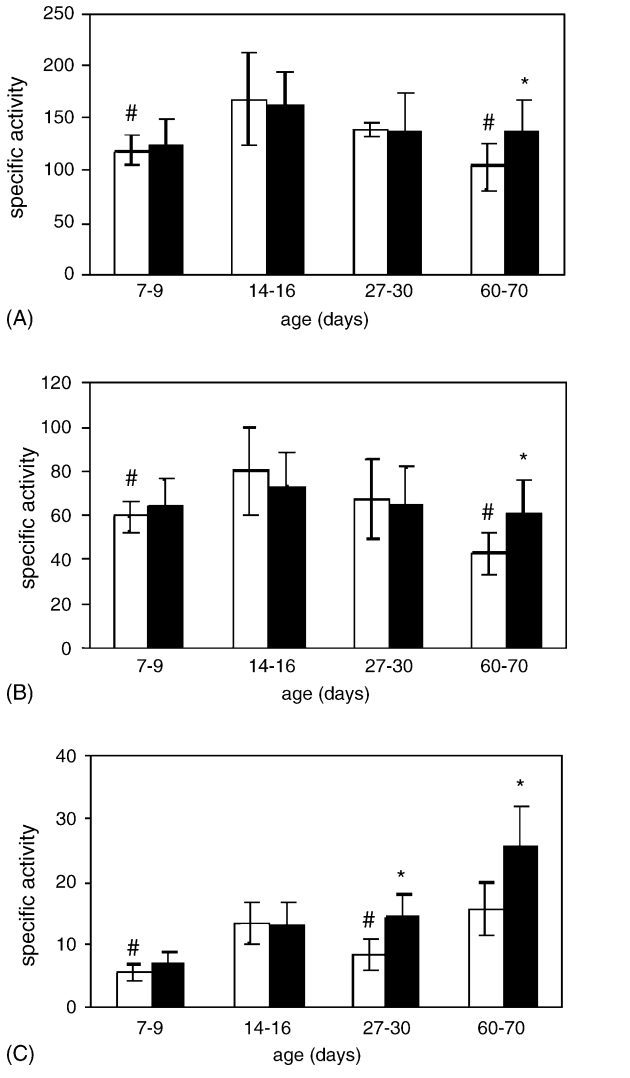

Fig. 3. Influence of pilocarpine model of epilepsy on (A) ATP, (B) ADP and (C) AMP hydrolysis in cerebral cortical synaptosomes of male rats at 7–9 (*n* saline = 8/*n* pilocarpine = 10), 14–16 (*n* saline = 7/*n* pilocarpine = 8), 27–30 (*n* saline = 7/*n* pilocarpine = 9) and 60–70-day old (*n* saline = 10/*n* pilocarpine = 16). Specific enzyme activities were expressed as nmol Pi min^{-1} mg^{-1} protein. * Indicates difference between control (white bars) and pilocarpine (black bars)-treated groups at the same age. # Indicates difference from control group at 14–16 days ($P < 0.05$: one-way ANOVA followed by Duncan multiple range test).

addition, ADP hydrolysis in 14–16-day-old rats was significantly increased in relation to 7–9 (50%), 27–30 (31%) and 60–70-day old (92%). In contrast, AMP hydrolysis in male rats at 7–9-day old was significantly different in relation to the AMP hydrolysis of control male rats at 14–16, 27–30 and 60–70-day old (Fig. 4C).

4. Discussion

Previous studies from our laboratory have shown increased ectonucleotidase activities in adult female rats submitted to the pilocarpine model of epilepsy at different times after administration of the convulsant (Bonan et al., 2000). We used animals at 7 days after the induction of the pilocarpine model, since this corresponds to the latent period, a phase in which several molecular changes occur and may contribute to the process of epileptogenesis (Priel et al., 1996). In addition, the latent period promotes the most pronounced increase in ectonucleotidase activities in the hippocampus and cerebral cortex in pilocarpine-treated female rats (Bonan et al., 2000).

Our results demonstrated increased ectonucleotidase activities in cerebral cortical and hippocampal synaptosomes of male adult rats after pilocarpine treatment. This finding is in agreement with a previous study from our laboratory (Bonan et al., 2000) that showed increased ectonucleotidase activities in the synaptosomes of the same brain regions in female adult rats.

Development and sex hormones are important determinants of seizure susceptibility. Male children experience a higher incidence of epilepsy or unprovoked seizures than do female

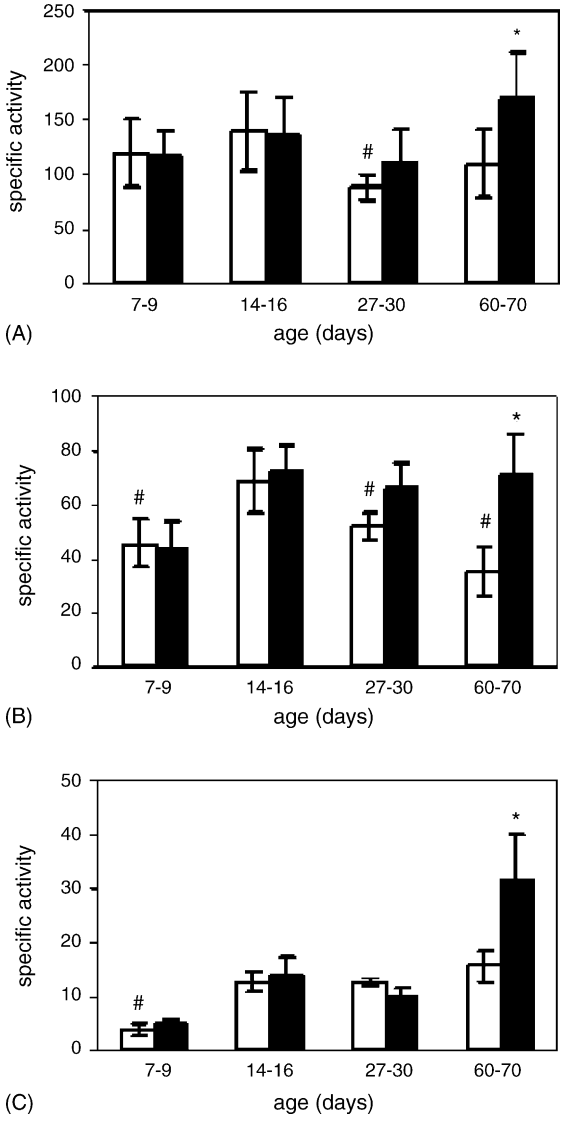

Fig. 4. Influence of the pilocarpine model of epilepsy on (A) ATP, (B) ADP and (C) AMP hydrolysis in the hippocampal synaptosomes of male rats at 7–9 (*n* saline = 7/*n* pilocarpine = 8), 14–16 (*n* saline = 7/*n* pilocarpine = 8), 27–30 (*n* saline = 7/*n* pilocarpine = 9) and 60–70-day old (*n* saline = 10/*n* pilocarpine = 16). Specific enzyme activities were expressed as nmol Pi $min^{-1}mg^{-1}$ protein. * Indicates difference between control (white bars) and pilocarpine (black bars)-treated groups at the same age. # Indicates difference from control group at 14–16 days ($P < 0.05$: one-way ANOVA followed by Duncan multiple range test).

children. Sex-specific differences in the development of seizure-suppressing neuronal networks may account, at least in part, for this increased age- and sex-related susceptibility to seizures (Veliskova et al., 2004). Thus, here we have studied ectonucleotidase activities in the cerebral cortical and hippocampal synaptosomes of young female and male rats (7–30 days) submitted to the pilocarpine model of epilepsy. Female and male rats present similar ontogenetic and synaptosomal ectonucleotidase activity profiles following pilocarpine treatment. The pilocarpine treatment profile observed suggests that ectonucleotidase activities are not altered by the plasticity associated with epileptogenesis in young rats, at least 7 days after induction of the pilocarpine model. Only ecto-5′-nucleotidase activity, however, was increased by pilocarpine in the cerebral cortical synaptosomes of 27–30-day old male rats, suggesting that the plastic events induced by pilocarpine promote alterations in this enzyme activity. This effect could be involved in the susceptibility to this epilepsy model that increases with age, as previously described (Priel et al., 1996). It has been reported that rats submitted to pilocarpine treatment before 17 days of age do not undergo the chronic period of this model, characterized by spontaneous recurrent seizures (Priel et al., 1996). Furthermore, ectonucleotidase activities were increased in adult rats during the chronic phase of the pilocarpine model (Bonan et al., 2000). Thus, it may be hypothesized that the increase in ectonucleotidase activities could be related to the development of recurrent seizures in this model.

Several studies have demonstrated that adenosine can act as an endogenous anticonvulsant (Brundege and Dunwiddie, 1997; Cavalheiro et al., 1991), due to its neuromodulatory action and mediated by adenosine A_1 receptors (Dunwiddie and Masino, 2001). Higher adenosine levels have been observed in temporal lobe epilepsy patients (During and Spencer, 1992) and an increase in adenosine A_1 receptors has been reported in human neocortex following the same kind of epilepsy (Angelatou et al., 1993). The distribution and expression of adenosine A_1 receptors on gestational day 20 resembled the widespread, yet heterogeneous, pattern observed in the adult (Weaver, 1996) and these receptors are already coupled to a G-protein (Daval et al., 1991; Daval and Werck, 1991). Upregulation of adenosine A_1 receptors after bicuculline-induced seizures can contribute to improvement of the adenosine anticonvulsant effect, especially in newborns (Daval and Werck, 1991). Our study demonstrates, however, that there is no differential nucleotide hydrolysis in young rats, which could suggest that extracellular adenosine production is similar in saline and pilocarpine-treated young rats, at least 7 days after pilocarpine administration. Despite the neuroprotective role played by adenosine in adult rats, there is evidence that the activation of adenosine A_1 receptor with doses of CPA that mimic the effect of high adenosine levels results in damage to developing brain (Turner et al., 2002). The damage promoted by adenosine agonist during postnatal life includes ventriculomegaly, reduction in white and gray matter volumes, reduced myelin basic protein expression and diminished total axon volume (Turner et al., 2002). In addition, activation of adenosine A_1 receptors inhibits the development of axons and can lead to leukomalacia (Rivkees et al., 2001). Based on these considerations, it may be hypothesized that ectonucleotidase activities, at basal levels, could contribute to the maintenance of physiological adenosine levels and, consequently, avoid a possible neurodegenerative role of this signaling molecule in young rats submitted to the pilocarpine model of epilepsy.

It has been demonstrated that, in synaptosomal preparations of rat cerebral cortex, ATP and ADP hydrolysis increase significantly from birth until the second postnatal week (Müller et al., 1990). Furthermore, the ecto-5′-nucleotidase enzyme is expressed at the surface of developing nervous cells and is regarded as a marker of neural development (Braun and

Zimmermann, 1998). These findings are in agreement with our results, since we observed a significant increase in nucleotide hydrolysis in hippocampal and cerebral cortical synaptosomes of female and male rats at 14–16 days of age. This period coincides with an intense synaptogenesis and augmentation in the activities of several enzymes involved in neurotransmitter metabolism and neuronal functions (Fiedler et al., 1987). It should be mentioned that 15-day-old animals may develop convulsions following pilocarpine at lower doses that are non-convulsant in rats aged 10 or 20 days (Brundege and Dunwiddie, 1997). The different sensitivity of developing rats to the convulsant effect of pilocarpine may be related to the immaturity of neuronal networks in the brain engaged in the generation and spread of seizure activity.

In summary, our findings highlight differences between the purinergic system of young and adult rats submitted to the pilocarpine model of epilepsy. Further studies are required, however, to investigate ectonucleotidases and the role of adenosine A_1 receptor agonists and antagonists at different times following the administration of pilocarpine for the elucidation of the mechanisms involved in epileptogenesis in young rats.

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