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Inhibitory Avoidance Task Reveals Differences in Ectonucleotidase Activities between Male and Female Rats

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Studies demonstrated that endogenous levels of estrogen affect the long-term potentiation (LTP) and long-term depression (LTD). ATP and adenosine may play a role in the modulation of LTP. Our laboratory observed in previous studies that inhibitory avoidance task is associated with a decrease in hippocampal ectonucleotidase activities in adult male rats. To explore if ectonucleotidases are modulated in memory formation in female rats, as observed in males, we evaluated the effect of inhibitory avoidance training on synaptosomal NTP Dase and 5′-nucleotidase activities in rat hippocampus from both sexes. The results demonstrated a decrease in ATP, ADP and AMP hydrolysis (37%, 38% and 32%, respectively) immediately after training and a significant inhibition only in ATP hydrolysis (36%) 30 min post-training in male rats. There were no changes in ectonucleotidase activities from female rats. These findings provide support for the view that could exist biochemical differences in ectonucleotidase activities between males and females.

KEY WORDS: Estrogen; ectonucleotidases; memory; ATP; LTP; LTD.

INTRODUCTION

Research on the differential performance of males and females in memory tasks has been performed. In a spatial navigation task, there were no male–female differences during acquisition, but it has been observed a better performance of males during extinction of memory (1).

A fundamental difference between the two genders is that females are influenced by cyclic hormonal levels (2). Fluctuations in estrogen levels, either by hormone treatment or naturally across the reproductive cycle in intact females, lead to a host of morphological (3–5), neurochemical (6–8) and electrophysiological (9–11) changes in brain areas, which participate actively in learning and memory including hippocampus, striatum, amygdala and frontal cortex (12). Recent studies indicate that the female gonadal hormone, estradiol, enhances performance of learning and memory tasks both in animal models and in humans (13). Furthermore, it has been suggested that different cognitive abilities are more or less sensitive to the modulating effects of estrogen

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(14–16) and there is clear evidence that estrogen may affect cognition through direct or indirect actions on the hippocampus (15,17).

The long-term potentiation (LTP) is considered as a partial model of memory and may even be considered as a particular form of memory, measurable at the eletrophysiological level (18). In addition, the long-term depression (LTD), another plastic event, has often been proposed to underlie memory events (19,20). It has been demonstrated that cyclical changes in endogenous levels of estrogen modulate the induction of LTD and LTP in the hippocampal CA1 region (21), while chronic treatment with estradiol does not alter LTP *in vitro* in hippocampus (22).

ATP is known to exert potent effects on the central nervous system, where it can act as a neurotransmitter or as a modulator regulating the activity of other transmitter structures (23,24). There is evidence that extracellular ATP may play an important role in synaptic plasticity events, like LTP (25–27). The signalling actions induced by extracellular ATP are directly correlated by the activity of ectonucleotidases, because they trigger enzymatic conversion of ATP to adenosine, controlling the nucleotide and nucleoside levels in the synaptic cleft (28). ATP released at synapses can be hydrolyzed by a sophisticated pathway composed by ectoenzymes that include NTPDase1 or NTPDase3 (ecto-apyrase, ATP diphosphohydrolase, EC 3.6.1.5), which can transform ATP and ADP to AMP. ATP can also be hydrolyzed by NTPDase2 (ecto-ATPase, EC 3.6.1.3), producing ADP (29). The AMP produced by NTPDase1 or 3 is subsequently hydrolyzed to adenosine by an ecto-5′-nucleotidase (EC 3.1.3.5), a key enzyme in this pathway (30–32). Adenosine is an endogenous nucleoside that also exerts an important role in the regulation of neuronal excitability (33,34). It has previously been shown that adenosine can modulate synaptic plasticity in rats (34). The synchronic action of a NTPDase and a 5′-nucleotidase is able to regulate the extracellular ratio of nucleotides/nucleosides (35).

Recently, we observed that one-trial inhibitory avoidance task is associated with a learning-specific, time-dependent decrease in hippocampal ectonucleotidase activities in adult male rats (36). To explore if the ectonucleotidase pathway is modulated in the process of memory formation in female rats, as observed in males, we evaluated the effect of inhibitory avoidance training on synaptosomal NTPDase and 5′-nucleotidase activities in rat hippocampus from both sexes.

EXPERIMENTAL PROCEDURE

Chemicals. Nucleotides (ATP, ADP, AMP), HEPES, Trizma Base and EDTA were obtained from Sigma Chemical CO. (St. Louis, MO, USA). Percoll was obtained from Pharmacia (Uppsala, Sweden) and was routinely filtered through millipore AP 15 pre-filters to remove aggregated, incompletely coated particles. All others reagents were of analytical grade.

Subjects. Male and female Wistar rats (age 70–90 days; 200–280 g) from our breeding stock were used in the study. Animals were maintained at a constant temperature (23 ± 2°C) in a 12 h light–dark cycle and had free access to food and water throughout the experiment. Just females in the diestrus state were used. Determination of the phase of estrous cycle was made by the type of vaginal cells observed in the microscope (200×). Procedures for the care and use of animals were adopted according to the regulations of Colégio Brasileiro de Experimentação Animal (COBEA), based on the Guide for the Care and Use of Laboratory Animals (National Research Council).

Step-down inhibitory avoidance task. Rats were gently placed on a 2.5-cm high, 7.0-cm wide, 25.0 cm long Formica platform at the left side of a $50 \times 25 \times 25$-cm apparatus, the floor of which was series of parallel 0.1-cm caliber stainless-steel bars spaced 1.0 cm apart. Immediately after stepping down, placing the four paws on the grid, animals received a 0.5 mA, 2-s scrambled foot-shock and were removed from the training apparatus.

Isolated footshock. In order to examine the possibility that the foot-shock could alter the enzyme activities, rats (shocked group) were placed directly on the grid and received a 0.5 mA, 2-s scrambled foot-shock, after which they were removed. A barrier was placed in order to avoid animals seeing an escape route to the platform.

Synaptosomes preparation. The rats were killed by decapitation in two different times (0 and 30 min) and their hippocampi were removed to an ice-cold medium solution (320 mM sucrose, 5.0 mM HEPES, pH 7.5, and 0.1 mM EDTA). Structures were gently homogenized in five volumes of ice-cold medium solution with a motor-driven Teflon-glass homogenizer. The synaptosomes were isolated as described previously by Nagy and Delgado-Escueta (37). Briefly, 0.5 ml of the crude mitochondrial fraction were 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 then washed twice at $15{,}000 \times g$ for 20 min with the same ice-cold medium to remove the contaminating Percoll. 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 ATP and ADP hydrolysis was essentially as described previously (38) and 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 reaction medium used to assay 5′-nucleotidase activity contained 10 mM $MgCl_2$, 100 mM Tris–HCl, pH 7.5 and 0.15 M sucrose in a final volume of 200 µl. The synaptosomal fractions (10–20 µg protein) were added to the reaction mixture, preincubated for 10 min and incubated for 20 min at 37°C. The reaction was initiated by the addition of ATP, ADP or AMP to a final concentration of 1.0 mM and stopped by the addition of 200 µl 10% trichloroacetic acid. The samples were chilled on ice for 10 min and aliquots were taken for the assay of released inorganic phosphate (P_i) (39). Incubation times and protein concentration were chosen in order to ensure the linearity of the reaction. Controls with the addition of the enzyme preparation after addition of trichloroacetic acid were used to correct nonenzymatic hydrolysis of the substrates.

Protein determination. Protein was measured by the Coomassie Blue method using bovine serum albumin as standard (40).

Statistical analysis. The data are expressed as mean ± S.D. Data were analyzed by two-way ANOVA and one-way ANOVA, followed by the Duncan multiple range test. $P < 0.05$ was considered to represent a significant difference with statistical analyses used. All analyses were performed with an IBM compatible computer using the SPSSPC software.

RESULTS

ATP hydrolysis in hippocampal synaptossomes from male rats trained and killed immediately (0 min) or 30 min after the training session in step-down inhibitory avoidance task decreased 37% and 36%, respectively, when compared to the respective shocked group (Fig. 1a). These effects are in agreement with results published by Bonan et al. (36). However, in female rats, there were no observed changes in ATP hydrolysis either immediately or 30 minutes after training session (Fig. 1b).

The results obtained about the ADP hydrolysis showed a 38% decrease in this activity when male rats were trained and killed immediately after the training session (0 min), but there were no differences when the animals were trained and killed 30 min after the training. The effect was considered significant ($P < 0.05$) when compared to the respective shocked group (Fig. 2a). In this condition, the sex difference was also observed and there were no differences in ADP hydrolysis between shocked and trained groups for female rats (Fig. 2b).

We also compared the effects of the step-down inhibitory avoidance task on ecto-5′-nucleotidase activity in hippocampal synaptosomes from male and female rats. As observed for Bonan et al. (36), our results showed a significant inhibition of 5′-nucleotidase activity immediately after training session (0 min) for male rats (Fig. 3a). The results showed a 32% inhibition of 5′-nucleotidase activity for males, but there were no changes in the enzyme activity for females (Fig. 3b). The group killed at 30 min after the training session did not present significant changes on 5′-nucleotidase activity for both sexes. There were no significant differences either for males or for females in the enzyme activities studied between the control group (normal rats) and the shocked groups killed at the corresponding times.

DISCUSSION

The present results demonstrate that inhibitory avoidance task produces distinct effects on ectonucleotidase activities from hippocampal synaptosomes of male and female rats.

There is increasing evidence to suggest that memory function is linked to the female reproductive states and it may be due to altered function of the hippocampus (22). Step-down inhibitory avoidance learning in the rat triggers biochemical events in the hippocampus that are necessary for the retention of this task (41). The events are similar in many ways to those described for LTP and other forms of neural plasticity (42–44). It was observed that estrogen levels are involved in memory formation. Estradiol can enhance learning and memory (13,45,46), but an acute administration of estradiol is associated with impaired learning on hippocampal-dependent tasks, including the Morris water task (47,48) and avoidance memory tasks (49,50).

In the present work and in previous studies from our laboratory (36), we demonstrated that ATP, ADP and AMP hydrolysis decreased immediately after the training session (0 min) in hippocampal synaptosomes of male rats submitted to inhibitory avoidance task. It has also been demonstrated here and by Bonan et al. (36) that ATP hydrolysis decreased in hippocampal synaptosomes of male rats killed at 30 min after the training session. The inhibitory effect observed could be due to some allosteric modulation of the enzyme activities involved in the adenine nucleotides degradation or other possible mechanisms, like protein phosphorylation. The biochemical cascade of memory consolidation involves, beyond activation of signaling system, the pre- and postsynaptic activation of protein kinases A and C

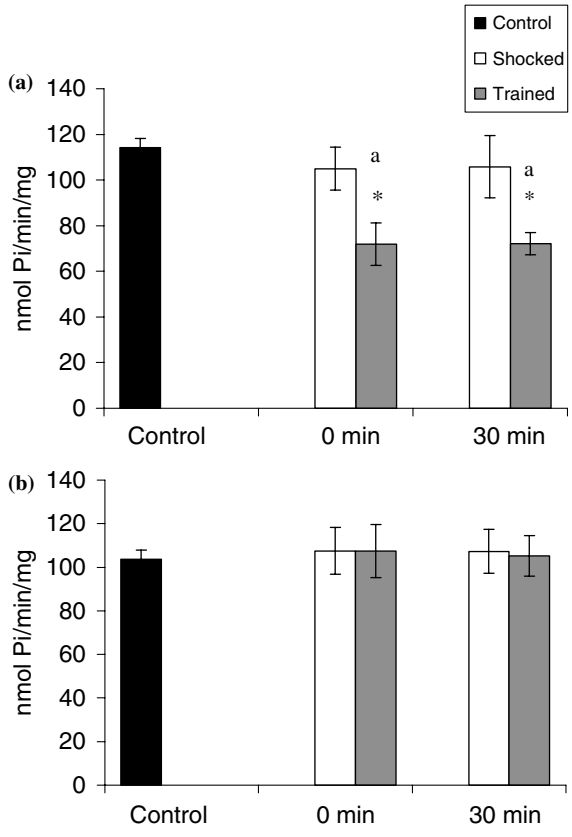

Fig. 1. Effect of training session in a step-down inhibitory avoidance task on ATP hydrolysis by synaptosomal NTPDase of hippocampus from male (a) and female (b) rats. Control represents normal rats; shocked group represents the animals that received only a foot-shock and were killed at 0 and 30 min after this conditioning. Trained group represents the animals that were trained in a step-down inhibitory avoidance task and were killed 0 and 30 min after the training. Bars represent the means ± SD of at least five animals. *Significantly different from control ($P < 0.05$). (a) Significantly different from shocked group ($P < 0.05$).

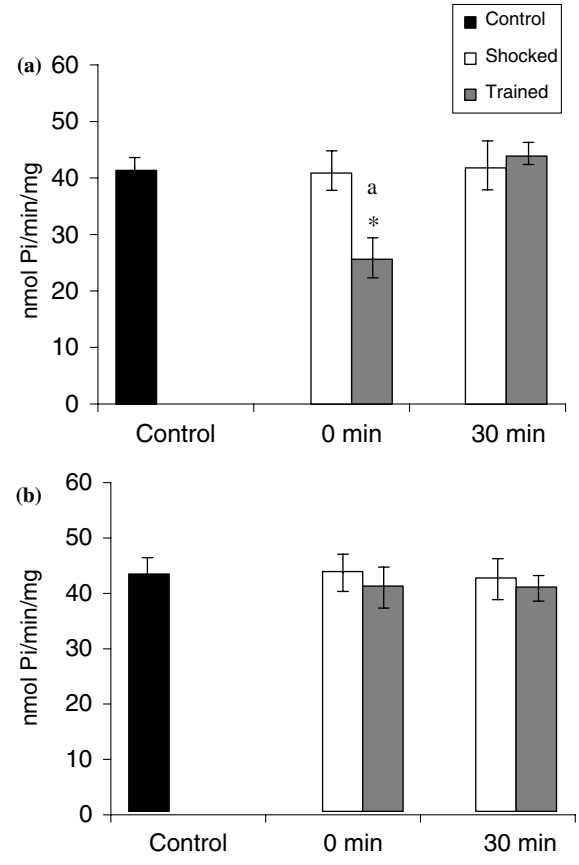

Fig. 2. Effect of training session in a step-down inhibitory avoidance task on ADP hydrolysis by synaptosomal NTPDase of hippocampus from male (a) and female (b) rats. Control represents normal rats; shocked group represents the animals that received only a foot-shock and were killed at 0 and 30 min after this conditioning. Trained group represents the animals that were trained in a step-down inhibitory avoidance task and were killed 0 and 30 min after the training. Bars represent the means ± SD of at least five animals. * Significantly different from control ($P < 0.05$). (a) Significantly different from shocked group ($P < 0.05$).

and calcium/calmodulin kinase II into hippocampus (41). If protein kinases play a role in the maintenance of the early stages of memory consolidation, the participation of extracellular ATP as a substrate in ectoprotein phosphorylation (36,42) could be necessary, at least, in male rats. However, our study has shown that there were no changes in ectonucleotidase activities from hippocampal synaptosomes of female rats trained and killed immediately and 30 min after inhibitory avoidance task. Then, in relation to the ectonucleotidases in central nervous system, there is a marked difference between sexes during the consolidation of an aversive memory.

It is becoming clear that these dichotomies fail to account for the complexity of estrogen actions. Some evidence indicate that neuroactive steroids, apart from their well-documented genomics effects, are potent modulators of the plasma membrane receptors that may interact with different effector systems in neuronal membranes (51,52). Subsequent research has identified that estrogen may influence neuronal excitability in the hippocampus through modulation of NMDA, AMPA and GABA receptor-mediated currents (53,54). In the proestrus, estrogen levels are high, the NMDA receptor-mediated Ca^{2+} transients are enhanced, LTP is augmented and LTD was severely attenuated (21). In this study, we evaluated the effect of inhibitory avoidance task on ectonucleotidase activities using females at diestrus of estrous cycle, when estrogen is relatively low. It has been demonstrated that at

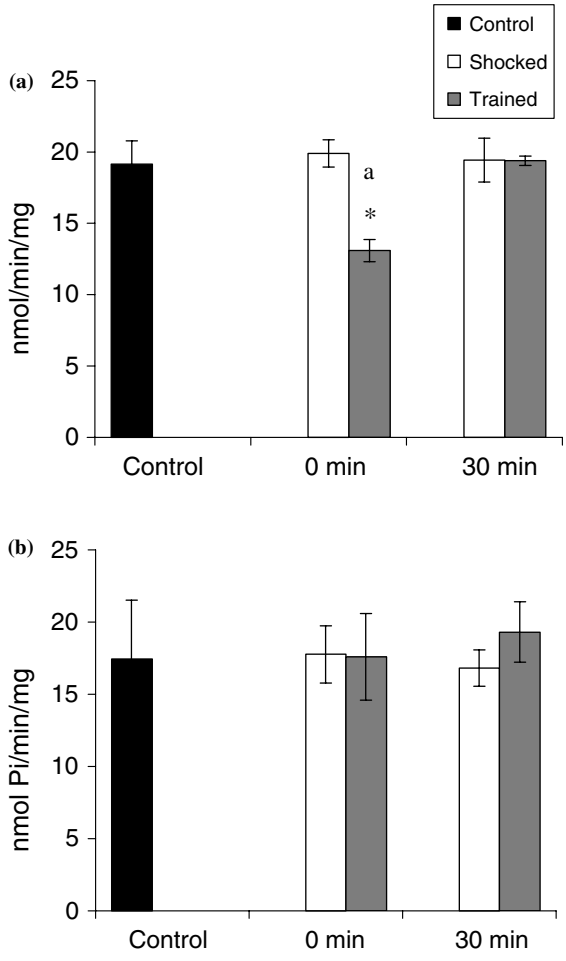

Fig. 3. Effect of training session in a step-down inhibitory avoidance task on AMP hydrolysis by synaptosomal ecto-5′nucleotidase of hippocampus from male (a) and female (b) rats. Control represents normal rats; shocked group represents the animals that received only a foot-shock and were killed at 0 and 30 minutes after this conditioning. Trained group represents the animals that were trained in a step-down inhibitory avoidance task and were killed 0 and 30 min after the training. Bars represent the means ± SD of at least five animals. * Significantly different from control ($P < 0.05$). (a) Significantly different from shocked group ($P < 0.05$).

diestrus the LTP induction was lower than at proestrus (21). Since at diestrus the LTP induction is decreased and it is a partial model of memory consolidation, it could contribute to the lack of an inhibitory effect on ATP hydrolysis in females, as observed in males. Then, we can suggest that the possible increase in ATP levels observed in males could be not necessary to the biochemical mechanisms related to synaptic plasticity in females, at least in the diestrus phase. Moreover, at diestrus, it has been shown that LTD was clearly manifested, but unlike LTP, LTD requires activation of protein phosphatases (55,56). These findings provide additional support for the idea that there are biochemical differences in the modulation of ectonucleotidase activities during synaptic plasticity events related to learning between males and females.

These biochemical differences between genders to inhibitory avoidance task led us to suggest that this distinction probably exists more by natural and sex-specific mechanisms than cognitive abilities, since we did not evaluate memory. It is hoped that the biochemical findings presented in this study can provide a framework for development of hypotheses and strategies for future studies about comparative memory processing in males and females.

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