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Habituation to an open field alters ecto-nucleotidase activities in rat hippocampal synaptosomes

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Abstract

ATP and adenosine may play a role in the mechanisms of synaptic plasticity and memory formation. Previous studies have shown that ecto-nucleotidase activities are altered during memory consolidation of an aversive task named step-down inhibitory avoidance. Here we investigate ecto-nucleotidase activities in hippocampal synaptosomes of rats submitted to training and test sessions of habituation to open field, which is one of the most elementary forms of learning. There were no significant alterations on ATP, ADP and AMP hydrolysis immediately after the training session. However, immediately after the test session (0 min), there was a significant increase of ATP hydrolysis (61%), but not of ADP and AMP hydrolysis. Sixty minutes after the test session, a significant increase of NTPDase (75% and 60.5% for ATP and ADP hydrolysis, respectively) and ecto-5′-nucleotidase (40%) activities was observed. This study reveals the involvement of ecto-nucleotidase activities in different learning paradigms during memory processing.

Keywords: Memory; Habituation; Open field; Retrieval; Ecto-nucleotidases; Adenosine

ATP and adenosine are important signalling molecules in the central nervous system [30,12]. ATP is synthesized, stored and released by the central and peripheral nervous systems upon depolarization [6,29]. Extracellular ATP acts through two subclasses of P2 purinoreceptors, P2X and P2Y [30]. P2X receptors are coupled to ligand-gated Ca^{2+}-permeable channels, whereas the P2Y receptors are linked to a G-protein [11].

The signalling actions induced by extracellular ATP are directly correlated to the activity of ecto-nucleotidases once they trigger enzymatic conversion of ATP to adenosine [39,31]. Ecto-nucleotidases comprise a group of ecto-enzymes involved in the control of nucleotide and nucleoside levels in the synaptic cleft, which includes nucleoside triphosphate diphosphohydrolase (NTPDase) family and ecto-5′-nucleotidase [39]. Four members of the NTPDase family are tightly bound to the plasma membrane via two transmembrane domains, and have a large extracellular region with an active site facing the extracellular side. NTPDase1, 3 and 8 slightly prefer ATP over ADP by a ratio of 1, 3 and 2, respectively. Meanwhile, NTPDase2 prefers triphosphonucleosides [5,31]. Adenosine, a product of ATP catabolism, can exert its neuromodulatory effects through four subtypes of P_1-purinoreceptors named A_1, A_{2A}, A_{2B} and A_3 [10,15,16].

Previous studies from our laboratory have shown that one-trial inhibitory avoidance training is associated with a learning-specific, time-dependent decrease of ecto-nucleotidase activities in hippocampus and entorhinal cortex of rats [7,8]. In addition, studies have observed that ATP and ADP hydrolysis are increased in anterior and posterior cingulate cortex after

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one-trial inhibitory avoidance training in rats [26]. These findings raise questions about the importance of the ecto-nucleotidase pathway in biochemical events related to the early phase of memory formation. Step-down inhibitory avoidance task involves the specific repression of the natural tendency of rats to explore beyond a safe platform without affecting their exploratory behavior while on the platform [20]. Recently, it has been shown that inhibitory avoidance training is restrained by treatments that inhibit long-term potentiation (LTP) in hippocampal area CA1 [18]. Moreover, inhibitory avoidance task is, indeed, accompanied by LTP in this brain area [37].

Another form of learning is habituation to an open-field, which has been very little studied in terms of pharmacological and biochemical mechanisms of memory consolidation and retrieval, clearly depending on the hippocampus [34,36]. Habituation to a novel environment is believed to be one of the most elementary forms of learning, in which the decreasing exploration, as a function of repeated exposure to the same environment, is taken as an index of memory [33,34]. This is normally studied in two or more brief sessions of exposure to an open field or similar environment [19,21].

Considering the involvement of the ecto-nucleotidase pathway in the formation of aversive memory induced by step-down inhibitory avoidance [7,8], we have evaluated ATP, ADP and AMP hydrolysis in rat hipocampal synaptosomes after habituation to an open-field. The influence of this enzyme pathway in a non-associative task [36], which presents biochemical differences in memory consolidation when compared to an aversive memory, was also discussed.

Male Wistar rats (age 60–90 days; weight 220–280 g) from our breeding colony were housed four to a cage, with water and food *ad libitum*. Animal were kept on a 12 h light/dark cycle (lights on at 07:00 h) at a constant temperature of 23 ± 1 °C. 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).

The animals were placed in a 40 cm × 60 cm open field surrounded by 50 cm high walls made of brown plywood with a frontal glass wall. The floor of the open field was divided by black lines into 12 equal rectangles. In the session of training, the animal is gently placed in front of the left posterior corner of the box and left to explore the arena during 5 min. After 24 h, the animals were submitted to a similar open field session, which corresponds to the test session. In both sessions, the rearings and crossings of the black lines were recorded. The decrease in the number of crossings and rearings between the two sessions was taken as a measure of the retention of habituation [33,34,36]. To analyze the effect of training session on the ecto-nucleotidases activities, the animals were submitted to the behavioral procedure described above and were sacrificed immediately (0 min) after the training session. In order to investigate the effect of test sessions on the ecto-nucleotidases activities, the animals were submitted to the training session and 24 h later exposed again to the behavioral apparatus to perform the test session. The animals were sacrificed 0, 60 or 120 min after the test session. All behavioral procedures were conducted at 8:00–11:00 am.

Synaptosomes from hippocampus were isolated [23] and ATP, ADP and AMP hydrolysis were assayed as described previously [4,7,17]. Released inorganic phosphate was determined according to Chan et al. [13]. Protein was measured by the Coomassie Blue method [9] using bovine serum albumin as standard. The data obtained for the enzyme activities are presented as mean ± S.E.M. of a number of animals studied in each condition. The statistical analysis used in these experiments was one-way ANOVA, followed by the Duncan multiple test. The differences between training and test performance were evaluated by Student *t*-test. $P < 0.05$ was considered to represent a significant difference in both statistical analysis used.

In the present study, we have demonstrated the effect of habituation in open field on NTPDases and ecto-5′-nucleotidase activities in rat hippocampal synaptosomes. The number of crossings was 93.3 ± 4.12 for training session and 45.3 ± 4.69 for test session in open field with a 24 h interval between the sessions (mean ± S.E.M.; $n = 18$; $P < 0.05$). Rearings of training and test session performances were 17.2 ± 1.35 and 7.33 ± 0.99, respectively (mean ± S.E.M.; $n = 18$; $P < 0.05$). There was a good evidence for habituation in all animals tested. There were no significant alterations on ATP ($n = 6$), ADP ($n = 6$) and AMP ($n = 6$) hydrolysis immediately after the training session (Figs. 1A, B and 2). However, immediately after test session (0 min), it has been observed a significant increase on ATP hydrolysis (208.5 ± 17.8 nmol Pi min^{-1}. mg $protein^{-1}$, $n = 6$), when compared to control group (129.2 ± 7.1 nmol Pi min^{-1} mg $protein^{-1}$), but none on ADP and AMP hydrolysis ($n = 6$). At 60 min after test session, our results have shown a significant increase of NTPDase activity (227 ± 14.6 and 56.4 ± 7.6 nmol Pi min^{-1} mg $protein^{-1}$ for ATP and ADP hydrolysis, respectively; $n = 6$), when compared to control group (129.2 ± 7.1 and 35.1

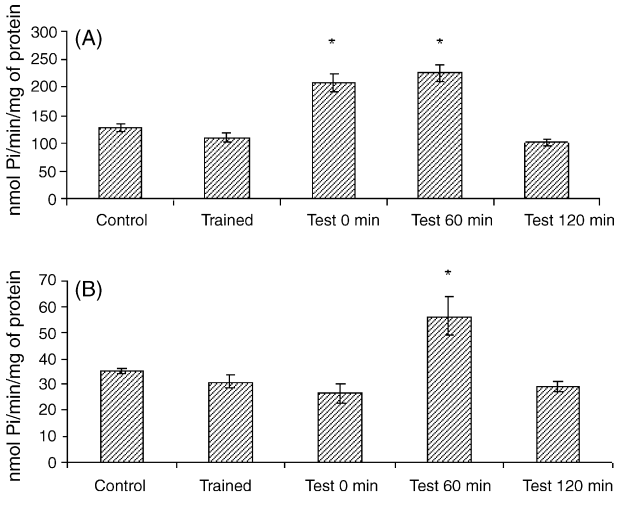

Fig. 1. Effect of training (trained group) and test sessions at 0 min (Test 0 min), 60 min (Test 60 min), 120 min (Test 120 min) in the habituation to an open field on the ATP (A) and ADP (B) hydrolysis in rat hippocampal synaptosomes. Control group represents naive rats. Bars indicate the means ± S.E.M for six different experiments. Data are expressed in specific activity (nmol Pi min^{-1} mg^{-1} protein). (*) Significantly different from the control group ($P < 0.05$; Duncan test).

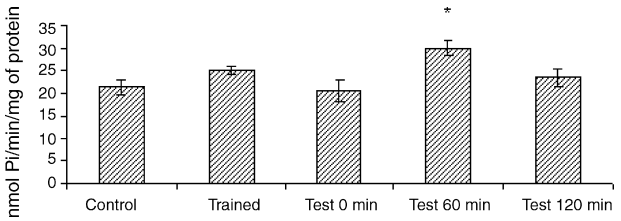

Fig. 2. Effect of training (trained group) and test sessions at 0 min (Test 0 min), 60 min (Test 60 min), 120 min (Test 120 min) in the habituation to an open field habituation on ecto-5′-nucleotidase activity in rat hippocampal synaptosomes. Control group represents naive rats. Bars indicate the means ± S.E.M. for six different experiments. Data are expressed in specific activity (nmol Pi min^{-1} mg^{-1} protein). (*) Significantly different from the control group ($P < 0.05$; Duncan test).

1.2 nmol Pi min^{-1} mg $protein^{-1}$ for ATP and ADP hydrolysis). In this same time period after test session, it has been also observed a significant enhancement of ecto-5′-nucleotidase activity (30 ± 1.5 nmol Pi min^{-1} mg $protein^{-1}$, $n = 6$) in rat hippocampal synaptosomes, when compared to control group (21.4 ± 1.5 nmol Pi min^{-1} mg $protein^{-1}$) (Fig. 2). In contrast, at 120 min after test session, there were no significant alterations on NTPDase (Fig. 1A and B) and 5′-nucleotidase (Fig. 2) ($n = 6$).

Here we have shown significant alterations of ecto-nucleotidase activities after the test session, but not after the training session of the habituation to open field. These results differ from other studies testing step-down inhibitory avoidance [7,8,27], in which ecto-nucleotidase activities had been altered at different time periods after the training session and had not changed in the test session. The results have indicated differences in molecular requirements between both habituation to an open field and step-down inhibitory avoidance [18,36]. Memory formation for habituation differs not only from one-trial avoidance, but also from contextual fear, spatial learning and other associative tasks in different species [20,35]. This suggests that associative and non-associative memories require different mechanisms for their formation. Furthermore, the biochemical changes underlying consolidation are not identical to those of retrieval, although both processes might involve the same synapses [3]. Vianna et al. [36] have shown that the calcium calmodulin protein kinase II inhibitor KN-62 is amnesic for habituation, but the NMDA antagonist AP5 (2-amino-5-phosphono pentanoic acid), the dopaminergic D1 antagonist SCH23390 and the MAP protein kinase inhibitor PD098059 have no effect on the retention of this task. In contrast, all of them have strongly affected memory of the avoidance task. The differences between the two tasks in relation to the effect of drugs acting upon CAMKII, PKA, MAPKK cascades and *de novo* protein synthesis, given in the hippocampus at different post-training intervals, remain throughout the consolidation period [35]. Therefore, our results indicate that the modulation of ecto-nucleotidase activities can be involved in the retrieval of non-associative memories.

These changes in the activities of ecto-nucleotidases may occur through transcriptional or post-transcriptional mechanisms. However, the significant changes on NTPDase and 5′-nucleotidase were observed at 0 and 60 min, but not at 120 min after test session. Therefore, it is possible to suggest that the modulation of these enzyme activities is more related to a control exerted by phosphorylation. Wink et al. [38] have presented evidence pointing out the possible modulation of rat brain NTPDase by phosphorylation. The sequence analysis of NTPDases activity present potential cAMP or cGMP-dependent protein kinase, PKC and casein kinase phosphorylation sites [32]. In addition, it has been described that protein kinase C can activate ecto-5′-nucleotidase [24]. Although the mechanism by which NTPDases altered their activities post-test session has not been elucidated, it is possible that their phosphorylation represents an important regulatory mechanism, controlling the extracellular levels of ATP and adenosine. In fact, both ATP, acting through P2 receptors [1] or as a substrate of ecto-protein kinases [14], as well as adenosine [22] modulate long-term synaptic plasticity phenomena, such as long-term potentiation, long-term depression and depotentiation.

The significant increase of ATP, ADP and AMP hydrolysis could induce an enhancement of adenosine levels immediately after the test session of habituation to an open field. It has been demonstrated that the purinergic system participates in different behavioral tasks, e.g. step-through and step-down inhibitory avoidance tasks [27], Morris water maze [2] and three-panel runway task [25]. Agonists and antagonists of adenosine A_1 and A_{2A} receptors impaired memory retrieval for inhibitory avoidance task [28]. Although adenosine plays an important role in neuromodulation of synaptic transmission, there are no studies with respect to the involvement of purinergic system on habituation. Our results have shown that ecto-nucleotidase activities can be altered during retrieval of habituation to an open field. Further studies are required to evaluate extracellular levels of ATP and adenosine, as well the density and efficiency of P1 and P2 receptors after consolidation and retrieval of memory.

Therefore, the present contribution focuses that different alterations in extracelluar nucleotide hydrolysis have been observed between non-associative and associative learning paradigms throughout memory processing.

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References

[1] T. Almeida, R.J. Rodrigues, A. de Mendonça, J.A. Ribeiro, R.A. Cunha, Purinergic P2 receptors trigger adenosine release leading to adenosine A_{2A} receptor activation and facilitation of long-term potentiation in rat hippocampal slices, Neuroscience 122 (2003) 111–121.

[2] M.E.M. Angelucci, C. Cesário, R.H. Hiroi, P.L. Rosalen, C. da Cunha, Effects of caffeine on learning and memory in rats tested in the Morris water maze, Braz. J. Med. Biol. Res. 35 (2002) 1201–1208.

[3] D.M. Barros, L.A. Izquierdo, J.H. Medina, I. Izquierdo, Pharmacological findings contribute to the understanding of the main physiological mechanisms of memory retrieval, Curr. Drug. Targets CNS Neurol. Disord. 2 (2003) 81–94.

[4] A.M. Battastini, J.B. da Rocha, C.K. Barcellos, R.D. Dias, J.J. Sarkis, Characterization of an ATP diphosphohydrolase (EC 3.6.1.5) in synaptosomes from cerebral cortex of adult rats, Neurochem. Res. 16 (1991) 1303–1310.
[5] F. Bigonnesse, S.A. Levesque, F. Kukulski, J. Lecka, S.C. Robson, M.J. Fernandes, J. Sevigny, Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase-8, Biochemistry 43 (2004) 5511–5519.
[6] P. Bodin, G. Burnstock, Purinergic signalling: ATP release, Neurochem. Res. 26 (2001) 959–969.
[7] C.D. Bonan, M.M. Dias, A.M. Battastini, R.D. Dias, J.J. Sarkis, Inhibitory avoidance learning inhibits ectonucleotidases activities in hippocampal synaptosomes of adult rats, Neurochem. Res. 23 (1998) 977–982.
[8] C.D. Bonan, R. Roesler, G.S. Pereira, A.M. Battastini, I. Izquierdo, J.J. Sarkis, Learning-specific decrease in synaptossomal ATP diphosphohydrolase activity from hippocampus and entorhinal cortex of adults rats, Brain Res. 31 (2000) 253–256.
[9] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[10] J.M. Brundege, T.V. Dunwiddie, Role of adenosine as a modulator of synaptic activity in the central nervous system, Adv. Pharmacol. 39 (1997) 353–391.
[11] G. Burnstock, Introduction P2 receptors, Curr. Top. Med. Chem. 4 (2004) 793–803.
[12] G. Burnstock, Pathophysiology and therapeutic potential of purinergic signaling, Pharmacol. Rev. 58 (2006) 58–86.
[13] K.M. Chan, D. Delfert, K.D. Junger, A direct colorimetric assay for Ca^{2+} stimulated ATPase activity, Anal. Biochem. 157 (1986) 375–380.
[14] W. Chen, A. Wieraszko, M.V. Hogan, H.A. Yang, E. Kornecki, Y.H. Ehrlich, Surface protein phosphorylation by ecto-protein Kinase is required for the maintenance of hippocampal long-term potentiation, Proc. Natl. Acad. Sci. 93 (1996) 8688–8693.
[15] R.A. Cunha, Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors, Neurochem. Int. 38 (2001) 107–125.
[16] T.V. Dunwiddie, S.A. Masino, The role and regulation of adenosine in the central nervous system, Annu. Rev. Neurosci. 24 (2001) 31–55.
[17] D. Heymann, M. Reddington, G.W. Kreutzberg, Subcellular localization of 5′ nucleotidase in rat brain, J. Neurochem. 43 (1984) 971–978.
[18] I. Izquierdo, L.R. Bevilaqua, J.I. Rossato, J.S. Bonini, J.H. Medina, M. Cammarota, Different molecular cascades in different sites of the brain control memory consolidation, Trends Neurosci. 29 (2006) 496–505.
[19] I. Izquierdo, C. da Cunha, R. Rosat, D. Jerusalinsky, M.B. Ferreira, J.H. Medina, Neurotransmitter receptors involved in memory processing by the amygdala, medial septum, and hippocampus of rats, Behav. Neural Biol. 58 (1992) 16–26.
[20] I. Izquierdo, J.H. Medina, Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures, Neurobiol. Learn. Mem. 68 (1997) 285–316.
[21] I. Izquierdo, N. Schroder, C.A. Netto, J.H. Medina, Novelty causes time-dependent retrograde amnesia for one-trial avoidance in rats through NMDA receptor- and CaMKII-dependent mechanisms in the hippocampus, Eur. J. Neurosci. 11 (1999) 3323–3328.
[22] A. de Mendonça, A.R. Costenla, J.A. Ribeiro, Persistence of the neuromodulatory effects of adenosine on synaptic transmission after long-term potentiation and long-term depression, Brain Res. 5 (2002) 56–60.
[23] A. Nagy, A.V. Delgado-Escueta, Rapid preparation of synaptosomes from mammalian brain using nontoxic isoosmotic gradient material (Percoll), J. Neurochem. 43 (1984) 1114–1123.
[24] K. Node, M. Kitakaze, T. Minamino, M. Tada, M. Inoue, M. Hori, T. Kamada, Activation of ecto-5′-nucleotidase by protein kinase C and its role in ischaemic tolerance in the canine heart, Br. J. Pharmacol. 120 (1997) 273–281.
[25] M. Ohno, S. Watanabe, Working memory failure by stimulation of hippocampal adenosine A_1 receptors in rats, Neuroreport 7 (1996) 3013–3016.
[26] G.S. Pereira, T. Mello e Souza, A.M. Battastini, I. Izquierdo, J.J. Sarkis, C.D. Bonan, Effects of inhibitory avoidance training and/or isolated foot-shock on ectonucleotidase activities in synaptosomes of the anterior and posterior cingulate cortex and the medial precentral area of adult rats, Behav. Brain Res. 22 (2002) 121–127.
[27] G.S. Pereira, T. Mello e Souza, E.R.C. Vinadé, H. Choi, C. Rodrigues, A.M.O. Battastini, I. Izquierdo, J.J.F. Sarkis, Blockade of adenosine A1 receptors in the posterior cingulate cortex facilitates memory in rats, Eur. J. Pharmacol. 437 (2002) 151–154.
[28] G.S. Pereira, J.I. Rossato, J.J. Sarkis, M. Cammarota, C.D. Bonan, I. Izquierdo, Activation of adenosine receptors in the posterior cingulate cortex impairs memory retrieval in the rat, Neurobiol. Learn Mem. 83 (2005) 217–223.
[29] J.W. Phillis, P.H. Wu, The role of adenosine and its nucleotides in central synaptic Transmission, Prog. Neurobiol. 16 (1981) 187–239.
[30] V. Ralevic, G. Burnstock, Receptors for purines and pyrimidines, Pharmacol. Rev. 50 (1998) 413–492.
[31] S.C. Robson, J. Sévigny, H. Zimmermann, The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance, Purinergic Signal. 2 (2006) 409–430.
[32] T. Smith, T.L. Kirley, Cloning, sequencing and expression of a human brain ecto-apyrase telated to both the ecto-ATPases and CD39 ecto-apyrase, Biochim. Biophys. Acta 1386 (1998) 65–78.
[33] C.M. Thiel, J.P. Huston, R.K. Schwarting, Hippocampal acetylcholine and habituation learning, Neuroscience 85 (1998) 1253–1262.
[34] C.M. Thiel, C.P. Muller, J.P. Huston, R.K. Schwarting, High versus low reactivity to a novel environment: behavioural, pharmacological and neurochemical assessments, Neuroscience 93 (1999) 243–251.
[35] M.R. Vianna, M. Alonso, H. Viola, J. Quevedo, F. de Paris, M. Furman, M.L. de Stein, J.H. Medina, I. Izquierdo, Role of hippocampal signaling pathways in long-term memory formation of a nonassociative learning task in the rat, Learn. Mem. 7 (2000) 333–340.
[36] M.R. Vianna, L.A. Izquierdo, D.M. Barros, M.M. de Souza, C. Rodrigues, M.K. Sant'Anna, J.H. Medina, I. Izquierdo, Pharmacological differences between memory consolidation of habituation to an open field and inhibitory avoidance learning, Braz. J. Med. Biol Res. 34 (2001) 233–240.
[37] J.R. Whitlock, A.J. Heynen, M.G. Shuler, M.F. Bear, Learning induces long-term potentiation in the hippocampus, Science 313 (2006) 1093–1097.
[38] M.R. Wink, G. Lenz, R. Rodnight, J.J.F. Sarkis, A.M.O. Battastini, Identification of brain ecto-apyrase as a phosphoprotein, Mol. Cell Biochem. 213 (2000) 11–16.
[39] H. Zimmermann, Ectonucleotidases: some recent developments and a note on nomenclature, Drug Dev. Res. 52 (2001) 44–56.