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Role of the ecto-nucleotidases in the cooperative effect of adenosine and neuropeptide-S on locomotor activity in mice

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ABSTRACT

Activation of adenosine receptors modifies the action of classic neurotransmitters (i.e. dopamine, glutamate and acetylcholine) and other neuromodulators, like vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP) and neuropeptide S (NPS). Similarly to adenosine, NPS is involved in the regulation of stimulus and response to fear and arousal. Thus, the present study investigates the effects of NPS on locomotor activity in mice treated with or without α,β-methylene adenosine 5′-diphosphate (AOPCP), the inhibitor of ecto-5′-nucleotidase. Additionally, we evaluate the activity of ecto-5′-nucleotidase in brain slices of mice treated with or without NPS. Male adult CF-1 mice received i.c.v. NPS as 0.1 nmol injection with or without pre-treatment with 1 nmol α,β-methylene adenosine 5′-diphosphate (AOPCP), the selective inhibitor of ecto-5′-nucleotidase, to evaluate locomotor activity. In another set of experiments, mice received i.c.v. infusion of 0.1 nmol NPS to assay enzymatic activity in brain slices. The results demonstrated that the pre-treatment with AOPCP, which was inactive per se, prevented NPS-induced hyperlocomotion in mice. The dose of 0.1 nmol NPS was efficient to induce hyperlocomotion in animals during the observation period in the activity cage. Regarding enzymatic activity, i.c.v. NPS injection did not induce any significant alterations in ATP and AMP hydrolysis in striatum and hippocampus brain slices of mice. The present study shows that the hyperlocomotor effect of NPS depends on the ecto-5′-nucleotidase activity.

1. Introduction

Neuropeptide S (NPS) is a 20-amino acid peptide recently identified in the brain and peripheral tissues of distinct species of vertebrates. This peptide is the endogenous ligand of a G-protein coupled receptor named NPSR receptor (Xu et al., 2004). In cells expressing the recombinant NPS receptor, NPS increases Ca^{2+} mobilization, intracellular cAMP formation and phosphorylation of extracellular signal regulated-kinase (ERK1/2) (Xu et al., 2004; Reinscheid et al., 2005). NPS receptor (NPSR) is expressed in the medial amygdala, substantia nigra pars compacta, subiculum, dorsal raphe, hypothalamus, thalamus, in the pyramidal cell layer of the ventral hippocampus, and was widely distributed in the cortex (Leonard and Ring, 2011). Higher NPS levels were found in cortex, hypothalamus, amygdala, endopiriform nucleus, subiculum, and nuclei of the thalamic midline, while moderate levels were found in substantia nigra (Xu et al., 2007). Conversely, NPS precursor mRNA is found highly expressed only in a cluster of neurons located between the locus coeruleus and Barrington's nucleus (Xu et al., 2004).

The neuroanatomical expression of NPS and its receptor NPSR supports the role played by this peptidergic system in physiological functions such as anxiety (Xu et al., 2004; Jungling et al., 2008; Leonard et al., 2008; Rizzi et al., 2008; Vitale et al., 2008), arousal (Xu et al., 2004; Rizzi et al., 2008), food intake (Beck et al., 2005; Smith et al., 2006), locomotion (Xu et al., 2004; Roth et al., 2006; Smith et al., 2006; Leonard et al., 2008; Okamura et al., 2008; Rizzi et al., 2008; Castro et al., 2009b), nociception (Li et al., 2009), memory (Han et al., 2009; Jungling et al., 2008) and drug addiction (Cannella et al., 2009,

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Pañeda et al., 2009). Additionally, distinct laboratories around the world have revealed that NPS promotes hyperlocomotion in mice (Xu et al., 2004; Roth et al., 2006; Leonard et al., 2008; Rizzi et al., 2008; Castro et al., 2009a, 2009b; Boeck et al., 2010), and rats (Smith et al., 2006).

Recently, a study demonstrated that the effects evoked by NPS on mouse locomotion are similar to those observed after the administration of stimulating caffeine doses (Rizzi et al., 2008). Also, NPS and caffeine act in the regulation of wakefulness states (Xu et al., 2004; Rizzi et al., 2008), and food intake (Beck et al., 2005). Probably, there is an interaction between the adenosinergic and NPS–NPSR receptor systems, because Lage et al. (2006), using the PCR technique, have shown alterations in expression of mRNA NPS and NPSR receptor in rat hypothalamus and brainstem after acute and repeated caffeine treatments. Very recently, our research group has demonstrated the inhibitory effect induced by caffeine and ZM 241385, a selective adenosine A_{2A} receptor antagonist, on hyperlocomotion induced by NPS in mice (Boeck et al., 2010).

Adenosine, operating via inhibitory adenosine A_1 receptors or excitatory adenosine A_{2A} receptors, has widespread modulatory actions in the nervous system and may interfere with the action of other classic neurotransmitters and neuromodulators (Ribeiro, 1999). Interestingly enough, in a synaptic cleft, adenosine available could have two distinct sources: (1) it may come from the release via bi-directional nucleoside transporter; (2) and/or from adenine nucleotides released, which are degraded by a chain of ecto-nucleotidases (Hoehn and White, 1990; Craig and White, 1993; Cunha et al., 1996). The most relevant ecto-enzymes involved in this chain are those of the ecto-nucleoside triphosphate diphosphohydrolases family (E-NTPDases), which hydrolyze nucleoside tri- and di-phosphates. At least eight different members of the NTPDase family have been discovered, cloned and studied over the last few years. NTPDases1, 2, and 3 are expressed in nervous tissue and mediate the termination of ATP signaling in the synaptic cleft (Zimmermann et al., 1998; Wink et al., 2006). The produced nucleoside monophosphates in the cleft are hydrolyzed by ecto-5′-nucleotidase (E.C. 3.1.3.5) (Fredholm et al., 2005; Robson et al., 2006), that is a pivotal step in extracellular adenosine production from the enzymatic chain (James and Richardson, 1993). It has been suggested that adenosine release leads to preferential A_1 receptor activation, while adenosine formed from the ecto-nucleotidase pathway leads to favored adenosine A_{2A} receptor activation (Cunha et al., 1996; Boeck et al., 2005). In this context, our group recently demonstrated the inhibitory effect induced by caffeine or ZM 241385, a selective A_{2A} receptor antagonist, on hyperlocomotion induced by NPS in mice (Boeck et al., 2010).

Thus, considering that adenosine plays a modulatory effect in the hyperlocomotion evoked by NPS, the present study aimed at investigating whether the involvement of adenosine in the hyperlocomotor effect of NPS is due to its production via ecto-nucleotidases pathway. To test this hypothesis we pre-treated mice with α,β-methylene-adenosine 5′-diphosphate (AOPCP), the specific ecto-5′-nucleotidase inhibitor, before NPS challenge. The present study also investigated whether NPS treatment is able to modify the E-NTPDase and ecto-5′-nucleotidase activities in hippocampal and striatal mouse brain slices.

2. Materials and methods

2.1. Materials

Adenosine 5′-triphosphate (ATP), adenosine 5′-monophosphate (AMP) and α,β-methylene adenosine 5′-diphosphate (AOPCP) were obtained from Sigma (St Louis, MO, USA). Human NPS was synthesized by Dr R. Guerrini, Department of Pharmaceutical Science and Biotechnology Center, University of Ferrara, according to published methods (Roth et al., 2006). NPS was dissolved in saline solution (NaCl 0.9 g%, w/v) and intracerebroventricularly (i.c.v.) administered in mice. AOPCP had its pH adjusted with NaOH 0.1 M and was diluted in saline solution. All other chemicals were of analytical reagent grade and purchased from local suppliers.

2.2. Animals and surgical procedure

Male albino CF-1 mice (2–3 months of age, 30–35 g) were obtained from our breeding colony (UNESC). Six animals were housed per cage with food and water freely available and were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.). All experimental procedures involving animals were performed in accordance with the National Institute of Heath's Guide for the Care and Use of Laboratory Animals and the recommendations of the Sociedade Brasileira de Ciências em Animais de Laboratório (SBCAL) for animal care, designed to minimize suffering and limit the number of animals used. To avoid circadian variations all experiments were carried out between 8:00 a.m. and 1:00 p.m. This study was approved by the local ethics committee (Comitê de Ética no Uso de Animais da Universidade do Extremo Sul Catarinense, no. 045/2009).

Surgery and i.c.v. infusion techniques were conducted according to Schmidt et al. (2000). Naïve mice were anesthetized with 7% chloral hydrate (w/v, 10 mL/kg body weight, i.p.). In a stereotaxic apparatus, the mouse skull skin was removed and an i.c.v. guide cannula (27-gauge) was unilaterally implanted 1.0 mm posterior to bregma, 1.0 mm to the right of the midline and 1.0 mm above the right lateral brain ventricle. The guide cannula was implanted 1.5 mm ventral to the superior surface of the skull and fixed with jeweler's acrylic cement. In the experiments, performed 48 h after surgery, an i.c.v. infusion was performed using a 30-gauge cannula that was fitted into the implanted guide cannula and connected by a polyethylene tube to a Hamilton microsyringe. The tip of the infusion cannula protruded 1 mm beyond the guide cannula, aiming at the lateral brain ventricle. After experiments, methylene blue (4 μL) was injected through the cannula and animals without dye in the lateral brain ventricle were discarded.

2.3. Treatments

Mice received i.c.v. vehicle injection (saline solution; 5 μL) or AOPCP (1 nmol; 5 μL) just before testing locomotor activity. Five minutes following AOPCP administration, mice received i.c.v. vehicle or NPS injection (0.1 nmol; 1 μL) and their locomotor activity was evaluated for 25 min. Previous studies performed in our laboratory showed that this dose of NPS produces a higher stimulatory effect on locomotion (Castro et al., 2009a; Castro et al., 2009b; Boeck et al., 2010). The dose of AOPCP employed in the present study was chosen based on the previous study in mice (Saute et al., 2006).

In another set of experiments, mice received vehicle or NPS (0.1 nmol) in the lateral ventricle as a constant volume of 1 μL, 5 min before the enzymatic assay.

2.4. Locomotor activity assay

An infrared beam array cage (Insight Equipments, Ribeirão Preto, Brazil) connected to a PC was used to assess locomotor activity in mice. The infrared beam array cage consists of a cubicle made of clear Perspex (48×50 cm) surrounded by 50-cm-high walls. Two blocks facing each other and containing an infrared array recorded horizontal activity, and a similar system assessed vertical activity. Just after i.c.v. vehicle or AOPCP injection non-habituated animals were gently placed on the center of the arena and were individually allowed to explore the apparatus for 5 min. Mice were gently removed from the cage, received an i.c.v. vehicle or NPS injection and were returned to the apparatus to explore it for another 25-min period (totaling 30 min of observation). All behavioral experiments were conducted in a well

lit (300 lx) and quiet room in the absence of the observer. After the behavioral evaluation of each mouse, the arena was cleaned with 10% ethanol solution. Each mouse was individually evaluated once for locomotor activity, for a 30-min period. The total distance covered by each animal was assessed based on 5-min time intervals.

2.5. Nucleotides hydrolyses assay

Five minutes after i.c.v. vehicle or NPS injection mice were killed by decapitation and the brains were rapidly removed and transferred to a pre-warmed HEPES-buffered salt solution with the following composition: 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 10 mM glucose (pH 7.4) and gassed with a 95% O_2/5% CO_2 mixture (incubation medium). The brains were cut longitudinally, their hippocampi and striatum dissected and sliced transversely as 400-μm-thick slices on a McIlwain tissue chopper. Two slices per tube (approx. 0.15 mg protein) were preincubated for 10 min at 37 °C with 400 μL of the incubation medium (described above). To measure ATP and AMP hydrolyses the slices were incubated with ATP or AMP (1 mM, final concentration) for 20 min at 37 °C. Following incubation, an aliquot of the assay medium was removed and mixed with trichloroacetic (TCA) to a final concentration of 5%. A sample of the supernatant was taken for the assay of inorganic phosphate (Pi) release by colorimetric determination (Chan et al., 1986). Non-enzymatic Pi released from nucleotide in the assay medium without cells and Pi released from cells incubated without nucleotide were subtracted from the total Pi released during nucleotide hydrolysis, to produce enzymatic activity values (nmol Pi/mg protein per min). All experimental conditions adopted were previously investigated in order to ensure the linearity of enzymatic reaction and the preservation of cellular viability until the end of the experiment (data not shown). Protein was determined using bovine serum albumin as standard (Bradford, 1976).

2.6. Statistical analysis

Behavioral data were presented as mean values ± S.E.M. (standard error of the mean) and each value reflected the mean of 10–14 animals per group. The effects of the combined administration of NPS and AOPCP were statistically analyzed using a two-way ANOVA with NPS and AOPCP as main factors, followed by the Tukey's post hoc test. Enzymatic data are presented as mean values ± S.E.M. (standard error of the mean) and each value reflected the mean of triplicate samples from 8 to 10 animals per group. The effect of the NPS was statistically analyzed using the Student's t-test. Differences were considered significant when $P<0.05$. Results were analyzed by STATISTICA® software version 7.0 (StatSoft, Inc., USA).

3. Results

Fig. 1 shows the effect of pre-treatment of ecto-5′-nucleotidase inhibitor AOPCP on NPS-induced hyperlocomotion. Non-habituated mice treated with AOPCP did not show any changes in locomotor activity, when compared to saline treated mice during the first 5-min period on the arena or even throughout the experimental period (Fig. 1A). Mice treated with NPS displayed increased locomotor activity, with significant effect beginning 10 min following the NPS injection ($F(1.41)=7.277$; $P=0.01$) (Fig. 1A). However, mice pre-treated with AOPCP before NPS administration did not display the NPS-induced hyperlocomotion, an effect statistically dependent on the interaction of AOPCP and NPS for all periods (20 min–$F(1.41)=5.771$; $P=0.02$; 30 min–$F(1.41)=7.839$; $P=0.008$). When the activity of mice was evaluated as cumulative distance traveled during 30 min, AOPCP effectively induced the prevention on NPS-induced hyperlocomotion ($F(1.41)=7.189$; $P=0.015$) reaching basal values (Fig. 1B).

The measurement of ecto-NTPDase activities was evaluated in slices of brain areas following NPS injection in mice (Fig. 2). ATP or AMP hydrolysis in striatum ($t_{ATP}=-0.806$; $P=0.432$; $t_{AMP}=-0.364$; $P=0.720$) (Fig. 2A) or hippocampus ($t_{ATP}=-1.998$; $P=0.062$; $t_{AMP}=-0.999$; $P=0.334$) (Fig. 2B) was not altered by NPS ($P>0.05$ for all nucleotides at each brain area).

4. Discussion

In the present study, we investigated the origin of adenosine that contributes to NPS-induced hyperlocomotion in mice using the combination of NPS and a selective inhibitor of the ecto-5′-nucleotidase, responsible for the inhibition of extracellular adenosine production from nucleotides released. The present data confirmed that 0.1 nmol NPS i.c.v injection substantially increased mouse spontaneous locomotion, according to other studies (Xu et al., 2004; Roth et al., 2006; Leonard et al., 2008; Rizzi et al., 2008; Castro et al., 2009a; Castro et al., 2009b). The present data are also in line with our

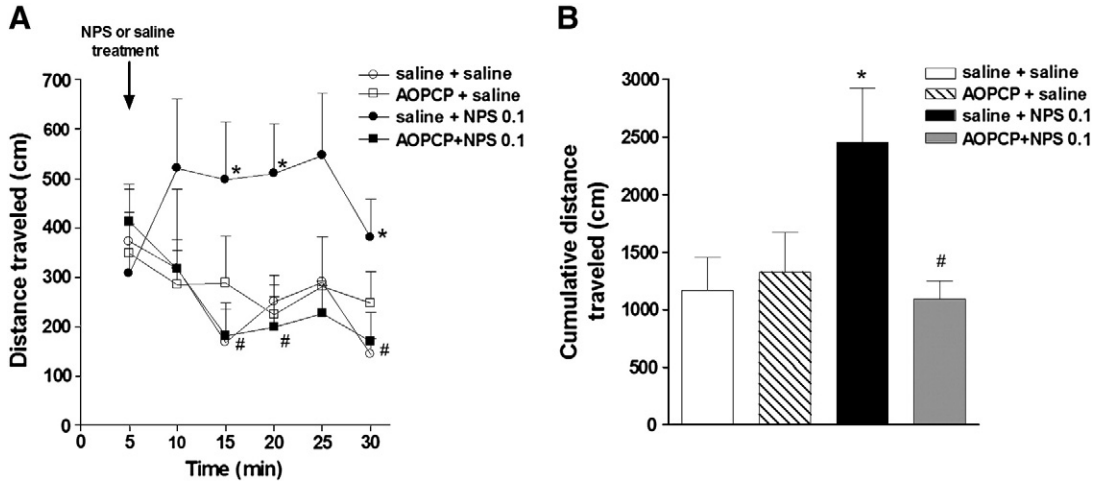

Fig. 1. Effect of the i.c.v. NPS injection (0.1 nmol; 1 μL) with or without AOPCP (1 nmol; 5 μL) on the spontaneous locomotor activity assessed in mice kept in infrared beam array cages for 30 min. Five minutes after the administration of saline or AOPCP, mice were treated with saline or NPS and (A) the locomotor activity was recorded for 25 min (B) to evaluate cumulative activity of mice. Data are shown as mean ± S.E.M. (10–14 mice/group). *$P<0.05$ vs. control group and #$P<0.05$ vs. NPS group, according to two-way ANOVA followed by the Tukey's test.

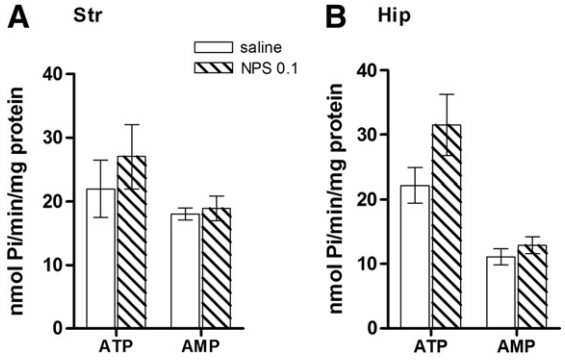

Fig. 2. Effect of the i.c.v NPS injection (0.1 nmol; 1 μL) on the ecto-nucleotidase activities in slices from hippocampus or striatum of mice. Enzymatic data are presented as mean values ± S.E.M. and each value reflects the mean of triplicate samples from 8 to 10 animals per group. The effect of NPS was statistically analyzed using the Student's t-test.

previous findings, showing robust and consistent hyperlocomotor effects of NPS in rodents that can nevertheless be blunted by adenosine A_{2A} receptor antagonists (Boeck et al., 2010).

Ecto-nucleotidases break down the adenine nucleotides in stages to produce free extracellular adenosine at the terminal step (Zimmermann, 1999). For example, the extrasynaptic enzyme ecto-nucleoside triphosphate diphosphohydrolase 1 (EC 3.6.1.5; E-NTPDase1, previously identified as ecto-ATPDase, ecto-apyrase or CD39) converts ATP and ADP to AMP in cultured hippocampal neurons (Boeck et al., 2002). The final and critical step of conversion of AMP to adenosine is carried out by ecto-5′-nucleotidase (Dunwiddie et al., 1997). Thus, to fully define the roles that individual species play in purinergic signaling, it is useful to prevent the extracellular metabolism of purines such as AMP (Cunha et al., 2000; Mihaylova-Todorova et al., 2002). In the present study, AOCPC was employed in order to inhibit ecto-5′-nucleotidases activity at dose previously shown to inhibit the behavioral responses to guanine-based nucleotides on pain and memory in mice (Saute et al., 2006; Schmidt et al., 2008). Also, AOPCP induces decrease on extracellular adenosine levels after injection of NMDA in the striatum of rat (Delaney et al., 1998) and it blocks release of purines in response to hypothalamic defense area stimulation, which it was observed that adenosine is predominantly released in the nucleus tractus solitarii (Dale et al., 2002). Previous in vitro studies using AOPCP demonstrated that the nucleotides released are the source of adenosine in cortical slices and synaptosomes after glutamate incubation (Hoehn and White, 1990; Craig and White, 1993). Thus, the studies demonstrated that the nucleosides (adenosine or guanosine) were produced in the extracellular space by the prior release of nucleotide (i.e. ATP).

In the present study, we also investigated the participation of adenosine produced by ecto-5′-nucleotidase during the stimulatory effects of NPS in brain of mice. Our findings are the first evidence that the administration of AOPCP does not significantly alter per se mouse locomotor activity. However, the combined injection of AOPCP and NPS blocks the effect of NPS as regards the increase in the distance covered during locomotion, as induced by the neuropeptide (Fig. 1).

Regarding adenosine receptors activation, the ecto-5′-nucleotidase activity is critical. It has been proposed that one of the possible mechanisms for extracellular adenosine to preferentially activate either inhibitory or facilitatory receptors depends on the manner in which extracellular adenosine was generated. If adenosine is released, as such, through bidirectional non-concentrative adenosine transporters, it could favor adenosine A_1 receptor-mediated inhibition. On the other hand, if adenosine is formed from the extracellular catabolism of ATP, it could favor adenosine A_{2A} receptor-mediated facilitation (Cunha et al., 1996). The preferences of adenosine for its receptors may be due to the proximal brain distribution of ecto-5′-nucleotidadase and adenosine A_{2A} receptors. In a recent paper we have demonstrated that the stimulatory effects of NPS on locomotion depend on the activation of adenosine A_{2A} receptor, while the activation of A_1 receptors plays a minor role in the modulation of NPS effects (Boeck et al., 2010). Taken together, these results suggested that the adenosine involved in the hyperlocomotor effects of NPS could be produced by extracellular catabolism of nucleotides.

In the present study we also investigated whether NPS treatment could affect the activity of ecto-nucleotidases in the striatum and hippocampus. Even though we did not observe a significant modification in ecto-nucleotidase activities following NPS administration (Fig. 2), the participation of this cascade pathway in the adenosine effect upon NPS cannot be excluded. One of the limitations in the protocol used in the present study was that the assessment of enzyme activities in brain slices was performed at the onset of the NPS effects (i.e. animals were euthanized 5 min after NPS injection). Thus, in this context, we cannot completely exclude the effects of NPS (directly or indirectly) on ecto-nucleotidases activity. However, it is interesting to note that the AOPCP effect is clear; thus, our results demonstrate that adenosine conversion is also necessary for the in vivo model of hyperlocomotion induced by NPS.

A recent study revealed that NPS suppressed the increase in extracellular acetylcholine levels induced by MK-801 (NMDA receptor antagonist) in the retrosplenial cortex (Okamura et al., 2010). Also, adenosine modulates extracellular acetylcholine levels, because adenosine A_{2A} receptors have been shown to facilitate the release of most neurotransmitter types, including acetylcholine, in different extra-striatal brain regions (Jin and Fredholm, 1997). Thus, the NPS modulation probably depends on the activation of adenosine A_{2A} receptors. Other studies have explored this idea, showing that the peptidergic modulation of excitatory synaptic transmission in the hippocampus by G-protein coupled receptors, operated by calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP), is strictly dependent on the activation of A_{2A} receptors (Cunha-Reis et al., 2008; Sebastião et al., 2000). Conversely, Ruzza et al. (2010) recently showed that the NPSR receptor antagonist SHA68 did not affect the hyperlocomotor effect of caffeine in mice. These findings suggest that the excitatory effects of caffeine are not dependent on NPSR receptors.

Thus, the possible role of adenosine depends on physiological needs, i.e. to contribute for the facilitation of synaptic transmission, possibly through an A_{2A} receptor-induced A_1 receptor desensitization (Lopes et al., 1999), which in turn would contribute to the effect of NPS on locomotor activity. We hypothesized that the modulatory effect of adenosine receptors under NPS-induced stimulation could be due to a common protein-G signaling pathway shared by both systems, since both adenosine A_{2A} and NPSR receptors are positively coupled to adenylate cyclase. However, the interaction of adenosine and NPS in the stress, anxiety, arousal and sleep, and food intake as being likewise modulated by NPS should be further investigated.

In conclusion, the present results demonstrate that the NPS-induced hyperlocomotion in mice is dependent on ecto-5′-nucleotidase activity; however, it should be mentioned that this assay was performed 5 min after NPS injection, thus longer periods after NPS-injection are required to assess possible effects. Finally, these data corroborate the view that an adenosinergic tonus is essential for the hyperlocomotor effect evoked by NPS.

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