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Ecto-nucleotidase pathway is altered by different treatments with fluoxetine and nortriptyline

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Abstract

Depression is one of the most disabling diseases and causes a significant burden to both individual and society. Selective serotonin reuptake inhibitors and tricyclic antidepressants, such as fluoxetine and nortriptyline, respectively, are commonly used in treatment for depression. These antidepressants were tested on cerebral cortex and hippocampal synaptosomes after acute and chronic *in vivo* and *in vitro* treatments. In chronic treatment, fluoxetine and nortriptyline decreased ATP hydrolysis (17.8% and 16.3%, respectively) in hippocampus. In cerebral cortex, nortriptyline increased ATP (32.3%), ADP (51.8%), and AMP (59.5%) hydrolysis. However, fluoxetine decreased ATP (25.5%) hydrolysis and increased ADP (80.1%) and AMP (33.3%) hydrolysis. Significant activation of ADP hydrolysis was also observed in acute treatment with nortriptyline (49.8%) in cerebral cortex. However, in hippocampus, ATP (24.7%) and ADP (46.1%) hydrolysis were inhibited. Fluoxetine did not alter enzyme activities in acute treatment for both structures. In addition, there were significant changes in NTPDase activities when fluoxetine and nortriptyline (100, 250, and 500 μM) were tested *in vitro*. There was no inhibitory effect of fluoxetine and nortriptyline on AMP hydrolysis in cerebral cortex and hippocampus. The findings showed that these antidepressant drugs can affect the ecto-nucleotidase pathway, suggesting that the extracellular adenosine levels could be modulated by these drugs.

Keywords: Fluoxetine; Nortriptyline; Ecto-nucleotidase; NTPDase; Ecto-5′-nucleotidase; Depression

1. Introduction

Depression is one of the most disabling diseases and causes a significant burden to both individual and society. Moreover, depression is a multifaceted disease in terms of symptoms, comorbidities and health complications and the treatment is difficult due to the heterogeneity of the disease (Rosenzweig-Lipson et al., 2007). Evidence suggests that brain regions as the prefrontal cortex, amygdala, related parts of the striatum, cingulate cortex, and hippocampus are involved in the functional neuroanatomy of depression (Drevets et al., 1992; Walsh et al., 2007; McEwen et al., 2002). The treatments for depression comprise monoamine oxidase (MAO) inhibitors, tricyclic antidepressants, serotonin reuptake inhibitors (SSRIs) and both serotonin and noradrenaline reuptake inhibitors (SNRIs) (Galeotti et al., 2002; Serra et al., 2006; Rosenzweig-Lipson et al., 2007). Many investigators have reported that administration of tricyclic antidepressants, such as nortriptyline,

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can result in inhibition of the presynaptic uptake of serotonin (5-HT) and/or noradrenaline (NA) (Morishita and Aoki, 2002; Stoll and Gentile, 2005; Su et al., 2007). In contrast, fluoxetine, a selective inhibitor of serotonin reuptake, has little effects on other neurotransmitters (Rossi et al., 2004; Cecconi et al., 2007; Chen et al., 2007).

Serotonin and noradrenaline can be co-released with ATP, which is considered a neurotransmitter and neuromodulator in central nervous system (CNS) (Burnstock, 2004). Extracellular ATP evokes responses by two subclasses of P2 purinoreceptors, P2X and P2Y (Ralevic and Burnstock, 1998; Burnstock, 2007). Inhibition of release via P2 receptors has been previously shown for dopamine (neostriatum), noradrenaline (brain cortex and hippocampus), and serotonin (cerebral cortex) (Koch et al., 1997; von Kügelgen et al., 1997). The signaling actions induced by extracellular ATP are directly correlated to the activity of ecto-nucleotidases (Zimmermann, 2001; Robson et al., 2006). Ecto-nucleotidases are involved in the control of nucleotide and nucleoside levels in the synaptic cleft and include NTPDase (nucleoside triphosphate diphosphohydrolase) family and ecto-5′-nucleotidase (Zimmermann, 2001). Four members of the NTPDase family (NTPDase1–3 and 8) 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 (Bigonnesse et al., 2004; Robson et al., 2006). These enzymes reveal a wide and partially overlapping tissue distribution. Northern hybridization suggests that the three related family members NTPDase1 to NTPDase3 are expressed in mammalian brain (Zimmermann and Braun, 1999). Moreover, NTPDase1 is a major ecto-nucleotidase of both microglia and blood vessels in the brain (Braun et al., 2000). Adenosine, a product of ATP catabolism, can evoke its neuromodulatory effects by four subtypes of P_1-purinoreceptors named A_1, A_{2A}, A_{2B} and A_3 (Brundege and Dunwiddie, 1997; Cunha, 2001; Dunwiddie and Masino, 2001; Cunha, 2005). Studies have shown that adenosine modulates cognitive states and is associated with affective and mood disorders, such as anxiety and depression (Ledent et al., 1997; Florio et al., 1998; Kaster et al., 2004). Moreover, it has been shown that hippocampal serotonergic neurotransmission is modulated by hippocampal adenosine receptor subtypes (Okada et al., 1999).

Considering that (i) adenosine and ATP are able to modulate 5-HT release, (ii) 5-HT and NA can be co-released with ATP and (iii) the action of ecto-nucleotidases represents one of the most important sources of extracellular adenosine, the aim of this study was to evaluate the effect *in vivo* and *in vitro* of fluoxetine and nortriptyline on the ecto-nucleotidases in synaptosomes from hippocampus and cerebral cortex of rats.

2. Materials and methods

2.1. Chemicals

Fluoxetine, nortriptyline, nucleotides, Trizma Base, malachite green, ammonium molybdate, polyvinyl alcohol, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, calcium, and magnesium chloride were purchased from Sigma (USA). All other reagents used were of analytical grade.

2.2. Animals

Male Wistar rats (age around 90 days, with 260–320 g) from our breeding stock were housed four to a cage, with food and water *ad libitum*. The animal house temperatures were kept between 22 and 23 °C with a 12-h light/dark cycle (lights on at 07:00). Animal care followed the Guide for the Care and Use of Laboratory Animals (NIH, USA), the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee (CEP 06/03016) of the Pontificia Universidade Católica do Rio Grande do Sul, Brazil.

2.3. In vivo treatments

2.3.1. Acute treatment

Animals received one single injection intraperitonially (i.p.) (10 mg/kg) of fluoxetine or nortriptyline 1 h before they were killed (Zanatta et al., 2001; Borelli et al., 2004; Ejsing and Linnet, 2005; Drapier et al., 2006; Marx et al., 2006). Control animals received saline injections (0.9% NaCl) in the same volume as those applied to antidepressant-treated rats.

2.3.2. Chronic treatment

The antidepressant drugs were administered daily for 14 days (10 mg/kg, i.p.) (Silva and Brandão, 2000; Zanatta et al., 2001; Borelli et al., 2004; Bonanno et al., 2005). Control animals received saline injections (0.9% NaCl) in the same volume as those applied to antidepressant-treated rats.

2.4. In vitro treatments

Antidepressants, fluoxetine or nortriptyline, were added to reaction medium before the preincubation with synaptosomal preparation and maintained throughout the enzyme assays. Antidepressants were tested at final concentrations of 100, 250, and 500 μM (Dhalla et al., 1980; Zanatta et al., 2001; Pedrazza et al., 2007).

2.5. Synaptosomal preparation

The rats were killed by decapitation, and their cerebral cortex and hippocampus were dissected, homogenized in 10 and 5 volumes, respectively, in an ice-cold medium consisting of 320 mM sucrose, 0.1 mM EDTA and 5.0 mM HEPES, pH 7.5. The synaptosomes were isolated as described previously (Nagy and Delgado-Escueta, 1984). Briefly, 0.5 ml of the crude mitochondrial fraction was mixed 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.

2.6. Determination of ecto-nucleotidase activities

The reaction medium used to assay ATP and ADP hydrolysis was essentially as described previously (Battastini et al., 1991), 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 $MgCl_2$, 0.1 M Tris–HCl, pH 7.5 and 0.15 M sucrose to final volume of 200 µl (Heymann et al., 1984). The synaptosomal fraction (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 1 mM ATP, ADP or AMP as substrate and stopped by the addition of 200 µl 10% trichloroacetic acid. The samples were chilled on ice for 10 min and 100 µl samples were taken to assess the released inorganic phosphate (Pi) (Chan et al., 1986). In enzyme assays, incubation time and protein concentration were chosen in order to ensure the linearity of the reaction. Controls, with the addition of the enzyme preparation after the addition of trichloroacetic acid, were used to correct non-enzymatic hydrolysis of the substrates. All samples were assayed in duplicate (*in vitro* assays) and triplicate (*in vivo* assays). Enzyme activities were expressed as nanomoles of Pi released per minute per milligram of protein.

2.7. Protein determination

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

2.8. Analysis of gene expression by semi-quantitative RT-PCR

The analysis of the expression of NTPDase 1, 2, 3, and ecto-5′-nucleotidase was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. After acute and chronic treatments, hippocampus and cerebral cortex of rats were isolated for total RNA extraction using Trizol reagent (Invitrogen) in accordance with manufacturer instructions. RNA purity was quantified spectrophotometrically and tested by eletrophoresis in a 1.0% agarose gel containing ethidium bromide. The cDNA species were synthesized with SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen) from 3 µg of total RNA following suppliers. RT reactions were performed for 50 min at 42 °C. cDNA (1 µl) was used as a template for PCR with specific primers for NTPDase1, 2, 3, and 5′-nucleotidase. β-actin was used for normatization as a constitutive gene. PCR reactions have a volume of 25 µl using a concentration of 0.4 µM of each primer indicated below and 200 µM and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer. Conditions for all PCR were as follows: Initial 1 min denaturation step at 94 °C, 1 min annealing step (NTPDase 1, 3 and 5′-nucleotidase: 65 °C; NTPDase2: 66 °C; β-actin: 58.5 °C), 1 min extension step at 72 °C for 35 cycles and a 10 min final extension a 72 °C. The amplification products were: NTPDase1 — 543 bp; NTPDase2 — 331 bp; NTPDase3 — 267 bp; 5′-nucleotidase — 403 bp; β-actin — 210 bp. The primers were described previously (Vuaden et al., 2007). For each set of PCR reactions, negative controls were included. Five microliters of the PCR reaction was analyzed on a 1% agarose gel, containing ethidium bromide and visualized with ultraviolet light. 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.

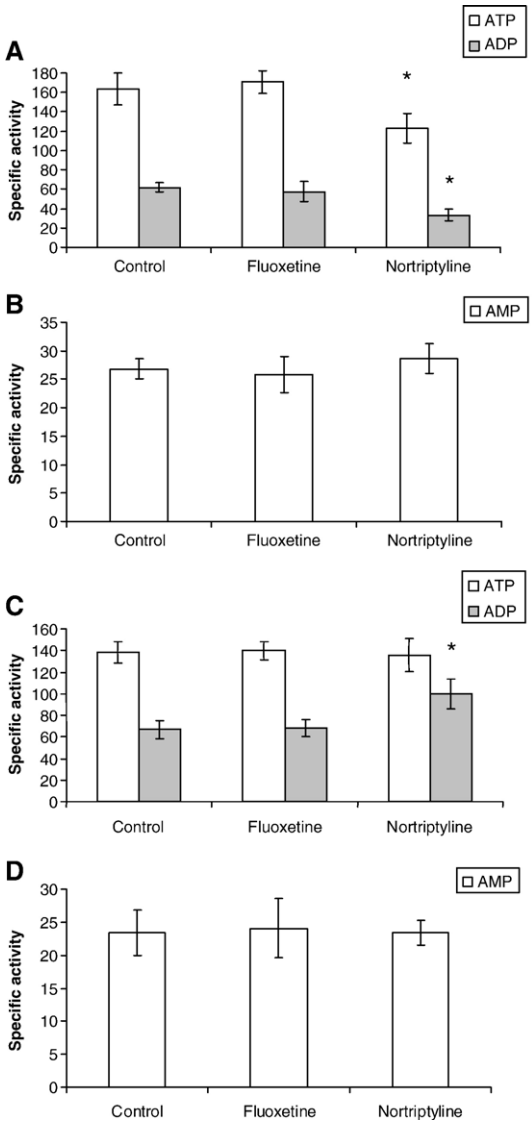

Fig. 1. Effect of acute treatment with fluoxetine or nortriptyline on ATP and ADP and AMP hydrolysis in hippocampus (A and B) and cerebral cortex (C and D) of rats. Bars represent the mean ± S.D. of five different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test ($p \leq 0.01$, when compared to control group).

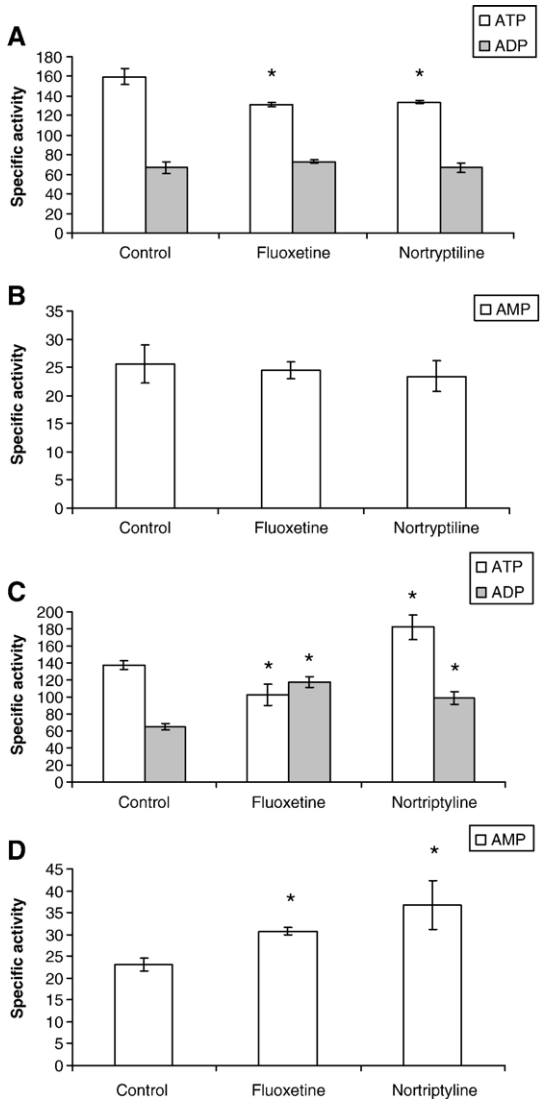

Fig. 2. Effect of chronic treatment with fluoxetine or nortriptyline on ATP and ADP and AMP hydrolysis in hippocampus (A and B) and cerebral cortex (C and D) of rats. Bars represent the mean±S.D. of five different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test ($p \leq 0.01$, when compared to control group).

2.9. Statistical analysis

Data were expressed by mean+S.D and analyzed by one-way analysis of variance (ANOVA) followed by the Tukey multiple range test considering $p<0.05$ as significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer.

3. Results

We evaluated the effect *in vivo* (acute and chronic treatments) and *in vitro* of antidepressant drugs on ATP, ADP, and AMP hydrolysis from hippocampus and cerebral cortex of rats.

In acute treatment with nortriptyline, ATP (24.7%) and ADP (46.1%) hydrolysis were inhibited in hippocampus (Fig. 1A). However, a significant activation of ADP hydrolysis was observed in acute treatment with nortriptyline (49.8%) in cerebral cortex (Fig. 1C). AMP hydrolysis was not affected by nortriptyline (Fig. 1B and D). Fluoxetine did not alter enzymes activities in acute treatment for both structures (Fig. 1 A, B, C and D).

In chronic treatment, nortriptyline and fluoxetine decreased ATP hydrolysis (17.8% and 16.3%, respectively) (Fig. 2A) in hippocampus, but these drugs did not alter ADP and AMP hydrolysis in both structures (Fig. 2A, B). However, in cerebral cortex, these drugs promote different effects on nucleotide hydrolysis. Nortriptyline increased ATP, ADP, and AMP hydrolysis (32.3%, 51.8% and 59.5%, respectively) (Fig. 2C, D) and fluoxetine decreased ATP (25.5%) hydrolysis and increased ADP (80.1%) and AMP (33.3%) hydrolysis.

The effect *in vitro* of nortriptyline on nucleotide hydrolysis was also tested in cortical and hippocampal synaptosomes. A significant inhibition in ATP hydrolysis (21.1–74.5%) was observed in hippocampal synaptosomes for all concentrations tested, but ADP hydrolysis was inhibited only at 250 and 500 μM (39.8%–60.4%) (Fig. 3A). A similar decrease of cortical NTPDase activity was observed in the all concentrations tested (37.5–73% for ATP hydrolysis and 41.4–80.3% for ADP hydrolysis) (Fig. 3B). Nortriptyline failed to inhibit AMP hydrolysis in both brain regions tested (data not shown).

In addition, for *in vitro* treatment, fluoxetine inhibited both ATPase and ADPase activities in all concentrations tested (100 to 500 μM). The inhibition promoted by fluoxetine in hippocampal synaptosomes varied from 66.8% to 82.2% for ATP hydrolysis and from 54.7% to 68.3% for ADP hydrolysis (Fig. 4A). The inhibitory effect in cortical synaptosomes varied

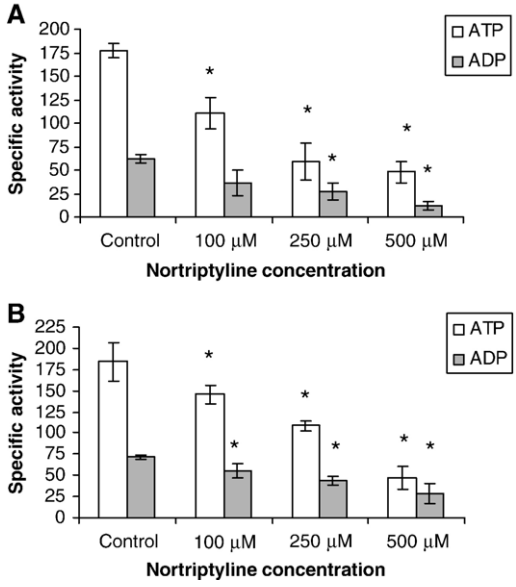

Fig. 3. *In vitro* effect of nortriptyline on NTPDase activities in hippocampus (A) and cerebral cortex (B) of rats, respectively. Bars represent the mean±S.D. of five different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test ($p \leq 0.01$, when compared to control group).

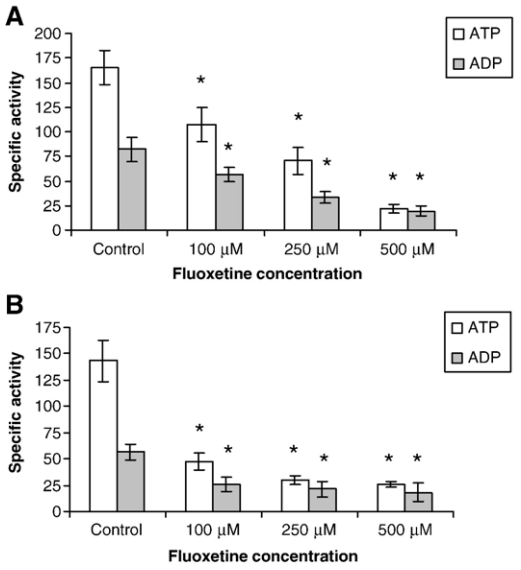

Fig. 4. *In vitro* effect of fluoxetine on NTPDase activities in hippocampus (A) and cerebral cortex (B) of rats, respectively. Bars represent the mean ± S.D. of five different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test ($p \leq 0.01$, when compared to control group).

from 34.7% to 86.9% in ATP hydrolysis and from 31.2% to 76.4% for ADP hydrolysis (Fig. 4B). There was no observed effect of fluoxetine on AMP hydrolysis in both hippocampus and cerebral cortex (data not shown).

The effects promoted by antidepressant drugs could be a consequence of transcriptional control. We have evaluated the gene expression for NTPDase1, NTPDase2, NTPDase3, and 5′-nucleotidase. The constitutive gene was normalized to β-actin expression to allow the comparison in different experimental conditions. The semi-quantitative RT-PCR analyses were performed when kinetic alterations had occurred. For this reason, NTPDases and 5′-nucleotidase were not analyzed after acute treatment with fluoxetine in hippocampus and cerebral cortex. The acute treatment with nortriptyline produced an increase in the NTPDase3 transcript levels in hippocampus (Fig. 5A, B). Interestingly, NTPDase1 and NTPDase 2 demonstrated an increase in the transcript levels in cerebral cortex (Fig. 6A, B).

The chronic treatment with nortriptyline promoted a decrease in NTPDase1, NTPDase2, and NTPDase3 transcript levels in hippocampus (Fig. 5C, D). In contrast, NTPDase1 and 5′-nucleotidase presented an increase of gene expression for NTPDase1 and 5′-nucleotidase in cerebral cortex (Fig. 6C, D). The chronic treatment with fluoxetine produced an enhancement for NTPDase1 and NTPDase3 transcript levels in hippocampus (Fig. 5E, F) and

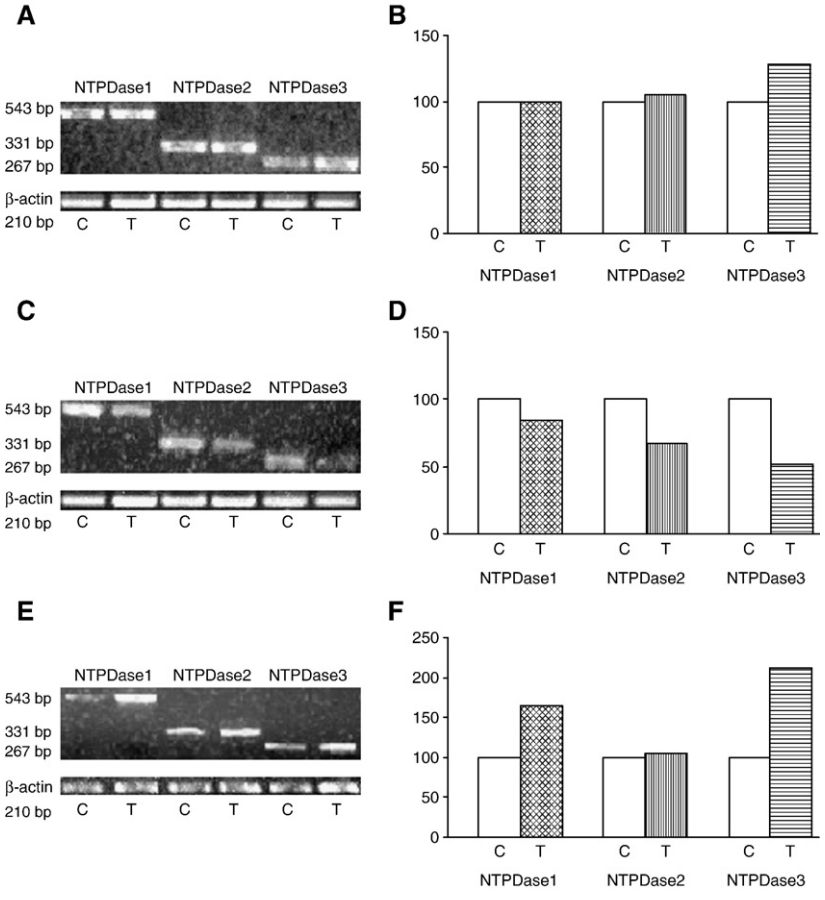

Fig. 5. Gene expression patterns after acute treatment with nortriptyline (A and B) and chronic treatment with nortriptyline (C and D) and fluoxetine (E and F) in hippocampus of rats. C represents control group and T represents treated group. Three independent experiments were performed, with entirely consistent results.

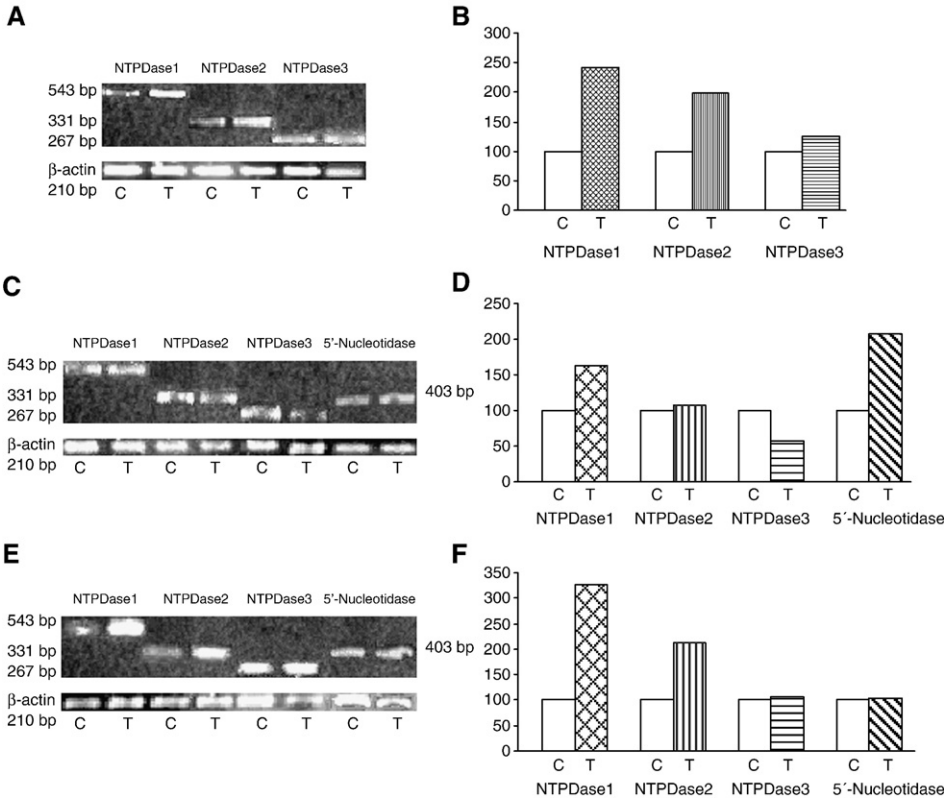

Fig. 6. Gene expression patterns after acute treatment with nortriptyline (A and B) and chronic treatment with nortriptyline (C and D) and fluoxetine (E and F) in cerebral cortex of rats. C represents control group and T represents treated group. Three independent experiments were performed, with entirely consistent results.

NTPDase 1 and NTPDase2 transcript levels were increased in cerebral cortex (Fig. 6E, F).

4. Discussion

NTPDase and ecto-5′-nucleotidase activities were sensitive to fluoxetine and nortriptyline during chronic treatment in cerebral cortex. In contrast, in acute treatment, ATP and ADP hydrolysis was decreased after administration of nortriptyline in hippocampus whereas only ADP hydrolysis was increased in cerebral cortex. In addition, for *in vitro* treatment, fluoxetine and nortriptyline inhibited the NTPDase activities in both structures tested. Furthermore, antidepressant drugs promoted changes in the transcript levels for NTPDase1, NTPDase2, NTPDase3, and 5′-nucleotidase.

Since these enzymes contribute to maintenance of physiological effects of extracellular ATP, ADP, AMP, and adenosine, the influence of the enzymatic cascade involved in the control of these nucleotides and nucleosides have been proposed in several pathophysiological situations (Agteresch et al., 1999). Chronic treatment with antidepressant drugs was able to alter the nucleotide hydrolysis in cerebral cortex whereas only ATP hydrolysis was inhibited in hippocampus. This effect shows a modulatory role of fluoxetine and nortriptyline on nucleotidase pathway in cerebral cortex, suggesting that the increase in the ATP, ADP and AMP hydrolysis could induce an increase in the levels of extracellular adenosine. However, fluoxetine and nortryptiline decrease ATP hydrolysis in hippocampus, which could induce an increase of ATP levels and a delayed production of adenosine. Okada et al. (1999) clearly showed that hippocampal serotonergic neurotransmission is modulated by hippocampal adenosine receptor subtypes. Tricyclic antidepressants are potent inhibitors of neuronal uptake of adenosine, which may raise the endogenous adenosine levels (Phillis and Wu, 1982; Phillis, 1984). Moreover, a dysregulation of the adenosine A_{2A} receptor may be present in depression, since a blunted intracellular calcium response to A_{2A} receptor stimulation is present in platelets in patients with depression (Berk et al., 2001). Recent findings have demonstrated that adenosine A_{2A} receptor antagonists produce an antidepressant-like effect in two models predictive of clinical antidepressant activity, the forced swimming test and the tail suspension test (El Yacoubi et al., 2001). Studies have shown that adenosine administration produces an antidepressant-like effect in the forced swimming test (FST) and in the tail suspension test, mediated through an interaction with A_1 and A_{2A} receptors (Kaster et al., 2004). Therefore, changes in the ecto-nucleotidase pathway induced by antidepressant drugs could modulate the adenosine levels and, consequently, the neuromodulation promoted by this nucleoside in depressive patients treated with these drugs.

In order to verify the direct effect of antidepressant drugs on ecto-nucleotidase activities, *in vitro* assays were performed in synaptosomes from hippocampus and cerebral cortex of rats. All drugs promoted significant changes on NTPDase activities after *in vitro* exposure. Previous studies demonstrated that

imipramine and fluoxetine decreased Na^+, K^+-ATPase activity in synaptic plasma membranes from the cerebral cortex of rats in a dose-dependent manner (Zanatta et al., 2001). Moreover, our laboratory has shown that NTPDase, but not ecto-5′-nucleotidase activities from cerebral cortex and hippocampus are decreased by the antidepressants sertraline and clomipramine after *in vitro* exposure (Pedrazza et al., 2007). Barcellos et al. (1998) have demonstrated that imipramine, desipramine and amitriptyline *in vitro* decreased ATP and ADP hydrolysis in synaptosomes from cerebral cortex of rats. It has been suggested that changes in membrane bilayer environment promoted by the interaction with antidepressant may be able to promote the inhibitory effect observed on NTPDase activity. The different effects promoted by antidepressant drugs on NTPDase and ecto-5′-nucleotidase activities can be related to the differences in membrane anchorage of these enzymes.

The kinetic effect promoted by antidepressant drugs could be a consequence of transcriptional control and/or post-translational mechanisms. For acute treatment, nortriptyline promoted an increase of ADP hydrolysis and a simultaneous increase of NTPDases transcript levels. However, some changes on nucleotide hydrolysis promoted by chronic treatment with fluoxetine are not in accordance with the changes observed in the transcript levels for NTPDases and ecto-5′-nucleotidase. The transcription machinery is continuously controlled by a complex signaling system, creating a set of signals able to adjust gene expression profile of the cell. This signal transduction can be exerted by proteins, products of enzyme reactions or even toxins able to regulate transcription factors (Krishna et al., 2006). The phenomena known as positive feedback loop (Pomerening et al., 2003, 2005), which is situated at the interface of genetic and metabolic networks, could explain the concomitant decrease of ATP hydrolysis and the increase of NTPDase1, NTPDase2, and NTPDase3 mRNA levels after chronic fluoxetine treatment.

In summary, we have shown that fluoxetine and nortriptyline can affect the ecto-nucleotidase pathway, suggesting that the extracellular adenosine levels could be modulated by these drugs.

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