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Influence of antidepressant drugs on Ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs) from salivary glands of rats

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ABSTRACT

Xerostomia is commonly caused by antidepressant drugs and ATP can influence the saliva production. Adenosine is the product of extracellular hydrolysis of adenine nucleotides in submandibular gland cells, which occurs by the action of ectonucleotidases. In this study, we have evaluated the effect of three different antidepressants in ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP1–3) activities in cultured cells from salivary glands. Rats received imipramine (10 mg/ml), fluoxetine (20 mg/ml) or moclobemide (30 mg/ml) by oral gavage. The drugs were administered once a day for 14 days. Our results have shown that the hydrolysis of *p*-nitrophenyl-5'-thymidine monophosphate increased in all treatments. These effects were not consequence of transcriptional control of E-NPP1–3 genes. The results reported here can highlight the importance of ectonucleotidases in the most common side effect caused by antidepressant therapy.

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1. Introduction

Several studies have discussed the role of adenosine triphosphate (ATP) as an extracellular mediator and neurotransmitter in various systems, including salivary glands.^{1,2} ATP and other extracellular nucleotides influence epithelial cell functions via P2 receptors.³ In the salivary glands, ATP can regulate important secretory processes by two distinct receptor families: P2X and P2Y.^{4,5} Four ATP receptors have been identified in the ductal and acinar cells: P2Y₁, P2Y₂, P2X₄, and P2X₇.^{6,7} The inactivation of ATP signalling is promoted by a cascade of cell-surface-bound enzymes constituted by ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs), alkaline phosphatases, and ecto-5'-nucleotidase (EC 3.1.3.5).¹

sphohydrolases (E-NTPDases), ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs), alkaline phosphatases, and ecto-5'-nucleotidase (EC 3.1.3.5).¹

The family of E-NPPs consists of seven structurally related enzymes that are located at the cell surface, either expressed as transmembrane proteins or as secreted enzymes.⁸ Only NPP1–3, which have a common ancestor, have been implicated in the hydrolysis of nucleotides^{9–12} whereas NPP6–7 are only known to hydrolyse phosphodiester bonds in lysophospholipids or other choline phosphodiester.¹²

Current evidence suggests that E-NPPs have multiple physiological roles, including nucleotide recycling, modula-

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tion of purinergic receptor signalling, regulation of extracellular pyrophosphate levels, stimulation of cell motility, activity of ecto-protein kinases and probably regulation of insulin receptor.¹³ These families of enzymes reveal not only overlapping tissue distributions, but also overlapping substrate specificities and functions.¹

Various types of enzymes mediate the saliva production and we have described in a previous study the presence of an E-NTPDase, an ecto-5'-nucleotidase¹⁴ and an E-NPP¹⁵ in acinar cells. NTPDase1 showed weak immunoreactivity whereas NTPDase2 activity was slightly higher in plasma membranes of nerve terminals and membranes of nerve fibres in salivary glands.²

Pharmacological therapy is the most common cause of reduced salivation and the most frequently drugs implicated in dry mouth are the tricyclic antidepressants, antipsychotics, atropine, beta-blockers, and antihistamines.¹⁶ Dry mouth is a common side effect caused by antidepressant therapy.¹⁷ The main function of antidepressants is to increase the extracellular neurotransmitter concentrations, inhibiting the metabolism and reuptake.¹⁸ Antidepressants include monoamine oxidase inhibitors, tricyclic compounds, selective serotonin, and norepinephrine reuptake inhibitors as well as some atypical drugs.¹⁹

Selective serotonin reuptake inhibitors (SSRIs) specifically prevent the reuptake of serotonin (thereby increasing the level of serotonin in synapses of the brain) whereas earlier monoamine oxidase inhibitors (MAOIs) block the degradation of neurotransmitters by enzymes. Tricyclic antidepressants (TCAs) prevent the reuptake of various neurotransmitters, including serotonin, norepinephrine and dopamine.¹⁷ The SSRIs, such as fluoxetine, tend to have fewer side effects than other antidepressants. Some of the side effects that can be caused by SSRIs include dry mouth, nausea, nervousness, insomnia, sexual problems, and headache. Imipramine, a tricyclic antidepressant, and moclobemide have also dry mouth as a common side effect.^{20,21} Several studies have shown a relationship between antidepressants and reduction of salivary flow, but the mechanisms involved in this process remain unclear.^{17,22-24} In this study we have evaluated the activities and expression of ecto-pyrophosphatase/phosphodiesterases from salivary glands of rats submitted to antidepressant therapy.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM) was purchased from Grand Island Biological Company (Grand Island, NY, USA). Collagenase I-S, nucleotides, Hepes and EDTA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Foetal bovine serum (FBS) was purchased from Cultilab Ltda (São Paulo, SP, Brazil). All the other chemical reagents were of the highest available quality.

2.2. Animals

Male Wistar rats ($n = 52/45$ -day-old rats) were maintained in groups of four animals per cage. The animals were kept at

room temperature of 22 ± 2 °C and light cycle from 7 a.m. to 7 p.m. receiving rat chow (Nutrilab1, Brazil) and water *ad libitum*. 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).

2.3. Antidepressant therapy

The animals were divided in four groups (with 13 animals for each group): saline, imipramine (IMI; Tofranil1, Biogalênica) 10 mg/ml, fluoxetine (FLU; Prozac, Eli Lilly) 20 mg/ml or moclobemide (MOC; Aurorix, Roche) 30 mg/ml, suspended in saline. All solutions were administered by oral gavage, 1 ml/kg. Antidepressant doses were chosen according to Kopittke et al.²⁴ Animals received the antidepressant treatment once a day for 14 days and subsequently (15 days after the beginning of therapy) they were euthanised for culture procedure.

2.4. Cell isolation and culture

Submandibular gland cell (SGC) clusters were obtained from 45-day-old rats. The animals were euthanised and the submandibular glands were removed quickly, trimmed of fat and fascia tissues and minced in a small volume of Hank's Buffer Saline Solution (HBSS), pH 7.4. The minced salivary glands were dispersed in HBSS supplemented with collagenase I-S (1 mg/ml). The gland cells were dissociated by pipetting 10 times every 20 min with a Pasteur pipette for 2 h. The collagenase was removed by centrifugation at $700 \times g$ (5 min) and the cell clusters were then washed with HBSS and centrifuged twice at $40 \times g$ (5 min) to remove the lysed cells and contaminants (red and endothelial cells).

Clusters with 4-5 cells (as observed by phase-contrast microscopy) were maintained in a water-saturated atmosphere with 95% air and 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) with 5% foetal bovine serum (FBS), pH 7.4 for 24 h.^{25,26}

2.5. Assay of ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) activity

After 24 h in culture, the SGC were washed and centrifuged three times at $1000 \times g$ for 3 min with a medium containing 135 mM NaCl, 5.0 mM KCl, 10 mM glucose and 10 mM Hepes (pH 7.4). The cell clusters were filtered through a nylon filter (100 mesh) to homogenise the cluster size. The artificial substrate for E-NPPs, *p*-nitrophenyl 5'-thymidine monophosphate (*p*-Nph-5'-TMP), was used as a substrate marker to evaluate the enzymatic activity, producing *p*-nitrophenol.²⁷ The reaction medium, containing 135 mM NaCl, 5.0 mM KCl, 10 mM glucose and 50 mM Tris-HCl buffer (pH 8.9), was preincubated with aliquots of 20 μ l of cell suspension (approximately 10-15 μ g of protein per tube) at 37 °C for 10 min in a final volume of 200 μ l. The enzyme reaction was started by the addition of *p*-Nph-5'-TMP to a final concentration of 0.5 mM. After 6 min of incubation, the reaction was stopped by the addition of 200 μ l 0.2N NaOH and the samples were chilled on ice. Incubation times and protein concentration were chosen to ensure the

linearity of the reaction. In order to correct non-enzymatic hydrolysis, we performed controls by adding the SGC after the reaction was stopped with 0.2N NaOH. All assays were carried out in triplicate. The amount of *p*-nitrophenol released from the substrate was measured at 400 nm using a molar extinction coefficient of $18.8 \times 10^{-3}/\text{M}/\text{cm}$. Enzyme activities were generally expressed as nmol *p*-nitrophenol released per minute per milligram of protein.

2.6. Protein determination

Protein was measured by the Comassie Blue method, according to Bradford,²⁸ using bovine serum albumin as standard.

2.7. Statistical analysis

The data obtained are expressed as means \pm S.D. of at least five experiments. The results of antidepressant treatments were analysed by Student's *t*-test or one-way ANOVA followed by Tukey test as *post hoc*. Values of $P < 0.05$ were considered significant.

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

Rat DNA sequences encoding to E-NPP1 (NM_022587.1), E-NPP2 splice isoforms (Q64610), and E-NPP3 (NM_178106) was retrieved from GenBank database and aligned using ClustalX program. Regions with low scores of similarity among sequences were used for searching specific primers, which were designed using the program Oligos 9.6. NPP2 splice isoforms were identified using primers localised upstream and downstream of the splice junction resulting in different PCR products. NPP2 isoform 1 PCR product is 587 bp long (with the intron) whereas NPP2 isoform 2 PCR product is 512 bp long (without the intron) (Table 1). In order to confirm the primers specificity, each primer was blasted against rat genome and it was able to recognise only its specific target sequence. Thus, the strategy adopted to construct the primers did not allow cross-amplification.

The analysis of the expression of E-NPP1, E-NPP2, and E-NPP3 was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Fourteen days after treatment with different antidepressants, the

salivary glands of rats were dissected and immediately frozen with liquid nitrogen for storage in -80°C freezer. The total RNA extraction, free of protein and DNA contamination, were obtained using TRIzol[®] Reagent (Invitrogen) in accordance with the manufacture instructions. The cDNA species were synthesised with SuperScript First-Strand Synthesis System for RT-PCR from 1 μg of total RNA and oligo (dT) primer in accordance with the suppliers. RT reactions were performed for 50 min at 50°C . cDNA (1 μl) was used as a template for PCR with specific primers for E-NPP1, E-NPP2, and E-NPP3. β -Actin PCR was performed as a control for cDNA synthesis. PCR conditions were optimised in order to determine the number of cycles that would allow product detection within the linear phase of mRNA transcripts amplification. PCR reactions were performed (total 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 PCRs were as follow: Initial 1 min denaturation step at 94°C , 1 min at 94°C , 1 min annealing step (NPP1: 60°C ; NPP2: 67°C ; NPP3: 65°C ; β -actin: 58.5°C), 1 min extension step at 72°C for 35 cycles and a final 10 min extension at 72°C . PCR products were submitted to electrophoresis using a 1% agarose gel. 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 three times using RNA isolated from independent extractions. The expression analysis was performed in replicate and representative findings were shown. The normalised expression levels of E-NPP1–3 genes were expressed as mean \pm S.E.M. and statistically compared by Student's *t*-test. P values ≤ 0.05 were considered significant.

3. Results

In order to verify if male Wistar rats undergo biochemical changes induced by antidepressant therapy, we have studied the influence of this treatment on *p*-Nph-5'-TMP hydrolysis from cells of SGC. Fig. 1 demonstrates the *p*-Nph-5'-TMP nucleotide hydrolysis in cells cultured of salivary submandibular glands for three different antidepressants. The *p*-Nph-5'-TMP hydrolysis (Fig. 1) was significantly enhanced for all treatments ($P < 0.05$). The treatment of IMI, FLU and MOC

Table 1 – PCR primer design.

NPP	GenBank accession number	Primers sequences	PCR product
NPP1 ^a	NP_445987	F 5'-GAATTCTTGAGTGGCTACAGCTTCCTA-3' R 5'-CTCTAGAAATGCTGGGTTTGGCTCCCGGCA-3'	410 bp
NPP2 (1) ^b	Q64610	F 5'-CCATGCCAGACGAAGTCAGCCGACC-3'	587 bp
NPP2 (2)		R 5'-CCAAACACGTTTGAAGCGGGGTAC-3'	512 bp
NPP3	AAH97326	F 5'-GAGAAGACAAATTTGCCATTTGGGAGG-3' R 5'-TCTCATTATTCCTTTGATTGCGGGAG-3'	301 bp
β -Actin	NP_112406	F 5'-TATGCCAACACAGTGTCTGG-3' R 5'-TACTCCTGCTTCTGATCCACAT-3'	210 bp

^a Primer sequences were obtained from Vollmayer et al.⁴⁸

^b Splice isoforms of NPP2.

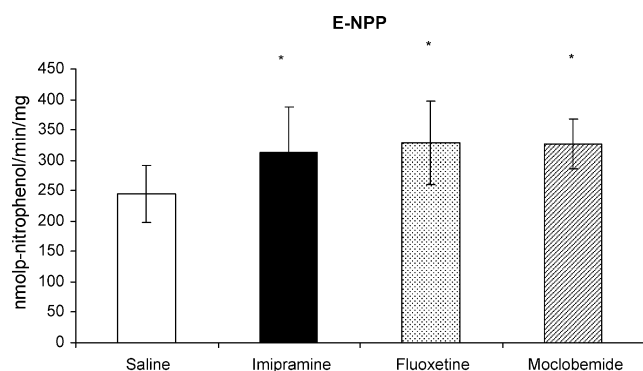


Fig. 1 – Effect of antidepressant drugs on E-NPP activity in submandibular gland cells. Bars represent mean \pm S.D. for five different experiments. *Represents significant statistical difference by one-way ANOVA followed by Tukey test as post hoc.

induced an increase of 29% (314 ± 72 nmol Pi min^{-1} mg^{-1}), 35% (329 ± 68 nmol Pi min^{-1} mg^{-1}) and 34% (326 ± 41 nmol Pi min^{-1} mg^{-1}) when compared to the control (244 ± 47 nmol Pi min^{-1} mg^{-1}).

The upregulation of E-NPPs activities could be consequence of transcriptional control and/or post-translational modifications. We evaluated E-NPP1–3 transcripts in SGC cells after antidepressant treatments. Although the mRNAs of both NPP2 splice isoforms could be easily identified in different brain structures with the strategy adopted (data not shown), it was not possible to detect NPP2 isoform 1 (587 bp) in SGC cells. The transcriptional control of E-NPP1–3 genes was not contributing to the upregulation of E-NPP1–3 observed after antidepressant treatments since their transcripts were not increased. Interestingly, the NPP1 mRNA was slightly decreased after FLU treatment (Fig. 2). These results suggest that the increased activity observed after IMI, FLU and MOC treatments in SGC cells could be consequence of changes in phosphorylation state. In order to verify this hypothesis, the E-NPP1 (NP_445987), E-NPP2 splice isoform 2 (Q64610) and E-NPP3 (AAH97326) amino acid sequences were analysed in NetPhosk, a kinase-specific prediction of protein phosphorylation sites tool (<http://www.cbs.dtu.dk>). The results obtained from E-NPP1 sequence indicated the residues Ser271 and Thr713 as potential Protein Kinase C phosphorylation sites. The same approach with E-NPP2 splice isoform 2 sequence indicated the residues Ser176, Thr412, Thr493 and Thr866 for Protein Kinase C and Ser396 for Protein Kinase B phosphorylation sites. The

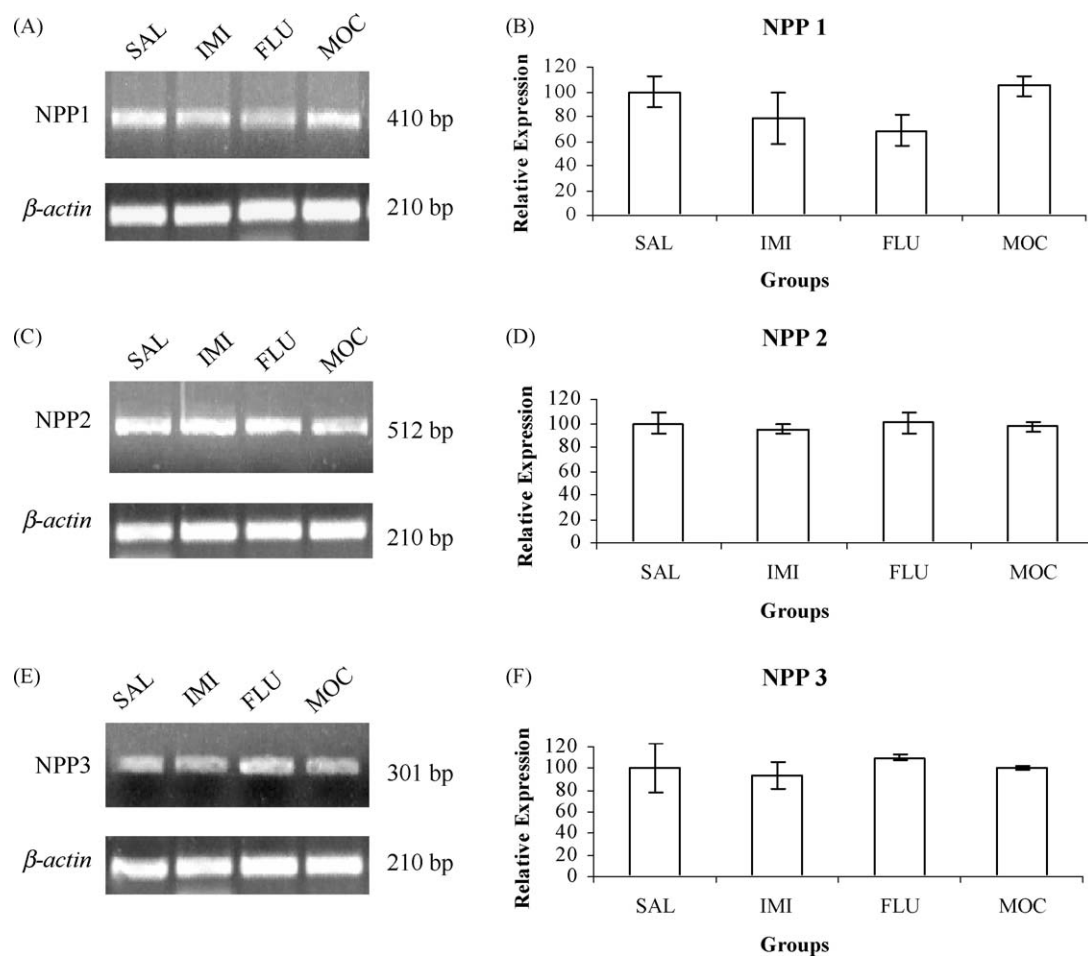


Fig. 2 – Relative gene expression patterns after imipramine (IMI), fluoxetine (FLU), and moclobemide (MOC) chronic treatment for NPP1 (A and B), NPP2 (C and D), NPP3 (E and F), and β -actin in salivary glands of rats. Three independent experiments were performed, with entirely consistent results.

residues Ser17, Thr647 and Ser786 as potential Protein Kinase C phosphorylation were recognised in E-NPP3 sequence. The residues were identified with high prediction scores in E-NPP1-3 sequences. The potential phosphorylation sites identified suggest that increase of E-NPP1-3 activities observed in SGC cells induced by antidepressant treatments could involve post-translational regulation by phosphorylation.

4. Discussion

The results of the present study demonstrated that IMI, FLU and MOC are able to alter the activities whereas the E-NPP1-3 genes expression was not changed in salivary gland.

Antidepressants have been frequently studied in the central nervous system, but in other organs their effects remain unclear. A common side effect caused by antidepressant treatment is dry mouth.¹⁷ Systemic diseases, radiation therapy, prescription and non-prescription drugs are important causes of salivary gland disturbances.²⁹ Saliva components are critical in maintaining oral health and supporting other oral functions. It is well known that several neurotransmitters, such as acetylcholine, substance P, vasoactive intestinal polypeptide, and ATP are co-released in salivary glands.^{30,31} Besides the ACh and ATP co-release following parasympathetic stimulation, extracellular ATP also functions as a regulator of salivary gland function, probably by the action in four distinct subtypes of P2 receptors identified in the ductal and acinar cells: P2Y₁, P2Y₂, P2X₄, and P2X₇.⁶

Degradation of ATP and other nucleotides can occur through a cascade of cell-surface-bound enzymes named ectonucleotidases, including E-NTPDase family, E-NPP family and ecto-5'-nucleotidase.³ Extracellular hydrolysis of ATP to adenosine promoted by ectonucleotidases has been reported for several cell types,³²⁻³⁴ including salivary glands.^{14,15}

Tricyclic antidepressants have shown common side effects, that can occur indirectly (central nervous system) and/or directly (salivary glands).^{22,35} In this study, we observed an increase in enzyme activity when *p*-Nph-5'-TMP was used as substrate in rats submitted to IMI treatment. However, there was no significant changes on the expression of E-NPPs. Scarpace et al.^{36,37} observed that the tricyclic antidepressant desipramine altered signal transduction in rat parotid and submandibular glands. It has been observed that salivary flow was significantly decreased when rats were submitted to IMI treatment.²⁴ In addition, it has been demonstrated that IMI alters cortical membrane fluidity for *in vitro* and *in vivo* studies, and that chronic IMI treatment affects brain membrane architecture and enzyme activities, such as Na⁺,K⁺-ATPase.³⁸ Our findings have also shown that fluoxetine and moclobemide treatment induced an increase on *p*-Nph-5'-TMP hydrolysis in acinar cells. Similar results were observed in evaluating the effect of fluoxetine on Na⁺,K⁺-ATPase activity.³⁸ It has been suggested that activation of purinergic receptors by ATP is a more effective stimulus than activation of muscarinic receptors in terms of elevating [Ca²⁺]_i. Thus, ATP can effectively regulate secretory processes in salivary glands.^{5,39} Therefore, considering that ATP and adenosine are important signalling molecules, it is possible to suggest that the control of ATP levels by ectonucleotidases during antidepressant

therapy can be involved in the regulation of salivary flow. One possible hypothesis could involve the activation of ectonucleotidases, leading to an enhanced degradation of extracellular ATP and its metabolites ADP and AMP. For this reason, the stimulus for Ca²⁺ entrance in the acinar cells is diminished, promoting a reduction in the salivary flow.

The alterations observed in ectonucleotidases could be a consequence of transcriptional control and/or post-translational mechanisms. The antidepressant drugs have been studied in the central nervous system and it is known that chronic treatment by various classes of these drugs may result in a common, final pathway of changes in gene expression in the brain.⁴⁰ We have studied the relative expression of E-NPP 1, 2, 3 from salivary glands of antidepressant-treated rats, since this pharmacological therapy seems to affect the kinetic of the ectonucleotidases. The results have shown that animals submitted to antidepressant treatments did not present significant changes in the relative expression for E-NPP1, E-NPP2 and E-NPP3 in SGC. However, a slight decrease of E-NPP1 expression was observed for the fluoxetine treatment (Fig. 2A and B).

The plausible explanation for the changes in the enzyme activities after antidepressant treatment may involve post-translational events. According to analysis performed in NetPhosK, E-NPP1-3 sequences showed high prediction scores for PKC phosphorylation sites. In fact, accumulating evidence suggested that signal transduction cascade including protein phosphorylation is implicated in the neurochemical action of antidepressant agents.^{41,42} In addition, chronic antidepressant therapy has been shown to induce changes in the function of protein kinase C, cyclic AMP-dependent protein kinase, and calcium/calmodulin-dependent protein kinase in the brain.⁴³ In addition, there is some evidence that the expression of protein kinase C (PKC) is upregulated by fluoxetine.⁴⁴ The activity of PKA can be altered in rat cerebral cortex following 2 or 3 weeks of treatment with different antidepressant agents such as tricyclics, monoamino oxidase inhibitors and selective serotonin reuptake inhibitors.⁴⁵⁻⁴⁷ Despite all data cited above are related to the rat brain, it could lead us to the hypothesis that phosphorylation may exert a modulation on these enzyme activities in the salivary glands of antidepressant treated rats.

The effects of nucleotides on second messenger levels, ion fluxes, and protein secretion in salivary cells suggest important roles for P2 receptors in modulating the production and composition of saliva.⁷ Recent studies have demonstrated that ATP-sensitive P2X(7) receptor regulates fluid secretion in the mouse submandibular gland.⁴⁸ ATP and other nucleotides can promote an increase in membrane conductance, and this fact contributes to the production of saliva. In our study, nucleotide hydrolysis by E-NPPs was enhanced; this fact can modify the membrane conductance and may be influencing the common side effects of antidepressant therapy, including the xerostomia. Further studies are necessary to understand the different extracellular signalling pathways involved in salivary secretion.

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