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# REGULATION OF SEROTONIN TRANSPORTER ACTIVITY BY ADENOSINE IN INTESTINAL EPITHELIAL CELLS

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## ABSTRACT

Serotonin plays a critical role in the regulation of intestinal physiology. The serotonin transporter (SERT) expressed in the intestinal epithelium determines 5-HT availability and activity. The serotonergic system and SERT activity have been described as being altered in chronic intestinal pathologies such as inflammatory diseases. Adenosine has also been shown to be involved in a variety of intestinal functions and to play a central role in the regulation of inflammatory responses of injured tissue. Since the modulation of SERT by adenosine in the intestine remains unknown, the aim of the present work was to study the effect of adenosine on SERT activity and expression and to determine the molecular mechanism involved. The study has been carried out using human enterocyte-like Caco-2 cells which endogenously express SERT. The results show that adenosine diminishes SERT activity in both the apical and basal membranes by acting in the intrinsic molecule with no alteration of either SERT mRNA or protein levels. The effect of adenosine appears to be mediated by A<sub>2</sub> receptors and activation of the cAMP/PKA signalling pathway. Moreover, the adenosine effect did not seem to involve the activation of AMP activated protein kinase. Adenosine effects are reached at high concentrations, which suggests that adenosine modulation of SERT may be significant under conditions of inflammation and tissue injury.

Keywords: serotonin transporter, adenosine, Caco-2 cells, regulation

## 1. Introduction

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4 Serotonin (5-hydroxytryptamine, 5-HT) is a neuromodulator that is mainly produced in the  
5 gastrointestinal (GI) tract, where it plays a critical role in the regulation of several  
6 physiological processes, mainly by acting as a paracrine signalling molecule [1-4]. The  
7 serotonergic system in the GI tract includes the serotonin transporter (SERT). This  
8 transporter is responsible for 5-HT uptake to enable its clearance from the extracellular  
9 milieu, thus determining 5-HT availability. SERT expression and activity have been described  
10 in the epithelial cells of the GI mucosa [5-8]. Changes in SERT activity can lead to alteration  
11 in the GI function, as evidenced by the GI side-effects associated with SERT selective  
12 inhibitors [9] that are widely used in disturbances in which serotonergic activity is affected.

20 Numerous studies have concluded that the alteration of the GI serotonergic system may  
21 be involved in the origin and/or prevalence of chronic GI pathologies such as Irritable Bowel  
22 Syndrome (IBS) and Inflammatory Bowel Disease (IBD) [10, 11]. Recent results have  
23 demonstrated that SERT expression is downregulated and SERT activity is impaired in  
24 intestinal epithelial cells treated with pro-inflammatory factors [12, 13], in animal models and  
25 human forms of intestinal inflammation [10, 14-16].

31 Adenosine is an endogenous purine nucleoside that plays a key role in nucleic acid,  
32 energy and protein metabolism. It has also been shown to be a powerful mediator of cellular  
33 responses. In the GI tract, adenosine has been demonstrated to modulate neurotransmission  
34 [17] and to be involved in a variety of intestinal functions [18]. Although adenosine is present  
35 at low concentrations in the extracellular space, adenosine availability increases under  
36 conditions of cellular stress, hypoxia and inflammation [19]. However, the role of adenosine  
37 in inflammatory processes is contradictory. Although increasing adenosine concentrations  
38 might be useful in the treatment of some forms of inflammation (due to its anti-inflammatory  
39 properties) [20], in certain disease states associated with long-lasting high endogenous levels  
40 of adenosine (such as sepsis and colitis), adenosine may act as a proinflammatory mediator  
41 [21].

51 Inflammation involves interdependent responses in a given tissue; including infiltration of  
52 leukocytes and macrophages, release of proinflammatory mediators and changes in the  
53 metabolism of resident cells. As indicated above, it is well established that intestinal  
54 inflammation coincides with decreased SERT activity and inflammatory mediators inhibit  
55 SERT function and expression in epithelial cells [12, 13]. Adenosine also plays a role in  
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intestinal inflammatory response although its role as a mediator that may evoke SERT alteration remains unclear. The aim of this work was to assess whether adenosine affects SERT activity and expression in human enterocyte-like Caco-2 cells and thus to elucidate possible mediators of SERT alteration in intestinal inflammation. The Caco-2 cell line has been used to carry out this study as it has been described as constitutively expressing SERT [6, 7] and results obtained in our laboratory have shown that these cells are an excellent model for the study of intestinal SERT regulation [22, 23].

The results obtained indicate that adenosine diminishes SERT activity by mainly affecting the transporter molecule itself, in the membrane. This effect appears to involve A<sub>2</sub> receptor activation and the cAMP signalling pathway. Since adenosine effects on SERT are significant at high concentration (similar to the adenosine availability in tissue injury and inflammation), these results may contribute to a better understanding of the involvement of adenosine and the serotonergic system in intestinal physiology and pathology.

## 2. Material and Methods

### 2.1. Materials

All generic reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Roche Applied Sciences (Sant Cugat del Valles, Barcelona, Spain). Reagents for proteins and western blotting were bought from Bio-Rad (Hercules, CA, USA). Cell culture media and supplements, M-MLV reverse transcriptase and Taq DNA polymerase were supplied by Invitrogen (Invitrogen, Carlsbad, CA, USA). Tissue culture plastic flasks and culture plates by Sarstedt, (Nümbrecht, Germany) and 12 well permeable polyester (PET) membranes were from Millipore (Billerica, MA, USA). Serotonin (5-HT), adenosine, dibutyl-cAMP (d-cAMP), (9S,10S,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester (KT 5720), 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), 2-phenylaminoadenosine (CV-1808), 8-[4-[(4-cyanophenyl) carbamoylmethyl] oxy]phenyl]-1,3-di(n-propyl)xanthine hydrate (MRS 1754), erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA), and specific primers were also from Sigma-Aldrich. [<sup>3</sup>H]-5-HT (specific activity 20 Ci mmol<sup>-1</sup>) was obtained from Perkin-Elmer (Boston, MA, USA) and Acrylamide came from AppliChem (Darmstadt, Germany). Mouse monoclonal antibody anti-

1 human SERT was supplied by Abcam (Cambridge, UK). The rabbit polyclonal anti-actin  
2 antibody and secondary antibodies coupled to horseradish peroxidase were from Santa Cruz  
3 Biotechnology (Santa Cruz, CA, USA).  
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## 7 **2.2. Cell culture**

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10 Caco-2/TC7 [24] cells were kindly provided by Dr. Edith Brot-Laroche (INSERM, VMR S  
11 872). The cells were cultured at 37 °C in an atmosphere of 5% CO<sub>2</sub> and maintained in high  
12 glucose DMEM supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml  
13 streptomycin, 1% non-essential amino acids and 20% foetal bovine serum (FBS). The cells  
14 were passaged enzymatically (0.25% trypsin-1 mM EDTA) and sub-cultured in 25 or 75 cm<sup>2</sup>  
15 plastic culture flasks. The medium was changed 48 h after seeding and daily thereafter. The  
16 cells were always used between passages 19 and 35. For 5-HT uptake assays, cells were  
17 seeded in 24-well plates at a density of 4x10<sup>4</sup> cells/well and uptake measurements were  
18 carried out 14 days after seeding (9 days after confluence). Previous results have shown that  
19 Caco-2 cells express SERT and its activity reaches a plateau on the fifth day after confluence  
20 [6]. Adenosine and the different modifiers were added to the culture medium at different  
21 concentrations and periods, depending on the determination. In the experiments, the cell  
22 medium was free of FBS 24 h before using the cells. This condition did not affect either the  
23 functional differentiation status or the SERT activity of Caco-2 cells (data not shown).  
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## 38 **2.3. 5-HT uptake studies**

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42 Uptake measurements were performed on cells attached to 24-well plates, either under control  
43 or after different experimental conditions. The transport medium composition in mM was as  
44 follows: 137 NaCl, 4.7 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, 2.5 CaCl<sub>2</sub>, 10 HEPES pH 7.4, 4  
45 Glutamine, 0.1% BSA, and both 5-HT 2x10<sup>-7</sup> M and [<sup>3</sup>H]-5-HT. Before measuring uptake,  
46 cells were pre-incubated at 37 °C in an atmosphere of 5% CO<sub>2</sub> with substrate-free transport  
47 medium for 30 min. The cells were immediately washed with substrate-free transport medium  
48 at 37 °C and then incubated with transport medium at 37 °C for 6 min. Transport was stopped  
49 by removing the transport medium and washing the cells twice with ice-cold substrate-free  
50 transport medium containing 2x10<sup>-5</sup> M 5-HT. The cells were solubilised in 0.1 M NaOH and  
51 samples were taken for radioactivity counting. Protein was measured using the Bradford  
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1 method [25], with BSA as standard. Results were calculated in pmol 5-HT/mg protein and  
2 were expressed as a % of control value (100%).

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4 In experiments in which 5-HT fluxes were measured, Caco-2 cells were seeded in 12 well  
5 permeable polyester (PET) membranes with porous size 0.4  $\mu\text{m}$  and 1  $\text{cm}^2$  growth area. These  
6 inserts establish apical (A) and basal (B) compartments. Bidirectional fluxes, A to B and B to  
7 A, were measured at intervals of 10 min after adding  $10^{-7}$  M 5-HT plus [ $^3\text{H}$ ]-5-HT either to  
8 the apical or basal compartment, respectively. The results were calculated in pmol 5-HT/10  
9 min and were expressed as a % of control (100%). At the end of the experiment, intracellular  
10 5-HT accumulation was determined by cell disruption in 0.1 M NaOH overnight and samples  
11 were taken for radioactivity counting and protein measurement. The results were calculated as  
12 pmol/mg protein, and were expressed as a % of control (100%). Cell monolayer integrity and  
13 confluence were checked by measuring transepithelial resistance (TER) with an Epithelial  
14 Voltohmmeter (Millicell Electrical resistance system, Millipore) before the beginning of each  
15 experiment.  
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#### 27 **2.4. RNA extraction, Reverse Transcription and Semi-quantitative-PCR analysis**

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30 RNA extractions were carried out with the RNeasy mini kit (Qiagen, Hilden, Germany)  
31 following the manufacturer's instructions, as previously described [22]. In short, total RNA  
32 was extracted from cells cultured in 25  $\text{cm}^2$  flasks, 14 days after seeding and under different  
33 experimental conditions. The extracted RNA (1  $\mu\text{g}$ ) was used as a template for first-strand  
34 cDNA synthesis using Oligo(dT) primers and a modified M-MLV reverse transcriptase.  
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36 Negative amplification control was performed in the absence of reverse transcriptase. One  
37 tenth of the resultant cDNA was used for PCR amplification of human adenosine receptors,  
38 human adenosine transporters and human SERT with human  $\beta$ -actin as an internal control.  
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40 The sequence of specific primers used and amplified fragment sizes are indicated in Table 1.  
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42 In the case of adenosine receptors, PCR amplification was carried out after 35 cycles under  
43 the following conditions: 94  $^{\circ}\text{C}$  for 1 min; 52  $^{\circ}\text{C}$  ( $A_3$ ) or 55  $^{\circ}\text{C}$  ( $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ ) for 30 s; 72  $^{\circ}\text{C}$   
44 for 1 min. In the case of adenosine transporters, PCR amplification was carried out after 40  
45 cycles and conditions were as follows: 94  $^{\circ}\text{C}$  for 1 min; 55  $^{\circ}\text{C}$  (ENT1, ENT2) or 50  $^{\circ}\text{C}$   
46 (CNT1, CNT2) or 52  $^{\circ}\text{C}$  (CNT3) for 1 min; and 72  $^{\circ}\text{C}$  for 3 min. In the case of SERT PCR  
47 amplification, 27 cycles were carried out as follows: 94  $^{\circ}\text{C}$  for 30 s; 56  $^{\circ}\text{C}$  for 30 s; and 68  $^{\circ}\text{C}$   
48 for 2 min 30 s. PCR amplification of  $\beta$ -actin was obtained after 25 cycles under the following  
49 conditions: 94  $^{\circ}\text{C}$  for 30 s; 60  $^{\circ}\text{C}$  for 30 s; and 68  $^{\circ}\text{C}$  for 30 s. These SERT and  $\beta$ -actin PCR  
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1 conditions were determined after screening (twenty to forty cycles) and final PCR  
2 amplification conditions were chosen so that neither the SERT nor actin RNAs (cDNAs)  
3 analysed reached a plateau at the end of the amplification protocol. PCR products were  
4 electrophoresed on 1% agarose gel and visualized under UV light after ethidium bromide  
5 staining. The images from the gels were captured with Biodoc-It Imaging System (UVP Inc,  
6 Upland, CA, USA). SERT/ $\beta$ -actin ratio in densitometric units was calculated with the  
7 Quantity One Analysis Software (Bio-Rad).  
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### 13 **2.5. Preparation of the brush border purified fraction from Caco-2 cells and western** 14 **blotting**

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20 Caco-2 cells were cultured in 75 cm<sup>2</sup> flasks and were used 14 days after seeding. The cells  
21 were washed twice with PBS and immediately resuspended with cold Tris-manitol buffer  
22 (Tris 2 mM, Manitol 50 mM, pH 7.1) containing protease inhibitors and 0.02% sodium azide.  
23 The suspension was homogenized and the cells were disrupted by sonication (fifteen, 1-  
24 second bursts, 60W). One sample was taken from the lysate for total protein analysis and  
25 protein quantification. CaCl<sub>2</sub> (final concentration 20 mM) was added to the cell lysate and  
26 after standing for 10 min in ice, the mixture was centrifuged for 10 min at 950 x g. The  
27 supernatant was taken and centrifuged at 33500 x g for 30 min. Then the pellet (brush border  
28 purified fraction) was resuspended in phosphate buffer (KH<sub>2</sub>PO<sub>4</sub>/ K<sub>2</sub>HPO<sub>4</sub> 10 mM pH 6.8)  
29 and a sample was taken for protein analysis. Protein was measured using the Bradford  
30 method.  
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40 Brush border purified samples and cell lysate (total protein 60  $\mu$ g) from Caco-2 cells were  
41 electrophoresed in 9% SDS-PAGE gels, and then transferred to PVDF membranes (Millipore)  
42 by electroblotting. The membranes were blocked with 5% non-fat dried milk plus 1% BSA  
43 and probed with mouse monoclonal anti-human SERT 1:500. The primary antibody was  
44 detected using a secondary goat anti-mouse Ig coupled to horseradish peroxidase and the ECL  
45 Plus detection kit (GE Healthcare, Buckinghamshire, UK) and was visualized with X-ray  
46 films (Hyperfilm MP, GE Healthcare). The blots were reprobbed, after stripping, with a rabbit  
47 polyclonal anti-actin antibody to determine differences in the sample load. The SERT/ $\beta$ -actin  
48 protein ratio was calculated in units from the films with the Quantity One Analysis Software  
49 (Bio-Rad) and the results were expressed in densitometric units.  
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## 2.6. Statistical analysis

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3 All results are expressed as means  $\pm$  SEM. Statistical comparisons were performed by  
4 unpaired t-test, with a confidence interval of 95% ( $p < 0.05$ ). Kinetic study of the 5-HT  
5 transport values was performed by non-linear regression, fitting the results to an equation  
6 containing a saturable (Michaelis-Menten) plus a non-saturable (diffusion) component.  
7 Statistical analysis was carried out by the computer-assisted Prism GraphPad Program (Prism  
8 version 4.0).  
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## 3. Results

### 3.1. Effect of adenosine on the 5-HT uptake

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24 The effect of adenosine on 5-HT uptake mediated by SERT was determined. Different  
25 adenosine concentrations were assayed and 5-HT uptake was measured after either acute (30  
26 min) or chronic (1 day) treatment with adenosine. The results are presented in Fig. 1A and  
27 show that adenosine diminishes 5-HT uptake. This effect was significant at adenosine  
28 concentration  $10^{-4}$  and  $10^{-3}$  M in acute treatment. However, in chronic treatment, adenosine  
29  $10^{-5}$  M showed a significant effect on 5-HT uptake. The adenosine effect appears to be  
30 dependent on the dose and the period of treatment.  
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37 Breakdown of extracellular adenosine by adenosine deaminase yields inosine, which has  
38 been described as being a potent immunomodulator [26]. In order to determine whether  
39 inosine was involved in adenosine effects, 5-HT uptake was measured in the presence of  
40 EHNA, a specific inhibitor of adenosine deaminase. The results showed that treatment with  
41 EHNA for 1 day did not affect the diminution of 5-HT uptake yielded by adenosine treatment  
42 (Fig. 1B).  
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### 3.2. Effect of adenosine treatment on 5-HT transepithelial flux

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53 5-HT fluxes were measured in Caco-2 cells treated with adenosine  $10^{-3}$  M for 1 day.  
54 Adenosine was added to the apical (A) or basal (B) side and apical to basal (A-B) or basal to  
55 apical (B-A) 5-HT fluxes were measured. The results showed that both A-B and B-A were  
56 significantly diminished by adenosine treatment (Fig. 2A) with the effect on A-B flux being  
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more significant. 5-HT cell accumulation was also measured under the above conditions and the results showed that both apical and basal adenosine significantly reduces 5-HT accumulation in the cells mediated by SERT from the apical or basal membrane (Fig. 2B). Transepithelial resistance (TER) was determined after treatment with adenosine and the results showed that TER was not affected by apical or basal adenosine treatment (data not shown).

### 3.3. Kinetic study of the adenosine effect on 5-HT uptake

The kinetic study was carried out in order to determine the effect of adenosine treatment on the kinetic constants  $V_{\max}$  and  $k_t$ , which inform the capacity and affinity of SERT respectively. 5-HT concentration ranged between  $5 \times 10^{-8}$  and  $5 \times 10^{-6}$  M. The results showed that the treatment of the cells for 1 day with adenosine  $10^{-3}$  M significantly diminished  $V_{\max}$  (in pmol 5-HT/mg protein; control =  $38.82 \pm 0.91$  and adenosine =  $24.48 \pm 0.62$ ). However,  $k_t$  was not affected by the treatment (in  $\mu\text{M}$ ; control =  $0.37 \pm 0.06$  and adenosine =  $0.36 \pm 0.06$ ).

### 3.4. Effect of adenosine on the SERT mRNA level and the SERT protein expression

To gain an in-depth knowledge of the molecular effects of adenosine, SERT mRNA and protein levels were measured in Caco-2 cells treated for 1 day with adenosine  $10^{-4}$  or  $10^{-3}$  M. The mRNA level was determined by semi-quantitative RT-PCR and the results showed that adenosine treatment did not alter the level of mRNA (Fig. 3). In addition, the adenosine treatment did not modify protein expression, either in the whole cell (cell lysate) or in the brush border membrane (apical membrane) measured by western blot (Fig. 4).

### 3.5. Signalling pathways involved in adenosine effect on SERT

To characterize the pathways which may mediate adenosine effects on SERT activity in Caco-2 cells, the expression of adenosine receptors and transporters was determined by RT-PCR. The results showed that Caco-2 cells express the adenosine receptors  $A_{2A}$  and  $A_{2B}$ , the concentrative nucleoside transporters CNT2, and CNT3 and the equilibrative nucleoside transporters ENT1 and ENT2 (Fig. 5). Therefore, both adenosine  $A_2$  receptors and intracellular adenosine carried by specific transporters may be involved in the effects on SERT activity.

1 The intracellular adenosine has been shown to activate AMP activated kinase (AMPK)  
2 [27], therefore, the role of AMPK on adenosine effect on SERT was assessed. Cells were  
3 treated for 1 day with AICAR, a known pharmacological activator of AMPK [27]. The results  
4 showed that AMPK activation reduced SERT activity; however, this effect was additive to the  
5 inhibition yielded by adenosine treatment (Fig. 6).  
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9 To determine the involvement of A<sub>2</sub> adenosine receptors, SERT activity was measured  
10 after treating the cells for 1 day with a selective A<sub>2</sub> receptor agonist (CV-1808) or with  
11 adenosine plus a selective A<sub>2B</sub> receptor antagonist (MRS 1754). The results showed that CV-  
12 1808 significantly reduced 5-HT uptake and the adenosine effect was reversed by MRS 1754  
13 (Fig. 7A). As the predominant signalling pathway of the adenosine receptors A<sub>2</sub> is through  
14 increased cAMP as well as PKA activation [28, 29], the mediation of cAMP in adenosine  
15 effects was assessed. The cells were treated with d-cAMP, cell-permeable cAMP analogue,  
16 and KT 5720, a specific PKA inhibitor. Results showed that the inhibition yielded by  
17 adenosine was reverted by KT 5720 and that KT 5720 alone did not affect 5-HT uptake.  
18 Moreover, the effect of cAMP was not additive to the effect of adenosine (Fig. 7B).  
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#### 29 **4. Discussion**

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33 5-HT effects on intestinal physiology depend on 5-HT availability, which is, in part, regulated  
34 by SERT expressed in enterocytes [6, 7]. Recent reports have confirmed the relevance of the  
35 SERT function for the control of intestinal activity [30, 31]. SERT expression and activity are  
36 affected in intestinal inflammation [10, 14-16] and inflammatory mediators have been shown  
37 to inhibit SERT function and expression in epithelial cells [12, 13].  
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42 Adenosine, which modulates intestinal activity, is increasingly recognized as modulating a  
43 wide variety of inflammatory responses. Indeed, adenosine concentration in the extracellular  
44 space increases under conditions of inflammation [19]. As indicated above, it is well  
45 established that intestinal inflammation coincides with decreased SERT activity. However,  
46 the effect of adenosine on SERT activity and expression in the intestinal epithelium remains  
47 unknown.  
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53 The results obtained in the present work show that adenosine diminishes SERT activity in  
54 a dose and period dependent way. A noteworthy aspect of this effect is the high adenosine  
55 concentration necessary to obtain an inhibitory effect on 5-HT uptake at both acute (30 min)  
56 and chronic (1 day) treatment. Adenosine is metabolized in the cells by adenosine deaminase  
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1 and it is converted into inosine. This molecule, which was thought to have no biological  
2 effects, acts as an immunomodulator [26]. To determine whether adenosine metabolism  
3 interferes with the adenosine effects on SERT, adenosine deaminase activity was inhibited  
4 and SERT activity measured in cells treated with adenosine. Our results show that the effect  
5 of adenosine on SERT was not modified when adenosine deaminase was inhibited. This  
6 suggests that in our cellular model, adenosine deaminase activity was not significant or, in  
7 case of metabolism, that the activity of the enzyme did not modify the final adenosine effect  
8 on SERT.  
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10 Adenosine physiological concentrations have been described as being lower than  $10^{-6}$  M.  
11 However, the adenosine effect on SERT is effective under conditions in which adenosine  
12 levels are high (such as in tissue injury and inflammation), in which adenosine concentration  
13 can be as high as  $10^{-4}$  M [32]. As tissue injury is a long-term condition in which adenosine  
14 may be increased, the following experiments were carried out in cells treated with adenosine,  
15 for 1 day.  
16

17 To gain an in-depth characterization of the adenosine effect on SERT activity, 5-HT  
18 transepithelial fluxes were measured in cells treated with adenosine, either by the apical or  
19 basal side. In both cases, adenosine diminished the corresponding 5-HT flux and 5-HT cell  
20 accumulation, indicating that adenosine acts on SERT located in both apical and basal  
21 membranes. In addition, the kinetic study showed that adenosine diminished  $V_{max}$ , indicating  
22 a reduction of the SERT capacity which may be related to a diminution of SERT in the  
23 membrane. However, SERT mRNA and protein levels were not affected by adenosine. These  
24 results suggest a modulation of SERT yielded by affecting the transporter molecule itself, in  
25 the membrane, without altering protein synthesis, intracellular traffic, or its availability. This  
26 kind of SERT regulation by physiological modulators was recently reported [23].  
27

28 Adenosine may act from the extracellular medium by means of binding to specific  
29 adenosine receptors and (or) it may be internalized by specific transporters to act from inside  
30 the cell. The expression of adenosine receptors and transporters was assessed in Caco-2 cells  
31 and the results obtained showed the expression of  $A_{2A}$  and  $A_{2B}$  receptors, and CNT2, CNT3,  
32 ENT1 and ENT2 transporters. Therefore, adenosine from both the extracellular or  
33 intracellular medium might yield its effects on SERT. Our results agree with previous studies  
34 carried out in the human GI tract in which the expression of  $A_2$  adenosine receptors [33, 34]  
35 and concentrative and equilibrative transporters [35] were demonstrated.  
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37 Adenosine transporters are responsible for the transport of adenosine to the intracellular  
38 medium. A well documented intracellular effect of adenosine is the activation of AMPK [27]  
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1 and our results show that activation of AMPK reduces SERT activity. This result indicates the  
2 possibility of SERT regulation mediated by intracellular adenosine and AMPK. However, the  
3 effect of both adenosine and AMPK treatment was additive, suggesting that, in our model,  
4 adenosine and AMPK might affect SERT from a different pathway and that adenosine effects  
5 on SERT might not require adenosine transport.  
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9 Another possibility for adenosine effects is the mediation of adenosine receptors. Our  
10 results showed that the activation of A<sub>2</sub> receptors with the agonist CV-1808 reduced SERT  
11 activity, a result that may confirm this hypothesis. Both adenosine receptors, A<sub>2A</sub> and A<sub>2B</sub>, are  
12 expressed in Caco-2 cells. However, adenosine IC<sub>50</sub> values for A<sub>2A</sub> receptor ranged between  
13 10<sup>-8</sup> and 10<sup>-6</sup> M, whereas A<sub>2B</sub> receptor activation generally requires adenosine levels that  
14 exceed 10<sup>-5</sup> M [36]. Therefore, the effect of adenosine on SERT might mainly involve A<sub>2B</sub>  
15 receptor activation. In fact, the results obtained showed that A<sub>2B</sub> receptor blockade with MRS  
16 1754, a potent A<sub>2B</sub> antagonist, specifically reversed the effect of adenosine on SERT. Recent  
17 studies have demonstrated that A<sub>2B</sub> adenosine receptors are upregulated during human IBD  
18 and potentially play a role in inflammation and colitis [20, 37]. Although these results suggest  
19 the involvement of A<sub>2B</sub> receptors in SERT effect, the role of A<sub>2A</sub> receptors can not be  
20 discarded.  
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23  
24 Recent results obtained in transfected cells and in mouse brain synaptosomes showed that  
25 adenosine yielded an increase in SERT activity; however, in this case, the effect was mediated  
26 by A<sub>3</sub> adenosine receptors [38, 39]. These results are in contrast to ours; nevertheless, the  
27 tissue studied, the adenosine receptor and the signalling pathway involved were different.  
28 This suggests a tissue-specific adenosine regulation of SERT.  
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31  
32 Previous studies by our group have demonstrated that SERT is tightly controlled by long  
33 term stimulation of the cAMP/PKA signalling pathway [6]. A<sub>2</sub> adenosine receptors  
34 predominant signalling pathway is through increased cAMP as well as PKA activation [28,  
35 29]. The results obtained showed that adenosine effects on SERT are mediated by  
36 cAMP/PKA, as adenosine effects are reversed by KT 5720, a PKA inhibitor. Moreover,  
37 cAMP inhibition of SERT was not additive to the effect of adenosine. These results therefore,  
38 confirm the mediation of A<sub>2</sub> receptors in adenosine effects on SERT.  
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41  
42 In conclusion, the results of this work show that adenosine, acting at high concentration,  
43 inhibits SERT activity in Caco-2 cells. This effect is mainly mediated by adenosine A<sub>2</sub>  
44 receptors and the cAMP/PKA signalling pathway. This study shows, for the first time, the  
45 modulation of SERT by adenosine in intestinal epithelial cells and this may contribute to our  
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1 knowledge and understanding of the regulation of the intestinal serotonergic system under  
2 physiological and pathological conditions.  
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4

## 5 **Acknowledgements**

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8

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Table 1. PCR primers pairs for the amplification of SERT, adenosine receptors and transporters, and  $\beta$ -actin.

Product	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
A <sub>1</sub>	GCCACAGACCTACTTCCACA	CCTTCTCGAACTCACACTTG	304
A <sub>2A</sub>	AACCTGCAGAACGTCACCAA	GTCACCAAGCCATTGTACCG	275
A <sub>2B</sub>	GATCATTGCTGTCCTCTGG	TCCTCGAGTGGTCCATCAG	301
A <sub>3</sub>	ACCACTCAAAGAAGAATATG	ACTTAGCTGTCTTGAAGTCC	327
ENT1	GCTTGAAGGACCCGGGGAGC	TGGAGAAGGCAAAGGCAGCCA	503
ENT2	TCCCAGGCCCAAGCTCAGGA	GGAACCGCAGGCAGACCAGC	427
CNT1	CTGTGTGGGTCCTCACCTTCCTG	GGAGAGGGCCAAGGCACAAGGG	799
CNT2	CAAAGGCCAGAGCAGCTGATC	CTTTACCCCTCCTCACTCTT	683
CNT3	GAAACATGTTTGACTACCCACAG	GTGGAGTTGAAGGCATTCTCTAAAACGT	480
SERT	AAATCCAAGCACCCAGAGAT	AGACTGTGTCCCTGTGGAGA	2105
$\beta$ -actin	AGCACGGCATCGTCACCAACT	ACATGGCTGGGGTGTGAAGG .	193

A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> are adenosine receptors

ENT1, ENT2, CNT1, CNT2 and CNT3 are adenosine transporters

## FIGURE LEGENDS

**Fig 1.** Effect of the dose and period of treatment of adenosine on 5-HT uptake. (A) Uptake was measured after 6 min incubation of  $2 \times 10^{-7}$  M 5-HT. Adenosine concentrations assayed were  $10^{-5}$ ,  $10^{-4}$ ,  $10^{-3}$  M. The treatment periods were 30 min (short term) or 1 day (long term). (B) Effect of the treatment during 1 day with adenosine  $10^{-4}$  or  $10^{-3}$  M plus EHNA  $10^{-5}$  M on 5-HT uptake. The results are expressed as the % of the uptake control and are the mean  $\pm$  SEM of 5 independent experiments. Control absolute values were  $11.18 \pm 0.10$  and  $9.51 \pm 0.20$  pmol 5-HT/mg prot in A and B figures respectively. \* $P < 0.05$  compared with the control (untreated cells). Degrees of freedom were 30 and 38 in A and B figures respectively.

**Fig 2.** Effect of adenosine treatment on 5-HT transepithelial fluxes and cell accumulation. (A) Adenosine  $10^{-3}$  M was added to apical or basal side for 1 day, and 5-HT apical to basal (A – B) or basal to apical (B – A) fluxes were measured respectively. 5-HT concentration was  $10^{-7}$  M and samples were taken every 10 min. Control conditions correspond to untreated cells (absolute values in pmol 5-HT/10 min were: in A – B =  $0.56 \pm 0.05$  and in B – A =  $0.22 \pm 0.03$ ). (B) 5-HT cell accumulation values under different conditions are represented: A – B = transport medium was added to the apical side without (control) or with adenosine  $10^{-3}$  M. (Control absolute value was  $94.57 \pm 5.42$  pmol 5-HT/mg prot); B – A = Transport medium was added to the basal side without (control) or with adenosine  $10^{-3}$  M. (Control absolute value was  $53.20 \pm 2.56$  pmol 5-HT/mg prot). The results are expressed as the % of the control value and are the mean  $\pm$  SEM of 4 independent experiments. \* $P < 0.05$  compared with the control value. Degrees of freedom in both, fluxes and accumulation analysis were 22.

**Fig 3.** Effect of adenosine treatment on SERT mRNA levels. Cells were treated for 1 day with adenosine  $10^{-4}$  or  $10^{-3}$  M and the total RNA was extracted. (A) PCR amplification of SERT (2105 bp) and actin (193 bp) in control and adenosine treated cells was obtained under conditions described in the Material and Methods section. (B) Quantitation of the SERT mRNA expression using  $\beta$ -actin as internal control (SERT/ $\beta$ -actin ratio). The results are expressed in arbitrary densitometric units and are the mean  $\pm$  SEM of 3 independent experiments.

**Fig 4.** Effect of adenosine treatment on SERT protein expression. Caco-2 cells were treated with adenosine  $10^{-4}$  or  $10^{-3}$  M for 1 day. (A) Cell lysate and apical membrane samples were electrophoresed and SERT immunodetected. (B) Quantitation of SERT protein in both cell lysate and apical membrane using actin as an internal control of protein load (SERT/ $\beta$ -actin

ratio). The results are expressed in arbitrary densitometric units and are the mean  $\pm$  SEM of 3 independent experiments.

**Fig 5.** Expression of adenosine receptors and transporters in Caco-2 cells. Total RNA from Caco-2 cells was extracted and cDNA was obtained by reverse transcription. PCR amplification product was obtained with primers indicated in the Table 1 and under conditions described in the Material and Methods section. (A) Bands corresponding to the amplification of adenosine receptors A<sub>2A</sub> (275 bp), A<sub>2B</sub> (301 bp). (B) Bands corresponding to concentrative nucleoside transporters CNT2 (683 bp), CNT3 (480 bp) and equilibrative nucleoside transporters ENT1 (503 bp) and ENT2 (427 bp).

**Fig 6.** Role of AMPK in adenosine effect on SERT activity. Cells were treated for 1 day with adenosine  $10^{-3}$  M and/or AICAR  $10^{-3}$  M. 5-HT uptake was measured after 6 min of incubation and 5-HT concentration was  $2 \times 10^{-7}$  M. Control absolute value was  $11.89 \pm 0.11$  pmol 5-HT/mg prot. The results are expressed as the % of the control value and are the mean  $\pm$  SEM of 6 independent experiments. \*P<0.05 compared with control. @ p<0.05 compared with adenosine or AICAR treated cells. Degrees of freedom = 70.

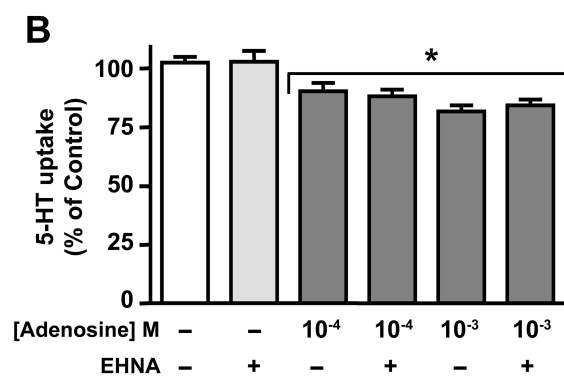
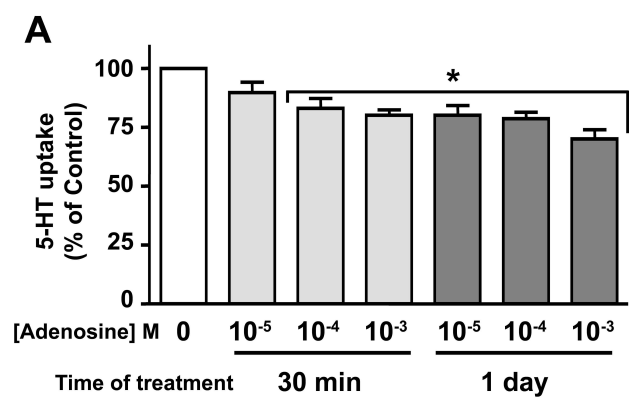
**Fig 7.** Role of adenosine receptors and involvement of the cAMP/PKA signalling pathway in adenosine effects on SERT activity. 5-HT uptake was measured after 6 min of incubation and 5-HT concentration was  $2 \times 10^{-7}$  M. Cells were treated during 1 day with adenosine and/or different modifiers. (A) Role of A<sub>2</sub> receptors. Cells were treated with CV-1808  $5 \times 10^{-8}$  M, adenosine  $10^{-4}$  M or  $10^{-3}$  M, and/or MRS 1754  $10^{-7}$  M or  $10^{-6}$  M. Control absolute value was  $10.18 \pm 0.09$  pmol 5-HT/mg prot. (B) Effect of cAMP pathway. Cells were treated with adenosine  $10^{-4}$  M or  $10^{-3}$  M, d-cAMP  $10^{-3}$  M and/or KT 5720  $10^{-6}$  M. Control absolute value was  $10.65 \pm 0.12$  pmol 5-HT/mg prot. The results are expressed as the % of the uptake control, and are the mean  $\pm$  SEM of 5 independent experiments. \*P<0.05 compared with the control. #P<0.05 compared with adenosine. Degrees of freedom were 28 in both experiments.

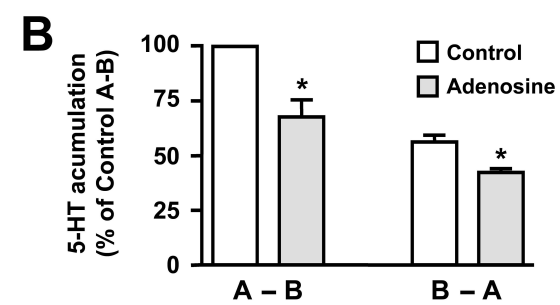
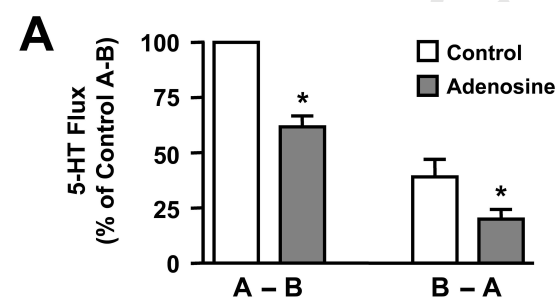
Table 1. PCR primers pairs for the amplification of SERT, adenosine receptors and transporters, and  $\beta$ -actin.

Product	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
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A <sub>2B</sub>	GATCATTGCTGTCCTCTGG	TCCTCGAGTGGTCCATCAG	301
A <sub>3</sub>	ACCACTCAAAGAAGAATATG	ACTTAGCTGTCTTGAAGTCC	327
ENT1	GCTTGAAGGACCCGGGGAGC	TGGAGAAGGCAAAGGCAGCCA	503
ENT2	TCCCAGGCCCAAGCTCAGGA	GGAACCGCAGGCAGACCAGC	427
CNT1	CTGTGTGGGTCCTCACCTTCCTG	GGAGAGGGCCAAGGCACAAGGG	799
CNT2	CAAAGGCCAGAGCAGCTGATC	CTTTACCCCTCCTCACTCTT	683
CNT3	GAAACATGTTTGACTACCCACAG	GTGGAGTTGAAGGCATTCTCTAAAACGT	480
SERT	AAATCCAAGCACCCAGAGAT	AGACTGTGTCCCTGTGGAGA	2105
$\beta$ -actin	AGCACGGCATCGTCACCAACT	ACATGGCTGGGGTGTGAAGG	193

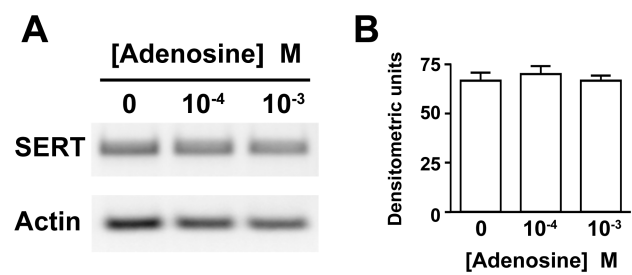
A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> are adenosine receptors

ENT1, ENT2, CNT1, CNT2 and CNT3 are adenosine transporters



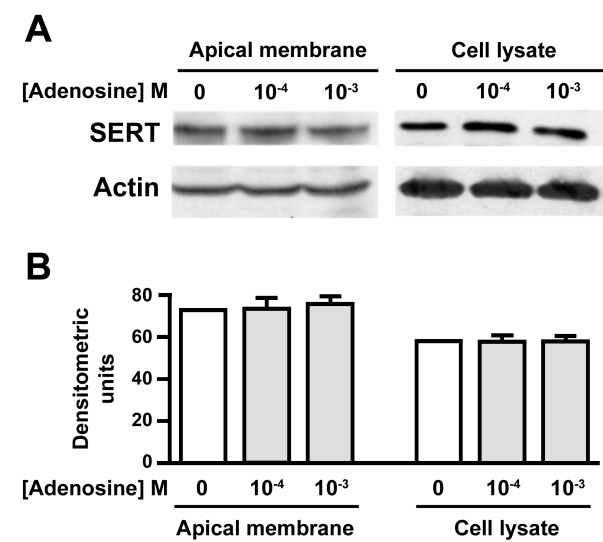


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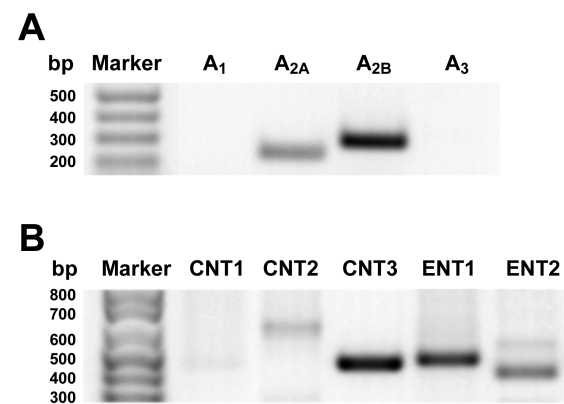


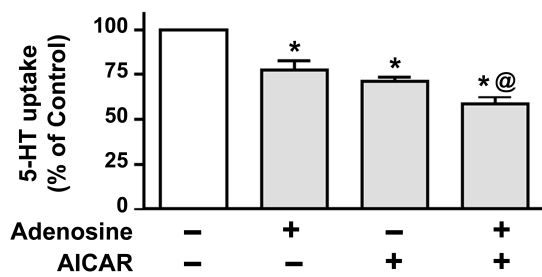


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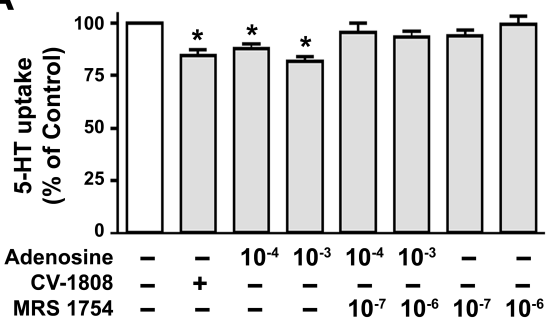


Accepted Manuscript

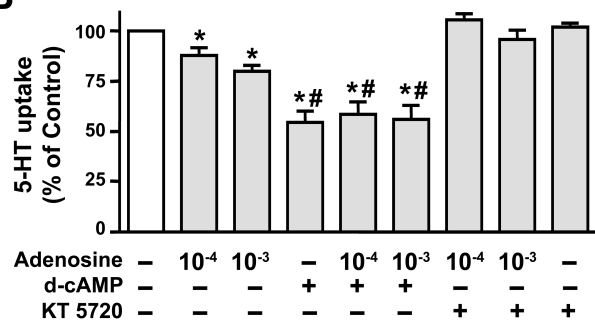




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