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**PURINERGIC MECHANISMS AS POTENTIAL
PHARMACOLOGICAL TARGETS FOR ENTERIC
INFLAMMATORY MOTILITY DISORDERS**

Tese de Candidatura ao grau de Doutor em
Ciências Biomédicas, submetida ao Instituto de
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do Porto.

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Dissertation in fulfilment of the requirements for the
PhD degree in Biomedical Sciences, submitted to
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I. ABBREVIATIONS

ABC proteins, ATP-binding cassette transporters
ACh, acetylcholine
ADA, adenosine deaminase
ADO, adenosine
ADP, adenosine diphosphate
AHP, prolonged after-hyperpolarization
AIN, ascending interneuron
AK, adenosine kinase
AMP, adenosine monophosphate
ANO-1, anoctamine 1
ANS, autonomic nervous system
AP, alkaline phosphatases
ATP, adenosine triphosphate
BDNF, brain-derived neurotrophic factor
Calb, calbindin
cAMP, cyclic adenosine monophosphate
CFTR, cystic fibrosis transmembrane conductance regular
CGRP, calcitonin gene related peptide
ChAT, choline acetyltransferase
C-Kit, tyrosine kinase receptor
CM, circular muscle
CNS, central nervous system
CNT, concentrative nucleoside transporters
CO, carbon monoxide
DAG, diacylglycerol
DIN, descending interneurons
DMPP, dimethylphenylpiperazinium
DMSO, dimethyl sulfoxide
DPCPX, 1,3-dipropyl-8-cyclopentylxanthine
DSS, dextran sodium sulphate
DYN, dynorphin

EFS, electrical field stimulation
EMN, excitatory motor neuron
ENK, enkephalins
E-NPP, ecto-nucleotide pyrophosphatases/phosphodiesterases
ENS, enteric nervous system
ENT, equilibrative nucleoside transporters
E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase
EPAN, extrinsic primary afferent neuron
ER, endoplasmic reticulum
fEPSPs, fast excitatory post-synaptic potentials
FLC, fibroblast-like cell
GABA, gamma-aminobutyric acid
GFAP, intermediate filament glial fibrillary acidic protein
GI, gastrointestinal
GPCR, G protein-coupled receptors
GPI, glycosylphosphatidylinositol
GRP, gastrin releasing peptide
HO1, heme oxygenase-1
HPLC, high performance liquid chromatography
HX, hypoxanthine
IBD, inflammatory bowel disease
ICC, interstitial cells of Cajal
IFN, intestinofugal neuron
IFN- γ , interferon γ
IL, interleukin
IMN, inhibitory motor neuron
INO, inosine
iNOS, inducible nitric oxide synthase
IP₃, inositol triphosphate
IPAN, intrinsic primary afferent neuron
KO, knockout
LGIC, ligand-gated ionotropic channel
LM-MP, longitudinal muscle-myenteric plexus

mAChR, muscarinic receptors of acetylcholine
Min, minutes
NA, noradrenaline
nAChR, nicotinic receptors of acetylcholine
NBTI, S-(4-Nitrobenzyl)-6-thioinosine
NGF, nerve grow factor
nNOS, nitric oxide synthase
NO, nitric oxide
NOS, nitric oxide synthetase
NT, nucleoside transporter
NT-3, neurotrophin-3
NT-4, neurotrophin-4
NYP, neuropeptide Y
PBS, phosphate saline buffer
PDGFR- α , a platelet derived growth factor receptor α
PGP 9.5, protein gene product 9.5
PLC, phospholipase C
PLP, periodate-lysine-paraformaldehyde
PM-ML, plexo mioentérico-músculo longitudinal
PVG, sympathetic prevertebral ganglia
ROS, reactive species
S100, calcium-binding protein
SAH, S-adenosyl-L-homocysteine hydrolase
SLC, solute carrier transporters
SM, submucosal plexus
SMC, smooth muscle cells
SMN, secretomotor/ vasomotor neuron
Sox 10, transcription factor SRY box-containing gene 10
SP, substance P
SNS, sympathetic nervous system
TNBS, 2,4,6-trinitrobenzenesulfonic acid.
TNF, tumour necrosis factor
TRC, T cell receptor

TTX, tetrodotoxin
UDP, uridine diphosphate
UTP, uridine triphosphate
UV, ultraviolet
VACHT, ACh vesicular transporter
VIP, vasoactive intestinal polypeptide
VNUT, vesicular nucleotide transporter
5-HT, 5-hydroxytryptamine

II. RESUMO

As purinas parecem desempenhar um papel fisiológico na regulação da motilidade intestinal, podendo afectar a actividade dos nervos entéricos, das fibras musculares, das células entéricas gliais, das células intersticiais de Cajal (ICC) e das células do tipo fibroblasto (FLC). A inflamação do intestino provoca alterações estruturais e funcionais mais ou menos duradouras no sistema nervoso entérico (SNE) e está associada à elevação dos níveis extracelulares das purinas, nomeadamente ATP e adenosina. Partindo de estudos anteriores controversos olhando para o controlo da inflamação pelas purinas, este projecto foi desenhado para definir o papel da cascata purinérgica no controlo da actividade do SNE após uma situação de inflamação aguda. O propósito do trabalho foi apontar possíveis alvos farmacológicos para o tratamento das doenças inflamatórias intestinais. Neste contexto, foram gerados modelos animais portadores de ileíte induzida através da instilação intraluminal de TNBS na ratazana e os mesmos foram utilizados para fins experimentais sete dias após a indução – modelo de ileíte pós-inflamatória. Todas as experiências foram realizadas em preparações de plexo mioentérico-músculo longitudinal (PM-ML) de íleo destes animais.

Neste modelo animal, colocou-se a hipótese da reacção inflamatória induzir alterações na densidade da população celular residente (para além da infiltração por células inflamatórias) capaz de gerar um desequilíbrio na libertação de purinas e, consequentemente, na libertação de ACh[³H] – o neurotransmissor mais relevante no controlo da motilidade intestinal. Para responder a essa questão, foram usadas preparações de PM-ML do íleo de ratazana marcadas com anticorpos específicos para cada um dos componentes celulares envolvidos. Tal como seria de esperar, verificou-se um aumento significativo do número de células inflamatórias marcadas com CD11B/Ox42 localizadas na região adjacente aos gânglios mioentéricos, poupando o seu interior. A reacção inflamatória fez-se acompanhar de um aumento da densidade das células gliais, marcadas positivamente com GFAP ou S100 β , e de uma perda de células intersticiais (ICC/FLC) imunoreactivas contra a vimentina e anoctamina-1 (Ano-1). Tal como

constatado por outros autores em modelos experimentais de ileíte pós-inflamatória, não se observaram alterações significativas na densidade e na distribuição de dois marcadores neuronais específicos, PGP9.5 e o NF200, contrariamente aos resultados obtidos em modelos mais severos de colite induzida pelo TNBS, onde a perda neuronal foi evidente.

Seguidamente exploraram-se as alterações funcionais subjacentes às modificações estruturais encontradas no modelo de ileíte pós-inflamatória. A estimulação das preparações de PM-ML do íleo de ratazanas submetidas ao tratamento com TNBS libertaram menor quantidade de ACh[³H]. Contudo, esta diminuição da transmissão colinérgica não foi acompanhada por uma perda significativa da imunoreactividade para o transportador vesicular de ACh, VaChT. Em animais controlo, a libertação de ACh resulta da exocitose vesicular em resposta à despolarização nervosa, sensível à TTX. No entanto, no tecido sujeito a inflamação a libertação de ACh para além de se encontrar significativamente reduzida, é também, menos sensível ao bloqueio pela TTX. A hipótese de que a libertação de ACh nos neurónios inflamados se pode dever à acção de um gliotransmissor excitatório capaz de activar directamente os terminais nervosos na ausência de potencial de acção foi comprovada através da inibição pelo fluoroacetato de sódio. Trata-se de uma toxina que afecta o metabolismo das células gliais entéricas e que reduziu significativamente os níveis de ATP libertados pelo plexo mioentérico do íleo de ratazana após tratamento pelo TNBS.

O efeito inibitório do fluoroacetato de sódio sobre a libertação de ACh[³H] nas preparações inflamadas pelo TNBS foi reproduzido pelo antagonista selectivo dos receptores P2X7, A438079, e pelo o inibidor dos hemicanais contendo panexina-1, carbenoxolona. O mesmo efeito não foi observado quando se preveniu a actividade das células intersticiais ICC/FLC através da inibição das correntes Ca_v3 (do tipo T) com mibefradil. Estes resultados sugerem que o ATP parece ser o gliotransmissor excitatório responsável pela activação directa dos terminais mioentéricos através da activação de receptores ionotrópicos do tipo

P2X2 e P2X2/3 e que a sua acumulação é dependente da actividade do complexo P2X7/panexina-1.

Contrariamente ao aumento da quantidade de ATP libertado pelo íleo de ratas tratadas com TNBS observou-se uma redução significativa dos níveis extracelulares do seu metabolito, adenosina, bem como da neuromodulação colinérgica mediada pela activação preferencial de receptores facilitatórios de subtipo A_{2A} localizados nos terminais nervosos colinérgicos. Os resultados mostram que a disparidade entre o aumento da libertação de ATP e o défice de formação de adenosina na ileíte pós-inflamatória pode ser atribuída, pelo menos em parte, à inibição anterógrada da ecto-5'-nucleotidase/CD73 (a enzima que converte AMP em adenosina) devido à acumulação de elevados níveis de ATP e ADP na sinapse mioentérica. Esta acumulação deve-se a uma redistribuição da localização da NTPDase2 (uma ATPase) da região ganglionar para a junção neuromuscular, sem afectar a densidade da NTPDase3 no plexo mioentérico dos animais tratados com TNBS, facto que condiciona a formação de ADP a partir do ATP naquela região intestinal. O défice de acumulação extracelular de adenosina na ileíte pós-inflamatória pode, ainda, dever-se ao aumento da sua inactivação em inosina pela ADA (frações membranas e solúvel). Existe um terceiro mecanismo passível de ser responsabilizado pela redução da neuromodulação adenosinérgica no plexo mioentérico inflamado, que deriva da redução significativa da densidade da população de células intersticiais ICC/FLC. De facto, a inibição da actividade das ICC/FLC através do bloqueio dos canais de Ca_v3 (do tipo T) característicos destas células, reduz consideravelmente os níveis de adenosina libertados pelo plexo mioentérico, tanto em animais controlo como injectados com TNBS, situação que contrasta com a ausência de efeito da gliotoxina, fluoroacetato de sódio. Sabe-se que as células intersticiais libertam quantidades significativas de adenosina por intermédio do transportador equilibrativo de nucleósidos sensível ao dipiridamol, mediante a activação de receptores muscarínicos do subtipo M_3 . Este mecanismo pode estar deficitário no intestino inflamado, já que a libertação de ACh se encontra atenuada e que a densidade de células intersticiais ICC/FLC também está diminuída, situação que

culmina no bloqueio do mecanismo de retroalimentação positivo operado pela activação de receptores facilitatórios A_{2A} nas terminações nervosas colinérgicas.

Em conclusão, os resultados mostram que a transmissão colinérgica está significativamente afectada no modelo de ileíte pós-inflamatória e que esse distúrbio pode resultar da existência de um desequilíbrio entre a neuromodulação adenosinérgica, influenciada pela actividade das células intersticiais ICC/FLC predominante em situações fisiológicas, e a excessiva activação dos mecanismos mediados pelo ATP resultantes da proliferação glial promovida pelo processo inflamatório. Se por um lado, a libertação de ATP através da actividade do complexo P2X7/panexina-1 pode levar à activação de receptores ionotrópicos dos subtipos P2X2 e P2X2/3 nos terminais nervosos colinérgicos mioentéricos favorecendo a libertação directa do neurotransmissor para manter o tónus colinérgico, por outro lado a metabolização do ATP em ADP, sem a consequente formação de adenosina no íleo inflamado, pode quebrar a facilitação da libertação de ACh operada pela activação sucessiva dos receptores facilitatórios M_3 e A_{2A} localizados, respectivamente, nas células intersticiais e nos terminais nervosos colinérgicos. Assim, os resultados deste trabalho sugerem que a manipulação da libertação diferencial das purinas, a intervenção sobre as suas vias de inactivação e a activação de certos subtipos de receptores purinérgicos podem constituir alvos terapêuticos interessantes para o tratamento das doenças inflamatórias intestinais.

III. ABSTRACT

Purines seem to play a physiological role in the regulation of gut motility by affecting the activity of enteric nerves, muscle fibers, enteric glial cells, interstitial cells of Cajal (ICC) and fibroblast cells (FLC). Intestinal inflammation may cause long-term structural and functional changes in the enteric nervous system (ENS), which might be related to increased levels of extracellular purines, namely ATP and adenosine. Taking into account controversies in the literature regarding the control of enteric inflammation by purines, this project was designed to investigate the role of the purinergic cascade in the control of SNE activity following acute inflammation. Our aim was to identify putative molecular targets for the pharmacological treatment of inflammatory enteric disorders. To achieve this goal, we used an animal model of postinflammatory ileitis due to intraluminal instillation of TNBS in the rat; experimental animals were used seven days after surgery. All experiments were performed in longitudinal muscle-myenteric plexus (LM-MP) preparations of rat ileum.

In this animal model, we hypothesized that inflammation may cause relevant changes in the density of resident cell populations (beyond the typical inflammatory infiltrates), which may unbalance the release of purines and, consequently, the release of [^3H]ACh – the most important neurotransmitter in the control of gut motility. To answer this question, LM-MP preparations were stained with specific antibodies directed against each specific cell type. As expected, we observed a significant increase in the number of inflammatory cells stained with CD11b/OX42, which were localized adjacent to myenteric ganglia, without penetrating this structure. The inflammatory insult, cause a significant increase in the density of enteric glial cells, exhibiting positive immunoreactivity against GFAP and S100 β , and a substantial loss of interstitial cells ICC/FLC expressing vimentin and anoctamine-1 (Ano-1). In agreement with the results obtained by other authors using the postinflammatory ileitis rat model, we failed to detect any changes in the density and distribution of two specific neuronal markers, PGP9.5

and NF200. These findings are in contrast with those obtained in more severe TNBS-induced colitis where neuronal loss is more evident.

Next, we investigated functional abnormalities reflected in the enteric nervous system that could be motivated by the structural changes detected in the postinflammatory ileitis rat model. Stimulation of the myenteric plexus of the ileum of rats treated with TNBS released significantly smaller amounts of [^{3} H]ACh than their control littermates. However, the reduction in the cholinergic tone was not accompanied by changes in the immunoreactivity against the ACh vesicular transporter, VACHT. In control animals, ACh release results from vesicle exocytosis of depolarized cholinergic nerves sensitive to TTX. Conversely, ACh release from inflamed myenteric nerve terminals was less sensitive to action potential blockade with TTX, indicating that it might occur through direct activation of axon terminals by an excitatory mediator released from non-neuronal cells. Implication of ATP released from proliferating glial cells was suggested because inhibition of enteric glial cells metabolism with sodium fluoroacetate decreased both the release of [^{3} H]ACh and ATP from LM-MP preparations of the ileum of TNBS-treated rats.

The inhibitory effect of sodium fluoroacetate on the release of [^{3} H]ACh was reproduced by the P2X7 receptor antagonist, A438079, and by the pannexin-1 hemichannel blocker, carbenoxolone, but the same effect was not observed in the presence of mibefradil, a Ca_v3 (T-type) channel blocker that prevents the activity of interstitial cells ICC/FLC. These results suggest that ATP might be the excitatory gliotransmitter responsible for the activation of ionotropic P2X2 and/or P2X2/3 receptors operating direct activation of cholinergic nerve terminals and that extracellular accumulation of the nucleotide may be dependent on the P2X7/pannexin-1 pathway.

In contrast to the increase in ATP overflow, the ileum of TNBS-treated rats accumulate smaller amounts of its metabolite, adenosine, resulting in a reduction in the preferential activation of facilitatory A_{2A} receptors located on cholinergic nerve terminals. The disparity between increased ATP overflow and adenosine

deficits in post-inflammatory ileitis was ascribed, at least in part, to feed-forward inhibition of ecto-5'-nucleotidase/CD73 (the enzyme that converts AMP into adenosine) by high amounts of ATP and ADP accumulated at the myenteric synapse. Redistribution of NTPDase2 (ATPase) from the ganglion region to the myenteric neuromuscular synapse, without much affecting NTPDase3, might explain surplus ADP accumulation near cholinergic nerve terminals in TNBS-treated rats. Deficits in extracellular adenosine in postinflammatory ileitis may also occur due to increases in adenosine inactivation into inosine by ADA (membrane-bound and soluble fractions). A third mechanism may also account for the loss of adenosine neuromodulation in the inflamed myenteric plexus, which derives from a reduction in the density of interstitial cells ICC/FLC population. As a matter of fact, inhibition of ICC/FLC activity by blocking Ca_v3 (T-type) channels, decreases significantly endogenous adenosine outflow by LM-MP preparations of both control and TNBS-treated rats. This situation was not observed in the presence of the gliotoxin, sodium fluoroacetate. It has been shown that interstitial cells release significant amounts of adenosine, via a dipyridamole-sensitive equilibrative nucleoside transporter, through the activation of muscarinic M_3 receptors. This mechanism may be deficient in postinflammatory ileitis, since ACh release is downregulated and the number of interstitial cells is reduced, leading to blockage to the positive feedback loop operated by facilitatory muscarinic M_3 (on ICC/FLC) and adenosine A_{2A} (on cholinergic nerve terminals) receptors that are responsible for maintaining the safety margin of myenteric cholinergic transmission.

In conclusion, data suggest that cholinergic neurotransmission is severely affected in postinflammatory ileitis, a situation that might result from an unbalance between adenosinergic neuromodulation (that is dominant in physiological conditions) and excessive ATP accumulation attributed to enteric glial cells proliferation promoted by the inflammatory insult. In principle, surplus ATP overflow gated by P2X7/pannexin-1 pathway may facilitate activation of ionotropic P2X2 and/or P2X2/3 receptors on cholinergic nerve terminals resulting in direct activation of neurotransmitter release independently of action potential generation, in order to keep the cholinergic tone at a critical minimum in the inflamed ileum.

But, on the other hand, ATP hydrolysis into ADP without significant adenosine formation detected in postinflammatory ileitis may break the facilitatory loop required to sustain neurotransmitter release operated by muscarinic M_3 and adenosine A_{2A} receptors localized on interstitial cells ICC/FLC and cholinergic nerve terminals, respectively. Thus, results presented here suggest that selective targeting the differential release of purines, the activity of the purinergic inactivation mechanisms and the activation of specific purinoceptor subtypes may offer novel pharmacological strategies for the treatment of inflammatory intestinal diseases.

IV. INTRODUCTION

1. Autonomic nervous system

Nervous system has an important role in all digestive processes; it's responsible for the control of motility, secretion, sensory perception, and immune function (Straub *et al.*, 2006). Autonomic nervous system (ANS) has two main functions, excitation and inhibition, of physiological processes. These functions are controlled through different kinds of nerve fibres, entitled excitatory and inhibitory. So, ANS is responsible for the unconscious control of essential functions for life being (e.g. blood circulation, heart function, digestion, temperature, stress). ANS has the purpose of preparing the body for stress and recovering the same level of activation following stressful conditions. It comprises three fundamental parts (figure 1): sympathetic, parasympathetic and enteric nervous system (ENS) described in 1921 by Langley (Lundgren *et al.*, 1989). Sympathetic and parasympathetic nervous system establish a connection between the central nervous system and peripheral organs. Sympathetic nervous system (SNS) together with the hypothalamic-pituitary-adrenal axis is known by the main role in the “fight and flight reaction” during stressful conditions. An efferent sympathetic pathway is activated in the brain, goes along the intermediolateral column of the spinal cord and come to sympathetic ganglia. Afterwards, the signal goes to postganglionic sympathetic nerve fibres, and enters in the intestine wall along arteries through the mesenteric serosa surface (Straub *et al.*, 2006). On the other hand, parasympathetic nervous system is known by the main role of “rest and digestion”; it works predominantly during relaxation conditions. It has one main contributor, the vagus nerve. Vagal branches comprise sensory fibres (afferent) and motor fibres (efferent). They are responsible for the communication between the brain and visceral organs (Stakenborg *et al.*, 2013). Simple functions like food intake, digestion and immunity are controlled by the interaction between the vagal nerve fibres and the gut. Extrinsic sympathetic and parasympathetic fibres terminate in vessel walls or in enteric plexuses that constitute the ENS. Like it was described, ENS can operate dependently of central nervous system, but unlike the other systems described so far, it can function independently of the central

nervous system, and this activity is exclusive of the gastrointestinal (GI) tract. For that reason, ENS was called by several authors the “second brain”. In this work we will focus our attention on the ENS.

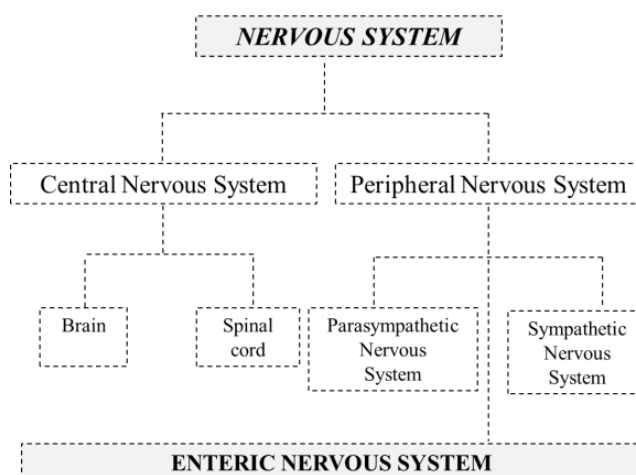


Figure 1: Diagram representative of Nervous system.

2. Enteric nervous system

The ENS has approximately 100 million neurons, a number equal to or greater than the number of neurons found in the spinal cord (Furness & Costa, 1980). The ENS is composed by three major ganglionated plexuses and several aganglionated plexuses. The myenteric (or Auerbach's), the submucosal (or Meissner's) and the mucosal are described as the ganglionated plexuses. The non-ganglionated plexuses, like the deep muscular plexus, inner submucosal plexus, periglandular plexus among others, supply all the layers of the intestine (Hansen, 2003).

The myenteric plexus is placed between longitudinal muscle and circular muscle layers and the submucosal plexus is located in the submucosa. Concerning to their functions in the ENS, submucosal plexus controls especially local gut secretion and local absorption, while myenteric plexus controls mainly GI movements and secretion of enzymes from adjacent organs, but these dominant functions may be mixed. When stimulated, ENS main effects include: (1) increase

of gut wall tonic contraction, (2) augmentation of the rhythmic contractions, (3) slight increase of the contraction rate, and (4) enhancement in the transmission velocity of excitatory waves along the gut wall. These effects originate an increase of the gut peristaltic waves.

The myenteric plexus is one continuous network of little nervous ganglia that are throughout the entire GI tract. Ganglia are sometimes called the nodes of plexus, because they are located in junction areas. So, they are usually called internodal or interganglionic strands. These ganglia can be distorted by muscle movements (Furness, 2006).

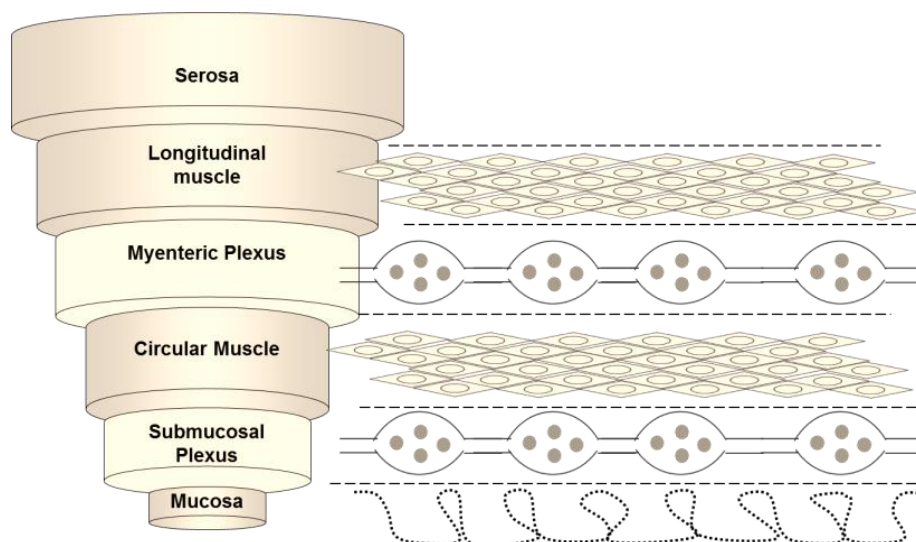


Figure 2: Schematic representation of the ENS. ENS is formed by two ganglionated plexuses: MP and Submucosal Plexus, located between two layers of muscle: the longitudinal muscle and circular muscle layer.

About 150 years ago, Auerbach described three essential components of the myenteric plexus (figure 3): (1) primary plexus, related to ganglia and internodal strands; (2) secondary (interfascicular) plexus, related to nerve strands which are parallel to the circular muscle; and a (3) tertiary plexus, found in the longitudinal muscle layer (Furness, 2006).

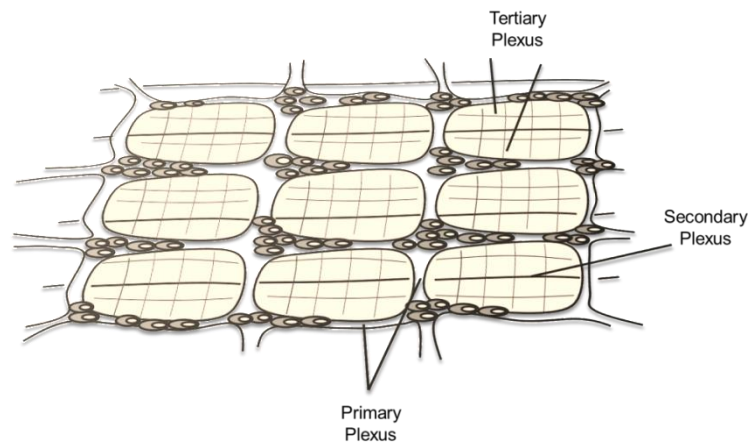


Figure 3: Schematic draw of components of the myenteric plexus.

2.1. Enteric neurons

Enteric neurons are organized in networks of enteric ganglia that are connected by intraganglionic strands that extend from the upper oesophagus to the internal anal sphincter (Costa *et al.*, 2000; Furness *et al.*, 1998). There are different classes of enteric neurons, classified based on their morphology, electrical properties, neurochemical code and function (Table 1). In that way, it was identified seventeen different types of neurons in the small intestine, but only 14 are described as functionally important (Furness, 2000; Hansen, 2003). The majority of the studies done on enteric neurons taxonomy were done in the guinea-pig small intestine (Costa *et al.*, 1996; Brehmer *et al.*, 1999; Furness, 2000; Costa, 2000).

2.1.1. Morphology of enteric neurons

Based on their morphology, in 1899, Dogiel proposed a classification to the enteric neurons. He classified enteric neurons as Dogiel type I, II and III. Later, were described four more types of enteric neurons (type IV, V, VI, and VII) (Brehmer *et al.*, 1999). Most of enteric neurons are from types I, II and III (Furness, 2000). How can these neurons be differentiated? Type I, is characterized by having a flattened cell body and numerous dendrites projecting into the musculature. The type II, possess an oval cell body with long processes projecting to ganglion cells. The type III, is characterized by a rectangular cell body

with irregular dendrites. In this latter neurons, the axons project to the mucosa (Brehmer *et al.*, 1999).

2.1.2. Electrophysiological properties of enteric neurons

Based on different electrophysiological properties, enteric neurons were classified in two possible ways, AH neurons and S neurons (Furness *et al.*, 1997). When an action potential is generated in the cell body of AH neurons, it is followed by a prolonged after-hyperpolarization (AHP), whereas if the same occurs in S neurons the action potential is followed by prominent fast excitatory post-synaptic potential (fEPSP). Morphologically S neurons are Dogiel type I, whereas AH neurons are Dogiel type II (Furness, 2006; Nurgali, 2009).

2.1.3. Neurochemical code of enteric neurons

Primary transmitters, transmitters synthesizing enzymes and neuromodulators released from enteric neurons include: acetylcholine (ACh), norepinephrine (NE), adenosine triphosphate (ATP), serotonin (5-HT), dopamine (DA), cholecystokinin (CCK), substance P (SP), vasoactive intestinal polypeptide (VIP), somatostatin (SOM), leu-enkephalin (leu-Enk), met-eukephalin (met-Enk) and bombesin. These compounds provide to the enteric neurons a neurochemical code. Based on that neurochemical code, most enteric neurons are classified essentially as nitrergic neurons, cholinergic neurons or peptidergic neurons (Furness, 2006). To make this distinction, immunolocalization studies using specifically markers, like nitric oxide synthase (nNOS), choline acetyltransferase (ChAT), SP, calcitonin-gene related peptide (CGRP) and VIP may be undertaken. Co-localization studies indicate the subclasses of neurons that co-express neurotransmitters and neuromodulators (Chiocchetti *et al.*, 2009). The neurochemical code and density of specific neurons differ between gut regions and between animal species (Furness, 2006).

2.1.4. Function of enteric neurons

Functionally, enteric neurons were classified as sensory, inhibitory and excitatory motor neurons, interneurons, secretomotor/ vasomotor neurons and

intestino-fugal neurons (figure 4) (Costa *et al.*, 1996; Furness, 2000; Costa *et al.*, 2000; Hansen, 2003). The majority of enteric neurons responsible for controlling motor activity of the gut are located in the myenteric plexus (Costa *et al.*, 1996).

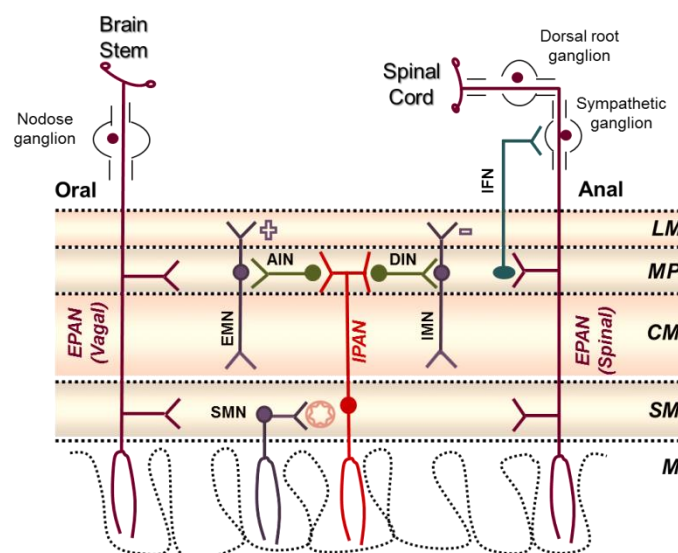


Figure 4: Types of neurons in the small intestine of the guinea-pig: **EPAN**- Extrinsic primary afferent neuron; **IPAN**- Intrinsic primary afferent neuron; **EMN**- excitatory motor neuron; **IMN**- inhibitory motor neuron; **AIN**- Ascending interneuron; **DIN**-Descending interneurons; **SMN**- Secretomotor/ vasomotor neuron; **IFN**- intestino-fugal neuron

2.1.4.1. Sensory neurons

Sensory neurons are established by a dense network of extrinsic (EPANs) and intrinsic (IPANs) primary afferent neurons (figure 5). They are present in myenteric ganglia (30% of myenteric neurons) and in submucosal ganglia (14% of submucosal neurons). Morphologically, they are entitled Dogiel type II and they present the electrophysiological properties of AH neurons (Costa *et al.*, 2000).

EPANs are the vagal and spinal afferents with their cell bodies outside the gut wall and IPANs are multipolar and their cell bodies are within the gut wall. Both types of neurons communicate with each other. A wide range of membrane receptors are expressed by sensory nerves to modulate their sensitivity. They include mechanoreceptors, thermoreceptors and chemoreceptors (Hansen, 2003).

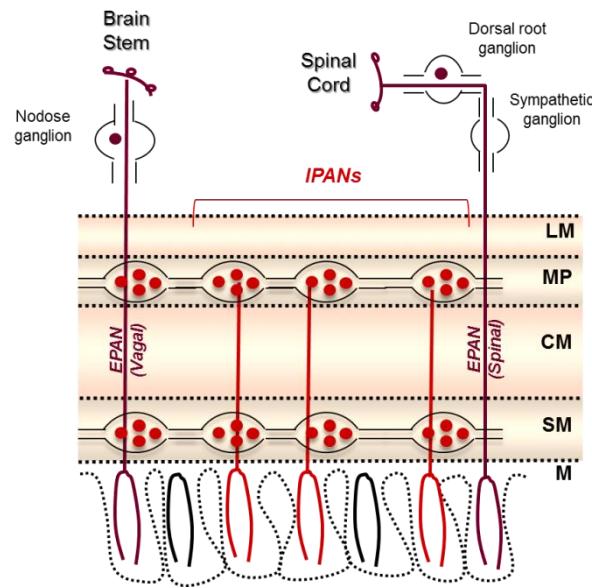


Figure 5: Types of sensory neurons, extrinsic (EPANs) and intrinsic (IPANs) primary afferent neurons in the ENS. **LM**, longitudinal muscle; **MP**, myenteric plexus; **CM**, circular muscle; **SM**, submucosal plexus; **M**, mucosa.

2.1.4.2. Motor neurons

Are described five types of motor neurons: excitatory muscle motor neurons, inhibitory muscle motor neurons, secretomotor/vasodilator neurons, secretomotor neurons that are not vasodilator and neurons that are innervating entero-endocrine cells (Furness, 2000). Motor neurons belongs to the Dogiel type I morphology and presents the electrophysiological properties of S neurons (Costa *et al.*, 2000) Longitudinal and circular muscles and the muscularis mucosae are innervated by muscle motor neurons. They can be excitatory or inhibitory. In that sense, they release neurotransmitters that promote muscle contractions or relaxations (Hansen, 2003). Motor neurons are immunoreactive to ChAT (enzyme responsible for the synthesis of ACh) and to SP (Costa *et al.*, 2000; Furness 2000). Thus, the excitatory muscle motor neurons release transmitters like ACh and tachykinins (SP and neurokinin A). On the other hand, inhibitory muscle motor neurons release substances like nitric oxide (NO), VIP, ATP, GABA, neuropeptide Y (NPY) and carbon monoxide (CO) (Costa *et al.*, 2000; Hansen, 2003).

2.1.4.3. Interneurons

The interneurons have both electrophysiological properties described above, may be AH neurons (morphologically, Dogiel type II) or S neurons (morphologically, Dogiel type I) (Costa *et al.*, 2000; Hansen, 2003). Ascending interneurons (one type of interneurons oriented in the oral direction) and descending interneurons (three types of interneurons oriented in the anal direction) are the types characterized in the small intestine of the guinea-pig (Furness, 2000). Ascending interneurons are essentially cholinergic, while the descending interneurons use as a neurotransmitters VIP, NO and some opioid peptides (Costa *et al.*, 2000). The three types of descending neurons express different chemical codings: ChAT/NOS/VIP, ChAT/SOM and ChAT/5-HT (Furness, 2000).

2.1.4.4. Secretomotor and vasomotor neurons

Secretomotor neurons are responsible for the control of secretions, while vasomotor neurons are responsible for the control of blood flow (Hansen, 2003). The majority of secretomotor neurons are projected to the mucosa (with their cell bodies in the mucosal plexus). Some few neurons are projected from the submucosa to the myenteric plexus (Furness, 2000). There are only 1% of these neurons in the myenteric ganglia, whereas most of them are located in the submucosous ganglia (32-42%) (Costa *et al.*, 2000). There are two classes of secretomotor neurons: cholinergic and non-cholinergic. The cholinergic neurons release ACh as a transmitter. ACh will act on muscarinic receptors located on mucosal epithelium. The non-cholinergic neurons release VIP and mediate most of the local reflex responses (Hansen, 2003; Costa *et al.*, 2000).

2.1.4.5. Intestinofugal neurons

Intestinofugal neurons are the single neurons that are projected from the myenteric ganglia to the sympathetic prevertebral ganglia (PVG), and that relay mechanosensory information to the sympathetic prevertebral ganglia neurons. They release ACh at the sympathetic prevertebral ganglia and evoke fast

excitatory postsynaptic potentials (fEPSPs) (Costa *et al.*, 2000; Szurszewski *et al.*, 2002).

Table 1: Morphologic, electrophysiological and functional characterization of enteric neurons (Furness, 2000; Hansen, 2003, Furness *et al.*, 2003, Sharkey *et al.*, 1998, Wong *et al.*, 2008)

FUNCTION	MORPHOLOGY	ELECTROPHYSIOLOGY	NEUROCHEMICAL CODING
Sensory (IPANs/EPANs)	Dogiel type II	AH	ChAT, Calb, CGRP, SP
Excitatory motor neurons	Dogiel type I	S	ChAT, SP, Calret
Inhibitory motor neurons			DYN, ENK, NOS, and VIP
Ascending interneurons	Dogiel type I/ II	AH/ S	ChAT, Calret, ENK, SP
Descending interneurons	Dogiel type I/ II	AH/ S	5-HT, DYN, GRP, NOS, somatostatin, and VIP
Secretomotor neurons (Cholinergic)	Type IV (Stach)	S	ChAT, CCK, CGRP, DYN, NYP, somatostatin, and VIP.
Secretomotor neurons (Non-Cholinergic)	Dogiel type I	S	
Intestinofugal neurons	Dogiel type I	S	ChAT, VIP, CCK, ENK, DYN, GRP

ChAT, coline acetyltransferase; **Calb**, calbindin; **CGRP**, calcitonin generelated peptide; **SP**, substance P; **DYN**, dynorphin; **GRP**, gastrin releasing peptide, **ENK**, enkephalins, **NOS**, nitric oxide synthetase; **VIP**, vasoactive intestinal polypeptide; **5HT**, 5-hydroxytryptamine; **NYP**, neuropeptide Y.

2.1.5. Enteric neurons in the longitudinal muscle-myenteric plexus (LM-MP)

The experimental work presented in this thesis is based on the use of the longitudinal muscle-myenteric plexus (LM-MP) preparation of rat ileum described by Paton and Vizi (1969). This preparation is highly enriched in cholinergic neurons, especially excitatory motor neurons that are projected to the LM (25%). They receive inputs from the IPANs (26%) and from the ascending and

descending interneurons (17%) (Costa *et al.*, 1996). Although ACh can be originated in the pre-ganglionic cholinergic nerve endings, this only represents a small proportion of the total content of ACh measured experimentally. This is due to the existence of an excessive number of fibers in the ganglion LM-MP compared to extrinsic pre-ganglionic fibers in the same preparation (Paton & Vizi, 1969).

2.2. Enteric Glial Cells

Enteric glial cells have huge morphological and molecular similarities to astrocytes found in the central nervous system (CNS). Enteric glial cells and astrocytes share electrophysiological properties and express two common proteins: the GFAP-intermediate filament glial fibrillary acidic protein and a calcium-binding protein S100 (Gulbransen & Sharkey, 2012).

Until very recently, it was thought that enteric glial cells had only a supportive function to neuronal cells. More recently, countless papers demonstrate that enteric glial cells are able to modulate enteric neural circuits, but their full role on this matter is yet to be defined experimentally. Mounting evidences also point towards enteric glial cells being neuroprotective and able to communicate with neighbouring neurons (Neunlist *et al.*, 2014; Boesmans *et al.*, 2013). They supply neurons with neurotransmitter precursors, release neuroactive substances (called gliotransmitters), express neurotransmitter receptors and respond to neurotransmitters/ neuromodulators, and have the machinery for sequestering and degrade neuroactive substances, namely ATP, glutamate, GABA (Rühl, 2005). In addition, these cells may exert a housekeeping role by expressing enzymes and transporters in order to control the availability of neuroactive substances that are toxic to the enteric neurons.

Around 1899, enteric glial cells in the gut were first described by Dogiel. They display an irregular, stellate-shaped, with an extensive array of highly branched processes. These cells present a small cell body, with a big nucleus occupying the all cell body leaving a little cytoplasmic volume (Gulbransen, 1994).

Enteric glial cells are present in all layers of the gut wall and are closely associated with neuronal cells within the ganglia, interganglionic fiber strands and intramuscular nerve fibres (Neunlist *et al.*, 2014). There are several types of enteric glial cells throughout the GI tract (figure 6).

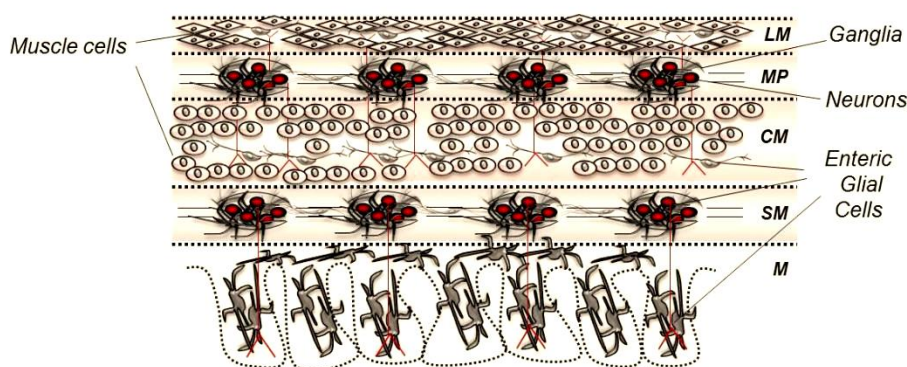


Figure 6: Distribution of enteric glial cells along the GI tract. They form a large and extensive network throughout all gut layers.

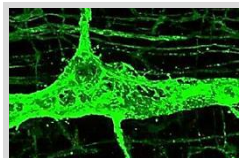
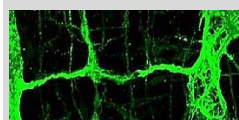
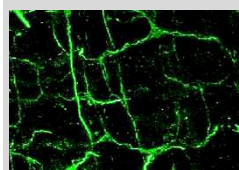
Hanani was the first to propose a classification for these cells, based only on morphological differences (Hanani and Reichenbach, 1994). He classified enteric glial cells in four types that are described in more detail in table 2 below.

To study enteric glial cells there are different markers, such as GFAP, S100 β and Sox-10 (transcription factor SRY box-containing gene 10) (Neunlist *et al.*, 2014). To the quantification of glial cells versus neurons, the preferential markers are Sox-10 (which labels the nucleus of glial cells) and HuC/D (which labels the cell bodies of neurons) (Boesmans *et al.*, 2013). However, nuclear stainings are unable to define cell boundaries where neurotransmitter receptors and ion channels are located and, therefore, GFAP and S100 β were preferred in this study (see Table 2). It is also possible to discriminate glial cells from enteric neurons by their functional characteristics. For instance, enteric neurons display a strong and fast Ca^{2+} response to high K^{+} depolarization, electrical field stimulation (EFS) and to the activation of nicotinic receptors with 1,1-dimethyl-4-phenylpiperazinium (DMPP), while enteric glial cells do not respond to these stimuli directly. They show delayed responses compared to neurons, which can be modulated by

several components of the purinergic signalling cascade (Boesmans *et al.*, 2013), namely NTPDase2 catalysing extracellular ATP hydrolysis and ADP formation favouring P2Y₁ receptor activation, and the danger signal represented by activation of the ionotropic P2X7 receptor secondary to high levels of ATP (reviewed in Gulbransen & Sharkey, 2012).

Despite these functional differences, our knowledge about the pathophysiological significance of enteric glial cells communication to neurons and *vice versa* is extremely limited although these cells are ideally positioned to participate in diverse aspects of GI functions. Regional particularities, intricate distribution and intimate relationship between enteric glial cells, neurons and other non-neuronal cell types like interstitial cells of Cajal and fibroblast-like cells (see below) do not facilitate the study of these complexities, but emphasize the importance of any single report concerning this issue.

Table 2: Types and localization of enteric glial cells (according to Boesmans *et al.*, 2015; Gulbransen & Sharkey, 2012).

Type of enteric glial cells	Localization	Image
Type I (protoplasmic enteric gliocytes)	Ganglia (between the neurons)	
Type II (fibrous enteric gliocytes)	Interganglionic	
Type III (mucosal enteric gliocytes)	Extraganglionic region along the nerve fibers; can also be present along small blood vessels	
Type IV (intramuscular enteric gliocytes)	Intramuscular (circular and longitudinal muscle), along the nerve fibers.	

Original immunofluorescence confocal microscopy images of enteric glia from LM-MP of the rat ileum using an antibody against calcium-binding protein S100 β (green) (obtained in collaboration with Doctor Fátima Ferreirinha).

2.3. Interstitial cells of Cajal

Interstitial cells of Cajal (ICC's) were discovered by Cajal around 1889. In that year, he thought that these cells were primitive neurons. But after more than 100 years of studies by several authors, it was shown that they were not neurons and do not originate from the neural crest (Garcia-Lopez *et al.*, 2009). These cells have been target of scientific discussion for years, because of their similarities with neurons and fibroblasts (Sanders *et al.*, 2014). The most important discover in this field was the expression of the tyrosine kinase receptor (c-Kit) at the surface of these cells; this receptor plays a crucial role in their development (Maeda *et al.*, 1992). Nowadays it is recognized that ICC's are of mesenchymal origin (Wards & Sanders, 2001). We also know that ICC's can be differentiated from other cells expressing high amounts of anoctamin 1, Ano-1 (Tmem16A), which is a calcium-activated chloride channel (Gomez-Pinilla *et al.*, 2009).

ICC's are dispersed around nerve plexuses and between smooth muscle fibres of the gut. They function as pacemaker cells, because of their important role in the control of spontaneous motility of the gut. ICC's are responsible for the propagation of electrical slow waves (Burnstock & Lavin, 2002). These cells have no basal lamina and form gap junctions with smooth muscle cells (SMC) (Komuro *et al.*, 1996).

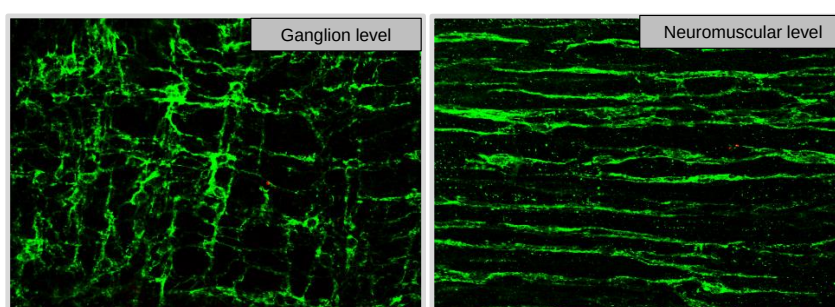


Figure 7: Original immunofluorescence confocal microscopy images of ganglionic (multipolar) and intramuscular (bipolar) ICC from LM-MP of the rat ileum using an antibody against Ano-1 (obtained in collaboration with Doctor Fátima Ferreirinha).

Depending of their location within the various layers of the GI tract, ICC's have a specific arrangement and cell shape (Komuro, 2006) (see table 3).

Table 3: Nomenclature, localization and morphology of ICC (Komuro, 2006)

Type of ICC	Localization	Morfology
<i>ICC's of the subserosa (ICC-SS)</i>	In the subserosal layer.	Stellate cells
<i>ICC's of longitudinal muscle (ICC-LM)</i>	Barely distributed in association with rather thicker nerve bundles.	Bipolar cells
<i>ICC's of the myenteric plexus (ICC-MP)</i>	Around the myenteric plexus in the space between the circular and longitudinal muscle layers.	Multipolar cells
<i>ICC's of the circular muscle (ICC-CM)</i>	Along the long axis of surrounding smooth muscle cells.	Bipolar cells
<i>ICC's of the deep muscular plexus (ICC-DMP)</i>	Closely associated with the nerve bundles and circular muscle fibers.	Multipolar cells
<i>ICC's of the submucosa and submucosal plexus (ICC-SM and ICC-SMP)</i>	At the interface between the submucosal connective tissue and the innermost circular muscle layer.	Multipolar cells

2.4. Fibroblast like cells (FLC)

FLC are another mesenchymal cell type, which is described as a kind of interstitial cells located on the smooth muscle layer of the GI tract (Kim, 2011). FLC are located along processes of enteric neurons, between the longitudinal and circular muscle layers, in juxtaposition with ICC's, and in the subserosal layer. These cells are in the vicinity of nerve varicosities and can also form gap junctions with SMCs (Kurahashi *et al.*, 2011; Iino & Nojyo, 2009). The gap junctions between FLC and SMCs are smaller than those found between ICC's and SMCs (Fujita *et al.*, 2003). This proximity suggests that FLC can be regulated by enteric nerves and transmit electrical and molecular signals to the smooth muscle fibres and, ultimately, influence muscle tone (Fujita *et al.*, 2003; Iino & Nojyo, 2009). The phenotypic characterization of FLC implies the presence of SK3, a small conductance Ca^{2+} -activated K^{+} channel (SK channel) (Fujita *et al.*, 2003), of the

platelet-derived growth factor receptor α (PDGFR- α) (Kurahashi *et al.*, 2011), and the cell-cell adhesion factor, CD34 (only found in humans) (Iino & Nojyo, 2009).

According to their morphology, FLC found in the myenteric layer are multipolar with thin processes, while FLC found in the muscle layers (circular and longitudinal) are bipolar with two main processes (Iino & Nojyo, 2009). SMCs are electrically coupled to ICC and PDGFR α -positive cells, forming an integrated unit called the SIP syncytium (figure 8) (Sanders *et al.*, 2014). SIP cells are known to provide pacemaker activity, propagation pathways for slow waves, transduction of inputs from motor neurons, and mechanosensitivity. Loss of interstitial cells has been associated with motor disorders of the gut. Nevertheless, in most cases, the physiological and pathophysiological roles for SIP cells have not been clearly defined.

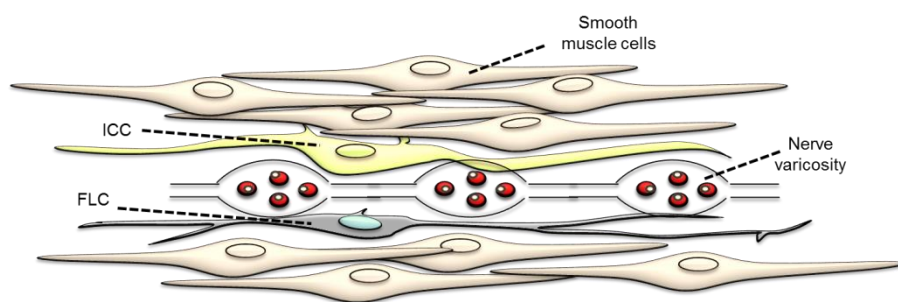


Figure 8: SIP syncytium, constituted by SMCs, ICC's and FLC's.

2.5. Smooth muscle cells

SMCs are responsible for the contractility in the GI tract. They have the main function to mix and propel intraluminal contents, to make a progressive absorption of nutrients and evacuation of residues. These functions are modulated by electrical and mechanical properties of these cells. SMC are constituted by three types of filaments: (1) thin actin filaments; (2) thick myosin filaments; (3) intermediate filaments (Bitar, 2003).

As reported previously, SMCs are connected by gap junctions to other cells, like ICC's and FLC's, forming the SIP syncytium. Thus, the contractile activity

produced by SMCs may occur via a mechanism called electromechanical coupling or via a pharmacomechanical coupling.

The first one, the electromechanical coupling, occurs through several steps represented in the following sequence:

- (1) Depolarization of membrane electrical potentials;
- (2) Opening of voltage-gated calcium channels;
- (3) Elevation of cytosolic calcium;
- (4) Activation of contractile proteins.

The second one, the pharmacomechanical coupling, occurs when chemical substances released as signals from nerves or from non-neuronal cells located near to the muscle act as a ligand to its receptor on the muscle membrane. As a consequence of that occurs the following sequence:

- (1) Opening of calcium channels;
- (2) Elevation of cytosolic calcium, without any change in the membrane electrical potential.

3. Purinergic Transmission

The first description of the extracellular action of adenine nucleotides and nucleosides was published in 1929 by Drury and Szent-Györgyi. These authors studied the effects of purines in the cardiovascular system of mammals. Despite of the clearness of the evidences, the recognition of the physiological role of purines as regulators of intercellular communication has generated some resistance for many years. In 1972, Burnstock and his collaborators were able to definitively establish the importance played by adenosine triphosphate (ATP). He coined the terms "purinergic" (relative to the extracellular mechanisms mediated by purines) and "purinergic transmission" (denoting to the role of ATP as a neurotransmitter) (Abbracchio & Burnstock, 1998). Dale's principle described that the neurons can synthesize, store and release substances. According to Dale's principle it was thought that each neuron released only one neurotransmitter (reviewed in Otsuka,

1988). Around 1976, Burnstock introduced the cotransmitter hypothesis; he demonstrated that ATP is a cotransmitter in the most nerves with classical neurotransmitters. Nowadays, it is recognized that ATP is released with ACh, NE, glutamate, GABA, 5-HT and DA in different types of neurons in the CNS (Burnstock, 2008). ATP can be accumulated into vesicles mediated by a Cl⁻ dependent vesicular nucleotide transporter (VNUT) (Abbracchio *et al.*, 2008).

Although, the concept that ATP is only stored within synaptic vesicles and released by exocytosis from nerve terminals and some non-neuronal cells upon stimulation, has been changing over the past few years. ATP can also be released by other mechanisms, as follows:

- (1) ATP can be released through the activation of ABC proteins (ATP-binding cassette transporters) (Linton, 2007). This mechanism remains a subject of controversy. Even though some studies reported that there are systems that express these ABC proteins capable of transporting ATP, others showed the inability of these proteins to carry this purine. ABC proteins are the cystic fibrosis transmembrane conductance regular (CFTR) (Bodin & Burnstock, 2001).
- (2) ATP can be released *via* connexin- and pannexin-containing hemichannels (Abbracchio *et al.*, 2008). The majority of hemichannels display permeability to ATP and small dyes, like propidium and ethidium iodide, YoPro and Lucifer yellow. Membrane depolarization can promote the opening of connexins and pannexins, but unlike pannexin-1, connexin hemichannels close at normal millimolar Ca²⁺ concentrations (e.g. 1.8 mM CaCl₂ in physiological solutions) and open under Ca²⁺ depletion conditions (Fasciani *et al.*, 2013). This particularity leaves room for pannexin-1 hemichannels playing a dominant role in ATP translocation under normal physiological conditions. Pannexins and connexins have a similar protein structure: four transmembranar domains, with N and C-terminal located in the cytosol (Lazarowski *et al.*, 2011).
- (3) Huge amounts of ATP can be released from damaged or dying cells upon membrane cell breaking events.

ATP released from cells is quickly hydrolysed by ectonucleotidases (ecto-enzymes) present in the synaptic cleft, giving rise to biologically active metabolites, such as ADP, AMP and adenosine (ADO) (Zimmermann, 2000). Burnstock, in 1978, reported that the physiological effects of adenosine (ADO), ATP and ADP were mediated by ADO-sensitive P1 receptors and nucleotides-activated P2 receptors (Abbrancchio & Burnstock, 1998).

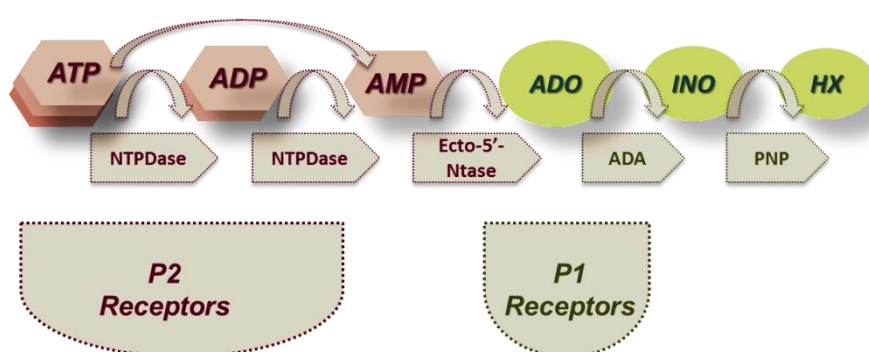


Figure 9: Purinergic signalling pathways. ATP is sequentially metabolised into ADP and AMP, by NTPDases. AMP is then hydrolysed into ADO via ecto-5'-nucleotidase. ADO is deaminated into inosine (INO) by adenosine desaminase (ADA). The nucleosides can activate P1 receptors and the nucleotides can activate P2 receptors.

3.1. Ectonucleotidases

Extracellular nucleotides (ATP, ADP and AMP) are hydrolysed into nucleosides (ADO) through the action of ectoenzymes. The product final of this metabolic chain, ADO, can be further inactivated by deaminase adenosine (ADA) into INO. Ectonucleotidases include several members of ENTPDase (ecto-nucleoside triphosphate diphosphohydrolases), E-NPP (ecto-phosphodiesterase/nucleotide phosphohydrolases), ecto-alkaline phosphatase and ecto-5'-nucleotidase (CD73) (Robson *et al.*, 2006). The distribution of these enzymes throughout tissues is very diverse. They are usually anchored to the membrane, but some of them can detach from their membrane link and keep activity in the soluble form, being called exonucleotidases (Zimmermann, 2000). NTPDases are

generally cell surface-located enzymes with an extracellular catalytic site, but some of them can exhibit an intracellular localization (Robson *et al.*, 2006). Nucleoside triphosphates (ATP and UDP) and diphosphates (ADP and UDP) are desphosphorylated by NTPDases in the presence of divalent cations (Ca^{2+} and Mg^{2+}) (Kukulski *et al.*, 2005).

Eight NTPDases (NTPDase 1 to 8) have been discovered, cloned and characterized functionally. NTPDases 1, 2, 3 and 8 are expressed on cell surface. NTPDases 5 and 6 are also expressed intracellularly. NTPDases 4 and 7 are located exclusively intracellularly (Yegutkin, 2008). NTPDases 4, 5, 6 and 7 are associated with intracellular organelles, such as the Golgi (Cardoso *et al.*, 2015). In this work, we will focus in four the ENTPDases that are located on the cell surface. ENTPDases are extremely important to control signalling through P2 receptors and they can influence ADO formation rate and, subsequently, P1 receptors activation (Kukulski *et al.*, 2005).

Ecto-5'-nucleotidase is an ecto-enzyme responsible for the hydrolysis of AMP into ADO and phosphate. This enzyme is considered the limiting step for ADO formation in the majority of the tissues (Cardoso *et al.*, 2015). Ecto-5'-nucleotidase is attached to the plasma membrane via a glycosyl phosphatidylinositol (GPI) anchor, which may be cleaved from cell membranes through hydrolysis of the GPI anchor by phosphatidylinositol-specific phospholipases or by proteolysis, retaining its catalytic activity in the soluble form (Sträter, 2006). Ecto-5'-nucleotidase, in both membrane and soluble forms, can be inhibited by micromolar concentrations of ATP and ADP - feed-forward inhibition (Vieira *et al.*, 2014). These nucleotides act as competitive inhibitors of this enzyme because they have the ability to bind to the catalytic site of ecto-5'-nucleotidase without being hydrolysed.

Adenosine deaminase (ADA) is another important enzyme that is located predominantly in the cytosol, but can also appear bound to the plasma membrane in inumerous cells. The irreversible deamination of ADO and 2'-deoxyadenosine into INO and 2'-deoxyinosine, respectively, is catalysed by ADA (Yegutkin, 2008).

Like ecto-5'-nucleotidase, active soluble forms of ADA (exo-ADA) may be released from activated cells (Correia-de-Sá *et al.*, 2006) or result from membrane cell damage (Vieira *et al.*, 2014). ADO acts in all P1 receptor subtypes, while INO can activate A₁ and A₃ adenosine receptors under certain conditions (Cardoso *et al.*, 2015).

Alkaline phosphatases (AP) represent a family of enzymes with low affinity for nucleotides. They are usually attached to the plasma membrane via a GPI anchor (Zimmermann, 2000). These enzymes promote the release of inorganic phosphates, pyrophosphates and adenine nucleosides, with a pH optimum for this catalytic reaction from 8 to 11 (Yegutkin, 2008).

The ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family has seven structurally-related ecto-enzymes (NPP1-NPP7). They hydrolyse 5'-phosphodiester bonds in nucleotides and their derivatives, with the subsequent release of 5'-nucleoside monophosphates (Goding *et al.*, 2003). This family has a high substrate specificity to hydrolyse pyrophosphate and phosphodiester bounds in nucleotides, nucleic acids and nucleotide sugars. However, only the first three enzymes of this family (NPP1-NPP3) are relevant in the hydrolysis of extracellular nucleotides, being important to the purinergic signalling cascade. NPP enzymes are considered really important for the metabolism of extracellular diadenosine polyphosphates in vertebrate tissues (Yegutkin, 2008). Structurally, they are type II transmembrane metalloenzymes, characterized by a short intracellular domain N-terminus, a single transmembrane domain and an extracellular domain containing a conserved catalytic site (Zimmermann, 2000; Goding *et al.*, 2003). These enzymes can act as ecto-enzymes, anchored to the cellular membrane, or upon proteolytic cleavage from cell membranes as exo-enzymes (Goding *et al.*, 2003).

Table 4: Role of enzymes of the purinergic cascade and their molecular characteristics (Yegutkin, 2008; Cardoso *et al.*, 2015; Kukulski *et al.*, 2005; Vieira *et al.*, 2014; Robson *et al.*, 2006).

ENZYME	STRUCTURE	FUNCTION
ENTPDase 1 (ATPDase, CD39 or Apyrase)	Contain two transmembranar domains at the N- and C-terminus	Dephosphorylates ATP directly into AMP, with minimal accumulation of ADP; Hydrolyses ATP, ADP, UTP and UDP with similar efficacy; Converts ATP to ADP and ADP to AMP; Active in pH ranging from 7 to 10.
ENTPDase 2 (ecto-ATPase, CD39L1)	Contain two transmembranar domains at the N- and C-terminus	Preferential triphosphonucleosidase; Hydrolyses ADP 10 to 15 times less efficiently than ATP; Converts ATP and UTP to ADP and UDP; Active at physiological pH as well in acidic conditions. Is inactive at pH > 9
ENTPDase 3 (CD39L3 or HB6)	Contain two transmembranar domains at the N- and C-terminus	Functional intermediate between NTPDase 1 and 2; Active in pH ranging from 5 to 11.
ENTPDase 8	Contain two transmembranar domains at the N- and C-terminus	Functional intermediate between NTPDase 1 and 2.
Ecto-5'-Nucleotidase (CD73)	Contain a glycosyl phosphatidylinositol (GPI) anchor, by which the mature protein is attached to the cell membrane via the C-terminal serine residue linked to a complex oligoglycan and a sphingolipidinositol group.	Dephosphorylates AMP into ADO. Usually the rate limiting step for ADO formation from released adenine nucleotides.
ADA (CD26)	More than 90% intracellular It is also located at the cell surface.	Converts ADO to the inactive metabolite, INO.

3.2. Purinergic receptors

Adenine nucleotide and nucleosides modulate cell functions through the activation of receptors bound to the plasma membrane. Purinoceptors are subdivided in P1 and P2 receptors. P1 receptors are preferentially activated by ADO, while P2 receptors are activated by both adenine (ATP, ADP, adenosine polyphosphates) and uracil (UTP, UDP) nucleotides. Receptor classification has been coined by Burnstock (1978) and further modified to the actual nomenclature

adopted by the *International Union of Pharmacology* (IUPHAR) (Abbrachio & Burnstock, 1998).

3.2.1. Adenosine Receptors (P1 receptors)

Adenosine receptors are classified in four subtypes, A_1 , A_{2A} , A_{2B} and A_3 , based on molecular cloning, pharmacologic profile and their ability to couple with different second messenger pathways, including the adenylyl cyclase cyclic AMP system (table 5). A_1 and A_{2A} receptors exhibit high affinity for ADO (activated by low ADO amounts), while A_{2B} and A_3 possess low affinity for the nucleoside and require higher levels of ADO to become activated, at least in rodents (Fredholm *et al.*, 2001). It has been proposed that high-affinity P1 receptors are predominantly activated under physiological processes, while the low-affinity receptors may be relevant only during pathological conditions, when the extracellular ADO levels become elevated (Antoniolli *et al.*, 2008; Bozorov *et al.*, 2009). All adenosine receptors belong to the family of G protein-coupled receptors (GPCR). Structurally, they show a seven transmembrane α -helical, with an extracellular amino-terminus and an intracellular carboxy-terminus (Fredholm *et al.*, 2001; Sheth *et al.*, 2014).

Table 5: Classification of adenosine receptors, based on their differential coupling to second messenger transduction systems (Fredholm *et al.*, 2001; Sheth *et al.*, 2014)

ADENOSINE RECEPTORS	G PROTEIN COUPLING (PRIMARY)	EFFECTS OF G PROTEIN COUPLING
A_1 (High affinity)	$G_{i/o}$ proteins	Adenylyl cyclase inhibition; $\downarrow$ cAMP Inhibition of Ca^{2+} currents Increase in GIRK currents
A_{2A} (High affinity)	G_s proteins	Adenylyl cyclase activation; $\uparrow$ cAMP
A_{2B} (Low affinity)	G_s proteins/ $G_{q/11}$ proteins	Adenylyl cyclase activation; $\uparrow$ cAMP, Stimulation of phospholipase C / IP_3 /diacylglycerol; $\uparrow$ IP_3 /DAG (PLC)
A_3 (Low affinity in rodents, high affinity in humans)	$G_{i/o}$ proteins/ $G_{q/11}$ proteins	Adenylyl cyclase inhibition; $\downarrow$ cAMP/ Stimulation of phospholipase C / IP_3 /diacylglycerol; $\uparrow$ IP_3 /DAG (PLC)

As all other metabotropic receptors, activation of adenosine receptors can lead to desensitization by several mechanisms. The A_1 receptor may be phosphorylated and internalized gradually, with a half-life of several hours; A_{2A} and A_{2B} receptors desensitization lasts about one hour, and that of the A_3 receptor recovers within a few minutes (Sheth *et al.*, 2014).

3.2.1.1. Adenosine

Production, metabolism and transport are the three main pathways for generating ADO in living cells. ADO can be produced intracellularly and extracellularly. ADO produced inside nerve cells is originated from its immediate precursor, the 5'-adenosine monophosphate (5'-AMP), through the action of intracellular 5'-nucleotidase. Then, ADO can either be inactivated into INO by ADA (Sheth *et al.*, 2014) or transported to the extracellular milieu, via equilibrative nucleoside transporters (ENT) (figure 10).

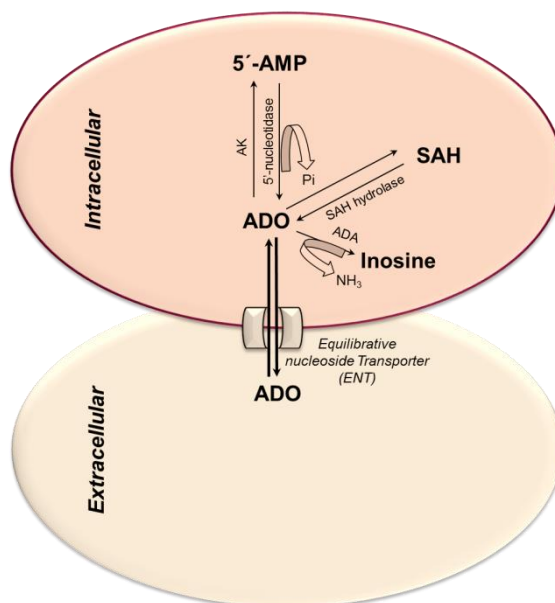


Figure 10: Intracellular production of ADO.

Extracellularly, ADO can be generated from the catabolism of ATP co-released with classical neurotransmitters. Released ATP is sequentially hydrolyzed by ecto-nucleotidases into ADP, AMP and, finally to ADO (figure 11). All these metabolites can act on membrane-bound purinoceptors to modulate the activity of

both neuronal and non-neuronal cells in the vicinity (Sheth *et al.*, 2014; Latini & Pedata, 2001).

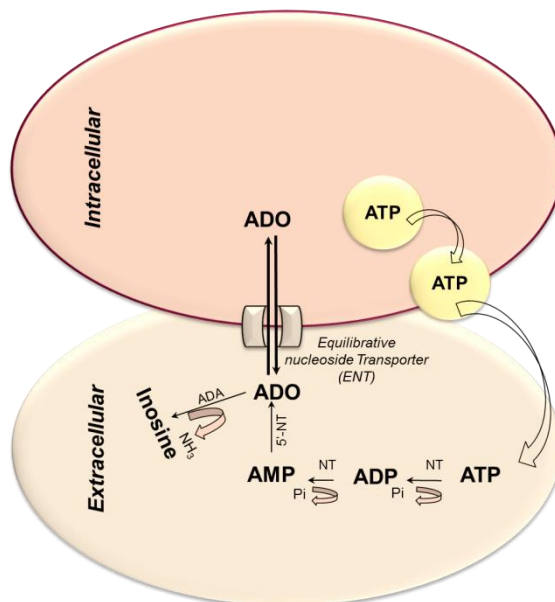


Figure 11: Extracellular production of ADO.

Reduction of ADO levels can occur in three different ways. Inside the cell, ADO can either be phosphorylated to AMP by adenosine kinase (AK) or deaminated into INO by ADA. Outside the cell, ADO is inactivated to INO through the action of adenosine deaminase (ADA) (figure 10 and 11), because AK is located exclusively in the cytoplasm. This enzyme presents a higher affinity for ADO (10 to 100 times) than ADA, which makes ADO phosphorylation the preferential pathway responsible for maintaining low intracellular concentrations of the nucleoside under normal physiological conditions. Intracellular deamination of ADO by ADA only becomes relevant when cytoplasmic levels of ADO rise and the phosphorylation pathway becomes saturated (Latini & Pedata, 2001). Thus, inhibition of both AK and ADA results in the increase of intracellular ADO levels forcing the exit of the nucleoside via equilibrative transporters. The same occurs in metabolically stressed cells, in which decreases in the cellular energy charge is

accompanied by extensive catabolism of adenine nucleotides leading to increased formation of ADO.

The third pathway used for intracellular ADO inactivation is the reversible reaction catalysed by S-adenosyl-L-homocysteine hydrolase (SAH hydrolase) requiring NAD^+ as a cofactor. This reaction results in the formation of S-adenosyl-L-homocysteine from ADO and L-homocysteine (Latini & Pedata, 2001).

3.2.1.2. Adenosine Transport

ADO transport across the plasma membrane is really important to regulate the extracellular ADO levels. Nucleoside transporters (NT) are members of the superfamily of solute carrier (SLC) transporters (Köse & Schiedel, 2009). In the literature, are described two broad families of adenosine transporters: (1) SLC29 family of equilibrative nucleoside transporter (ENT), which are facilitated-diffusion nucleoside transporters (sodium independent manner), and (2) SLC28 family of concentrative nucleoside transporters (CNT), which transport nucleosides actively against their gradient (sodium dependent manner) (Thorn & Jarvis, 1996; Köse & Schiedel, 2009).

Four types of ENTs exist, which are classified as ENT1, ENT2, ENT3 and ENT4. All these members of the SLC29 family share the ability of transport ADO, but they differ in the ability of transport nucleosides and nucleobases (Thorn & Jarvis, 1996; Yao *et al.*, 1997; Köse & Schiedel, 2009).

Classically, ENTs were classified based in their sensitivity to the inhibition by the synthetic nucleoside analogue, nitrobenzylthioinosine (NBTI). ENT1 (equilibrative-sensitive transporters, es) are inhibited at low nanomolar concentrations of NBTI and dipyridamole, while ENT2, ENT3 and ENT4 (equilibrative-insensitive transporters, ei) are inhibited only by micromolar concentrations of NBTI (Latini & Pedata, 2001). ENT1, ENT2 and ENT4 can be found at the plasma membrane, while ENT3 is mostly intracellularly (Köse & Schiedel, 2009).

Concerning the SLC28 family of CNT, three members were identified, CNT1, CNT2 and CNT3. They differ in their substrate specificities, being CNT1 more specific for pyrimidine-nucleosides, CNT2 for purine-nucleosides and CNT3 for both pyrimidine and purine nucleosides (Gray *et al.*, 2004).

3.2.2. P2 Receptors

Extracellular purine and pyrimidine nucleotides (ATP, UTP, ADP, UDP) are originated in response to tissue stress, cell damage, and pore formation, but can also be released from non-damaged cells through several mechanisms, including vesicle exocytosis (Lazarowski *et al.*, 2011; Bodin & Burnstock, 2001). They exert their action through the activation of two different families of P2 receptors, namely P2X and P2Y receptors. P2X receptors are trimeric ligand-gated ionotropic channels (LGIC), while P2Y receptors are metabotropic G-protein-coupled receptors (GPCR). Cellular responses to ionotropic P2X receptors are faster than to activation of P2Y receptors (Jacobson, 2010; Burnstock & Williams, 2000; Abbrachio *et al.*, 2006).

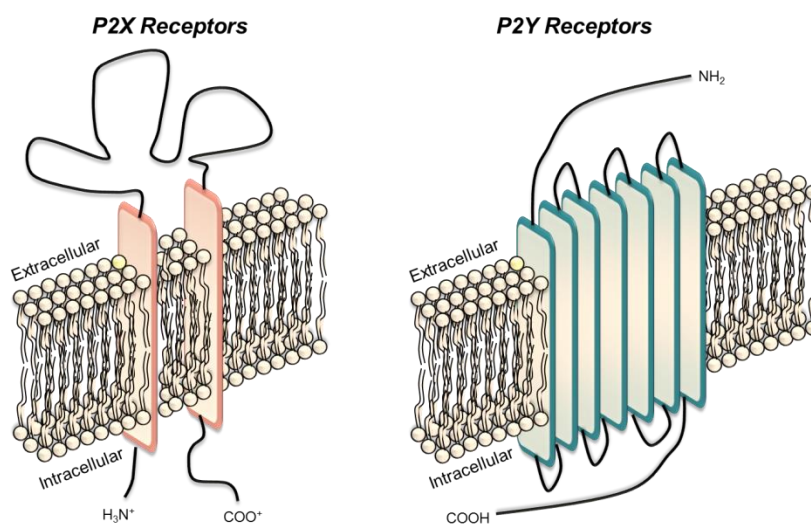


Figure 12: Schematic representation showing the membrane topology of a typical P2X and P2Y receptor. P2X receptors are ion channels with two transmembrane domains, while P2Y receptors are G-protein-coupled receptors with seven transmembrane domains.

3.2.2.1. P2X Receptors

There are 7 subtypes of P2X receptors: P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 and P2X7. Activation of P2X receptors by binding to extracellular ATP generates an influx of cations, such as sodium and calcium, leading to depolarization of excitable cells and activation of cytosolic enzymes (Jacobson, 2010). In neurons, they modulate neurotransmitter release acting presynaptically or trigger fast excitatory signals in the postsynaptic membrane (Kaczmarek-Hájek *et al.*, 2012).

The subcellular distribution of the seven P2X receptor subtypes may differ from cell to cell. Most of them are expressed at the plasma membrane, but some of them may be in the endoplasmic reticulum (ER) or within endosomes and lysosomes. Localization of these receptors depends on their trafficking properties (Robinson & Murrell-Lagnado, 2013). P2X receptors are able to form heteromers and homomers (Jacobson, 2010). All P2X receptor subunits can form homomers, with the exception of the P2X6; the same occurs regarding heteromer formation, the exception in this case is the P2X7 receptor subtype (Ren & Bertrand, 2008). All these receptors have a similar topology consisting of two transmembrane domains, a large extracellular ligand binding loop, and intracellular N and C terminals (Kaczmarek-Hájek *et al.*, 2012).

All subtypes of P2X receptors are activated by ATP, but they can be distinguished by the required concentrations of the nucleotide. Based on that, P2X receptors can be grouped in three main classes (Jacobson, 2010; Burnstock & Williams, 2000):

- (1) Receptors with high affinity for ATP ($EC_{50}=1\ \mu\text{M}$), which are rapidly activated and desensitized: P2X1 and P2X3 receptors.
- (2) Receptors with lower affinity for ATP ($EC_{50}=10\ \mu\text{M}$), showing slow desensitization and sustained depolarizing currents: P2X2, P2X4, P2X5 and P2X6 receptors.
- (3) Receptors with very low affinity for ATP ($EC_{50}=300\text{-}400\ \mu\text{M}$), exhibiting little or no desensitization and capable of forming large pores: P2X7 receptors.

3.2.2.2. P2Y Receptors

Purine nucleotides (ATP and ADP) and pyrimidine nucleotides (UTP and UDP and UDP-glucose) activate metabotropic P2Y receptors. Abbracchio and collaborators (2006) described eight distinct mammalian subtypes of P2Y receptors, namely P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄.

According to the pharmacological profile, the P2Y receptors can be subdivided into four main groups:

- (1) Adenine nucleotide-preferring receptors, responding essentially to ATP and ADP: P2Y₁, P2Y₁₂, P2Y₁₃ and human P2Y₁₁;
- (2) Uracil nucleotide-preferring receptors, responding respectively to UTP and UDP: P2Y₄ and P2Y₆;
- (3) Receptors with mixed selectivity: P2Y₂, P2Y₄ and P2Y₁₁;
- (4) Receptors responding to sugar nucleotides, UDP-glucose and UDP-galactose: P2Y₁₄.

Table 6: Main characteristics of P2Y receptor subtypes: agonist preference and G protein-coupling (Abbracchio *et al.*, 2006; White *et al.*, 2003).

Receptor	Agonist (preferential)	Primary G protein coupling/ Second Messenger
P2Y ₁	ADP (ATP partial agonist)	G _{q/11} ; PLC activation; G _{i/o} ; Potassium channel
P2Y ₂	UTP=ATP	G _{q/11} ; PLC activation
P2Y ₄	UTP>ATP	G _{q/11} ; PLC activation
P2Y ₆	UDP>>UTP (partial agonist)	G _{q/11} ; PLC activation
P2Y ₁₁	ATP>UTP	G _s ; AC stimulation; G _{q/11} ; PLC activation
P2Y ₁₂	ADP (selective)	G _{i/o} ; AC inhibition
P2Y ₁₃	ADP>>ATP	G _{i/o} ; AC inhibition
P2Y ₁₄	UDP>UDP-glucose/UDP galactose	G _{i/o} ; AC inhibition; G _{q/11} ; PLC activation; can activate PLCβ via beta/gamma subunits.

4. Cholinergic Transmission

Acetylcholine (ACh) is the prime neurotransmitter of autonomous nervous system and its release originates a type of transmission, entitled cholinergic. The discovery of pharmacological effects of ACh was done in studies in adrenal glands by Reid Hunt around 1900. In 1914, in a study of the pharmacological actions of ACh, Dale distinguished two types of activity, muscarinic and nicotinic. When injecting muscarine (active ingredient of mushroom *Amanita muscaria*) in animals he reproduced the same effects of ACh. These effects were blocked with small doses of atropine (poisonous constituent of *Atropa belladonna*). After blocking the muscarinic effects with atropine, higher doses of ACh produced similar effects to nicotine (reviewed in Rang *et al.*, 2004).

In 1953, Dale was the responsible for the establishment of the term cholinergic to refer to any neuron that releases ACh as a neurotransmitter.

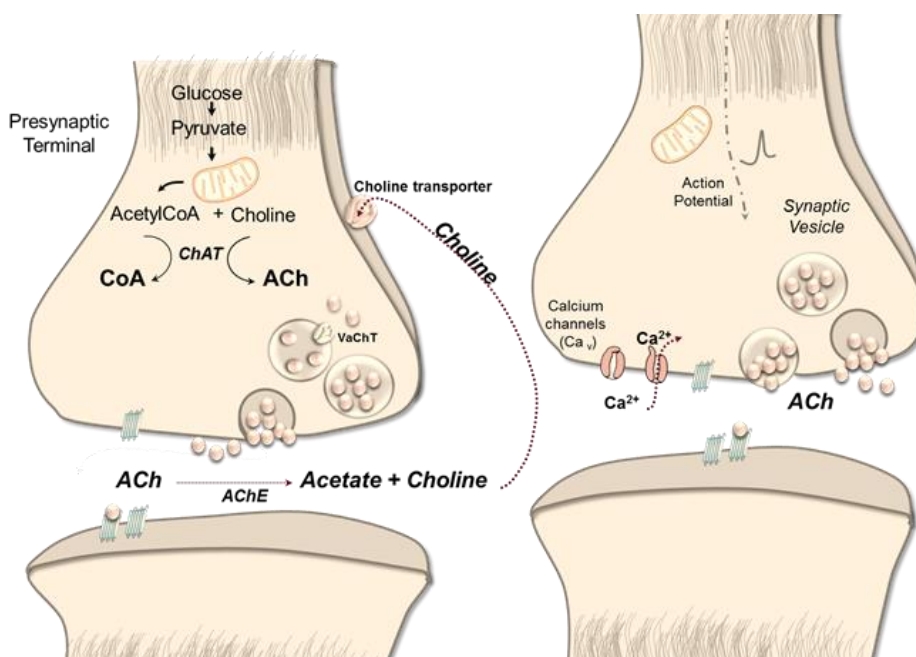


Figure 13: ACh metabolism in cholinergic nerve terminals. ACh is synthesized from choline and acetyl-coenzyme A (acetyl-CoA) through the action of ChAT. ACh is stored in synaptic vesicles by means of a vesicular ACh transporter (VaChT). Following its release into the synaptic cleft, ACh is metabolized by the acetylcholinesterase (AChE). Choline originated from ACh inactivation by AChR is re-uptaken by the terminal through a high-affinity sodium-dependent choline transporter. ACh exocytosis occurs when an action potential reaches the presynaptic nerve terminal, which promotes calcium influx via voltage-sensitive channels and, consequently, vesicles fusion to the plasma membrane releasing the neurotransmitter.

ACh is synthesized in axonal terminals of cholinergic neurons by the action of ChAT. ChAT catalyses the reaction between acetyl coenzyme-A (acetyl-CoA) and choline. ACh and coenzyme-A (CoA) result as reaction products. Acetyl-CoA is originated in the mitochondria from the complete oxidation of pyruvate. The formation of acetyl-CoA is catalyzed by an enzyme complex called pyruvate dehydrogenase complex. Choline that is needed for the synthesis of ACh comes from various sources, including the circulating choline in the plasma, the hydrolysis of phospholipids of cell membranes and from the recycling of choline resulting from the action of acetylcholinesterase (AChE) on ACh released into the synaptic cleft. Reuptake of choline is mediated by a concentrative transporter existing in cholinergic nerve terminals, which exhibits high-affinity for choline (K_m between 1 and 5 μM) and is dependent on temperature and transmembrane Na^+ gradient. Synthesis of ACh is limited by the availability of choline. Synthesized ACh is transported into synaptic vesicles by an antiport mechanism. When an action potential reaches nerve terminals, it triggers the influx of Ca^{2+} from the extracellular milieu via voltage-sensitive channels, which promotes the fusion of synaptic vesicles to the plasma membrane and, consequently, the exocytotic release of ACh to the synaptic cleft (Rang *et al.*, 2004). Once in the extracellular space, released ACh may activate two subtypes of cholinergic receptors, muscarinic (mAChR) and nicotinic (nAChR).

5. Purinergic and cholinergic transmission in the Myenteric Plexus

ACh is the prime regulator of intestinal motility and the dominant excitatory neurotransmitter of the myenteric plexus (Costa *et al.*, 1996). In this tissue, ATP may be co-released with ACh, but also with other neurotransmitters (White, 1982). Both excitatory and inhibitory effects may result from activation of purinoceptors by ATP and its metabolites, ADP and ADO, depending on the receptor subtype being activated, their location, and target cells.

ACh release from cholinergic enteric nerve terminals is modulated by purines via the activation of both P1 and P2 purinoceptors, which can promote or inhibit ACh release (De Man *et al.*, 2003).

5.1. P1 Receptors

All subtypes of P1 receptors (adenosine A₁, A_{2A}, A_{2B} and A₃ receptors) were found in whole-mount preparations of the LM-MP of rat ileum (Vieira *et al.*, 2011). Results from this study demonstrate substantial heterogeneity in the distribution of P1 receptors in this tissue. Concerning high-affinity adenosine receptor subtypes, the adenosine A₁ receptor is predominantly located in neuronal cell bodies of myenteric ganglia, while the A_{2A} receptor is expressed mainly on VACHT-positive cholinergic nerve fibres. Regarding to the low affinity adenosine receptor subtypes, our group showed that the A_{2B} receptor is dominantly expressed on myenteric glial cells, while the A₃ receptor is co-localized with the A₁ receptors on neuronal cell bodies of myenteric ganglia.

From the four adenosine receptor subtypes found in the LM-MP of the rat ileum, only the A_{2B} receptor is not directly involved in cholinergic neurotransmission. Besides the well-characterized inhibitory effect of ADO in the GI tract mediated by A₁ receptors, our group demonstrated that endogenously generated ADO facilitates [³H]ACh release from myenteric neurons through preferential activation of prejunctional A_{2A} receptors (Duarte-Araújo *et al.*, 2004a). Upregulation of ACh release was also obtained by stimulating low affinity A₃ receptors localized at myenteric cell bodies way from the synaptic region, which activation may facilitate action potential generation; synergism between A₃ receptors activation and the A_{2A} facilitatory receptors localized on axon terminals, has been observed (Vieira *et al.*, 2011).

The co-existence of high-affinity inhibitory A₁ and excitatory A_{2A} receptor subtypes on cholinergic neurons prompted the question of how endogenous ADO discriminates between these two receptors to regulate synaptic transmission in the myenteric plexus of the rat ileum. At the rat myenteric plexus, ADO may be originated from the extracellular catabolism of released adenine nucleotides through the ectonucleotidase pathway (Duarte-Araújo *et al.*, 2009). However, hydrolysis of released ATP contributes only partly to the total extracellular concentration of the nucleoside; the other component is probably carried out by

the outflow of ADO as such via dipyridamole-sensitive ENTs (Correia-de-Sá *et al.*, 2006). Results from these studies also demonstrate that extracellular ADO deamination by ADA secreted into the extracellular milieu (Correia-de-Sá *et al.*, 2006) is the most efficient mechanism to regulate the endogenous levels of the nucleoside at the myenteric synapse (Duarte-Araújo *et al.*, 2004a). Thus, extremely high extracellular ADA activity, along with a less-efficient ADO uptake mechanism, may restrict endogenous ADO actions to the synaptic region near the release/production sites channelling to the activation of facilitatory A_{2A} receptors (Duarte-Araújo *et al.*, 2004a; Correia-de-Sá *et al.*, 2006).

5.2. P2X Receptors

P2X receptors are expressed on myenteric and submucosal motor, sensory, and secretomotor neurones; they are also found on enteric glial cells, ICCs and smooth muscle fibres (Burnstock, 2012) (see table 7).

In the myenteric plexus, ATP that acting on P2X receptors contributes to facilitate electrically-evoked fast excitatory postsynaptic potentials (fEPSPs) (Galligan, 2002). fEPSPs in the myenteric plexus of guinea-pigs have a dominant purinergic component (67%) mediated by P2X2 and P2X3 receptor subtypes. Purinergic fEPSPs are more evident in the ileum when compared with other regions of the intestine. P2X2 receptors (20%) and P2X3 receptors (80%) are also located in intrinsic sensory neurons (Ren & Bertrand, 2008).

Functional interactions between P2X and nicotinic ACh receptors have been described. Both ionotropic receptors are co-expressed in myenteric neurons, indicating that their interaction might be physiologically relevant (Ren & Bertrand, 2008). In this context, our group showed that ATP transiently activates facilitatory P2X receptors mediating non-stimulated ACh release from myenteric motor neurons (Duarte-Araújo *et al.*, 2009).

Burnstock & Lavin (2002) identified P2X2 and P2X5 receptors in ICCs of the guinea-pig myenteric plexus, but no immunostaining was detected for P2X₁, P2X₃, P2X₄, P2X₆ or P2X₇. These authors proposed that ATP released from

enteric nerves, enteric glial cells or during smooth muscle contractions can activate P2X2 or P2X5 receptors in ICCs, leading to increases in intracellular $[Ca^{2+}]$, impacting the activity of slow waves.

Table 7: P2X receptor subtypes and respective locations in the gut (based on data from the following papers: Burnstock, 2012; Galligan, 2002; Liña-Rico *et al.*, 2015; Van Crombruggen *et al.*, 2007; Silva *et al.*, 2015; Burnstock & Lavin, 2002).

RECEPTORS	LOCATION
P2X1	Smooth muscle of rat submucosal arterioles. Smooth muscle of the rat distal colon. Smooth muscle of enteric blood vessel. Enteric glial cells (rat distal colon) Resident macrophages Highly expressed in human submucous plexus. Co-localized with P2X2 in the majority of submucous neurons.
P2X2	Myenteric neurons: descending interneurons, NOS-containing motor neurons; Nerve fibres in the myenteric plexus (rat distal colon) Nerve fibres in the submucosal ganglia: inhibitory motor neurons, VIP-containing secretomotor; intrinsic sensory neurons. Longitudinal and circular muscle of the colon. Epithelial cells (rat distal colon). ICC's.
P2X3	Small subpopulation of myenteric ganglion neurons, but in a larger number of ganglion neurons in the submucous plexus; Cholinergic neurons of submucous plexus of the guinea-pig ileum. Intrinsic sensory neurons; AH-type neurons. Nerve fibres of submucous and myenteric plexus (rat distal colon). Neurons of the human myenteric plexus, but <u>not</u> in the submucous plexus. Longitudinal smooth muscle fibres. Epithelial cells (rat distal colon).
P2X4	Only present in resident macrophages (rat distal colon).
P2X5	Longitudinal smooth muscle fibres. Myenteric plexus: nerve fibers surrounding ganglion cell bodies and possibly on glial cell processes. Submucous plexus: intracellularly and on the plasma membranes of neurons. ICC's.
P2X6	Intrinsic sensory neurons. Enteric Glial cells of myenteric and submucous plexus (rat distal colon).
P2X7	Myenteric and submucosal ganglia in stomach, small intestine, and colon. Surrounding ganglion cell bodies in both myenteric and submucous plexuses. Enteric glial cells of myenteric and submucous plexus (rat distal colon). <u>Not</u> present in smooth muscle cells, ICCs, and fibroblast-like cells.

5.3. P2Y Receptors

ATP acting on metabotropic P2Y receptors is responsible for slow EPSPs in the ENS (Ren & Bertrand, 2008). Slow EPSPs are modulated by ACh acting on muscarinic receptors and by tachykinins acting on neurokinin receptors. Slow EPSPs is also mediated by ATP acting at P2Y receptors.

It has been proposed that P2Y₁ and P2Y₂ receptors have a dominant neuronal localization, while the P2Y₄ receptor is located in another cell types, like enteric glial cells and ICCs. Our group demonstrated that activation of P2Y₁ receptors by ADP generated from released ATP via NTPDase2 restrains ACh release during prolonged stimulation periods of myenteric motor nerves (Duarte-Araújo *et al.*, 2009). Please refer to table 8 for the distribution of P2Y receptors along the GI tract.

Mounting evidences are now available about the physiological impact of neuron-to-glia communication (Boesmans *et al.*, 2013; Gulbransen & Sharkey, 2009; Gulbransen *et al.*, 2010). Enteric glial cells respond to ATP and UTP released by neurons, via P2 receptors, by increasing intracellular $[Ca^{2+}]_i$. Previous studies implicated P2Y₄, and possibly the P2Y₁₁, receptors in ATP-mediated effects in enteric glial cells (Gulbransen & Sharkey, 2009; Gulbransen *et al.*, 2010), but the P2Y₁ receptor activated by ADP generated from the extracellular hydrolysis of ATP may also be involved (Gulbransen & Sharkey, 2012). Neuron-to-glia neurotransmission has been proposed in the myenteric plexus of the guinea pig distal colon. In this model, the stimulation of primary afferent fibres releases neurotransmitters, such as ATP, CGRP and SP, resulting in the activation of presynaptic enteric nerve terminals leading to ACh and ATP co-release. These compounds act respectively on ionotropic nicotinic ACh and P2X receptors on postsynaptic enteric neurons. Evidence suggest, however, that enteric glia respond mainly to ATP released from discrete populations of enteric sympathetic nerves under specified circumstances, which by activating P2Y₄ receptors stimulated Ca^{2+} responses in the surrounding enteric glia (Gulbransen *et al.*, 2010).

Table 8: P2Y receptor subtypes and respective locations in the gut (based on data from the following papers: Ren & Bertrand, 2008; Burnstock, 2014; Van Crombruggen *et al.*, 2007; Van Nassauw *et al.*, 2006).

RECEPTORS	LOCATION
P2Y ₁	Myenteric and submucous plexus (mouse); NOS immunoreactive neurons (descending interneurons or inhibitory motor neurons); Secretomotor neurons in submucous plexus. ICC's, in human and murine small intestine. FLC. Enteric glial cells in rat distal colon. Smooth muscle cells in rat distal colon.
P2Y ₂	Myenteric plexus of the rat colon (Dogiel type I neurons); Almost a quarter of the neurons co-expressed nNOS. Co-localize with a majority of calretinin immunoreactive neurons in the myenteric plexus (ascending interneurons and excitatory motor neurons). All NPY-immunoreactive neurons in the submucous plexus (secretomotor neurons) Enteric glial cells.
P2Y ₄	ICC's, in human and murine small intestine. Enteric glial cells (in rat distal colon and in guinea-pig colon).
P2Y ₆	Myenteric plexus (all regions of the guinea pig GI tract); <u>Not</u> in the submucous plexus. Co-localize with NOS immunoreactive neurons (32%), descending interneurons and inhibitory motor neurons. Co-localize to calretinin immunoreactivity neurons (45%), ascending interneurons and excitatory motor neurons. Enteric glial cells and in enteric smooth muscle cells (in rat distal colon).
P2Y ₁₁	Enteric glial cells (in rat distal colon). Smooth muscle cells of the guinea-pig colon.
P2Y ₁₂	Localized to calbindin immunoreactive neurons (AH-type sensory neurons), in the guinea pig small intestine. In the myenteric plexus, in the rat colon. In neurons exhibiting a Dogiel type II morphology and in uniaxonal neurons (in rat distal colon).
P2Y ₁₃	Enterocytes (in rat distal colon).
P2Y ₁₄	Some neurons of submucous plexus (in rat distal colon).

ICCs and FLCs are electrically coupled to smooth muscle fibres via gap junctions from a multicellular functional syncytium termed the SIP Syncytium. Cells that make up the SIP syncytium are highly specialized containing unique receptors, ion channels and intracellular signalling pathways, which may be affected by neurotransmitters to regulate the excitability of GI muscles (reviewed in Blair *et al.*, 2014). For instance, purines activating P2Y₁ receptors in FLCs stimulate the PLC transduction pathway, which culminates in IP₃ production and Ca²⁺ recruitment from intracellular stores leading to increases in SK3 channels permeability and cells hyperpolarization (Don Koh & Rhee, 2013).

5.4. Nicotinic and Muscarinic Receptors

ACh activates directly two subclasses of receptors, muscarinic and nicotinic receptors. Because nicotinic ACh receptors are ligand-gated ion channels, they mediate fast synaptic transmission in the ENS. It was shown that nicotinic receptors-mediated neurotransmission prevails in ascending pathways, whereas it has only a minor contribution in descending pathways (Galligan, 2002). Schneider and Galligan (2000) showed distinct populations of presynaptic nicotinic receptors in the myenteric plexus. They demonstrated that nicotinic receptors are located in the nerve terminals and in the somatodendritic region of AH and S neurons. Our group provided evidences suggesting that ACh mediates a positive feedback mechanism via the activation of prejunctinal nicotinic receptors, presumably by mediating the influx of Ca²⁺ directly through the receptor pore and, thus, independently of voltage-sensitive calcium channels (Duarte-Araújo *et al.*, 2004b). Interestingly, this mechanism seems to be tonically regulated by endogenous ADO through the activation of A_{2A} receptors coupled to the adenylate cyclase/cyclic AMP transducing system.

Immunocytochemistry studies showed that nicotinic receptors containing $\alpha 3$ -, $\alpha 5$ -, and $\beta 2$ - subunits are most abundant primary cultured myenteric neurons, whereas the $\alpha 7$ - subunit stained only a small number of these neurons (Zhou *et al.*, 2002). Glushakov and colleagues (2004) showed that submucosal neurons of the guinea-pig ileum express nicotinic receptors containing $\alpha 3$ -, $\alpha 5$ - and $\alpha 7$ -

subunits, but not $\alpha 4$ subunits. Moreover, Obaid and colleagues (2005) using radioimmunoassays and immunocytochemistry reached the conclusion that guinea pig enteric ganglia express $\alpha 3$ -, $\alpha 5$ -, $\beta 2$ -, $\beta 4$ - and $\alpha 7$ -subunits.

In addition to nicotinic receptors, ACh can also activate muscarinic receptors (North *et al.*, 1985). The GI tract of healthy dairy cows contains M_1 receptors in the submucosal and myenteric plexus, M_2 receptors in SMCs, M_3 receptors in the lamina propria, nerve terminals and intermuscular nerve fibers, and in microvessel myocyte, and M_5 in microvessel myocyte and SMCs. M_4 receptors were not found in these tissues (Stoffel *et al.*, 2006). At the LM-MP of the mouse ileum, M_2 and M_4 receptors may mediate muscarinic autoinhibition in ACh release (Takeuchi *et al.*, 2005). Using the rat ileum, we demonstrated that ACh exerts a dual neuromodulatory effect on its own release through the activation of inhibitory M_2 receptors and excitatory M_3 receptors, depending on the stimulation train length (Vieira *et al.*, 2009). The participation of M_1 receptors in the control of ACh release was discarded.

Goyal (2013) revised the role of ICCs in cholinergic neurotransmission in the gut. It was proposed that ICCs may be intermediates in cholinergic neuromuscular transmission at the SIP syncytium. ICC's are endowed with muscarinic receptors (mainly M_3 receptors), which have a prime role in this process. Activation of muscarinic receptors in ICCs generates a depolarizing potential and activates different signalling pathways that are driven to smooth muscle fibres leading to muscle contraction. M_3 receptors are coupled to $G_{q/11}$ leading to activation of phospholipase C (PLC) resulting in IP_3 and DAG formation, with subsequent recruitment of intracellular Ca^{2+} and protein kinase C activation, respectively (Koh & Rhee, 2013). Increases in intracellular Ca^{2+} can strengthen activation of the protein kinase C, which may subsequently stimulate ADO outflow by equilibrative transporter ENT1. Increase in protein kinase C activity may also stimulate the activity of 5'-nucleotidase inside the cell and/or inhibit AK, enhancing intracellular accumulation and efflux of ADO from the cell. Thus, the muscarinic M_3 positive feedback mechanism facilitating ACh release from stimulated myenteric

neurons may be indirectly mediated by the release of ADO from ICCs leading to activation of prejunctional facilitatory A_{2A} receptors in a novel neuron-to-glia and back glia-to-neuron communication pathway operated by muscarinic M_3 and adenosine A_{2A} receptors.

Increasing evidences have been produced showing that ICCs are crucial in the transduction of cholinergic signals to smooth muscle fibres in the GI tract. However, in the absence of ICCs ACh can couple to $G_{q12/13}$ protein and trigger activation of the Rho-kinase (RhoK) pathway to cause contraction in SMCs (Koh & Rhee, 2013).

6. Inflammatory bowel diseases

In Portugal, inflammatory bowel diseases (IBD) have a prevalence of 146 patients per 100 000 inhabitants (Azevedo *et al.*, 2010). IBDs include Crohn's disease, ulcerative colitis and indeterminate colitis. Ulcerative colitis occurs only in the colon and within the gut wall tissue damage is restricted to the mucosa and lamina propria. On the other hand, Crohn's disease can occur in any region of the gut and tissue damage may penetrate the entire thickness of the organ (Geboes & Collins, 1998). Peristaltic changes are common clinical symptoms in IBS. Since in these chronic GI disorders etiopathogenesis remains unclear, therapeutic management is usually empiric, extrapolated from ulcerative colitis and Crohn's disease. The most typically used drugs are intestinal anti-inflammatory drugs, systemic corticosteroids, immunosuppressants, biologic therapy and antibiotics (Azevedo *et al.*, 2010). The determination of inflammatory activity is a really important step for the assessment of disease activity, for choosing an adequate therapy, and for contributing to ameliorate the quality of patients' life (Vilela *et al.*, 2012).

Structural abnormalities of the ENS are observed in IBD. In Crohn's Disease are often observed nerve fibre modifications, such as damage, hypertrophy, and reactive hyperplasia, and enteric glial cells hyperplasia. Neuronal

hypertrophy of the Meissner's plexus is a common pathological feature of ulcerative colitis (Geboes & Collins, 1998). In both diseases, neutrophils and lymphocytes (positive for CD4) are the predominant cells in the inflamed mucosa. Increases in the number of B cells, macrophages, dendritic cells, plasma cells, eosinophils and mast cells are also very common (Roberts-Thomson *et al.*, 2011).

During an inflammatory insult in the gut, activated leukocytes infiltrate the gut wall. Various cells of the mucosal immune system produce a number of pro- and anti-inflammatory cytokines, which induce chemotaxis and leukocytes proliferation (Neurath, 2014; Schepper *et al.*, 2008). Cytokine levels and cytokine expression are similar in Ulcerative colitis and Crohn's disease. Increases in TNF- α and IFN- γ are consistent with a Th1 response. The proinflammatory cytokines described in these disorders are: TNF α , IL-12, IL-23, and perhaps IL-17 and IFN- γ (Roberts-Thomson *et al.*, 2011). The released cytokines also induce morphological and functional alterations in the neural apparatus, such as sprouting, neuronal necrosis, and neuronal sensitization (Schepper *et al.*, 2008).

Enteric plasticity comprises a wide range of structural and/or functional changes in enteric neurons, glial cells and pacemaker cells of Cajal. As a result of adaptive responses to different types of pathophysiological conditions, enteric neurons are able to rapidly change their structure, function and chemical phenotype in order to maintain homeostasis of gut functions. These plasticity changes occur in distinct situations as a consequence of the wide array of pathological conditions, ranging from enteric neuropathies to extra-intestinal (e.g. Parkinson's disease) or intestinal diseases (e.g. IBD) (Vassina *et al.*, 2006).

Even though the period of inflammation may be brief, and the region of overt inflammatory damage is circumscribed, its effects on enteric neurons are long-lasting and significant changes in organ function are observed in regions that are remote from the inflammatory site (Pontell *et al.*, 2009).

6.1. Ileitis

Inflammation of the ileum is called ileitis. It is often caused by Crohn's disease, although it may be caused by other diseases, such as ischemia, neoplasms, medication-induced, eosinophilic enteritis, and others. Inflammation of the ileum may be acute or in more severe conditions, can be chronic (DiLauro *et al.*, 2010).

6.2. Animal models of inflammatory bowel diseases

A wide variety of animal models of IBD appeared in last years (table 9). IBD animal models have proven to be important tools for detecting potential therapeutic agents and for investigating the mechanisms involved in its pathogenesis (Jurjus *et al.*, 2004; Wirtz and Neurath, 2007a,b). It is considered an appropriate animal model, the one that display certain key characteristics:

- (1) Morphologic alterations;
- (2) Inflammation;
- (3) Pathophysiology and course similar to human IBD.

The most widely accepted models are those resulting from the instillation of chemical irritants using trinitrobenzene sulfonic acid (TNBS) or dextran sodium sulphate (DSS) (Jurjus *et al.*, 2004; Wirtz and Neurath, 2007a,b). In the present work, it will be used the chemically-induced model by the instillation of TNBS into the lumen of the ileum. So, now I will focus mostly in this model.

Table 9: Animal models categories and subcategories of IBD.

Models (categories)	Subcategories
<i>Gene Knockout (KO) models</i>	(1) Interleukin-2 KO/IL-2 receptor (R)α KO mice (2) IL-10 KO mice models (3) T cell receptor (TCR) mutant mice (4) TNF-3' untranslated region (UTR) KO mice (5) Trefoil factor-deficient mice
<i>Transgenic mouse and rat models</i>	(1) IL-7 transgenic mice (2) Signal transducer and activating transcription (STAT)-4 transgenic mice (3) HLA B27 transgenic rats
<i>Spontaneous Colitis models</i>	(1) C3H/HeJBir mice (2) SAMP1/Yit mice
<i>Inducible models (Chemically induced)</i>	(1) Acetic acid (2) Iodoacetamide (3) Indomethacin (4) TNBS (5) Oxazolone (6) DSS (7) Peptidoglycan –polysaccharide (PG-PS) colitis
<i>Adoptive transfer models</i>	(1) Colitis induced by transfer of heat shock protein (hsp) 60-specific CD8 T cells. (2) CD45RB transfer model

6.2.1. TNBS-Induced ileitis

Ileitis can be induced by a intraluminal instillation of 2,4,6-trinitrobenzenesulfonic acid (TNBS) in ethanol, which is required to break the mucosal barrier (Martinolle *et al.*, 1997; Wirtz *et al.*, 2007).

This model is also frequently used to induce colitis. The model in the colon seems to be more aggressive than in the ileum. In colon, the chronic phase is more easily achieved than in the ileum. This difference may be due to the absence of specific ileum resident bacteria found in the colon. The resident flora is needed to keep the inflammatory response to TNBS (Moreels *et al.*, 2001). These authors sustain that TNBS-induced ileitis in the rat lacks the chronic phase and it is

accompanied by functional hypocontractility of longitudinal SMCs during the acute inflammatory phase. They claim that the contractility of the longitudinal muscle is increased in this phase due to structural changes. Moreels and colleagues (2001) concluded that the rat model of TNBS-induced ileitis is characterized by an acute transmural inflammation.

In 1992, Selve & Wöhrmann published a study where they described a model of chronic IBD with TNBS, in the rat ileum. In this work, chronic inflammation was only achieved 3 to 4 weeks after starting TNBS application. The protocol implicates a previous immunization and a daily application of TNBS (via a catheter). This chronic phase in the ileum is characterized by the following pathological alterations:

- (1) Intense hyperemia and oedema of the mucosal surface;
- (2) Inflammatory cell infiltration (by lymphocytes and histiocytes in all layers of the gut wall;
- (3) Without neutrophil infiltration;
- (4) Transmural granulomatous inflammation of the gut wall.

In the model of TNBS-induced ileitis used more often, pathological alterations are the following:

- (1) Substantial increase in the thickness of the intestinal wall (Tsujikawa *et al.*, 1999);
- (2) Transmural lesions, that involves Th-1 mediated macrophage expression of cytokines, such as TNF- α , IFN- γ and IL-12 (Merritt *et al.*, 2002).
- (3) Mucosal infiltration by mononuclear and polynuclear cells (Martinolle *et al.*, 1997);
- (4) Increased production of inflammatory mediators such as leukotrienes, prostaglandins, platelet-activating factor, SP and NO (Martinolle *et al.*, 1997).
- (5) T lymphocytes and eosinophils are found more close to myenteric ganglia (Pontell *et al.*, 2009).

- (6) Increased neuronal excitability persists for at least 8 weeks (Pontell *et al.*, 2009).
- (7) Critical changes in spontaneous contractile activity of circular and longitudinal muscle (Martinolle *et al.*, 1997).

TNBS-induced ileitis results in different effects on the electrophysiological properties of distinct types of neurons of the small intestine. Dogiel type I neurons from inflamed ileum of the guinea-pig had prominent late afterhyperpolarizing potentials (AHPs) after a single action potential triggered by an intracellular current pulse. Dogiel type II neurons were hyperexcitable compared to the controls (Nurgali *et al.*, 2007). It was described also by Stewart and colleagues, in 2003, that there is an increase of excitability of intestinal sensory neurons by modulation of ion channels (Na^+ and K^+) in guinea-pig ileitis. The appearance of tetrodotoxin-resistant sodium currents is increased, while potassium currents are suppressed.

One group has provided insights on the effects of inflammation on cell proliferation in the myenteric plexus of the guinea-pig ileum (Bradley *et al.*, 1997). These authors concluded that inflammation of the ileum does not stimulate the appearance of new myenteric neurons, but stimulates significantly enteric glia cells proliferation. They also observed an increase in the number of macrophages and T-cells in the tissue. It appears that the increased number of macrophages is not a result of cells proliferation, but may be a consequence of the infiltration of resident cells. Apparently inflammatory cell infiltrates spare the myenteric ganglia and, for this reason, they designate myenteric ganglia as an immune-privileged tissue where immunocytes do not penetrate.

Reports from the literature refer to impairments of the purinergic modulation of enteric neurotransmission during chronic inflammation of the mouse ileum (De Man *et al.*, 2003). During an inflammatory insult, the P2Y_{11} receptor is activated in immune cells by low concentrations of ATP, while the P2X_7 receptor requires high concentrations of ATP ($>100 \mu\text{M}$) to become activated. The release of inflammatory mediators may be accomplished by the activation of P2X_3 and P2X_7 receptors (Sanger, 2012). Increases of the P2X_3 receptor expression has been

observed in human patients with IBD, which implicates a potential role for this receptor in enteric dysmotility and pain (Yiangou *et al.*, 2001). Increase expression of the P2Y₆ receptor has also been observed in epithelial cells, thus promoting the epithelial release of cytokines and chemokines that indirectly modulate the severity of GI inflammation (Grbic *et al.*, 2008; Roberts *et al.*, 2012)

Nowadays, there is an increasing interest on the involvement of ADO in the pathophysiology of intestinal disorders (Antonioli *et al.*, 2008). In addition, an active role of extracellular ATP and its receptors in the alterations motivated by intestinal inflammation has been suggested (Burnstock, 2008). In IBD models, not only morphological but also changes in the excitability and synaptic properties of enteric neurons might justify the GI dysfunction.

V. GOALS

The main goals of this study were to investigate the following aspects in a model of postinflammatory ileitis caused by intraluminal instillation of TNBS in the rat:

- (1) The kinetics of the extracellular catabolism of ATP and ADO formation/inactivation cycle in order to comprehend the overall homeostatic role of the purinergic cascade on enteric excitability.
- (2) The action of endogenous adenosine on electrically evoked [³H]ACh release from myenteric motor neurons in postinflammatory conditions, using selective antagonists to adenosine receptors.
- (3) Changes in the electrically-evoked release of purines (ATP and ADO) and ACh in the myenteric plexus of control and TNBS-treated rats.
- (4) Changes in purine releasing sites, their relation to cell type and density changes, in order to propose novel therapeutic targets for inflammatory intestinal diseases.

Understanding how ubiquitous neuromodulators, such as adenine nucleotides and ADO, affect plastic adaptations of enteric neurotransmission during inflammatory insults may prompt for novel therapeutic targets to manage inflammatory intestinal diseases.

VI. ORIGINAL RESEARCH PAPERS

The results presented in this thesis were published/ submitted for publication as original research papers, as follows:

- **Paper 1:** Vieira C, Magalhães-Cardoso MT, Ferreirinha F, Silva I, Dias AS, Pelletier J, Sévigny J & Correia-de-Sá P (2014). **Feed-Forward Inhibition of CD73 and Upregulation of Adenosine Deaminase Contribute to the Loss of Adenosine Neuromodulation in Postinflammatory Ileitis**. Mediators of Inflammation. Volume 2014 (2014), Article ID 254640, 19 pages. (DOI: 10.1155/2014/254640)
- **Paper 2:** Vieira C, Magalhães-Cardoso MT, Ferreirinha F, Silva I & Correia-de-Sá P (2015). **Postinflammatory ileitis induces neurochemical adjustments of purine cellular resources in the myenteric plexus**. (*Manuscript submitted for publication*)

Data from the following papers published by the author were also used and/or discussed in this thesis:

- Vieira C, Ferreirinha F, Silva I, Duarte-Araújo M, Correia-de-Sá P. **Localization and function of adenosine receptor subtypes at the longitudinal muscle-myenteric plexus of the rat ileum**. Neurochem Int. 2011. 59(7):1043-1055.
- Vieira C, Duarte-Araújo M, Adães S, Magalhães-Cardoso T, Correia-de-Sá P. **Muscarinic M₃ facilitation of acetylcholine release from rat myenteric neurons depends on adenosine outflow leading to activation of excitatory A_{2A} receptors**. Neurogastroenterol Motil. 2009. 21(10):1118-e95.
- Correia-de-Sá P, Adães S, Timóteo MA, Vieira C, Magalhães-Cardoso T, Nascimento C, Duarte-Araújo M. **Fine-tuning modulation of myenteric motoneurons by endogenous adenosine: On the role of secreted adenosine deaminase**. Auton Neurosci. 2006. 126-127:211-224.

Author contributions to the papers:

Author participated in all aspects of the work and performed autonomously the surgical procedures, *in vivo* and *in vitro* experiments, analyzed the data, discussed the results and drafted the papers until final approval. Paulo Correia-de-Sá (MD, PhD) supervised the project, designed the experiments, analyzed the data, discussed the results and drafted/approved the papers. The author received collaborations from Isabel Silva (MSc, PhD Student) – quantification of ATP by the luciferin-luciferase bioluminescence assay; Fátima Ferreirinha (PhD) - image acquisition and analysis of immunofluorescence confocal micrographs; and Teresa Magalhães-Cardoso (PharmD) – quantification of adenine nucleotides and nucleosides by HPLC.

PAPER 1

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Feed-forward inhibition of CD73 and upregulation of adenosine deaminase contribute to the loss of adenosine neuromodulation in postinflammatory ileitis

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ABSTRACT

Purinergic signalling is remarkably plastic during GI inflammation. Thus, selective drugs targeting the “purinome” may be helpful for inflammatory GI diseases. The myenteric neuromuscular transmission of healthy individuals is fine-tuned controlled by ADO acting on A₁ inhibitory and A_{2A} excitatory receptors. Here, we investigated the neuromodulatory role of ADO in TNBS-inflamed LM-MP of the rat ileum. Seven-day postinflammation ileitis lacks ADO neuromodulation, which may contribute to accelerate GI transit. The loss of ADO neuromodulation results from deficient accumulation of the nucleoside at the myenteric synapse despite that increases in ATP release were observed. Disparity between ATP outflow and ADO deficit in postinflammatory ileitis is ascribed to feed-forward inhibition of ecto-5'-nucleotidase/CD73 by high extracellular ATP and/or ADP. Redistribution of NTPDase2, but not of NTPDase3, from ganglion cell bodies to myenteric nerve terminals leads to preferential ADP accumulation from released ATP, thus contributing to prolong inhibition of muscle-bound ecto-5'-nucleotidase/CD73 and to delay ADO formation at the inflamed neuromuscular synapse. On the other hand, depression of endogenous ADO accumulation may also occur due to enhancement of ADA activity. Both membrane-bound and soluble forms of ecto-5'-nucleotidase/CD73 and ADA were detected in the inflamed myenteric plexus. These findings provide novel therapeutic targets for inflammatory gut motility disorders.

Keywords: Ectonucleotidases; enteric nervous system; intestinal inflammation; myenteric plexus; purinergic receptors; soluble enzymes.

INTRODUCTION

The ENS undergoes a series of adaptive responses to different pathological conditions (e.g. inflammatory and/or ischemic insults) (Sharkey *et al.*, 2001; Vassina *et al.*, 2006). For instance, enteric neurons rapidly change their structure, function or chemical phenotype in order to maintain gut homeostasis. Even if the inflammatory insult is brief and the damage circumscribed, its repercussion on enteric neurons may be long-lasting leading to significant changes in intestinal function, which can be observed in remote regions from the inflammation site. The postinflammatory status is frequently accompanied by substantial increases in enteric motility (Pontell *et al.*, 2009).

Inflammation of the GI tract causes marked changes in the release of purines leading to subsequent adaptive modifications of purinoceptors expression and/or function (reviewed in Roberts *et al.*, 2012). The underlying mechanisms of disturbed purinergic modulation are not completely understood, in part because the study of purinoceptors may be hampered by the presence of distinct nucleotide release sites and cell surface enzymes that rapidly break down extracellular nucleotides into nucleosides (Kukulski *et al.*, 2011). In healthy individuals, ATP is released predominantly from stimulated enteric neurons (White & Leslie, 1982), but its release from non-neuronal cells (e.g. smooth muscle fibres, ICC) might also occur (Nitahara *et al.*, 1995). Four members of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family, namely NTPDase1, NTPDase2, NTPDase3 and NTPDase8, and two members of the ecto-nucleotide pyrophosphatases/phosphodiesterases (E-NPP) family, NPP1 and NPP3, are located at the plasma membrane and hydrolyse extracellular nucleotides (Kukulski *et al.*, 2011; Kukulski *et al.*, 2005; Stefan *et al.*, 2005). The relative contribution of distinct ecto-enzymes to the modulation of purinergic signalling depends on

differential tissue and cell distribution, regulation of expression, targeting to specific membrane domains, but also on substrate preference and availability.

Regarding the substrate preference, NTPDase1 (CD39 or apyrase) dephosphorylates ATP directly into AMP, with minimal accumulation of ADP. NTPDase2 (ATPase) is a preferential nucleoside triphosphatase hydrolysing ADP 10 to 15 times less efficiently than ATP, leading to minimal AMP accumulation (Kukulski *et al.*, 2005). NTPDase3 and NTPDase8 are functional intermediates between NTPDase1 and NTPDase2 (Kukulski *et al.*, 2005). Because of their involvement in physiological processes, namely blood clotting, vascular inflammation, immune reactions and certain types of cancer, NTPDases are now considered as potential drug targets (Gendron *et al.*, 2002). As for NPP1 and NPP3, they release nucleoside 5'-monophosphate from a variety of nucleotides, but intriguingly, their phosphorylated product (e.g. AMP) bind to NPPs with a higher affinity than substrates do, and thus inhibit catalysis (Stefan *et al.*, 2009). Finally, AMP is hydrolysed to ADO and inorganic phosphate by ecto-5'-nucleotidase (CD73), which is concentrated in the myenteric smooth muscle cell layer (Nitahara *et al.*, 1995). Interestingly, ecto-5'-nucleotidase (CD73) may be cleaved from cell membranes through hydrolysis of the GPI anchor by phosphatidylinositol-specific phospholipases or by proteolysis while retaining its catalytic activity in the soluble form (Sträter *et al.*, 2006).

At the myenteric neuromuscular synapse, ATP is primarily metabolized into AMP, which is then dephosphorylated into ADO; alternative conversion of ATP into ADP is more relevant at high ATP concentrations (Duarte-Araújo *et al.*, 2009). Thus, gradients of ATP and its breakdown products (ADP and ADO) may provide fine tuning control of peristaltic motor performance in the gut during stressful situations, such as sustained neuronal activity, ischemia and inflammation, when extracellular ATP reaches high levels (see Milusheva *et al.*, 1990; De Man *et al.*, 2003). ATP transiently facilitates ACh release from non-stimulated nerve terminals via prejunctional P2X (probably P2X₂) receptors. Hydrolysis of ATP directly into AMP and subsequent formation of ADO activates inhibitory A₁ receptors in smooth

muscle fibres (Nicholls *et al.*, 1996) and in ganglionic cell bodies of myenteric neurons (Vieira *et al.*, 2011), where it downmodulates the release of excitatory neurotransmitters, such as SP (Moneta *et al.*, 1997) and ACh (Duarte-Araújo *et al.*, 2004). Besides the well characterized inhibitory A₁ receptors, myenteric nerve terminals are endowed with prejunctional A_{2A} receptors mediating facilitation of ACh release (Duarte-Araújo *et al.*, 2004; Correia-de-Sá *et al.*, 2006; Vieira *et al.*, 2011).

Previous findings from our group demonstrated that the ecto-nucleotidase pathway contributes only partially to the total interstitial ADO concentration in the myenteric plexus (Correia-de-Sá *et al.*, 2006). ADO released as such from either neuronal or non-neuronal cells seems to be the main source of endogenous ADO in the ENS (Duarte-Araújo *et al.*, 2004). This release is sought to be mediated by facilitated diffusion via nucleoside transporters, which can be regulated by endogenous signaling molecules, such as ACh via muscarinic M₃ receptors (Correia-de-Sá *et al.*, 2006; Vieira *et al.*, 2011). On the other hand, we demonstrated the extracellular deamination by ADA represents the most effective mechanism regulating synaptic ADO levels in the LM-MP of the ileum from healthy animals (Duarte-Araújo *et al.*, 2004; Correia-de-Sá *et al.*, 2006). ADA is a ubiquitous purine metabolic enzyme localized on the cell surface (ecto-ADA) (Pettengil *et al.*, 2013). Upon proteolytic cleavage from cell membranes, soluble forms of ADA (exo-ADA) retain their catalytic activity and may be found in interstitial fluids (Franco *et al.*, 1997). We detected a relatively high ADA activity (~0.6 U/ml) in superfusates collected after stimulating LM-MP preparations, which is in keeping with the hypothesis that “ADA secretion” may restrict endogenous ADO actions to the synaptic region near the release / production sites (Correia-de-Sá *et al.*, 2006). ADA secretion increases in various diseases and may be originated from monocyte/macrophage lineages, thus reflecting the involvement of the cellular immune system. De Man and colleagues (2003) reported that chronic intestinal inflammation enhanced the enteric contractile activity in part due to an impairment of purinergic modulation of cholinergic nerve activity (De Man *et al.*, 2003).

Because, adenine nucleotides and nucleosides (and ADA itself) may be released from activated inflammatory cells (Marquard *et al.*, 1984), as well as from neighbouring neuronal and non-neuronal enteric cells (Stead *et al.*, 1989; Bogers *et al.*, 2000), there is an increasing interest in the neuromodulatory effects exert by ADO during inflammatory insults. The therapeutic potential of ADO-related compounds for controlling intestinal motility and inflammation (De Man *et al.*, 2003; Antonioli *et al.*, 2008), prompted us to investigate the kinetics of ADO formation/inactivation cycle in order to understand the overall homeostatic role of the nucleoside on enteric excitability in postinflammatory ileitis caused by intraluminal instillation of TNBS in the rat.

MATERIAL AND METHODS

Animals

Rats of either sex (Wistar, ~200 g) (Charles River, Barcelona, Spain) were kept at a constant temperature (21°) and a regular light (06.30-19.30 h)-dark (19.30-06.30 h) cycle, with food and water) and a regular light (06.30-19.30 h)-dark (19.30-06.30 h) cycle, with food and water *ad libitum*. All studies involving animal experiments are reported in agreement with ARRIVE guidelines. Animal handling and experiments were in accordance with the guidelines prepared by Committee on Care and Use of Laboratory Animal Resources (National Research Council, USA) and followed the European Communities Council Directive (86/609/EEC).

TNBS-induced intestinal inflammation model

Intestinal inflammation was produced by the instillation of 2,4,6-trinitrobenzenesulfonic acid (TNBS) into the lumen of the rat ileum. After a fasting period of 4-8 hours with free access to drinking water, rats undergo median laparotomy under anaesthesia with an association of medetomidine (10 mg/Kg) and ketamine (75 mg/Kg) given subcutaneously. At the end of the procedure animals were retrieved with atipamezole (10 mg/Kg). The terminal ileal loop was

gently exteriorized, and TNBS (40 mM) was injected through the enteric wall into the lumen of the ileum, 10 centimeters proximal to the ileocolonic junction. Controls received 1 ml of 0.9% saline. Sixty minutes after surgery, the rats were allowed to eat and drink *ad libitum*. After surgery, pain was controlled with tramadol hydrochloride (10 mg/Kg). To have a control of time course of body weight loss and recovery after injection of TNBS, rats were weighed prior to TNBS administration and daily following surgery. Animals with intestinal inflammation (TNBS) transiently lose weight for three to four days after surgery, and regain weight thereafter. Seven days after surgery, animals were sacrificed following an overnight fasting period.

Histological findings and gastrointestinal motility

The postinflammatory phase of TNBS-induced ileitis was characterized in haematoxylin-eosin stained sections using Pontell and Jergens criteria, which is based in the loss of normal tissue architecture, epithelial damage and infiltration of inflammatory cells (Pontell *et al.*, 2009; Jergens, 1999; Engel *et al.*, 2009; Wirtz & Neurath, 2007). Evaluation of GI motility was performed *in vivo* by oral administration of a gavage containing the methylene blue dye. Dye progression in the GI tract was evaluated after 30 minutes, both in control and TNBS-injected animals: the total length of the small intestine was used to normalize data.

[³H]-Acetylcholine release experiments

Sections of the rat ileum not including the terminal five centimetres were used. The longitudinal muscle strip with the myenteric plexus attached was separated from the underlying circular muscle according to the method of Paton and Vizi (1969). This preparation is highly enriched in cholinergic neurons, mainly excitatory neurons projecting to the longitudinal muscle (25%) that receive inputs from intrinsic primary afferents (26%) and from ascending and descending pathways (17%) (Costa *et al.*, 1996).

The procedures used for labelling the preparations and measuring evoked [³H]-Acetylcholine ([³H]-ACh) release were previously described (Duarte-Araújo *et*

al., 2004; Duarte-Araújo *et al.*, 2009; Correia-de-Sá *et al.*, 2006; Vieira *et al.*, 2011) and used with minor modifications. LM-MP strips were mounted in 3 ml capacity vertical perfusion chambers heated at 37 °C. After a 30 min equilibration period, myenteric neurons were labelled for 40 min with 1 µM [³H]-choline (specific activity 2.5 µCi nmol⁻¹) under EFS (1 Hz-frequency, 1 ms pulse width). Following loading, the washout Tyrode's solution contained hemicholinium-3 (10 µM) to prevent choline uptake. After a 60 min period of washout, bath samples (3 ml) were automatically collected every 3 min using a fraction collector (Gilson, FC203B, France). Tritium content of the samples was measured by liquid scintillation spectrometry (% counting efficiency: 40±2%).

Test drugs were added 15 min before S₂. The change in the ratio between the evoked [³H]-ACh release during the two stimulation periods (S₂/S₁) relative to that observed in control situations (in the absence of test drugs) was taken as a measure of the effect of the tested drugs. None of the drugs changed significantly (*P*>0.05) basal tritium outflow.

Kinetic experiments of the extracellular catabolism of purines and HPLC analysis

For the kinetic experiments of the extracellular catabolism of adenine nucleotides and ADO, strips from the longitudinal muscle with the myenteric plexus attached (LM-MP) were mounted in a 2 ml organ bath. All experiments were performed at 37°C. Preparations were superfused with gassed (95% O₂ and 5% CO₂) Tyrode's solution containing (mM): NaCl 137, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1, NaH₂PO₄ 0.4, NaHCO₃ 11.9 and glucose 11.2. After a 30 min equilibration period, the preparations were incubated for 45 min with Tyrode's solution to eliminate endogenous interfering compounds. Following a washout period of 10 min, the preparations were then incubated with 30 µM of ATP, ADP, AMP or ADO (zero time). Samples of 75 µl were collected from the organ bath at different times up to 45 min for HPLC (with UV detection) analysis of the variation of substrate disappearance and product formation (Duarte-Araújo *et al.*, 2004; Duarte-Araújo *et al.*, 2009; Correia-de-Sá *et al.*, 2006; see also Pinheiro *et al.*, 2013). The

concentrations of the substrate and products were plotted as a function of time (progress curves). In some experiments, we measured unbound 5'-nucleotidase and ADA activities in the fluid retained in the bath after removing the LM-MP preparation. We followed a similar experimental protocol to that used for studying the kinetics of the extracellular catabolism of adenine nucleotides and ADO in the presence of the preparations.

Release of ATP and adenine nucleosides (adenosine plus inosine)

Experiments were performed using an automated perfusion system for sample collecting for given time periods, therefore improving the efficacy of HPLC (with diode array detection) and bioluminescence analysis. After the 30 min equilibration period, the preparations were incubated with 1.8 ml gassed Tyrode's solution, which was automatically changed every 15 min by emptying and refilling the organ bath with the solution in use. The preparations were electrically stimulated once, 15 min after starting sample collection (zero time), using 3000 square wave pulses of 1 ms duration delivered at a 5 Hz frequency. In these experiments, only the sample collected before stimulus application and the two samples collected after stimulation, were retained for analysis. Bath aliquots (50-250 μ L) were frozen in liquid nitrogen immediately after collection, stored at -20°C (the enzymes are stable for at least 4 weeks) and analysed within 1 week of collection by HPLC with diode array detection (Finigan Thermo Fisher Scientific System LC/DAD, equipped with an Accela Pump coupled to an Accela Autosampler, a diode array detector and an Accela PDA running the X-Calibur software chromatography manager). Chromatographic separation was carried out through a Hypersil GOLD C18 column (5 μ M, 2.1 mm x 150 mm) equipped with a guard column (5 μ m, 2.1 mm x 1 mm) using an elution gradient composed of ammonium acetate (5 mM, with a pH of 6 adjusted with acetic acid) and methanol. During the procedure the flow rate was set at 200 μ L/min and the column temperature was maintained at 20°C. The autosampler was set at 4°C and 50 μ L of standard or sample solution was injected, in duplicate, for each HPLC analysis. In order to obtain chromatograms and quantitative analysis with maximal sensibility,

the diode array detection wavelength was set at 259 nm for ADO and 248 nm for INO. In parallel, the ATP content of the same samples was evaluated with the luciferin-luciferase ATP bioluminescence assay kit HS II (Roche Applied Science, Indianapolis, Indiana) (see e.g. Pinheiro *et al.*, 2013; Timóteo *et al.*, 2014). Luminescence was determined using a multi detection microplate reader (Synergy HT, BioTek Instruments). Stimulation-evoked release of adenine nucleotides and nucleosides was calculated by subtracting the basal release, measured in the sample collected before stimulation, from the total release of adenine nucleotides and nucleosides determined after stimulus application.

Immunofluorescence staining and confocal microscopy observation

LM-MP fragments were isolated from the rat ileum as previously described. The LM-MP fragments were stretched to all directions and pinned onto Petri dishes coated with Sylgard®. The tissues, then, were fixed in PLP solution (paraformaldehyde 2%, lysine 0.075 M, sodium phosphate 0.037 M, sodium periodate 0.01 M) for 16 h at 4 °C. Following fixation, the preparations were washed three times for 10 min each using 0.1 M phosphate buffer. At the end of the washout period, tissues were cryoprotected during 16 h with a solution containing anhydrous glycerol 20% and phosphate buffer 0.1 M at 4 °C and, then, stored at -20 °C for further processing. Once defrosted, tissue fragments were washed with tamponated phosphate saline buffer (PBS) and incubated with a blocking buffer, consisting in foetal bovine serum 10%, bovine serum albumin 1%, Triton X-100 0.3% in PBS, for 2 h; washout was facilitated by constant stirring of the samples. After blocking and permeabilization, samples were incubated with selected primary antibodies (see Table 10) diluted in the incubation buffer (foetal bovine serum 5%, serum albumin 1%, Triton X-100 0.3% in PBS), at 4 °C, for 48 h. For double immunostaining, antibodies were combined before application to tissue samples. Following the washout of primary antibodies with PBS supplemented with Triton-X 0.3% (3 cycles of 10 min), tissue samples were incubated with species-specific secondary antibodies in the dark for two hours, at room temperature. Finally, tissue samples were mounted on optical-quality glass

slides using VectaShield as mounting media (VectorLabs) and stored at 4 °C. Observations were performed and analyzed with a laser-scanning confocal microscope (Olympus FluoView, FV1000, Tokyo, Japan).

Table 10: Primary and secondary antibodies used in immunohistochemistry experiments.

ANTIGEN	CODE	SPECIES	DILUTION	SUPPLIER
<u>Primary antibodies</u>				
Adenosine receptor A ₁	AB1587P	Rabbit (rb)	1:50	Chemicon
Adenosine receptor A _{2A}	A2aR21-A	Rabbit (rb)	1:150	Alpha Diagnostics
NTPDase1	rN1-6 _L I ₄	Rabbit (rb)	1:1000	http://ectonucleotidases-ab.com
NTPDase2	rN2-6 _L	Rabbit (rb)	1:400	http://ectonucleotidases-ab.com
NTPDase3	rN3-1 _L I ₅	Rabbit (rb)	1:150	http://ectonucleotidases-ab.com
NTPDase8	rN8-8 _C I ₅	Guinea-pig (gp)	1:600	http://ectonucleotidases-ab.com
Ecto-5'-nucleotidase	rNu-9 _L I ₅	Rabbit (rb)	1:1000	http://ectonucleotidases-ab.com
ADA	sc-25747	Rabbit (rb)	1:50	Santa Cruz
<u>Secondary antibodies</u>				
Alexa Fluor 488 anti-rb	A-21206	Donkey	1:1000	Molecular probes
TRITC 568 anti-gp	706-025-148	Donkey	1:150	Molecular probes

Antibody production

All primary antibodies used in this study have previously been validated (Enjyoji *et al.*, 1999; Heine *et al.*, 2001; MartínSantué *et al.*, 2009; Bartel *et al.*, 2006; Fausther *et al.*, 2007): rabbit rN1-6L to rat NTPDase 1, rabbit rN2-6L to rat NTPDase2, rabbit rN3-1L to rat NTPDase3, guinea pig rN8-8C to rat NTPDase8, rabbit rNU-9L and rabbit rNU-4L to rat ecto-5'-nucleotidase/CD73. Genetic immunization protocol was carried out with plasmids (pcDNA3.1) encoding each protein, in New Zealand rabbits for antibodies against rat NTPDase1, rat NTPDase2, rat NTPDase3, rat ecto-5'-nucleotidase and Hartley guinea pigs for rat NTPDase8 antibodies. All procedures were approved by the Canadian Council on Animal Care and the Université Laval Animal Welfare Committee.

Materials and solutions

Adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), ADO, INO, hypoxanthine (HX); 2,4,6-trinitrobenzenesulphonic acid (TNBS); choline chloride, paraformaldehyde (prills), lysine, sodium periodate, anhydrous glycerol, fetal bovine serum (Sigma, St Louis, MO, USA); serum albumin, Triton X-100, metanol, potassium dihydrogen phosphate (KH_2PO_4) (Merck, Darmstadt, Germany); dipyridamole (Boehringer Ingelheim, Germany); 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (Research Biochemicals, Natick, MA, USA); mibefradil dihydrochloride, tetrodotoxin (TTX), (4-(2-[7-amino-2-(2-furyl{1,2,4}-triazolo{2,3- α {1,3,5}triazin-5-yl-aminoethyl) phenol (ZM 241385) (Tocris Cookson Inc., UK); [methyl- ^3H]-choline chloride (ethanol solution, 80 Ci mmol $^{-1}$) (Amersham, UK); Pic®A reagent (Waters corporation, Milford, USA); ATP bioluminescence assay kit HS II (Roche Applied Science, Indianapolis, Indiana); medetomidine hydrochloride (Domitor®, Pfizer Animal Health); atipamezole hydrochloride (Antisedan®, Orion, Espoo, Finland); ketamine hydrochloride (Imalgene®, Merial, Lyon, France); Sodium chloride 0,9%, tramadol hydrochloride (Labesfal, Santiago de Besteiros, Portugal).

All drugs were prepared in distilled water. All stock solutions were stored as frozen aliquots at -20°C . Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used (0.5 % v/ v), were observed. The pH of the perfusion solution did not change by the addition of the drugs in the maximum concentrations applied to the preparations.

Presentation of data and statistical analysis

The values are expressed as mean $\pm$ SEM, with n indicating the number of animals used for a particular set of experiments. Statistical analysis of data was carried out using paired or unpaired Student's t-test or one-way analysis of

variance (ANOVA) followed by Dunnett's modified t-test. $P < 0.05$ represents significant differences.

RESULTS

Postinflammatory ileitis led to increases in gastrointestinal transit in the rat

Histology sections of the ileum of TNBS-injected rats were characterized using the criteria proposed by Pontell and Jergens (see Materials and Methods) (Pontell *et al.*, 2009; Jergens, 1999). One week after the TNBS treatment we observed a proliferative regeneration of the mucosal integrity. Postinflammatory infiltrates consisting mainly with eosinophils, lymphocytes and macrophages were scarcely seen, but when present extended to the submucosa, enteric plexuses and muscular layers. At that time, the total ileal wall thickness of TNBS-treated rats was increased due to increase in thickness of both mucosal and muscular layers.

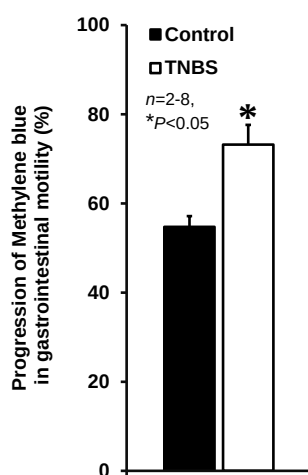


Figure 14: Progression of methylene blue dye gavage along the GI tract during 30 minutes in control and TNBS-injected animals. The total length of the small intestine was used to normalize data. The vertical bars represent SEM. $*P < 0.05$ (unpaired Student's t-test) represents significant differences as compared to the control situation.

The GI motility was evaluated prior to sacrifice of the animals. This was done by measuring the progression of methylene blue dye gavage during 30 min. Figure 14 shows that methylene blue dye progression was significantly ($P < 0.05$)

faster in TNBS-injected animals than in control animals, indicating that GI propulsion is increased in postinflammatory ileitis. Although TNBS-ileitis in the rat lacks a typical chronic phase, the postinflammatory phase is characterized by increases in motility (see e.g. Moreels *et al.*, 2001).

Adenosine neuromodulation is impaired in postinflammation ileitis

Previous reports suggested that the purinergic control of cholinergic nerve activity may be significantly impaired during chronic intestinal inflammation, both in the colon of rabbits and guinea-pigs (Deporotere *et al.*, 2002; Strong *et al.*, 2010), and in mice ileum (De Man *et al.*, 2003). Despite the differences in the mechanisms used to produce intestinal inflammation among these groups, all are unanimous on the indication for the need of more studies in order to investigate the underlying pathways responsible for disturbed purinergic neuromodulation. Hence, we focused our attention on the net tonic action of endogenous ADO on electrically-evoked [³H]-ACh release from myenteric motoneurons seven days after an inflammatory insult to the rat ileum. In preparations from healthy rats, the A₁ receptor antagonist, DPCPX (10 nM) increased the release of [³H]-ACh by 27±4% (n=4) (Figure 15A). Conversely, selective blockade of adenosine A_{2A} receptors with ZM 241385 (50 nM) significantly decreased the evoked tritium outflow by 37±10% (n=6). The results indicate that endogenous adenosine exerts a dual role on evoked [³H]-ACh release via the activation of inhibitory A₁ and excitatory A_{2A} receptors in the rat ileum (cf. Duarte-Araújo *et al.*, 2004; Correia-de-Sá *et al.*, 2006). However, when similar experiments were conducted in the LM-MP seven days after ileal inflammation none of the adenosine receptor antagonists caused any measurable change on evoked transmitter release (Figure 15A). These results indicate that ADO modulation of cholinergic nerve activity is severely impaired in postinflammation ileitis, as previously suggested using both myographic recordings and electrophysiology methods (see above).

Several authors hypothesized that purinoceptors downregulation in the ENS can occur during prolonged contact with purines. This was predicted since purines, such as ATP and ADO, can be released from activated inflammatory cells

(Marquardt *et al.*, 1984) in the vicinity of myenteric neurons (Bogers *et al.*, 2000). To elucidate this contention, we performed immunolocalization studies by confocal microscopy to assess changes in the expression and localization of both A_1 and A_{2A} adenosine receptors that could contribute to explain the lack of endogenous ADO neuromodulatory tonus in the inflamed ileum. The immunoreactivity against A_1 receptors is located predominantly on cell bodies of myenteric neurons of the ileum in control as well as in TNBS-injected rats (Figure 15B). The LM fibers also stain positively with the adenosine A_1 receptor antibody (data not shown). Yet, no significant differences were observed between control and inflamed preparations (Figure 15B).

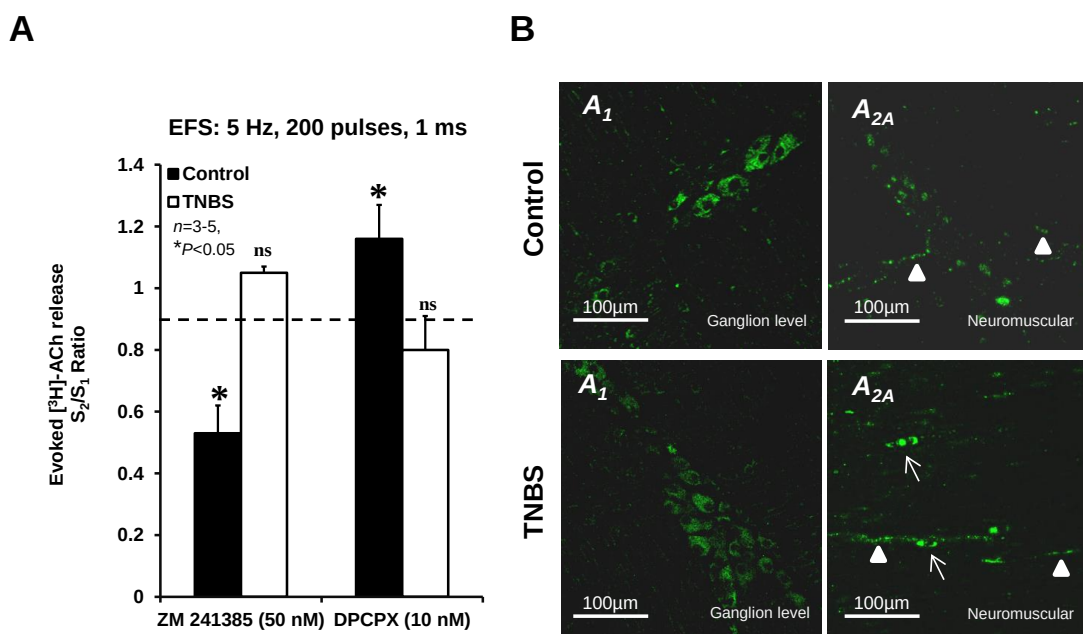


Figure 15: (A) Actions of selective adenosine A_1 and A_{2A} receptor antagonists on [^{3} H]-ACh release from myenteric motoneurons stimulated electrically (5 Hz, 200 pulses, 1 ms duration). DPCPX (10 nM, A_1 receptor antagonist) and ZM 241385 (50 nM, A_{2A} receptor antagonist) were added to the incubation media before the second period of stimulation (S_2) and were present throughout the assay. The ordinates are changes in S_2/S_1 ratios compared to the S_2/S_1 ratio obtained in control conditions, i.e. with no drugs added. The data are means $\pm$ SEM of three to five individual experiments. * $P < 0.05$ (one-way ANOVA followed by Dunnett's modified t-test) represent significant differences when compared to the situation where no drugs were added (dashed line). **(B)** Confocal images of whole-mount preparations of LM-MP preparations of the ileum from control and TNBS-injected rats. Adenosine A_1 receptor immunoreactivity is predominantly present in ganglion neuronal cell bodies in control and TNBS-injected preparations. Adenosine A_{2A} receptor immunoreactivity is mainly present in nerve bundles and axon terminals of myenteric neurons (triangles). In inflamed preparations, adenosine A_{2A} receptor staining is also observed in few mononuclear cells infiltrating the neuromuscular layer (arrows).

Likewise, the A_{2A} receptor immunoreactivity, which is more evident on myenteric nerve terminals, also did not significantly differ among control and inflamed tissues (Figure 15B). Interestingly, we observed staining against the A_{2A} receptor in few mononuclear cells (probably lymphocytes) infiltrating the neuromuscular level, which might be responsible for the immunosuppressant effect of A_{2A} receptors activation in experimental ileitis (Odashima *et al.*, 2005; Cavalcante *et al.*, 2006). Mononuclear infiltrates containing A_{2A}-positive immune cells may contribute to the increased mRNA expression of this receptor found in chronic inflamed intestinal tissues (Antonioli *et al.*, 2011). Although we cannot exclude at this stage differences in the intracellular signaling pathway triggered by activation of the two high affinity adenosine receptor subtypes, A₁ and A_{2A}, in the LM-MP of the rat ileum, our data suggest that receptors expression is fairly conserved in the ileum seven days following an inflammatory insult and, apparently, does not underscore the lack of ADO neuromodulation tonus in this condition (see e.g., De Man *et al.*, 2003; Antonioli *et al.*, 2011; Antonioli *et al.*, 2013).

Postinflammatory ileum releases more ATP, which is not followed by adenosine formation

It has been hypothesized, but not yet proven, that disruption in ATP release and/or breakdown as most likely explanations for the suppression of purinergic neuromuscular transmission in inflamed regions of the distal colon (Strong *et al.*, 2010). It is possible that inflammatory mediators affect mitochondrial function, and therefore ATP synthesis, in the ulcerated region of the chronic inflamed intestine. Another possibility is that there may be increased expression of ectonucleotidases, which are responsible for the breakdown of ATP (Duarte-Araújo *et al.*, 2009; Neshat *et al.*, 2009), as has been demonstrated in the purinergic sympathetic regulation of submucosal blood vessels (Neshat *et al.*, 2009). Here, we measured the extracellular accumulation of ATP (by bioluminescence) in parallel with ADO plus INO content (by HPLC with diode array detection) in samples collected immediately before and after electrical stimulation of LM-MP preparations of the

ileum of both control and TNBS-injected rats. Figure 16 shows that, in healthy animals, stimulation of the LM-MP at a frequency of 5 Hz (3000 pulses of 1 ms duration) yield increased amounts of ATP in the incubation fluid, which was followed by even higher extracellular accumulation of adenine nucleosides, consisting mostly of ADO and INO. This pattern was totally reversed seven days after inflammation of the rat ileum. Basal levels of ATP significantly ($P < 0.05$) increase (Figure 16A), whereas the baseline of endogenous ADO plus INO decreased (Figure 16B), in the fluid collected from TNBS-treated LM-MP of the rat ileum as compared to control tissues. Although the amount of ATP and adenine nucleosides increased from baseline following electric stimulation of LM-MP preparations in both animal groups, released ATP levels reached higher values in inflamed preparations than in controls (Figure 16A), but the opposite was observed regarding the content of ADO plus INO (Figure 16B).

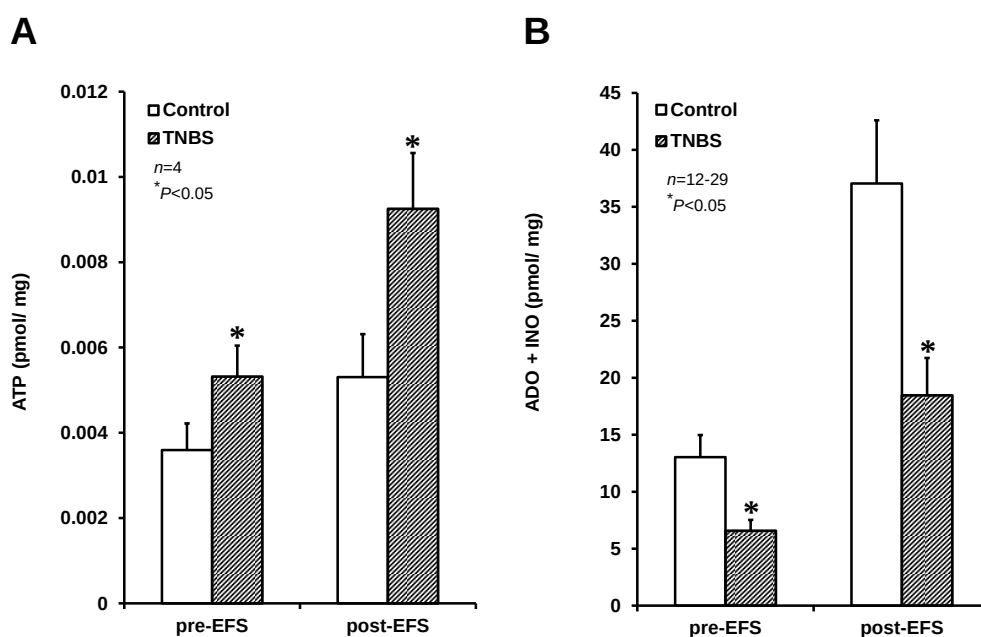


Figure 16: Effect of EFS on extracellular accumulation of purines in the LM-MP of control and inflamed rat ileum. The preparations were incubated with Tyrode's solution for 15 min and then subject to EFS (5 Hz frequency, 3000 pulses of 1ms duration). Samples collected from the incubation media were analysed by HPLC to separate and quantify purine nucleosides; ATP outflow was assessed using the luciferin-luciferase bioluminescence assay. Illustrated are ATP (**A**) and purine nucleosides (**B**) content of bath samples collected immediately before (Pre-EFS) and after (Post-EFS) LM-MP stimulation expressed in pmol/mg of tissue. Data are means $\pm$ SEM of an n number of individual experiments. *P < 0.05 (one-way ANOVA followed by Dunnett's modified t test) represent significant differences as compared to control animals.

These results indicate that, in contrast to previous hypothesis, the amount of ATP released in basal conditions and following electric stimulation of TNBS-treated ileal preparations was significantly higher than in control tissues, although one could not discard at this stage that ATP accumulation could also result from a decrease in the extracellular breakdown of the nucleotide (see below). Despite this increase in ATP accumulation, the content of ADO plus INO in the superfusates from TNBS-treated animals was severely decreased, which might contribute to explain the lack of endogenous ADO neuromodulatory tonus in postinflammatory ileitis (Figure 15; see also Antonioli *et al.*, 2011).

In healthy tissue, but not in the ileum following the inflammatory insult, stimulus-evoked ADO release was partially dependent on neuronal activity. This was evidenced because pretreatment of the preparations with tetrodotoxin (TTX), applied in a concentration (1 μ M) that essentially abolished evoked [^{3} H]-ACh release, reduced by about 50% adenine nucleosides outflow from control tissues with almost no effect in TNBS-treated preparations (Figure 17). These findings indicate that barely half of the extracellular ADO released from stimulated LM-MP preparations from healthy rats comes from TTX-sensitive neuronal cells, but this source may be severely affected in the postinflammation phase of TNBS-ileitis due to neuronal dysfunction, as demonstrated by several authors. Under conditions leading to myenteric neuronal dysfunction such as chronic inflammation, ATP release may be shifted from a neuronal origin towards other cell sources (e.g. smooth muscle fibers, glial cells, ICC, infiltrating immune cells) (see e.g., Nitahara *et al.*, 1995). As a matter of fact, evoked ATP release from TTX-resistant non-neuronal sources increased ($P<0.05$) above baseline from 7.4 ± 0.6 fmol/mg ($n=4$) in control animals to 13.2 ± 0.2 fmol/mg ($n=4$) in TNBS-treated preparations.

Smaller amounts of adenosine released from inflamed ileum originates from activated pacemaker interstitial cells of Cajal via equilibrative nucleoside transporters

Considering that ADO may also be released as such from non-neuronal cells at the tripartite myenteric synapse, we tested whether stimulus-evoked

smooth muscle contraction and activation of pacemaker ICCs affected the outflow of adenine nucleosides from the ileum of control and TNBS-injected rats. Blockade of smooth muscle contractions with the Ca_v1 (L-type) voltage-sensitive calcium channel inhibitor, nifedipine (1-5 μM), did not affect the accumulation of adenine nucleosides in bath samples collected immediately before and after electric field stimulation to the LM-MP from healthy animals (Correia-de-Sá *et al.*, 2006). However, selective blockade of Ca_v3 (T-type) calcium channels located predominantly in ICCs with mibefradil (3 μM) significantly ($P < 0.05$) depressed the outflow of adenine nucleosides from both control and TNBS-treated preparations (Figure 17). The results indicate that ICCs are the main source of ADO released from stimulated LM-MP of the ileum in the postinflammation phase. Data also indicate that ICCs cooperate with myenteric neurons to increase extracellular ADO in preparations from healthy rats.

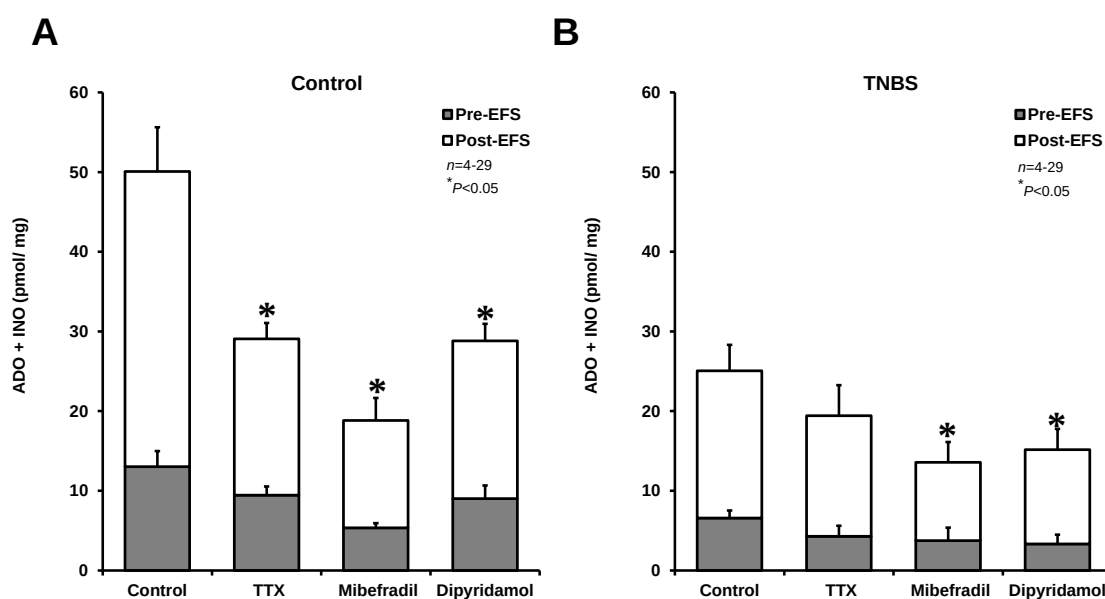


Figure 17: Influence of tetrodotoxin (TTX, 1 μM), mibefradil (3 μM) and dipyridamol (0.5 μM) on stimulation-evoked release of purine nucleosides (ADO plus INO) from the LM-MP of control and TNBS-injected rat ileum. Drugs were in contact with the preparations for at least 15 min before stimulus application (5 Hz frequency, 3000 pulses of 1 ms duration). Samples collected from the incubation media were analysed by HPLC with diode array detection. Illustrated is the purine nucleosides content of bath samples collected immediately before (Pre-EFS) and after (Post-EFS) stimulation of LM-MP expressed in pmol/mg of tissue. Data represent the means $\pm$ SEM of an n number of individual experiments. * $P < 0.05$ (one-way ANOVA followed by Dunnett's modified t test) represent significant differences from the evoked amount of purine nucleosides detected in control conditions where no drugs were added to the preparations.

Given that ADO is neither stored nor released as a classical neurotransmitter and previous findings from our laboratory demonstrated that the ecto-nucleotidase pathway contributes only partially to the total interstitial ADO concentration in the myenteric plexus (Correia-de-Sá *et al.*, 2006), we tested the involvement of ENTs to extracellular ADO released from tissues isolated from control and TNBS-injected rats. The nucleoside transport inhibitor, dipyridamole (0.5 μ M), decreased proportionally and by a similar magnitude to that obtained with mibefradil (3 μ M) the outflow of adenine nucleosides from both control and inflamed tissues (Figure 17). Thus, it is likely that extracellular ADO detected in ileum following an inflammatory insult originates predominantly from activated ICCs via ENTs.

Another potential source of endogenous ADO in the rat ileum may be adenosine 3',5'-cyclic monophosphate (cAMP) extruded from activated cells, which may be converted to AMP and then to ADO by ecto-phosphodiesterase and ecto-5'nucleotidase/CD73, respectively (Giron *et al.*, 2008). Despite the existence of a functional extracellular cAMP-adenosine pathway in the rat ileum, we were unable to detect any measurable amounts of cAMP by HPLC analysis (with diode array detection) of the samples used to quantify adenine nucleosides, even though we could identify a chromatographic peak corresponding to cAMP spectrum but with a higher retention time than ADO using a 30 μ M external standard (data not shown). These results suggest that, under the present experimental conditions, the cAMP-adenosine pathway does not account significantly for endogenous ADO formation in the LM-MP of the rat ileum.

The kinetics of the extracellular catabolism of adenine nucleotides (ATP and ADP) was not different in control and postinflammation preparations of the rat ileum

Despite the observation that the ileum in the postinflammation phase releases more ATP than control preparations, we observed a decrease in the extracellular ADO content in samples collected both under basal and stimulated conditions. It is, therefore, possible that disruption of ATP catabolism into ADO via ecto-NTPDases may occur in the inflamed LM-MP of the ileum. Figure 18 illustrates the time course of the extracellular catabolism of ATP and ADP in the LM-MP of the rat ileum from control and TNBS-treated rats. No significant differences ($P>0.05$) were observed among the half-degradation times of ATP (30 μ M) and ADP (30 μ M) in control and TNBS-treated preparations, i.e. the half degradation time of the two adenine nucleotides ranged from 6 to 7 min independently of the experimental condition.

Extracellular ATP (30 μ M) was metabolized into ADP, AMP, ADO, INO and HX, whose concentrations increased with time. Higher amounts of AMP as compared to ADP were detected in the bath at all time points following ATP (30 μ M) application (Figure 18A). ADP (30 μ M) catabolism led to AMP, ADO, INO and HX (Figure 18B). Interestingly, we detected differences in the accumulation of AMP and its degradation product, ADO, between control and postinflamed preparations incubated either with ATP (30 μ M) or ADP (30 μ M). The extracellular AMP content increased (orange bars), whereas ADO decreased (green bars), proportionally in the incubation fluid of TNBS-treated preparations as compared to those isolated from control rats following incubation with either ATP (30 μ M) or ADP (30 μ M) (Figure 18). Increases in AMP content of bath samples from postinflammation ileum reached the control levels 45 min after starting incubation with either ATP (30 μ M) or ADP (30 μ M), i.e. when the concentration of adenine nucleotides reached minimum (Figure 18).

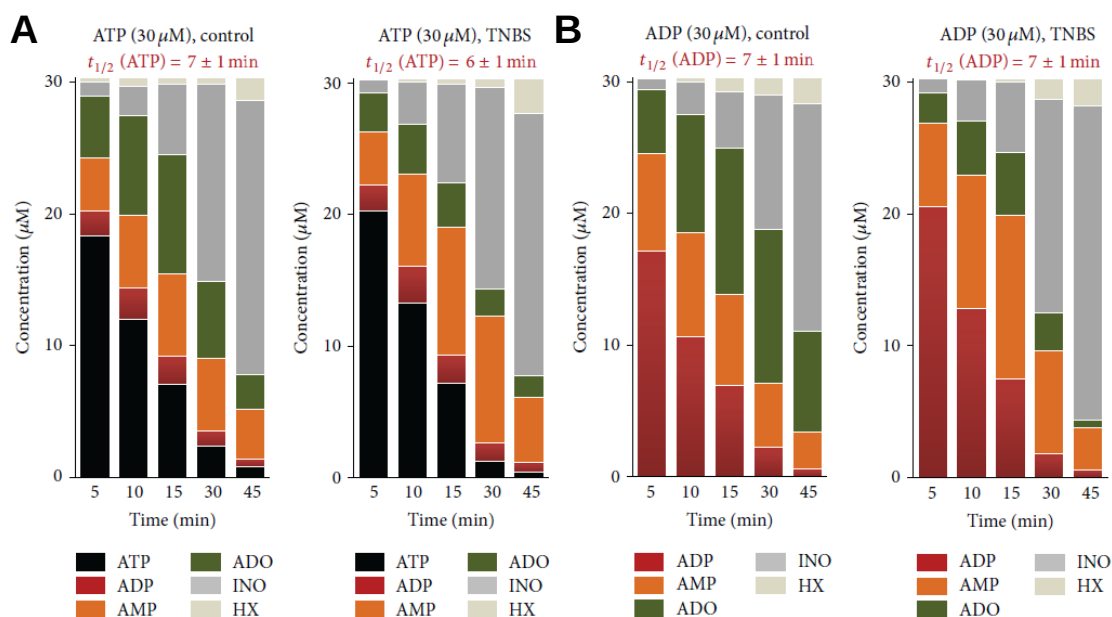


Figure 18: Kinetics of the extracellular catabolism of ATP (**A**) and ADP (**B**) in the LM-MP of the ileum of control and TNBS-injected rats. ATP and ADP (30 μM) were incubated at zero time (see Methods). Collected samples were analysed by HPLC with UV detection to separate and quantify ATP (black), ADP (red), AMP (orange), ADO (green), INO (dark grey) and HX (light grey). Average results obtained in four experiments.

Feed-forward inhibition of ecto-5'-nucleotidase/CD73 by adenine nucleotides controls adenosine formation in postinflammation ileitis

Data from Figure 18 clearly indicate that differences in ADO formation from the extracellular catabolism of adenine nucleotides in postinflammation ileitis implicate AMP dephosphorylation by ecto-5'-nucleotidase, which is the rate limiting enzyme for ADO formation in the rat myenteric plexus (Duarte-Araújo *et al.*, 2009). Surprisingly, we did not observe significant changes ($P > 0.05$) in the half degradation time of AMP (30 μM) between control and TNBS-treated preparations (Figure 19A). If any difference has to be mentioned, is the fact that the inflamed ileum accumulated proportionally less ADO in the extracellular milieu as compared to control tissues when AMP (30 μM) was used as substrate (Figure 19A). Overall, the results indicate that ADO is being rapidly converted into INO by ADA in the extracellular space of both control and TNBS-treated preparations (see below), given that stoichiometry of AMP conversion into ADO, INO and HX is kept

unaltered ruling out a significant contribution of the nucleoside uptake system (cf. Duarte-Araújo *et al.*, 2004).

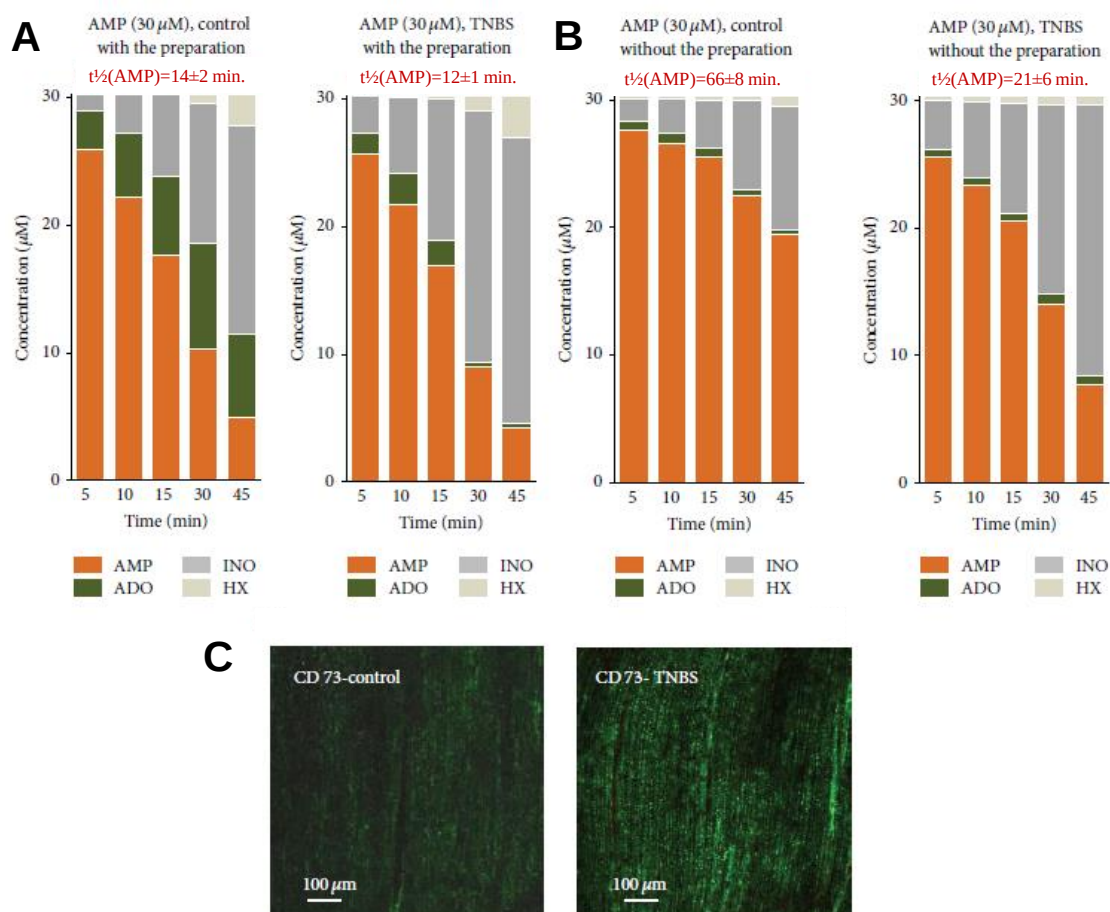


Figure 19: Kinetics of the extracellular catabolism AMP (30 μ M) in the LM-MP of the ileum of control and TNBS-injected rats (**A**). The residual ecto-5'-nucleotidase/CD73 activity (soluble form) in the incubation fluid after removing the preparations from the organ bath, is also shown (**B**). AMP (30 μ M) was incubated at zero time (see Methods). Collected samples were analysed by HPLC with UV detection to separate and quantify AMP (orange), ADO (green), INO (dark grey) and HX (light grey). Average results obtained in four experiments. Panel (**C**) shows that immunoreactivity against ecto-5'-nucleotidase/CD73 in longitudinal smooth muscle fibres of the ileum from TNBS-treated rats is much more evident than in control animals.

Since ecto-5'-nucleotidase/CD73 may be cleaved from its membrane GPI anchor by proteolysis retaining its catalytic activity in the incubation fluid, we decided to test whether binding of this enzyme to the plasma membrane was somehow affected during chronic inflammation in order to explain the differences detected in ADO formation from the hydrolysis of adenine nucleotides. To this end, we evaluated the 5'-nucleotidase activity in the fluid remaining in the bath after removing the preparation following a 45-min incubation period (Figure 19B). Under these experimental conditions, the half-life of AMP (30 μ M) was significantly ($P<0.05$) decreased in TNBS-treated preparations ($t_{1/2}=21\pm6$ min, two-times slower than with the tissue present) as compared to controls ($t_{1/2}=66\pm8$ min, almost five-times slower than with the tissue present). Data suggest that postinflammation ileitis causes an increase in unbound soluble forms of 5'-nucleotidase, which contribute to dephosphorylation of extracellular AMP into ADO without changing the global enzyme activity in the tissue. Notwithstanding, functional repercussions may be expected from this fact taking into consideration that ecto-5'-nucleotidase/CD73 is concentrated in the myenteric smooth muscle cell layer of healthy ileum (Nitahara *et al.*, 1995), whereas unbound soluble forms of the enzyme may generate ADO from extracellular AMP way from the most common site at the myenteric neuromuscular synapse in the inflamed ileum.

Given that we did not observe any significant differences in the AMP catabolism between control and TNBS-treated preparations (see Figure 19A) and we, still, detected increases in ecto-5'-nucleotidase/CD73 immunoreactivity in the longitudinal neuromuscular layer of the inflamed rat ileum (Figure 19C; see also (Antonioli *et al.*, 2011), we hypothesized that ecto-5'-nucleotidase/CD73 could be feed-forwardly inhibited by high extracellular levels of ATP and/or ADP which bind to the catalytic site of the enzyme blunting ADO formation from AMP in inflamed preparations (Figure 16; see also Magalhães-Cardoso *et al.*, 2003). The ecto-5'-nucleotidase activity was evaluated by quantifying the ratio [Nucleosides]:[Total nucleotides] per min, which is a direct measure of the activity of ecto-5'-nucleotidase, using either ATP (30 μ M) or ADP (30 μ M) as substrates. Figure 20A shows that this ratio increased progressively with time upon consumption of ATP

or ADP in the incubation media. The activity of ecto-5'nucleotidase/CD73 was significantly ($P<0.05$) impaired in the LM-MP of the ileum following an inflammatory insult, particularly when ADP (30 μ M) was used as substrate. These findings agree with data from Figure 18, showing that ADO formation is delayed with a compensatory increase in AMP accumulation in the inflamed ileum as compared to control preparations which is compatible with our hypothesis that ATP and/or ADP feed-forwardly inhibit ecto-5'-nucleotidase/CD73 in postinflammation ileitis.

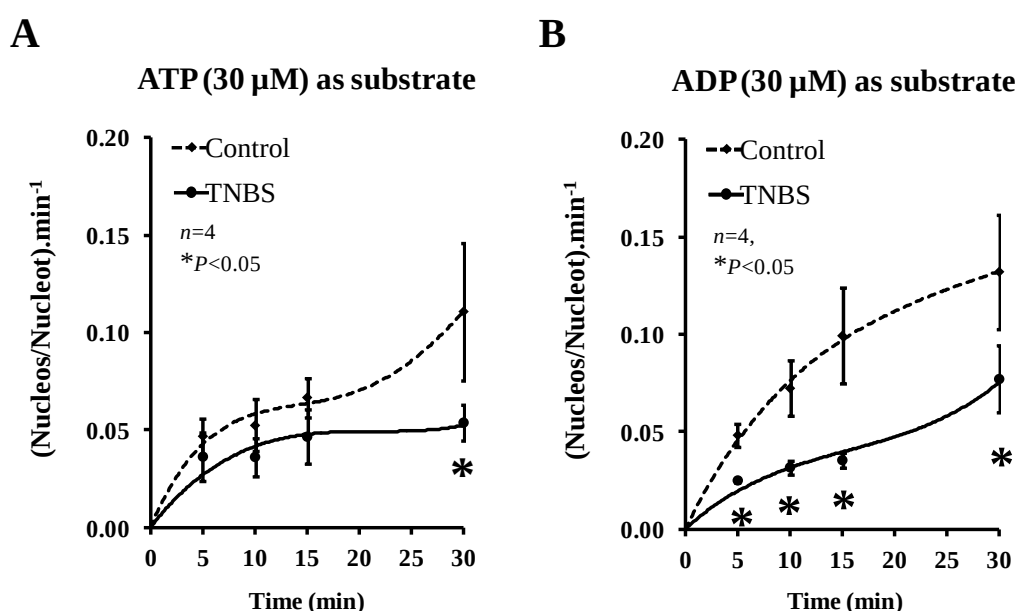


Figure 20: Activity of ecto-5'-nucleotidase/CD73 when ATP (**A**) and ADP (**B**) were used as substrates of the ectonucleotidase cascade in the LM-MP of the ileum of control and TNBS-injected rats. ATP and ADP (30 μ M) were incubated at zero time. Ecto-5'-nucleotidase/CD73 activity was evaluated by quantifying the ratio [Nucleosides]:[Nucleotides]/min. Average results obtained in four experiments; the vertical bars represent the SEM and are shown when they exceed the symbols in size. * $P < 0.05$ (one-way ANOVA followed by Dunnett's modified t test) represent significant differences as compared to control animals.

Tissue distribution changes of NTPDase2, NTPDase3 and NTPDase8 in postinflammation ileitis

The relative amounts of ATP and/or ADP near the ecto-5'-nucleotidase/CD73, which is concentrated in the myenteric smooth muscle layer

(Nitahara *et al.*, 1995; see also Figure 19C), is paramount to predict the magnitude of feed-forward inhibition of the enzyme by released adenine nucleotides. This may be determined by the expression and co-localization of NTPDases responsible for the kinetics of the catabolism of adenine nucleotides in control as well as in inflamed ileum.

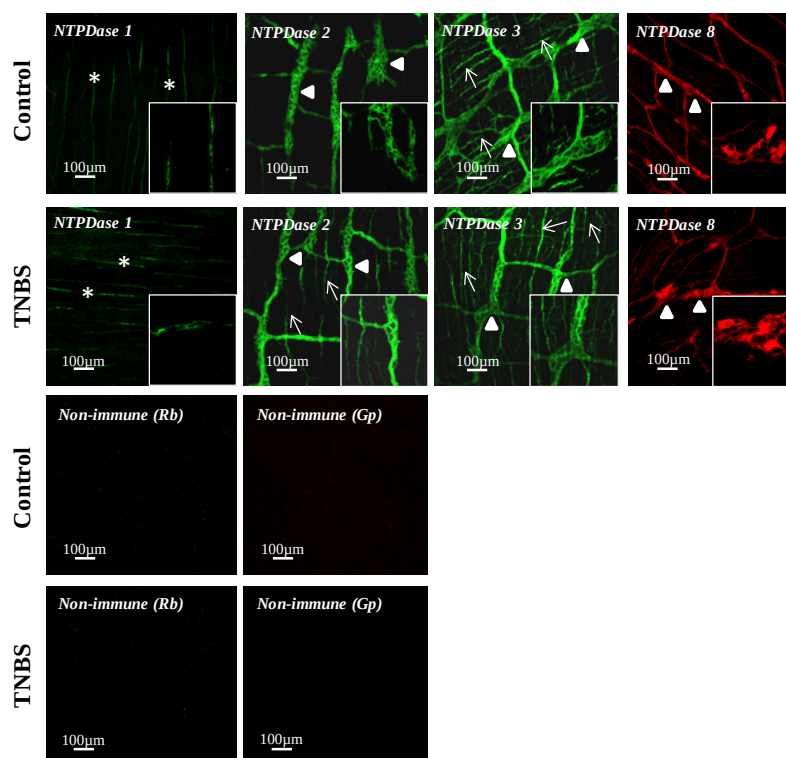


Figure 21: Localization of NTPDase1, NTPDase2, NTPDase3 and NTPDase8 immunoreactivity in single confocal images of whole-mount preparations of the LM-MP of the ileum of control and TNBS-injected rats. In healthy animals, NTPDase 1 immunoreactivity is present only in blood vessels (asterisks); NTPDase2 immunoreactivity is present predominantly in ganglion neuronal cell bodies (triangles) and large ramifications (primary meshwork) of the myenteric plexus, whereas NTPDase3 is also evident on myenteric axon terminals (arrows). In TNBS-treated preparations the NTPDase2 staining acquires a pattern that is very similar to that of NTPDase3, with NTPDase2 immunoreactivity also appearing in nerve bundles and axon terminals of myenteric neurons. NTPDase8 stains few ganglion neuronal cell bodies and large ramifications (primary meshwork) of the myenteric plexus; this pattern did not significantly change among control and TNBS-treated animals. No staining was obtained when non-immune sera from host species (rabbit, Rb, and guinea-pig, Gp) were used instead of interest primary antibodies.

Figure 21, shows that in control LM-MP preparations the immunoreactivity against NTPDase2 is restricted to ganglion cell bodies and large ramifications (primary meshwork) of the myenteric plexus (*cf.* Braun *et al.*, 2004). This contrasts

with the localization of NTPDase3 immunoreactivity, which is also evident on myenteric nerve trunks and terminals corresponding to secondary and tertiary neuronal meshworks, respectively (Lavoie *et al.*, 2011). At this time, we cannot exclude the presence of this enzyme on intramuscular ICCs. This pattern changes in the postinflammation ileum. In TNBS-treated preparations, the NTPDase2 immunoreactivity is also observed in the secondary and tertiary neuronal meshwork, meaning that ADP formation from released ATP by stimulated enteric neurons (White & Leslie, 1982) and smooth muscle fibres (Nitahara *et al.*, 1995) may contribute to prolong feed-forward inhibition of muscle-bound ecto-5'-nucleotidase/CD73 at the myenteric neuromuscular synapse following an inflammatory insult.

Apparently no significant differences were found in the expression and localization of NTPDase3 in the inflamed LM-MP of the rat ileum. Regarding NTPDase8, we were able to demonstrate its presence in few neuronal cell bodies of myenteric ganglia, with much lesser expression at the neuromuscular layer (Figure 21). Phenotypic characterization of NTPDase8 positive neuronal cell bodies deserves future investigations. Like NTPDase3, we found no gross changes in NTPDase8 immunoreactivity between control and TNBS-treated animals. We focused our attention on these three ectoenzymes, because we detected no immunoreactivity against NTPDase1 in the LM-MP of the rat ileum besides few blood vessels (Figure 21).

Postinflammation ileitis is accompanied by higher adenosine deaminase (ADA) activity: contribution of membrane-bound (ecto-ADA) and soluble (exo-ADA) forms of the enzyme

The bath concentrations of ADO (30 μ M) decrease progressively with time yielding to the formation of INO and HX in the LM-MP of the rat ileum (see Correia-de-Sá *et al.*, 2006). The extracellular catabolism of ADO (30 μ M) was faster ($t_{1/2}=24\pm3$ min, $n=4$) in TNBS-treated than in control preparations ($t_{1/2}=52\pm10$ min, $n=4$). The rate of extracellular ADO inactivation can be best appreciated by

calculating ADA activity in the present experimental conditions, which is represented in Figure 22A.

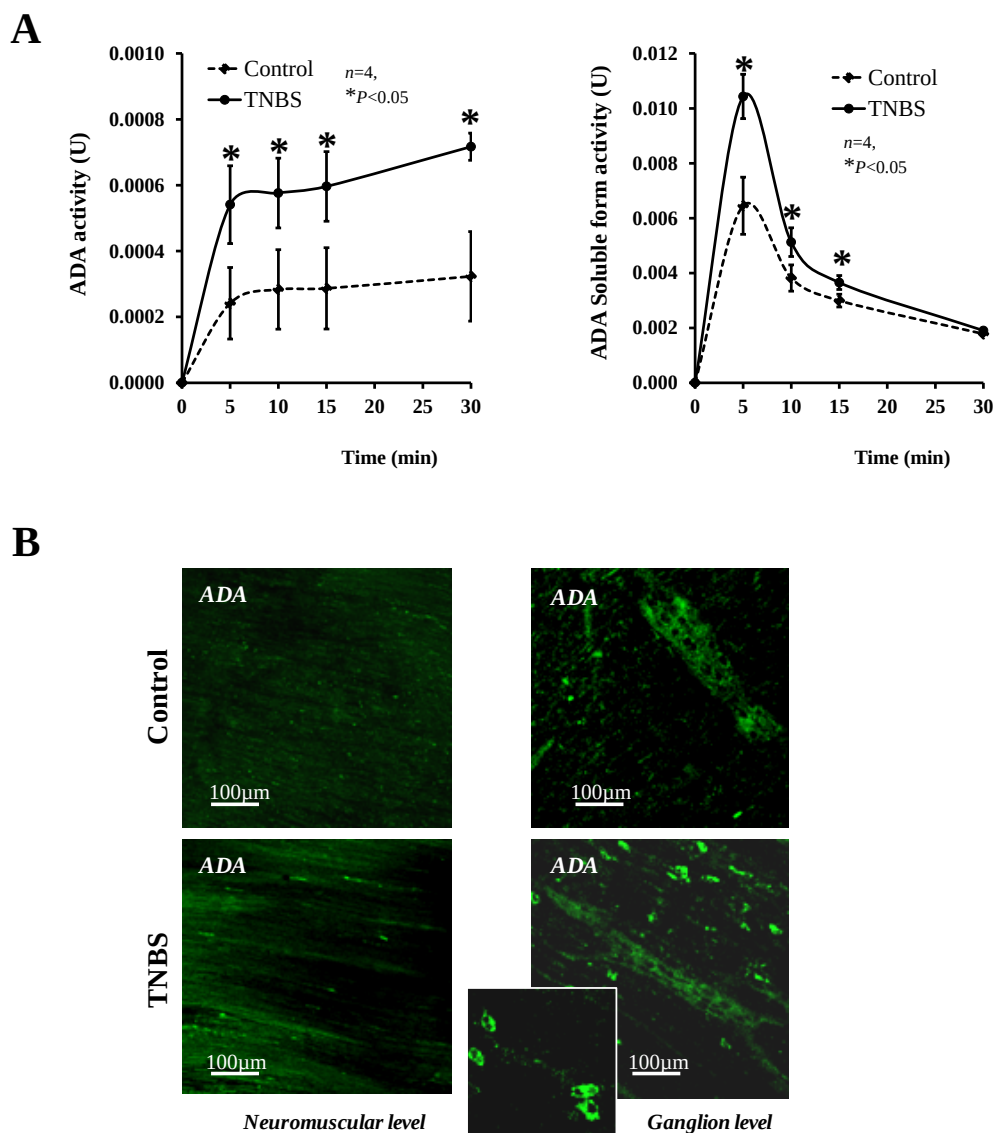


Figure 22: (A) Activity of ADA during the extracellular catabolism of ADO in the LM-MP of the ileum of control and TNBS-injected rats. ADO (30 µM) was incubated at zero time. Average results obtained in four experiments; the vertical bars represent the SEM and are shown when they exceed the symbols in size. * $P < 0.05$ (one-way ANOVA followed by Dunnett's modified t test) represent significant differences as compared to control animals. **(B)** Localization of ADA immunoreactivity in single confocal images of whole-mount preparations of the LM-MP of the ileum of control and TNBS-injected rats, taken both at ganglia and neuromuscular layers. Please note that ADA immunoreactivity is much more exuberant in preparations from TNBS-treated rats than in control animals. The figure insert details ADA staining in mononuclear inflammatory cells infiltrating myenteric ganglia.

Data indicate that the LM-MP of the ileum following an inflammatory insult has increasing amounts of ADA that we confirmed by immunofluorescence confocal microscopy (Figure 22B). ADA immunoreactivity was detected both at the ganglion level, as well as at the neuromuscular layer probably attached to smooth muscle fibers. In TNBS-treated preparations, ADA staining was also observed in mononuclear cells, most probably T lymphocytes and macrophages, infiltrating the myenteric plexus at the ganglion level (Martin *et al.*, 1995; Conlon & Law, 2004). Likewise, Antonioli and col. (2010) demonstrated by Western blot analysis increases in the expression of ADA at the level of inflamed colonic tissues. Like the ecto-5'-nucleotidase/CD73, extracellular ADA can be found attached to the plasma membrane (ecto-ADA) (Pettengill *et al.*, 2013), as well as in soluble forms after proteolytic cleavage from cell membranes (exo-ADA) (Franco *et al.*, 1997). Both forms retain catalytic activity. Figure 22A, also shows that the activity of the soluble form of ADA retained in the incubation fluid is highest during the first 5 min after removing the LM-MP preparation from the bath. Soluble ADA activity was not accompanied by any modification of lactate dehydrogenase activity, thus indicating that its activity is not due to damaged cells (see e.g. Correia-de-Sá *et al.*, 2006).

The soluble ADA activity was further increased in the incubation fluid which had been in contact for 45 min with preparations treated with TNBS. This implies that ADA is secreted in high amounts from the inflamed ileum, thus contributing to explain the lack of ADO neuromodulatory tonus. Even though INO may accumulate in postinflammation ileitis as a result of increased extracellular ADO deamination (see Figures 18 and 19), we failed to detect any variation on evoked [³H]-ACh release when INO was applied to the incubation fluid of control and TNBS-treated preparations in concentrations as high as 1 mM (data not shown). Catabolic degradation of ADO by soluble ADA may also result in the impairment of immune modulation by endogenous ADO (via A_{2A} receptors on mononuclear cells, see Figure 15B) and the consequent worsening of inflammation and tissue injury (Antonioli *et al.*, 2010).

DISCUSSION AND CONCLUSIONS

Enteric plasticity comprises a wide range of structural and/or functional changes in enteric neurons, glial cells and pacemaker ICCs, which are located between the muscle layers of GI tract. Different types of pathophysiological conditions drive to distinct adaptive responses. Enteric neurons are known to control all GI functions. They are able to rapidly change their structure, function or chemical coding to maintain the gut homeostasis (e.g. during inflammatory disorders) (Lakhan *et al.*, 2010). Despite heterogeneity of enteric adaptive responses, involvement of ubiquitous purine nucleotides and nucleosides provide fine tuning control of peristaltic motor performance in the gut during stressful situations, such as sustained neuronal activity, ischemia and inflammation (reviewed in Pettengill *et al.*, 2013). Numerous studies have described the potential role of purinergic function regulators in inflammation and immunity (Robson *et al.*, 2006; Antonioli *et al.*, 2013; Louis *et al.*, 2008; Sotnikov & Louis, 2010). There is recent evidence that blockade of adenosine kinase, which results in increased endogenous levels of ADO, downregulates the inflammatory response in experimental colitis (Siegmund *et al.*, 2001). In addition, Mabley *et al.* (2003) showed that INO resulting from the breakdown of ADO effectively reduced the inflammatory response in a murine model of colitis, despite we showed in this study that INO accumulation might not be sufficient to explain the purinergic neuromodulatory changes observed in postinflammation ileitis. These, and many other findings, suggest that ADO can mediate both pro-inflammatory (via A₁ or A_{2B} receptors) and/or anti-inflammatory/ immunosuppressant activities (mainly through A_{2A} and A₃ receptors) in inflammatory gut diseases, and they have potential for the treatment of these diseases (reviewed in Antonioli *et al.*, 2013). However, much is unknown to what extent the immune-modulatory role of purines during inflammation interferes with neuromodulation of the ENS (De Man *et al.*, 2003).

Control of cellular activity by purines depends on the activation of two families of membrane-bound purinoceptors, P1 and P2, which are sensitive to ADO and to adenine and/or uracil nucleotides, respectively. Interestingly, P1 and

P2 purinoceptors are also present on immune cells and there is evidence that ADO and ATP are generated at sites of inflammation (see, for a review (Cronstein, 1994). Purinoceptors activation is fine-tuned regulated by the extracellular conversion of released adenine and uracil nucleotides leading to the formation of other biologically active products, namely ADP/UDP and ADO, via a cascade of membrane-bound ectonucleotidases (Kukulski *et al.*, 2005; Yegutkin, 2008). Thus, cellular expression and topology of ectonucleotidase enzymes, as well as factors that extrinsically affects their catalytic activity (e.g. ionic concentration, nucleotide binding), create gradients of adenine nucleotides and their breakdown products, which may discriminate which nearby purinoceptor is more likely to be activated. For instance, under basal conditions ATP transiently facilitates ACh from non-stimulated myenteric neurons via prejunctonal P2X (probably P2X₂) receptors; however, increases in the amount of ATP released from stimulated neurons create conditions favourable to ADP accumulation at the myenteric synapse, leading to downregulation of transmitter release via the P2Y₁ receptor (Duarte-Araújo *et al.*, 2009). Strategic manipulation of the activity of ectonucleotidases has been proposed as a novel therapeutic approach to modify pathogenic purinergic signalling cascades (reviewed in Burnstock & Williams, 2000).

At the myenteric plexus, the rate limiting enzyme responsible for extracellular ADO formation from released adenine nucleotides is ecto-5'-nucleotidase/CD73 (Duarte-Araújo *et al.*, 2004, Duarte-Araújo *et al.*, 2009). This enzyme acts in a concerted manner with ADO inactivation pathways, both cellular uptake and ADA, to control extracellular levels of the nucleoside and, thereby, the activation of co-localized P1 receptors (Correia-de-Sá *et al.*, 2006). Interestingly, although much less has been explored in functional terms, the enzymes most relevant to control ADO levels in the extracellular milieu, both ecto-5'-nucleotidase/CD73 and ADA, may be cleaved from their anchor to the plasma membrane while retaining their catalytic activity in the soluble form (Pettengill *et al.*, 2013). Coincidentally or not, significant increases in ecto-5'-nucleotidase/CD73 and ADA mRNA expression were observed in inflamed colonic tissues (Antonioli *et al.*, 2014). Fragile docking of these enzymes to the plasma membrane is more

likely to occur within the context of inflammatory reactions, given that recruitment of inflammatory cells release a huge number of cytokines, chemokines and enzymes, some of these with proteolytic activity. One of these enzymes is glycosylphosphatidylinositol-specific phospholipase, which plays a role in inflammation since it can hydrolyse the GPI anchor of several membrane proteins and its hydrolytic products up-regulate cytokine expression in macrophages (O'Brien *et al.*, 1999). The widespread distribution of soluble forms of ecto-5'-nucleotidase/CD73 and ADA create conditions to unbalanced bulk production and/or inactivation of ADO way from its original location, thus affecting mass organ function. Likewise, inflammatory infiltrates including T lymphocytes, which are endowed with NTPDase1/CD39 and ecto-5'-nucleotidase/CD73 enzymes, may additionally contribute to ADO accumulation disturbance and to unpredictable P1-receptor-mediated responses in inflamed tissues. In light of these compelling hypotheses, this work was designed to investigate the kinetics of the catabolism of adenine nucleotides and ADO accumulation in the myenteric plexus of the ileum following an inflammatory insult.

Data from this study evidenced a disparity between higher amounts of ATP detected in the extracellular fluid released from TNBS-treated ileal preparations and deficits in the ADO levels measured in the same samples, both under baseline conditions and after electrical stimulation. This puzzling contradiction has not been detected before. On the contrary, indirect evidences made by other authors led to the hypothesis that the disruption of ATP release could be due to mitochondrial dysfunction induced by inflammatory mediators (Neshat *et al.*, 2009; Strong *et al.*, 2010), which was not confirmed in the present study. In postinflammation ileitis, higher ATP amounts can be originated from infiltrating immune and non-neuronal cells, such as glial cells, ICCs and smooth muscle contractions (see e.g., (Nitahara *et al.*, 1995); reviewed in Burnstock & Lavin, 2002, considering the proposed inflammatory neuronal dysfunction. Hemichannels containing pannexins may act as conduits for ATP release from non-neuronal cells in response to physiological and pathological stimuli. These channels are able to form signalling complexes with the purinergic P2X7 receptor, which activation leads to large pore formation

and consequently to further release of ATP (Diezmos *et al.*, 2013; Gulbransen *et al.*, 2012). These authors proposed a model where high extracellular levels of ATP chronically activate neuronal P2X7, pannexins and caspases. This relationship between P2X7 and pannexins has been related to the loss of enteric neurons during inflammatory conditions (Lakhan & Kirchgessner, 2010; Gulbransen *et al.*, 2012, Sanger, 2012). Moreover, our data add some information to explain the impairment of purinergic signalling during intestinal inflammation detected in other reports (De Man *et al.*, 2003, Depoortere *et al.*, 2002, Strong *et al.*, 2010) which has been expanded in this study by showing a lack of ADO neuromodulatory control of ACh release from postinflammation myenteric plexus. We provided compelling evidences that most of the ADO originated in the inflamed myenteric plexus of the rat ileum was derived from activated ICC's by the release of the nucleoside as such via dipyridamole-sensitive equilibrative transporters. This also agrees with previous findings from our group showing that ADO formation from released ATP, via the ecto-nucleotidase cascade, contributes only partially to the total interstitial ADO concentration in the myenteric plexus of healthy rats (Correia-de-Sá *et al.*, 2006).

The apparent disturbance of ADO generated from the extracellular catabolism of released ATP may be due to feed-forward inhibition of ecto-5'-nucleotidase/CD73 as a consequence of high extracellular levels of ATP, and particularly ADP, accumulated in the inflamed myenteric synapse [cf. Magalhães-Cardoso *et al.*, 2003]. ATP and ADP are competitive inhibitors of ecto-5'-nucleotidase/CD73 with inhibition constants in the low micromolar range. This indicates that they can bind to the active site of this enzyme, like AMP, but they cannot be hydrolysed (Sträter, 2006; Fausther *et al.*, 2012); recovery of ecto-5'-nucleotidase/CD73 activity occurs once extracellular ATP and ADP levels decrease below the micromolar range. Redistribution of NTPDase2 from ganglion cell bodies, which is the preferential localization of this enzyme in healthy animals, towards myenteric nerve terminals affords the most probable explanation for the unpredicted ADP accumulation in postinflammation neuromuscular synapse. This is possible because NTPDase2 is a preferential nucleoside triphosphatase

hydrolysing ADP 10 to 15 times less efficiently than ATP (Kukulski *et al.*, 2005), and we detected no changes in the distribution of NTPDase3 and NTPDase8 present in the myenteric plexus. Notably, no evidence of NTPDase1 expression was detected in the rat myenteric plexus, besides a few blood vessels. Thus, during postinflammation ileitis the hydrolysis of ATP into ADO at the myenteric synapse may be transiently interrupted by feed-forward inhibition of ecto-5'-nucleotidase/CD73, which makes a shift from P1 receptors activation (by ADO) to a preferential P2 receptors activation (by ATP or ADP). These data are in agreement with the hydrolysis of ATP by NTPDase2 and ecto-5'-nucleotidase/CD73 in the rat liver (Cunha, 2001). Indeed, depending with which NTPDase subtype ecto-5'-nucleotidase/CD73 co-localizes different amounts of ADO will be generated (Cunha, 2001). The functional interpretation of these findings in order to justify the increase in GI motility may be complicated by disturbances in compartmentalization originated by the release of significant amounts of soluble forms of the enzyme (Pettengil *et al.*, 2013; Sträter, 2006). Nevertheless, our results fully agree with other authors suggesting that ecto-5'-nucleotidase/CD73 might play a significant role in the modulation of purinergic signalling during enteric inflammation (Louis *et al.*, 2008; Antonioli *et al.*, 2013).

Previous findings from our group demonstrated that extracellular deamination by ADA is the most efficient mechanism regulating synaptic ADO levels and, consequently, tonic activation of facilitatory A_{2A} receptors in myenteric nerve terminals of healthy rats (Duarte-Araújo *et al.*, 2004). Besides the very high level of activity of ecto-ADA, a less-efficient nucleoside transport system sensitive to dipyridamole may also contribute to inactivation of extracellular ADO. Owing to the effectiveness of both inactivation mechanisms, endogenous ADO actions may be restricted to the release/production region at the myenteric cholinergic synapse, while exogenously added ADO seems to activate preferentially extrajunctional inhibitory A₁ receptors (Duarte-Araújo *et al.*, 2004; Vieira *et al.*, 2011). A question remains unanswered regarding the tonic effect of endogenous ADO on low affinity excitatory A₃ receptors, although selective agonists to this receptor have been shown to beneficially influence inflammation in experimental models (Guzma *et al.*,

2006, Antonioli *et al.*, 2010). Tandem localization of adenosine A₃ (on cell bodies) and A_{2A} (on nerve varicosities) receptors along myenteric neurons explains why the A₃ receptor activation may be prevented by ZM 241385 (50 nM), a selective A_{2A} receptor antagonist with low affinity for A₃ receptors ($K_i > 10 \mu\text{M}$). This implies that endogenous ADO acts preferentially on prejunctional A_{2A} under normal conditions making the activation of low affinity A₃ receptors by ADO high unlikely during the postinflammatory phase of TNBS-ileitis due to suppression/inactivation of the nucleoside (this study). The putative activation of A₃ receptors by endogenously formed INO via ADA also cannot explain the loss of the neuromodulatory influence of nucleosides in postinflammation ileitis. While we are uncertain regarding the extracellular levels of ADO at both particular locations, both *in vivo* and *in vitro* models suggest that the balance between inhibitory A₁ and excitatory A_{2A} may be important in regulating intestinal motility. For instance, De Man *et al.* (2003) reported that chronic intestinal inflammation enhanced enteric contractile activity in part due to a loss of the cholinergic neuromodulation mediated by A₁ receptors (De Man *et al.*, 2003). It is worth noting that human post-ganglionic myenteric neurons co-express adenosine A₁ and A_{2A} receptors, which exhibit a heterogeneous distribution (Christofi, 2001). Therefore, there is a considerable interest in the neuroprotective effects exerted by ADO during inflammatory (and ischemic) insults, and it is conceivable that adenosine accumulation disturbances may contribute to enteric excitability during pathological conditions.

Besides the proposed interruption of the ectonucleotidase cascade at the level of ecto-5'-nucleotidase/CD73 leading to AMP accumulation and low ADO formation in the inflamed myenteric plexus, our data also show that ADA activity is significantly enhanced in postinflammation ileitis thus contributing to decrease the extracellular ADO levels and, thereby, tonic activation of P1 receptors. ADA is the enzyme responsible for the deamination of ADO into INO. This enzyme has a wide phylogenetic distribution and its amino acid sequence is highly conserved, suggesting that ADA is a key enzyme in purine metabolism. Besides the classical intracellular form, ADA is reported to bind to the cell surface of T lymphocytes, via

the activation cell marker CD26 (Cristalli *et al.*, 2001). The presence of ADA in nerve terminals has also been demonstrated in many brain regions and it was hypothesized that ADA-containing terminals may release ADO or the enzyme itself, which could serve to regulate purinergic neurotransmission (Nagy *et al.*, 1990; Nagy *et al.*, 1996). In the rat myenteric plexus, we first described that ADA activity increased in bath samples collected after tissue stimulation in parallel with the interstitial accumulation of ADO and its metabolites (Correia-de-Sá *et al.*, 2006). This implies that the amount of ADO detected following stimulation of the preparation was largely underestimated as compared to the levels of the nucleoside in the biophase. Secretion of ADA may occur in several diseases, namely those affecting hematopoietic and immune (Ungerer *et al.*, 1992). To our knowledge, this is the first study reporting an increase in the activity of soluble ADA in the chronic inflamed myenteric plexus of the rat ileum. Our findings unravel the mechanism by which inhibition of ADA might attenuate inflammation in experimental colitis (Antonioli *et al.*, 2007).

In conclusion, data indicate that postinflammation ileitis suppresses ADO neuromodulation, which may contribute to increase GI motility. Impairment of ADO neuromodulation is most probably due to deficient accumulation of the nucleoside at the myenteric synapse despite the paradoxical increase in ATP release. Discrepancy between ATP outflow and ADO deficiency in the chronic inflamed ileum can be ascribed to feed-forward inhibition of ecto-5'-nucleotidase/CD73 by high extracellular levels of ATP and/or ADP. Interestingly, redistribution of NTPDase2, but not of NTPDase3, from ganglion cell bodies to myenteric nerve terminals leads to preferential ADP accumulation from released ATP, thus contributing to prolong inhibition of muscle-bound ecto-5'-nucleotidase/CD73 and to delay ADO formation at the inflamed neuromuscular synapse. On the other hand, depression of endogenous ADO accumulation may also occur due to enhancement of ADA activity. We observed a remarkably increase in the activity of soluble forms of ecto-5'-nucleotidase/CD73 and ADA in the postinflammatory phase of TNBS-ileitis, which may contribute to unbalanced bulk production and/or inactivation of ADO away from its location in the native tissue thus affecting organ

function. Thus, our findings suggest that pharmacological inhibition of ADA, and/or the use of AMP-derived prodrugs able to selectively activate P1 receptor co-localized with ecto-5'-nucleotidase/CD73, represent promising therapeutic strategies to ameliorate motility disturbances and immune reactivity inherent to inflammatory enteric disorders.

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PAPER 2

(Manuscript submitted for publication)

Postinflammatory ileitis induces neurochemical adjustments of purine cellular resources in the myenteric plexus

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ABSTRACT

Uncoupling between ATP overflow and extracellular adenosine formation changes purinergic signalling in postinflammatory ileitis. Loss of adenosine neuromodulation was ascribed to feed-forward inhibition of ecto-5'-nucleotidase/CD73 by high extracellular adenine nucleotides in the inflamed ileum. Here, we hypothesized that inflammation-induced changes in cellular density may also account to unbalance the release of purines and their influence on [³H]ACh release from TNBS-treated longitudinal muscle-myenteric plexus preparations of the rat ileum. The population of S100β-positive glial cells increase and a loss of ANO-1-positive interstitial cells of Cajal/fibroblast-like cells (ICC/FLC) were noted in the ileum 7-days after the inflammatory insult. In the absence of changes on VACHT-positive cholinergic nerves by confocal microscopy, the inflamed myenteric plexus released smaller amounts of [³H]ACh, which also became less sensitive to neuronal block with TTX (1μM). Instead, [³H]ACh release was attenuated by sodium fluoroacetate (5mM), carbenoxolone (10μM) and A438079 (3μM), which prevent activation of glial cells, pannexin-1 hemichannels and P2X7 receptors, respectively. Sodium fluoroacetate also decreased ATP release without significantly affecting adenosine levels indicating that surplus ATP overflow parallels reactive gliosis in postinflammatory ileitis. Conversely, loss of ICC/FLC may explain the lower adenosine levels in TNBS-treated preparations, since blockade of Ca_v3 (T-type) channels existing in ICC/FLC with mibefradil (3μM) or inhibition of equilibrative nucleoside transporters with dipyridamole (0.5μM), both decreased extracellular adenosine. Data suggest that postinflammatory ileitis

operates a shift on purinergic neuromodulation reflecting the upregulation of ATP-releasing enteric glial cells and the depletion of ICC/FLC that account for decreased adenosine overflow via nucleoside transporters.

Keywords: Postinflammatory ileitis; Acetylcholine release; Adenosine release; ATP release; Myenteric plexus; Enteric glia; Interstitial cells.

INTRODUCTION

Inflammation of the gastrointestinal (GI) tract triggers a series of adaptive morphological, chemical and functional changes in the cellular components responsible for maintaining gut homeostasis (Sharkey & Kroese, 2001). These involve the number and chemical coding of enteric neurons, but also the relative abundance and activity of adjacent non-neuronal cells such as enteric glia, interstitial cells of Cajal, fibroblast-like cells and smooth muscle fibers, directly or indirectly influenced by inflammatory infiltrates. Adaptive cellular responses may impact on the coordination of motor function, local blood flow, GI secretions and also on the endocrine and immune reactions (Costa *et al.*, 2000). As a matter of fact, the postinflammatory status is frequently accompanied by significant changes in enteric motility (Pontell *et al.*, 2009; Vieira *et al.*, 2014).

Changes in the release of purines together with adaptive modifications of purinoceptors expression and/or function are hallmarks of inflammatory reactions in most tissues, with the GI tract being no exception (Roberts *et al.*, 2012). Although purinergic signaling modifications underlying inflammatory responses of the GI tract are not fully understood, the extreme plasticity of the purinergic system and its pathophysiological impact on immune reactions, enteric neuronal networking and cellular communication make drugs targeting the purinergic cascade ideal candidates for treating inflammatory GI diseases. Purines, such as ATP and adenosine, are released from activated infiltrating inflammatory cells (Marquardt *et al.*, 1984), as well from resident neuronal and non-neuronal enteric cells (Stead *et al.*, 1989; Bogers *et al.*, 2000). ATP released in response to

inflammatory mediators is crucial for neutrophil activation and immune defense (Lazarowski *et al.*, 2011), but can also function as a danger signal preventing cells invasion of immune-privileged tissues, like myenteric ganglia (Bradley *et al.*, 1997).

In healthy individuals, ATP is co-released by vesicular exocytosis from enteric neurons with other neurotransmitters, like acetylcholine (ACh) (Burnstock, 1976). Mounting evidences indicate that ATP release from non-neuronal cells is also critical to control organ functions, in both normal and stressful conditions (Bodin & Burnstock, 2001; Lazarowski *et al.*, 2011; Mutafova-Yambolieva, 2012; Silva *et al.*, 2015). Non-neuronal release of ATP may be carried out by vesicular ATP transporters (VNUT) (Sawada *et al.*, 2008; Lazarowski *et al.*, 2011), as well as via other mechanisms involving activation of ABC proteins and hemichannels containing connexins and/or pannexins (Bodin & Burnstock, 2001; Lazarowski *et al.*, 2011; Timóteo *et al.*, 2014; Carneiro *et al.*, 2014; Silva *et al.*, 2015).

Once released from either neuronal or non-neuronal cells, ATP modifies organ functions by activating directly ionotropic P2X and metabotropic P2Y purinoceptors or indirectly, via P1 receptors, after being metabolized into adenosine through ecto-nucleotidases. At the myenteric neuromuscular synapse, ATP transiently facilitates [³H]ACh release from non-stimulated nerve terminals via the activation of P2X (most probably P2X₂ or P2X_{2/3}) receptors (Duarte-Araújo *et al.*, 2009). Fast conversion of ATP directly into AMP catalyzed by NTPDases 2 and 3 (Vieira *et al.*, 2015) and, subsequent, formation of adenosine by ecto-5'-nucleotidase/CD73, controls evoked [³H]ACh release through stimulation of high-affinity excitatory A_{2A} and/or inhibitory A₁ receptors located on nerve terminals and ganglion cells bodies of myenteric nerves, respectively (Duarte-Araújo *et al.*, 2004; 2009; Vieira *et al.*, 2011). However, inflammation may lead to overexpression of NTPDase2 at the myenteric synapse (Vieira *et al.*, 2014), which is a preferential nucleoside triphosphatase hydrolysing ADP 10 to 15 times less efficiently than ATP (Kukulski *et al.*, 2011). This promotes ADP accumulation and

subsequent downregulation of transmitter release via prejunctional inhibitory P2Y₁ receptors (Duarte-Araújo *et al.*, 2009).

In contrast to ATP, adenosine is not stored nor released from synaptic vesicles. Our group demonstrated that the ecto-nucleotidase pathway contributes only partially to the total interstitial adenosine concentration in the myenteric plexus (Correia-de-Sá *et al.*, 2006). Adenosine released as such from either neuronal or non-neuronal cells seems to be the main source of endogenous adenosine in the enteric nervous system (Duarte-Araújo *et al.*, 2004). This release is sought to be mediated by facilitated diffusion via equilibrative nucleoside transporters (Gu *et al.*, 1995; Duarte-Araújo *et al.*, 2004; Correia-de-Sá *et al.*, 2006) existing predominantly in interstitial cells of Cajal and/or fibroblast-like cells (ICC/FLC) (Vieira *et al.*, 2014). Another potential source of endogenous adenosine could be adenosine 3',5'-cyclic monophosphate (cAMP) extruded from activated cells, which can be converted to AMP and then to adenosine by ecto-phosphodiesterase and ecto-5'nucleotidase/CD73, respectively (Giron *et al.*, 2008), but this pathway does not account significantly for endogenous adenosine formation in the myenteric plexus of the rat ileum (Vieira *et al.*, 2014).

Uncoupling between ATP overflow and extracellular adenosine formation has been observed in postinflammatory ileitis (Vieira *et al.*, 2014). This situation, which disrupts the purinergic control of gut motility, has been ascribed to feed-forward inhibition of ecto-5'-nucleotidase/CD73 by high extracellular levels of adenine nucleotides together with an enhancement of adenosine deaminase (ADA) activity in the inflamed ileum. While these findings explain, at least partially, the loss of adenosine neuromodulation in the inflamed ileum, they miss the point regarding enhancement of the ATP-mediated tone. In this study, we hypothesized that inflammation-induced changes in the density of specific enteric resident cells could also account to unbalance the release of purines and, thus, their influence on evoked [³H]ACh release from longitudinal muscle-myenteric plexus preparations of rats with TNBS-induced postinflammatory ileitis.

MATERIAL AND METHODS

TNBS-induced intestinal inflammation rat model

Rats of either sex (Wistar, ~200 g) (Charles River, Barcelona, Spain) were kept at a constant temperature (21°) and a regular light- (06.30–19.30 h) dark (19.30–06.30 h) cycle, with food and water) and a regular light- (06.30–19.30 h) dark (19.30–06.30 h) cycle, with food and water *ad libitum*. Intestinal inflammation was produced by the instillation of 2,4,6-trinitrobenzenesulfonic acid (TNBS) into the lumen of the rat ileum. After a fasting period of 4–8 hours with free access to drinking water, rats undergo median laparotomy under anaesthesia with an association of medetomidine (10 mg/Kg) and ketamine (75 mg/Kg) given subcutaneously. At the end of the procedure animals were retrieved with atipamezole (10 mg/Kg). The terminal ileal loop was gently exteriorized, and TNBS (40 mM) was injected through the enteric wall into the lumen of the ileum, 10 centimeters proximal to the ileocolonic junction. Controls received 1 mL of 0.9 % saline. Sixty minutes after surgery, the rats were allowed to eat and drink *ad libitum*. After surgery, pain was controlled with tramadol hydrochloride (10 mg/Kg). To have a control of time course of body weight loss and recovery after injection of TNBS, rats were weighed prior to TNBS administration and daily following surgery. Animals with intestinal inflammation (TNBS) transiently lose weight for three to four days after surgery and regain weight thereafter. Seven days after surgery, animals were sacrificed following an overnight fasting period.

[³H]-acetylcholine release experiments

Sections of the rat ileum not including the terminal five centimetres were used. The longitudinal muscle strip with the myenteric plexus attached was separated from the underlying circular muscle according to the method of Paton and Vizi (1969). This preparation is highly enriched in cholinergic neurons, mainly excitatory neurons projecting to the longitudinal muscle (25%) that receive inputs from intrinsic primary afferents (26%) and from ascending and descending pathways (17%) (Costa *et al.*, 1996).

The procedures used for labelling the preparations and measuring evoked [^3H] acetylcholine ([^3H]ACh) release were previously described (Duarte-Araújo *et al.*, 2004, 2009; Correia-de-Sá *et al.*, 2006; Vieira *et al.*, 2011) and used with minor modifications. Longitudinal muscle-myenteric plexus (LM-MP) strips were mounted in 365 μL chambers of a semi-automated 12-sample superfusion system (SF-12 Suprafusion 1000, Brandel, Gaithersburg, MD, USA) heated at 37°C. After a 30-min equilibration period, nerve terminals were labelled during 40 min with 1 μM of [^3H]choline (specific activity 5 $\mu\text{Ci/nmol}$) under electrical field stimulation (1 Hz frequency, 1 ms pulse width) using two platinum-made grid electrodes placed above and below the muscle strip (transmural EFS stimulation). Following loading, the washout superfusion (1 ml/min) of the preparations was performed during 80 min with Tyrode's solution supplemented with the choline uptake inhibitor, hemicholinium-3 (10 μM). Tritium outflow was evaluated by liquid scintillation spectrometry (TriCarb2900TR, Perkin Elmer, and Boston, USA; % counting efficiency: 56 $\pm$ 2%) in 0.6 ml bath samples automatically collected every 1 min using the SF-12 suprafusion system. [^3H]ACh release was evoked by two periods of EFS (S_1 and S_2), each consisting of 200 square wave pulses of 1 ms duration delivered at a 5-Hz frequency.

Test drugs were added 8 min before S_2 and were present up to the end of the experiments. The change in the ratio between the evoked [^3H]ACh release during the two stimulation periods (S_2/S_1) relative to that observed in control situations (in the absence of test drugs) was taken as a measure of the effect of the tested drugs. Positive and negative values represent facilitation and inhibition of evoked [^3H]ACh release, respectively. None of the drugs significantly ($P>0.05$) changed the basal tritium outflow.

Release of ATP and adenine nucleosides (adenosine plus inosine)

The procedures used to measure ATP and adenine nucleosides were previously described (Vieira *et al.*, 2014). Experiments were performed using an automated perfusion system for sample collecting for given time periods, therefore improving the efficacy of HPLC (with diode array detection) and bioluminescence

analysis. After a 30-min equilibration period, the preparations were incubated with 1.8 mL gassed Tyrode's solution, which was automatically changed every 15 min by emptying and refilling the organ bath with the solution in use. The preparations were electrically stimulated once, 15 min after starting sample collection (zero time), using 3000 square wave pulses of 1ms duration delivered at a 5-Hz frequency. In these experiments, only the sample collected before stimulus application and the two samples collected after stimulation were retained for analysis. Bath aliquots (50–250 μ L) were frozen in liquid nitrogen immediately after collection, stored at -20°C (the enzymes are stable for at least 4 weeks) and analysed within 1 week of collection by HPLC with diode array detection (Finigan Thermo Fisher Scientific System LC/DAD, equipped with an Accela Pump coupled to an Accela Autosample, a diode array detector and an Accela PDA running the X-Calibur software chromatography manager). Chromatographic separation was carried out through a Hypersil GOLD C18 column (5 μ M, 2.1mm $\times$ 150 mm) equipped with a guard column (5 μ m, 2.1mm $\times$ 1 mm) using an elution gradient composed of ammonium acetate (5mM, with a pH of 6 adjusted with acetic acid) and methanol. During the procedure the flow rate was set at 200 μ L/min and the column temperature was maintained at 20°C . The autosampler was set at 4°C and 50 μ L of standard or sample solution was injected, in duplicate, for each HPLC analysis. In order to obtain chromatograms and quantitative analysis with maximal sensibility, the diode array detection wavelength was set at 259 nm for adenosine and 248 nm for inosine.

In parallel, the ATP content of the same samples was evaluated with the luciferin-luciferase ATP bioluminescence assay kit HS II (Roche Applied Science, Indianapolis, Indiana). Luminescence was determined using a multi detection microplate reader (SynergyHT, BioTek Instruments).

Stimulation-evoked release of adenine nucleotides and nucleosides was calculated by subtracting the basal release, measured in the sample collected before stimulation, from the total release of adenine nucleotides and nucleosides determined after stimulus application.

Myographic recordings of ileal contractile activity

The contractile activity of the LM-MP was recorded as previously described (Vieira *et al.* 2009, 2011; Mendes *et al.*, 2015). Ileum strips from control and TNBS-treated rats were mounted along the longitudinal axis in 14 ml capacity perfusion chambers connected to isometric force transducers. The changes in tension were recorded continuously with a PowerLab data acquisition system (Chart 5, v.4.2; AD Instruments, USA). Tissues were preloaded with 0.5 g of tension and allowed to equilibrate for 90 min under continuous superfusion with gassed (95% O₂ and 5% CO₂) Tyrode's solution at 37°C.

Immunofluorescence staining and confocal microscopy observation

LM-MP fragments were isolated from the rat ileum as previously described. The LM-MP fragments were stretched to all directions and pinned onto Petri dishes coated with Sylgard®. The tissues, then, were fixed in PLP solution (paraformaldehyde 2%, lysine 0.075M, sodium phosphate 0.037M, sodium periodate 0.01 M) for 16 h at 4°C. Following fixation, the preparations were washed three times for 10 min each using 0.1M phosphate buffer. At the end of the washout period, tissues were cryoprotected during 16 h with a solution containing anhydrous glycerol 20% and phosphate buffer 0.1M at 4°C and, then, stored at -20°C for further processing. Once defrosted, tissue fragments were washed with tamponated phosphate saline buffer (PBS) and incubated with a blocking buffer, consisting in foetal bovine serum 10%, bovine serum albumin 1%, triton X-100 1% in PBS, for 2 hours; washout was facilitated by constant stirring of the samples. After blocking and permeabilization, samples were incubated with selected primary antibodies (see Table 1) diluted in the incubation buffer (foetal bovine serum 5%, serum albumin 1%, Triton X-100 1% in PBS), at 4°C, for 48 h. For double immunostaining, antibodies were combined before application to tissue samples. Following the washout of primary antibodies with PBS supplemented with Triton X 1% (3 cycles of 10 min), tissue samples were incubated with species-specific secondary antibodies in the dark for two hours, at room temperature. Finally, tissue samples were mounted on optical-quality glass slides using

VectaShield as mounting media (VectorLabs) and stored at 4°C. Observations were performed and analyzed with a laser scanning confocal microscope (Olympus FluoView, FV1000, Tokyo, Japan).

Table 11: Primary and secondary antibodies used in immunohistochemistry experiments

ANTIGEN	CODE	SPECIES	DILUTION	SUPPLIER
<u>Primary antibodies</u>				
PGP 9.5	7863-1004	Mouse (ms)	1:500	Serotec
NF200	ab8135	Rabbit (rb)	1:1000	ABCAM
VaChT	AB1588	Guinea-pig (gp)	1:500	Chemicon
nNOS	ab1376	Goat (gt)	1:300	ABCAM
GFAP	MAB360	Mouse (ms)	1:600	Chemicon
S100	Ab868	Rabbit (rb)	1:400	ABCAM
Vimentin	M0725	Mouse (ms)	1/150	Dako
Ano-1	Ab53212	Rabbit (rb)	1:100	ABCAM
P2X7	APR-004	Rabbit (rb)	1/50	Alomone
CD11B (OX42)	Sc-53086	Mouse (ms)	1/50	Santa Cruz
<u>Secondary antibodies</u>				
Alexa Fluor 488 anti-rb	A-21206	Donkey	1:1000	Molecular probes
Alexa Fluor 488 anti-ms	A21202	Donkey	1:1000	Molecular probes
Alexa Fluor 568 anti-gt	A11057	Donkey	1:1000	Molecular probes
Alexa Fluor 568 anti-ms	A-10037	Donkey	1:1000	Molecular probes
Alexa Fluor 633 anti-ms	A21052	Goat	1:1000	Molecular probes
TRITC 568 anti-gp	706-025-148	Donkey	1:150	Jackson Immuno Research
Dylight 649 anti-gp	706-025-148	Donkey	1:100	Jackson Immuno Research

Materials and solutions

2,4,6-trinitrobenzenesulphonic acid (TNBS); carbenoxolone, choline chloride, paraformaldehyde (prills), lysine, sodium periodate, anhydrous glycerol, fetal bovine serum (Sigma, St Louis, MO, USA); serum albumin, triton X-100, metanol, potassium dihydrogen phosphate (KH_2PO_4) (Merck, Darmstadt, Germany); A438079, mibefradil dihydrochloride, L-NAME, tetrodotoxin (TTX)

(Tocris Cookson Inc., UK); Sodium Fluoroacetate (Supelco); [methyl-³H]-choline chloride (ethanol solution, 80 Ci mmol⁻¹) (Amersham, UK); ATP bioluminescence assay kit HS II (Roche Applied Science, Indianapolis, Indiana); medetomidine hydrochloride (Domitor, Pfizer Animal Health); atipamezole hydrochloride (Antisedan, Orion, Espoo, Finland); ketamine hydrochloride (Imalgene, Merial, Lyon, France); Sodium chloride 0.9%, tramadol hydrochloride (Labesfal, Santiago de Besteiros, Portugal).

All drugs were prepared in distilled water. All stock solutions were stored as frozen aliquots at -20°C. Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used (0.5% v/v), were observed. The pH of the perfusion solution did not change by the addition of the drugs in the maximum concentrations applied to the preparations.

Presentation of data and statistical analysis

The values are expressed as mean ± SEM, with *n* indicating the number of animals used for a particular set of experiments. Statistical analysis of data was carried out using paired or unpaired Student's *t*-test with Welch correction. *P*<0.05 represents significant differences.

RESULTS

Postinflammatory ileitis causes an increase in enteric glial cells (types III and IV) and a partial loss of pacemaker interstitial cells of Cajal / fibroblast-like cells

Structural changes accompanied by neuronal cell death have been observed in chronic intestinal inflammation (Sanovic *et al.*, 1999; Linden *et al.*, 2005; Venkataramana *et al.*, 2015). However, we found no obvious changes in the amount of neurons stained positively against (1) neurofilament NF200 expressed predominantly in Dogiel type I and II neurons (Hu *et al.*, 2002), and (2) the pan-neuronal marker, protein gene product 9.5 (PGP 9.5), in the myenteric plexus 7 days after instillation of TNBS into the lumen of ileum compared to control rats treated with saline (Figure 23c-f). This is compatible with Moreels *et al.* (2001) findings showing that TNBS-induced ileitis in the rat lacks the chronic inflammatory phase and is characterized by an acute transmural inflammation that is accompanied by functional abnormalities of neuronal activity which persists for at least 8 weeks without obvious neuronal loss (Stewart *et al.*, 2003; Nurgali *et al.*, 2007). Conversely, these authors found alterations in longitudinal muscle contractility which was attributed to structural thickness of the ileal wall.

Confocal micrographs show that neuronal cells are grouped in small clusters (enteric ganglia), which are interconnected by nerve fibre bundles from whom emerge small diameter nerve terminals (see *e.g.* Furness, 2006). Inflammatory infiltrates consisting of monocytes/ macrophages exhibiting immunoreactivity against the integrin CD11B/OX42 were found surrounding the myenteric plexus of TNBS-treated rats (Figure 23b), but not in preparations from control animals (Figure 23a). The absence of inflammatory cells inside myenteric ganglia has been observed before (see *e.g.* Figures 24B and 27B) and this is why myenteric ganglia are considered an immune-privileged tissue (Bradley *et al.*, 1997).

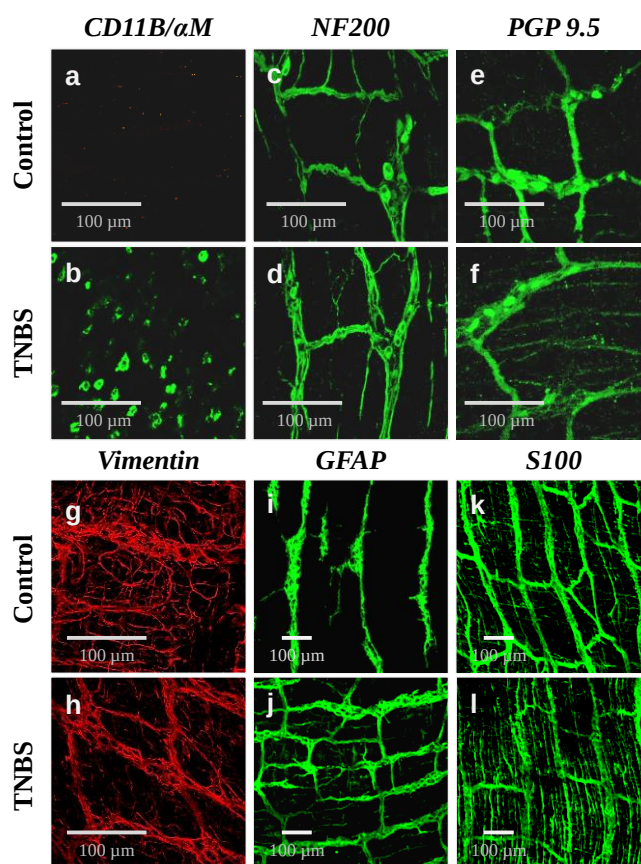


Figure 23: Confocal micrographs of whole-mount preparations of the longitudinal muscle-myenteric plexus of the ileum of control and TNBS-treated rats. Z-stacks illustrate the immunoreactivity against CD11B/αM (OX42) (marker of inflammatory cells) (a,b), NF200 (neurofilament expressed in neurons) (c,d), PGP9.5 (pan-neuronal cell marker) (e,f), vimentin (intermediate filament of mesenchymal cells, like ICCs and FLCs) (g,h), GFAP (i,j) and S100β (k,l) (enteric glial cells markers). Images are representative of at least four individuals per group. Scale bars = 100 μm.

Enteric glial cells express astrocytic cell markers, including the intermediate filament glial fibrillary acidic protein (GFAP) and the calcium-binding protein S100β (Gulbransen & Sharkey, 2012). Subtypes of enteric glia are classified in: type I, intraganglionic cells; type II, within interganglionic fibers; type III, form a matrix in the extraganglionic region remaining in close association with neuronal bundles; and type IV, elongated glia running with nerve fibres within the musculature (Boesmans *et al.*, 2015). Figure 23i-l shows that the myenteric plexus of rats treated with TNBS exhibits increased amounts of GFAP- and S100β-positive glial cells, which are particularly evident in extraganglionic (glial type III) and

intramuscular regions (glial type IV). Thus, in contrast to the absence of significant structural changes in the neuronal cell population, TNBS-induced ileitis stimulates enteric glial cells proliferation (Bradley *et al.*, 1997) or, at least, the increase in expression and/or synthesis of glial protein cell markers (Cabarrocas *et al.*, 2003), in a similar manner to that observed in astrocytes of the CNS (Lhermitte *et al.* 1980; Rühl *et al.*, 2004).

Interstitial cells of Cajal and fibroblast-like cells (ICC/FLC) are known to act as pacemaker cells and integrators of nerve activity and smooth muscle contraction, respectively (Sanders, 1996). The number of these cells can significantly change in pathological conditions (Ekblad *et al.*, 1998). Using vimentin, a characteristic intermediate filament of mesenchymal cells like ICC/FLC, we show here that the density of vimentin-positive cells decrease significantly in the myenteric plexus of TNBS-induced ileitis (Figure 23h) compared to control preparations (Figure 23g). Likewise, staining with anoctamine-1 (Ano-1), a calcium-activated chloride channel involved in the pacemaker activity of ICCs (Gomez-Pinilla *et al.*, 2009), also decreased in density 7-days after instillation of TNBS into the lumen of the rat ileum (Figure 24A). The partial loss of Ano-1 immunoreactivity was detected by confocal microscopy focusing in the ganglion layer (myenteric stellate cells; Figure 24Aa and 24Ab) and in the neuromuscular region (intramuscular spindle-shape cells, Figure 24Ac and 24Ad) of inflamed LM-MP preparations. The presence of auto-fluorescent inflammatory cells (coloured in red) was more visible surrounding the myenteric ganglia than at the intramuscular layer of TNBS-treated rats (see inset of Figure 24A).

The loss of ICC/FLC may affect the frequency of spontaneous enteric contractions (Kinoshita *et al.*, 2007). In fact, myographic recordings demonstrate that the frequency of spontaneous contractions of LM-MP preparations of the ileum of TNBS-treated animals were less frequent ($P<0.05$) than those observed in control animals (Figure 24A).

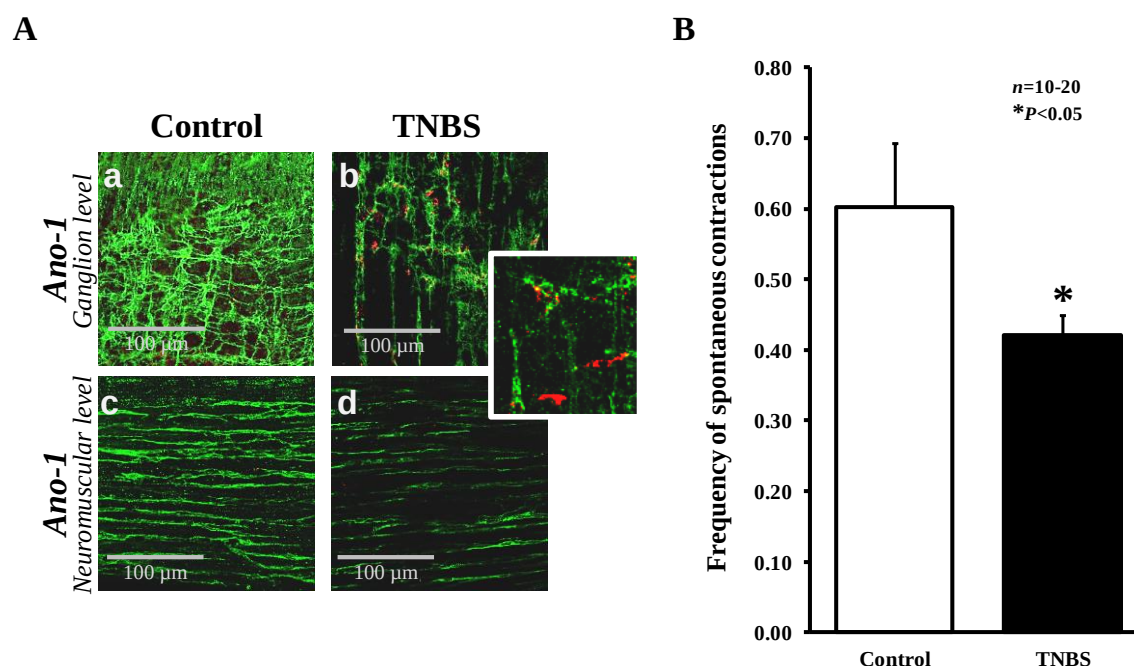


Figure 24: The partial loss of anoctamine-1 (Ano-1)-positive interstitial cells in the myenteric plexus of TNBS-treated rats correlates with the reduction in the frequency of spontaneous contractions of the rat ileum. **(A)** Confocal micrographs of whole-mount preparations of the ileum of control and TNBS-injected rats taken at the myenteric ganglion level (a,b) and at the longitudinal smooth muscle layer (c,d). Shown is the immunoreactivity against Ano-1, a calcium-activated chloride channel involved in the pacemaker activity of ICCs. At the ganglion level, but not at the neuromuscular junction layer, it is possible to visualize autofluorescent inflammatory cells (stained in red, inset) infiltrating the myenteric plexus of TNBS-treated rats. Images are representative of at least five individuals per group. Scale bars = 100 μm. **(B)** Histograms representing the frequency of spontaneous myographic contractions of the ileum from control rats and TNBS-treated animals. The data are means ± SEM of a *n* number of animals. **P*<0.05 (unpaired Student's *t*-test with Welch correction) represents a significant difference from control animals.

Myenteric neurons from the ileum of TNBS-treated rats release smaller amounts of [³H]ACh, but no relationship exists between cholinergic hypoactivity and nitrergic inhibitory signals

ACh is the prime regulator of intestinal motility and is the most important excitatory neurotransmitter in the myenteric plexus (Costa *et al.*, 1996). Figure 25A shows that cholinergic neurons from the myenteric plexus of TNBS-treated rats release significantly smaller amounts of [³H]ACh (see also Collins *et al.*, 1992a; Davis *et al.*, 1998), despite no obvious changes were detected by confocal microscopy in the density of cholinergic nerve fibres stained specifically against

the vesicular acetylcholine transporter (VACHT) (Arvidsson *et al.*, 1997) (Figure 25B).

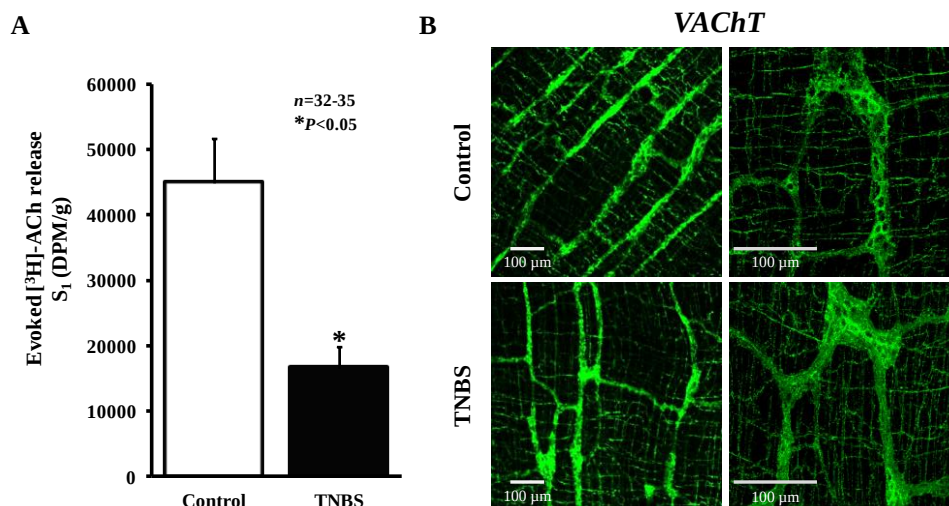


Figure 25: Electrically-stimulated myenteric neurons of TNBS-treated rats release smaller amounts of $[^3\text{H}]\text{ACh}$. **(A)** Shown is the amount of $[^3\text{H}]\text{ACh}$ released from electrically-stimulated (5 Hz, 1 ms, 200 pulses) myenteric neurons of the ileum of control and TNBS-treated rats during S_1 in disintegrations per min (DPM)/g of wet tissue. **(B)** Confocal micrographs of whole-mount preparations of the longitudinal muscle-myenteric plexus of the ileum from control and TNBS-treated rats stained against the vesicular ACh transporter (VACHT). The z-stacks shown are representative of at least five individuals per group. Scale bar = 100 μm .

These results prompted us to investigate whether cholinergic nerve hypoactivity had any relationship with increased inhibitory neurotransmission operated by NO, which can be released from neighbouring nitrergic nerves, enteric glia cells and/or infiltrating immunocytes (MacEachern *et al.*, 2015; see also Figure 26A). This was hypothesized because up-regulation of nitric oxide synthase (NOS) activity and enhancement of NO-mediated inhibitory neurotransmission were demonstrated in chronic inflammatory bowel diseases (Miller *et al.*, 1993). Figure 26B shows that inhibition of NOS activity with L-NAME (100 μM) had no effect on evoked $[^3\text{H}]\text{ACh}$ release from the LM-MP of the ileum of control and TNBS-treated rats, indicating that NO does not mediate inhibition of $[^3\text{H}]\text{ACh}$ release in TNBS-induced ileitis. This was concluded despite the relative increase in neuronal NOS-immunoreactivity in enteric ganglia cells of TNBS-treated animals (Figure 26Aii).

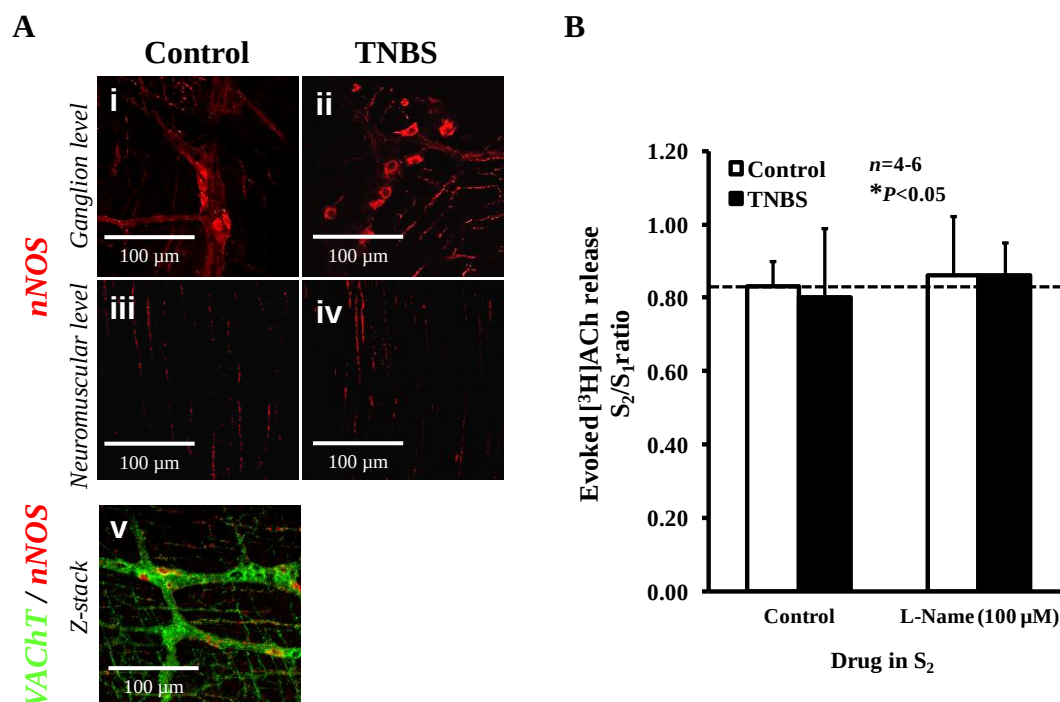


Figure 26: (A) Confocal micrographs of whole-mount preparations of the longitudinal muscle-myenteric plexus of the ileum from control and TNBS-treated rats taken at the myenteric ganglion level (i,ii) and at the longitudinal smooth muscle layer (iii,iv). Shown is the immunoreactivity against neuronal NOS (nNOS); myenteric ganglia from TNBS-treated rats exhibit a higher number of nNOS-positive neuronal cell bodies than control tissues. The z-stack presented in panel (v) shows that nNOS-positive nitrergic nerve fibres (red) are adjacent, but do not co-localize, with VACHT immunoreactivity (green). Images are representative of at least four individuals per group. Scale bar = 100 μM . **(B)** Effect of the nitric oxide synthase (NOS) inhibitor, L-Name (100 μM), on $[^3\text{H}]\text{ACh}$ released from stimulated myenteric neurons of the ileum of control and TNBS-treated rats. After loading and washout periods, $[^3\text{H}]\text{ACh}$ release was elicited by two trains (S_1 and S_2) of electrical field stimulation, each consisting of 200 pulses delivered at a 5 Hz frequency. L-Name (100 μM) was applied 8 min before S_2 . The ordinates are changes in S_2/S_1 ratios compared to the S_2/S_1 ratio obtained without addition of any drug. The data are means $\pm$ SEM of four to six individuals. $*P<0.05$ (unpaired Student's t -test with Welch correction) represent significant differences from control animals.

Postinflammatory shift from neuronal to non-neuronal control of evoked $[^3\text{H}]\text{ACh}$ release from stimulated myenteric neurons

Figure 27A shows that $[^3\text{H}]\text{ACh}$ release from electrical-stimulated ileal myenteric neurons of healthy rats was almost prevented ($P<0.05$) by the blockage of nerve action potentials with tetrodotoxin (TTX, 1 μM). This confirms our previous observations, where we also demonstrated that $[^3\text{H}]\text{ACh}$ exocytosis from

depolarized myenteric nerve terminals depend on Ca^{2+} influx through voltage-sensitive channels (Duarte-Araújo *et al.*, 2004a; 2004b; Correia-de-Sá *et al.*, 2006). Interestingly, inhibition of the electrically-driven [^3H]ACh outflow from TNBS-treated preparations was much less sensitive to TTX (1 μM) than controls (Figure 27A), suggesting that [^3H]ACh outflow in TNBS-induced ileitis is likely due to direct axonal activation by a non-neuronal released activator (Broadhead *et al.*, 2012; Gulbransen *et al.*, 2012).

Under the present experimental conditions, the influence of smooth muscle contractions was ruled out since the Ca_v1 (L-type) channel blocker, nifedipine (1 μM), depressed ACh-induced contractions of the longitudinal muscle without affecting the release of [^3H]ACh from stimulated LM-MP of both control (Correia-de-Sá *et al.*, 2006) and TNBS-treated rats (unpublished observations). Likewise, we discarded the participation ICC/FLC in the regulation of evoked [^3H]ACh release in both animal groups, because the transmitter release was not ($P>0.05$) affected when the experiments were performed in the presence of selective inhibitors of ICC/FLC activity, namely mibefradil (3 μM) (Figure 27A) and apamin (3 μM) (data not shown), which block specifically voltage-sensitive Ca_v3 (T-type) and small-conductance $\text{K}_{\text{Ca}2}$ (SK3) channels characteristic of these cells, respectively (Fujita *et al.*, 2003).

Therefore, we tested whether inhibition of enteric glial metabolism with sodium fluoroacetate (5 mM) (MacEachern *et al.*, 2015) could affect [^3H]ACh release from stimulated myenteric neurons. Figure 27A, shows that while sodium fluoroacetate (5 mM) was unable to change transmitter release in preparations from healthy rats, it significantly ($P<0.05$) decreased evoked [^3H]ACh release from myenteric neurons of TNBS-treated animals. These results led us to the hypothesis that maintenance of minimal cholinergic tone in postinflammatory ileitis depends on the release of an excitatory gliotransmitter from proliferating enteric glial cells.

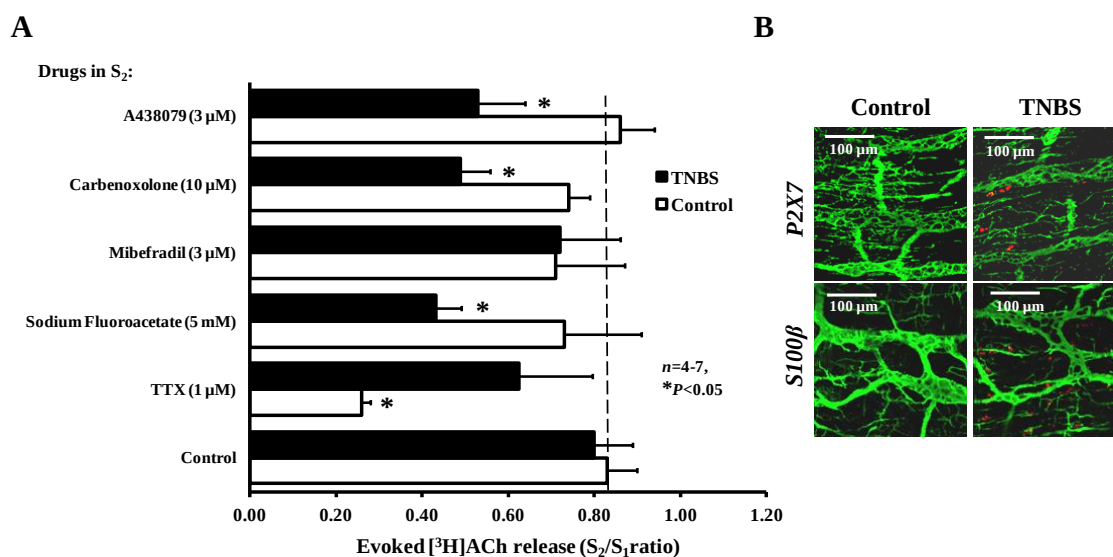


Figure 27: (A) Effects of TTX (1 μ M, a nerve action potential blocker), sodium fluoroacetate (5 mM, a glial cell metabolism uncoupler), mibefradil (3 μ M, an inhibitor of Ca_v3 (T-type) channels existing in ICC/FLC), carbenoxolone (10 μ M, a pannexin-1 inhibitor) and A438079 (3 μ M, a P2X7 receptor antagonists) on electrically-evoked [^{3} H]-ACh release from the LM-MP of the rat ileum of control and TNBS-treated rats. After loading and washout periods, [^{3} H]ACh release was elicited by two trains (S_1 and S_2) of electrical field stimulation, each consisting of 200 pulses delivered at a 5 Hz frequency. Drugs were applied 8 min before S_2 . Abscissa is changes in S_2/S_1 ratios compared to the S_2/S_1 ratio obtained without addition of any drug. The data are means $\pm$ SEM of four to seven individuals. * P <0.05 (unpaired Student's t -test with Welch correction) represent significant differences from the situation where no drugs were added (dashed vertical line). **(B)** Confocal micrographs of whole-mount preparations of the longitudinal muscle-myenteric plexus of the ileum from control and TNBS-treated rats. Shown is the immunoreactivity against the P2X7 receptor and the enteric glial cell marker, S100 β . Please note the presence of autofluorescent inflammatory cells (red dots) outside myenteric ganglia in TNBS-treated preparations. Z-stacks shown in the pictures are representative of at least four individuals per group. Scale bar=100 μ m.

ATP may be the putative excitatory gliotransmitter responsible for maintaining the cholinergic tone in postinflammatory ileitis

The nature of the putative excitatory gliotransmitter regulating ACh release from inflamed myenteric neurons is uncertain. Considering our previous observations that (1) ATP can be released from both neuronal and non-neuronal cells, and that (2) it can increase the release of [^{3} H]ACh from resting myenteric nerve terminals through the activation of ionotropic P2X receptors in the presence of TTX (Duarte-Araújo *et al.*, 2009), we designed experiments to test if

endogenous ATP could play any role in the regulation of ACh release in TNBS-induced ileitis.

Recently, our group demonstrated that ATP can be released from non-neuronal cells through hemichannels containing pannexin-1 and this mechanism can lead to a “vicious cycle” where ATP can induce the release of ATP via the activation of ionotropic P2X and metabotropic P2Y receptors under normal and pathological conditions (Pinheiro *et al.*, 2013; Timóteo *et al.*, 2014; Noronha-Matos *et al.*, 2014; Silva *et al.*, 2015; Certal *et al.*, 2015). High extracellular ATP levels, such as those detected after an inflammatory insult, can stimulate low-affinity P2X7 receptors, which often but not exclusively couple to pannexin-1 to promote ATP outflow, via the pannexin-1 hemichannel or the P2X7 receptor pore (Diezmos *et al.*, 2013; Gulbransen *et al.*, 2012). This prompted us to investigate the role of pannexin-1 hemichannels and P2X7 receptors on [³H]ACh release from stimulated LM-MP preparations of control and TNBS-treated rats using specific inhibitors.

Figure 27A, shows that selective blockage of pannexin-1 hemichannels and P2X7 receptors respectively with carbenoxolone (10 μM) and A438079 (3 μM) significantly ($P < 0.05$) decreased [³H]ACh release from myenteric neurons of TNBS-treated rats, but not of their control littermates. The magnitude of the inhibitory effects on transmitter release produced by carbenoxolone (10 μM) and A438079 (3 μM) was about the same that observed upon blocking glial cells metabolism with sodium fluoroacetate (5 mM) (Figure 27A). Moreover, the distribution of the P2X7 immunoreactivity in the myenteric plexus of both groups of animals is remarkably similar to that of the glial cell marker, S100β (Figure 27B). Co-localization of the P2X7 receptor and the S100β glial marker was demonstrated in the rat myenteric plexus before (Vanderwinden *et al.*, 2003; reviewed in Gulbransen & Sharkey, 2012).

Postinflammatory myenteric glial cells release higher amounts of ATP

In a previous study from our group, we demonstrated that ATP is released in higher amounts from electrically-stimulated TNBS-treated preparations than in control tissues (Vieira *et al.*, 2014). High postinflammatory ATP levels are

comparable to those obtained in control LM-MP preparations submitted to blockage of action potentials generation, of glial cells metabolism and of ICC/FLC activation respectively with TTX (1 μ M), sodium fluoroacetate (5 mM) and mibefradil (3 μ M) (Figure 28A). These findings indicate that all these cells contribute to keep low extracellular ATP levels in normal physiological conditions. However, this scenario changes considerably in TNBS-induced ileitis. From the inhibitors used to target specifically the three resident cell types in the myenteric plexus, only sodium fluoroacetate (5 mM) was able to decrease significantly ($P<0.05$) the release of ATP in postinflammatory ileitis, while TTX (1 μ M) and mibefradil (3 μ M) were both ineffective (Figure 28A).

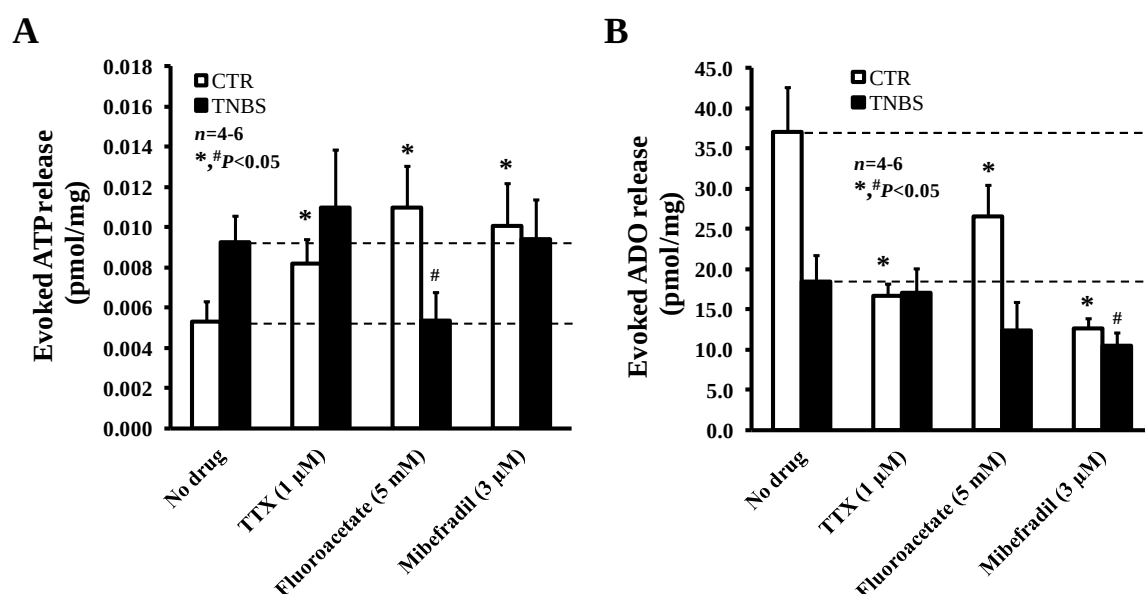


Figure 28: Differential effects of TTX (1 μ M), sodium fluoroacetate (5 mM) and mibefradil (3 μ M) on ATP and adenosine (plus inosine) outflow from stimulated longitudinal muscle-myenteric plexus preparations of the ileum from control and TNBS-treated rats. Drugs were in contact with the preparations for at least 15 min before stimulus application (5 Hz frequency, 3000 pulses of 1 ms duration). Samples from the incubation media were collected and analysed in parallel using the luciferin-luciferase bioluminescence assay and HPLC with diode array detection to evaluate their content in ATP (A) and adenosine (plus inosine) (B), respectively. Ordinates represent stimulation-induced increases in ATP (A) and adenosine (plus inosine) (B) above baseline levels determined immediately before the stimulus application; results are expressed in pmol/mg of tissue. The data are means $\pm$ SEM of four to six individuals. * $P<0.05$ (unpaired Student's *t*-test with Welch correction) represent significant differences from the amount of purines released in control conditions where no drugs were added to the preparations (dashed horizontal lines).

Thus, increased overflow of ATP after an inflammatory insult may possibly result from activation of proliferating glial cells. Interestingly, the pharmacology affecting ATP overflow from stimulated ileal preparations of TNBS-treated rats (Figure 28A) looks like that verified by measuring the release of [^3H]ACh (Figure 27A), further strengthening our hypothesis that ATP may be the putative excitatory gliotransmitter responsible for maintaining the cholinergic tone in TNBS-induced ileitis.

Deficient extracellular adenosine accumulation in postinflammatory ileitis parallels the loss of ICC/FLC

Although adenosine may be released from activated inflammatory cells (Marquardt *et al.*, 1984) in the vicinity of myenteric neurons (Bogers *et al.*, 2000), the loss of adenosine neuromodulatory control of evoked [^3H]ACh release in TNBS-induced ileitis detected in a previous report results mainly from deficient accumulation of the nucleoside at the myenteric synapse (Vieira *et al.*, 2014); this was verified despite the increase in ATP content of the same samples. Uncoupling between ATP overflow and adenosine levels in postinflammatory ileitis was ascribed to feed-forward inhibition of ecto-5'-nucleotidase/CD73 and upregulation of adenosine deaminase. Here, we decided to evaluate the putative contribution of inflammation-induced type-specific cell density changes in the myenteric plexus to adenosine deficiency. To this end, we measured the extracellular concentration of adenosine (plus inosine) in parallel with the ATP levels before and after electrical stimulation of LM-MP preparations of both control and TNBS-treated rats in the presence of cell-specific activity inhibitors. Figure 28B shows that the amounts of adenosine released into the extracellular fluid following stimulation of the LM-MP are much higher in healthy controls than in TNBS-treated rats.

Stimulus-evoked adenosine release was partially dependent on neuronal activity in preparations from healthy rats, but not in the ileum of TNBS-treated animals. This was assumed because pre-treatment of the preparations with TTX (1 μM) reduced by about 50% the outflow of adenine nucleosides in control tissues with no effect on TNBS-treated preparations. This means that the neuronal source

of adenosine may be severely affected in TNBS-induced ileitis due to neuronal cells dysfunction (see above). Even though we considered unlikely that proliferating glial cells could contribute to adenosine deficits in the inflamed myenteric plexus of the rat ileum, we tested the effect of the glial cell metabolic uncoupler, sodium fluoroacetate (5 mM). Inhibition of glial cells metabolism had only a limited effect on adenosine outflow from stimulated LM-MP preparations of both control and TNBS-treated rats (Figure 28B). The inhibitory action of sodium fluoroacetate (5 mM) was slightly more evident in healthy tissues than in TNBS-treated preparations, which is in agreement with the proposed uncoupling between ATP overflow and adenosine formation secondary to the inflammatory insult.

Prevention of smooth muscle contractions with the voltage-sensitive Ca_v1 (L-type) channel inhibitor, nifedipine (5 μM), did not affect the release of adenosine (plus inosine) in both animal groups (Correia-de-Sá *et al.*, 2006; Vieira *et al.*, 2009; 2014). However, blockade of Ca_v3 (T-type) channels expressed in ICC/FLC with mibefradil (3 μM) significantly ($P < 0.05$) attenuated the release of adenosine (plus inosine) from both control and TNBS-treated animals. Taking into consideration previous findings from our laboratory demonstrating that the nucleoside transport inhibitor, dipyridamole (0.5 μM), decreased proportionally and by a similar amount to that obtained with mibefradil (3 μM) the outflow of adenosine (plus inosine) from both control and inflamed tissues (Vieira *et al.*, 2014), it is very likely that adenosine accumulated in the ileum following an inflammatory insult originates predominantly from activated ICC/FLC via equilibrative nucleoside transport.

DISCUSSION AND CONCLUSIONS

A number of key cellular players may be involved in the reaction to inflammatory insults by the gastrointestinal system. These include inflammatory cells, such as granulocytes, lymphocytes, mast cells, monocytes and macrophages, which release numerous cytokines, pro-inflammatory peptides, neuroactive and neurotrophic factors, noxious compounds, inflammatory enzymes

and other danger signalling molecules. Enteric plasticity comprises a wide range of structural and/or functional changes in enteric neurons, glial cells and interstitial cells (ICC/FLC). As a result of adaptive responses to different types of pathophysiological conditions, enteric neurons are able to rapidly change their structure, function and chemical phenotype in order to maintain homeostasis of gut functions. In contrast to long-term functional abnormalities in the activity of neuronal cells described by several authors (Stewart *et al.*, 2003; Nurgali *et al.*, 2007) and confirmed in the present study by denoting hypoactivity of cholinergic neurotransmission, we found no obvious modifications in cellular density and distribution of specific neuronal cell markers (*e.g.* NF200, PGP 9.5) in TNBS-induced ileitis. In this sense, ileitis caused by TNBS differs from other chronic inflammatory disease models because it lacks the chronic inflammatory phase (Moreels *et al.*, 2001) that is usually accompanied by neuronal cell loss (Sanovic *et al.*, 1999; Linden *et al.*, 2005; Venkataramana *et al.*, 2015). Notwithstanding, this constrain may be turned into an advantage characteristic of this animal model if one wants to investigate the adaptive changes of neuronal to non-neuronal cells communication after a transient inflammatory insult, as we aimed in this work.

Results show that stimulated myenteric neurons release smaller amounts of [³H]ACh in the ileum of TNBS-treated rats compared to their control littermates, without evidence of any alteration of VACHT-positive cholinergic nerve fibres in the myenteric plexus. Likewise, significant decreases in [³H]choline uptake, acetylcholine release and contractile responses to stimulation of enteric nerves have been observed in rats with colitis induced by TNBS (Poli *et al.*, 2001). These results imply a functional loss, not corresponding to cellular depletion, of cholinergic neurotransmission in inflamed tissues (Collins *et al.*, 1992a). The mechanism underlying attenuation of ACh release in the inflamed rat intestine has been a matter of debate in the literature, but so far there is no unifying theory given the contradictory findings when looking at different immune cell players and inflammatory mediators (*e.g.* Davis *et al.*, 1998). This is so, even though evidences have been produced showing that cytokines may directly change neurotransmitters content and release (Collins *et al.*, 1992b). This strengthens our

hypothesis that functional and structural adaptations of resident myenteric cell populations of the rat ileum may also play a relevant role in the mechanisms underlying downregulation of cholinergic neurotransmission in TNBS-induced ileitis.

Using immunofluorescence confocal microscopy, we show here that the LM-MP of the rat ileum becomes deficient in Ano-1 positive ICCs, both in the ganglion layer (myenteric stellate cells) and at the neuromuscular region (intramuscular spindle-shape cells). The same occurred regarding immunostaining against vimentin, which is an intermediate filament protein also present in FLC. Notwithstanding the fact that vimentin can stain more strongly ICC compared to FLC, since the former is enriched in intermediate filament fibres (Fujita *et al.*, 2003; Komuro *et al.*, 1996), we were unable to conclusively distinguish the two cell types in this work and, for the sake of transparency, we prefer referring to these cells as interstitial cells or ICC/FLC. Investigations about many aspects of the role played by each of these cell types are deeply needed. In the more severe colitis rat model, muscularis resident macrophages have been implicated in the loss of ICC/FLC detected 7-days after instillation of TNBS (Kinoshita *et al.*, 2007). An association between low numbers of CD206-positive anti-inflammatory macrophages with a loss of interstitial cells have been observed in patients with diabetic gastroparesis (Bernard *et al.*, 2014). Porcher *et al.* (2002) demonstrated an almost complete abolition of interstitial cells in biopsy samples from patients with Crohn's disease. The loss of ICCs may account for the decrease in the frequency of spontaneous contractions of LM-MP preparations of the ileum of TNBS-treated rats (this study), since these cells have been implicated in the generation of electrical slow waves regulating the phasic contractile activity of the gastrointestinal smooth muscle (Kinoshita *et al.*, 2007).

To our knowledge, this is the first time that a loss of ICC/FLC in the myenteric plexus of the rat ileum following an inflammatory insult is associated with the depletion of the extracellular adenosine levels compared to normal physiological condition. This was suggested because the lowest extracellular

adenosine levels in the ileum were detected upon blocking ICC/FLC activity with the Ca_v3 (T-type) channel inhibitor, mibefradil, both in control conditions and after the inflammatory insult with TNBS. Therefore, it is highly likely that this mechanism, together with the feed-forward inhibition of ecto-5'-nucleotidase/CD73 and upregulation of adenosine deaminase shown in a previous study (see Vieira *et al.*, 2014), may concur to prevent adenosine-mediated actions in TNBS-induced ileitis. Considering that, under normal physiological conditions, endogenous adenosine facilitates [^3H]ACh release from stimulated myenteric neurons through the preferential activation of prejunctional $\text{A}_{2\text{A}}$ receptors (Duarte-Araújo *et al.*, 2004a), one may speculate that cholinergic hypoactivity in TNBS-induced ileitis is dependent on the loss of adenosine $\text{A}_{2\text{A}}$ receptor-mediated neurofacilitation. Moreover, one must not forget that neurons are also an important source of extracellular adenosine in the ileum of healthy animals (Correia-de-Sá *et al.*, 2006) and this resource may turn to be deficient accompanying inflammation-induced functional abnormalities of myenteric neurons, which cannot be compensated by the loss of ICC/FLC.

Purinergic re-enforcement to maintain cholinergic neurotransmission during high enteric nerve activity or whenever endogenous levels of adenosine become elevated has been observed in hypoxia, inflammation and postoperative ileum (Milusheva *et al.*, 1990; De Man *et al.*, 2003; Kadowaki *et al.*, 2003). In a previous study, we provided evidences suggesting that muscarinic M_3 receptors activation by neuronally-released ACh triggers a positive feedback loop leading to facilitation of transmitter release that is indirectly mediated by adenosine outflow and activation of prejunctional $\text{A}_{2\text{A}}$ receptors (Vieira *et al.*, 2009), but at that time we had no information regarding the cellular players of the SIP syncytium involved in this pathway. Nowadays, we learned that ICC/FLC are endowed with muscarinic M_3 receptors coupled to $\text{G}_{\text{q}/11}$ (reviewed in Goyal, 2013) and these receptors can be activated by inhibiting endogenous ACh breakdown with physostigmine resulting in adenosine overflow from the myenteric plexus of healthy rats (Vieira *et al.*, 2009). Activation of phospholipase C (PLC) by muscarinic M_3 receptors causes the formation of IP_3 and DAG with subsequent recruitment of intracellular Ca^{2+} and

protein kinase C activation, respectively (Koh & Rhee, 2013). Increases in intracellular Ca^{2+} can strengthen activation of protein kinase C, which subsequently stimulates adenosine outflow via the equilibrative transporter ENT1 (Coe *et al.*, 2002). Increase in protein kinase C activity might also stimulate 5'-nucleotidase inside cells (Obata *et al.*, 2001) and/or inhibit adenosine kinase (Sinclair *et al.*, 2000), enhancing intracellular adenosine accumulation and the efflux of the nucleoside from the cells. These hypotheses were confirmed in the myenteric plexus of healthy rats incubated with the protein kinase C activator, phorbol 12-myristate 13-acetate, whereas extracellular adenosine accumulation was prevented by inhibiting the equilibrative nucleoside transport system with dipyrindamole (Vieira *et al.*, 2009).

In this context, our current vision about these processes is that the loss of ICC/FLC expressing muscarinic M_3 receptors in TNBS-induced ileitis contributes to reduce extracellular adenosine accumulation, breaking down the amplification loop initiated by ACh release from myenteric neurons, mediated by muscarinic M_3 receptors-induced adenosine overflow from ICC/FLC, and concluded through the activation of facilitatory $\text{A}_{2\text{A}}$ receptors on cholinergic nerve terminals. To our knowledge, this is the first time adenosine release from myenteric ICC/FLC is implicated in functional cholinergic nerve abnormalities in postinflammatory ileitis.

While the findings discussed so far may justify the downregulation of cholinergic neurotransmission in TNBS-induced ileitis, they fail to explain why ACh release from inflamed myenteric neurons becomes resistant to blockade of nerve action potentials with TTX and the mechanism(s) underlying the TTX-resistant transmitter release that is still significant in inflamed preparations. The appearance of TTX-resistant sodium currents has been observed before in TNBS-induced ileitis (Stewart *et al.*, 2003). Interestingly, the pharmacology concerning the modulation of [^3H]ACh release from myenteric motor neurons of TNBS-treated rats is remarkably similar to that obtained when attempting to control ATP release in the same preparations. Notwithstanding the fact that inflammation-induced variations in the myenteric concentrations of ACh and ATP diverge (*i.e.* ACh

decreases and ATP increases), the release of the two transmitters were significantly reduced in the presence of the gliotoxin, sodium fluoroacetate, but it was not affected by the neuronal activation blocker, TTX. These results led us to suggest that enteric glia cells are crucial to maintain ACh release from inflamed myenteric neurons and that ATP released from these cells may be the excitatory gliotransmitter responsible for keeping a minimal cholinergic tone in TNBS-induced ileitis.

As a matter of fact, we observed increases in GFAP- and S100 β -immunoreactivities in TNBS-induced ileitis suggesting that the inflammatory insult may stimulate enteric glial cells proliferation (Bradley *et al.*, 1997) and/or activate the expression/synthesis of glial protein cell markers (Cabarrocas *et al.*, 2003). The release of cytokines and growth factors from inflammatory and immune cells may promote glial cells proliferation (Fields & Burnstock, 2006). On the other, enteric glial cells may secrete high amounts of neurotrophic factors, like NGF and GDNF, which may change the neurochemical code and content of neurotransmitters in myenteric neurons, even if they do not vary in terms of density (Von Boyen & Steinkamp, 2010). Enteric glia cells may also be a source of pro-inflammatory cytokines, such as IL-6 and IL-1 β (Stoffels *et al.*, 2014), with IL-1 β being associated with the suppression of ACh release from the myenteric plexus (Van Assche & Collins, 1996). However, if this were the case in TNBS-induced ileitis, blockade of enteric glia cells metabolism would enhance, rather than decrease, evoked [^{3} H]ACh release from myenteric motor neurons. Moreover, enteric glia contain neurotransmitter precursors, uptake and degrade neuroactive substances, express neurotransmitter receptors and provide neurosupportive actions (Vasina *et al.*, 2006). Regarding recovery from inflammatory adaptive cell changes, it appears that enteric glial dysfunction does not persist after the resolution of intestinal inflammation in TNBS-treated animals, indicating that attempts to correct glial cells dysfunction may be restricted to the course of inflammatory adaptations (MacEachern *et al.*, 2015).

The function of enteric glial cells is intimately tied to the levels of purines and membrane-bound ectonucleotidases, which regulate the availability of P2 receptor ligands (Braun *et al.*, 2004; Lavoie *et al.*, 2011). Data from this study suggest that surplus ATP released by the myenteric plexus after an inflammatory insult may originate from proliferating glial cells, because ATP accumulation was reduced to control levels in the presence of the glial metabolic uncoupler, sodium fluoroacetate. ATP released from glial cells, acting most likely via prejunctional homodimer P2X2 or heterodimer P2X2/3 receptors, may trigger the release of ACh directly from cholinergic nerve terminals (Galligan, 2002; Castelluci *et al.*, 2002; Ren *et al.*, 2003; Ohta *et al.*, 2005; Duarte-Araújo *et al.*, 2009). Ionotropic P2X receptor subtypes are Ca^{2+} permeable non-selective cation channels (also permeable to Na^+), which can also inhibit membrane potassium conductance in enteric neurons (Barajas-López *et al.*, 1994). Via these mechanisms, ATP can cause membrane depolarization leading to a secondary opening of voltage-gated Ca^{2+} channels and to transmitter release in the absence of action potential generation (Duarte-Araújo *et al.*, 2009; see also Barthó *et al.*, 2006). Redistribution of NTPDase2, but not NTPDase3 from ganglia to the neuromuscular region leads to preferential ADP accumulation from released ATP, particularly when extracellular ATP levels reach the V_{max} for NTPDase3 (Duarte-Araújo *et al.*, 2009) like that occurring in inflamed neuromuscular synapses (Vieira *et al.*, 2014). Inflammation-induced increase in the expression of NTPDase2 in intramuscular glia may also contribute to restrain nerve-evoked ACh release in TNBS-induced ileitis via the activation of ADP-sensitive inhibitory P2Y₁ receptors in myenteric nerve terminals (Duarte-Araújo *et al.*, 2009), yet this hypothesis needs experimental confirmation.

Next we tried to investigate the mechanism underlying the control shift from neuronal to non-neuronal of ACh release in TNBS-induced ileitis. Results show that evoked ACh release from inflamed myenteric neurons was attenuated by blocking the P2X7 receptor-pannexin-1 pathway respectively with A438079 and carbenoxolone in a similar manner to that verified with sodium fluoroacetate. Interestingly, the P2X7 receptor immunoreactivity in the myenteric plexus of the rat

ileum is remarkably similar to that obtained for the glial cell marker, S100 β , indicating that they may co-localize (Vanderwinden *et al.*, 2003; reviewed in Gulbransen & Sharkey, 2012). Despite this, the P2X7 receptor is also expressed in neuronal soma and nerve terminals, but it is absent from smooth muscle fibres, ICC and FLC.

The ionotropic P2X7 receptor is a non-selective cation channel allowing Na⁺ and Ca²⁺ influx and K⁺ efflux in the presence of high-micromolar ATP concentrations (Morandini *et al.*, 2014). This receptor has been largely associated with inflammatory diseases, including inflammatory bowel diseases (Diezmos *et al.*, 2013), and it can signal through caspase-1 to cause the production of IL-1 β and IL-18 (Kurashima *et al.*, 2012), as well as TNF- α and nitric oxide (Alves *et al.*, 2013), mostly from inflammatory cells. Likewise, pannexin-1 is expressed in a variety of immune cells and is required for caspase-1 cleavage in response to P2X7 receptor activation (Pelegriin & Surprenant, 2006). Interestingly, downregulation of pannexin-1 is associated with pro-inflammatory responses, whereas the opposite promotes anti-inflammatory reactions (Sáez *et al.*, 2014). Pannexin-1, along with pannexin-2, is expressed in all layers of the human colon, including the mucosa, muscularis mucosa, submucosa and muscularis externa, where it is found preferentially in enteric ganglia, but also in the endothelium of blood vessels, epithelial cells and goblet cells (Diezmos *et al.*, 2013; Maes *et al.*, 2015). The P2X7 receptor and pannexin-1 hemichannels form signalling complexes in several cell types and both structures may concur to release ATP from non-neuronal cells under physiological and pathological conditions. Interestingly, the selective P2X7 receptor antagonist, A438079, and the specific pannexin-1 inhibitor, carbenoxolone, did not modify evoked ACh release in the ileum of healthy rats where myenteric ATP levels are low, but they significantly decreased [³H]ACh overflow from preparations of TNBS-treated rats in conditions where extracellular ATP release from proliferating glial cells is assumed to be high. It has also been shown that activation of the P2X7 receptor in enteric neurons may elicit ATP release through pannexin-1 hemichannels and this may act as a danger signal for neurons (Gulbransen *et al.*, 2012). However, this mechanism is unlikely

to modulate [^3H]ACh outflow in postinflammatory ileitis because no substantial neuronal loss was observed in our experimental conditions and the transmitter release in TNBS-induced ileitis was TTX-resistant, yet fluoroacetate-sensitive, indicating a dominant participation of enteric glial cells.

It has been proposed that enteric glial cells may play a critical role in maintaining the integrity of the bowel and that their loss or dysfunction might contribute to cellular mechanisms of inflammatory bowel diseases. In this respect, a fulminating and fatal jejuno-ileitis is produced 7-14 days after ablation of enteric glia in adult transgenic mice, with almost none effects in other parts of the GI tract including the colon (Bush et al., 1998). The most striking finding from that study is that this pathology can be verified with only moderate degeneration of myenteric neurons, resulting in moderate distension of the bowel and minor changes in stool pellet propulsion, suggesting that neuronal dysfunction is more relevant than structural abnormalities in order to disrupt gut motility, as we detected in the TNBS-induced ileitis rat model. Pathological lesions are patchy and start always at the mucosal surface progressing to the muscularis externa, including pronounced dilation of capillaries with inflammatory cell infiltrates and erythrosthiasis, but are relatively independent of bacterial overgrowth resulting from disturbed gut motility due to neuronal degeneration. The functional defects observed in the transgenic animal model are comparable to those detected when glial ablation was produced with fluorocitrate, where alterations in the mucosal function were not seen (reviewed in Gulbransen & Sharkey, 2012). These findings suggest that it is the inflammatory insult, and not altered glial function, that underlies mucosal alterations. However, it remains to be explored whether deficits in ATP outflow from deficient glial cells play a role in triggering this disease process. This would be clinically relevant, since we predicted here that the nucleotide may be an important gliotransmitter responsible for sustaining cholinergic tone after an inflammatory insult and this might contribute to avoid degeneration of myenteric neurons as already proved for glutamate in the CNS (Bush et al., 1999). In this context, the vasoactive properties of released adenine nucleotides and

nucleosides may also contribute to microvascular disturbances and certain changes resembling ischemic bowel (see e.g. Mendes *et al.*, 2015).

Thus, similarities in the appearance of end-stage pathology in various bowel conditions (e.g. inflammation, hypoxia/ischemia, diabetes) suggest that the gut may have limited ways of responding when it is defective (Bush *et al.*, 1998). For this reason it is important to identify single events that are able to trigger complex pathological cascades. Our working hypothesis in this study was that inflammation-induced cell density changes in the myenteric plexus may unbalance the release of purines and, thus, their influence on ACh release from stimulated enteric nerves. We clearly demonstrated that inflammation-induced cholinergic hypoactivity is deeply dependent on the purinergic shift from a preferential adenosinergic tone under physiological conditions to a more prevalent ATP-mediated control of ACh release in TNBS-induced ileitis. The adenosine neuromodulation deficit parallels the partial loss of ICC/FLC population in the inflamed myenteric plexus and contributes to explain the brake of the cholinergic amplification loop operated by facilitatory muscarinic M_3 and adenosine A_{2A} receptors, which is responsible for keeping the safety margin of myenteric neuromuscular transmission under stressful conditions. On the other hand, proliferating and/or reactive enteric glial cells markedly influence the amount of ATP at the inflamed myenteric synapse by creating a vicious cycle that promote the release of the nucleotide through a mechanism involving the P2X7 receptor/pannexin-1 pathway. Data suggest that ATP may be the excitatory gliotransmitter responsible for keeping the cholinergic tone above a critical minimum in postinflammatory ileitis. Inflammation may, thus, recapitulate an ancestral mechanism by which ATP released by glial cells directly (via ionotropic P2X receptors) stimulates ACh release from cholinergic nerve terminals even when these cells are disturbed and unable to generate normal action potentials. In conclusion, this study provides a deeper understanding of the pathophysiological impact of purines in the communication between non-neuronal cells and neurons underlying the dysregulation of cholinergic neurotransmission in postinflammatory ileitis. Data suggest that the purinergic cascade (e.g. ATP release mechanisms,

ecto-nucleotidase enzymes and purinoceptors activation) may offer novel therapeutic targets to overcome long-term cholinergic neurotransmission deficits affecting motility in inflammatory bowel diseases.

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VII. CONCLUDING REMARKS

Inflammation of the intestine leads to long-lasting structural and functional changes in the ENS, which may be observed in remote regions from the inflammation site. These modifications are usually accompanied by notorious changes in the purinergic signalling cascade affecting both immune responses and synaptic neurotransmission, although the underlying mechanisms and consequences of this diversity are not completely understood. This study aimed at investigating the nature and activity of resident purine-releasing cells in the myenteric plexus, the mechanisms responsible for the release of these mediators from cells, the kinetics of their extracellular metabolism/ inactivation cycle and the purinoceptor subtypes most implicated in postinflammatory ileitis. Because adenine nucleotides and nucleosides can be released from infiltrating inflammatory cells, as well as from resident neuronal and non-neuronal enteric cells, there is an increasing interest in studying the mechanism by which purines can control inflammatory reactions, synaptic neurotransmission and motility/secretion in the gut following inflammatory insults. Fulfilling these objectives, one may be able to suggest novel therapeutic targets to control enteric inflammatory diseases.

Most studies pursuing this goal, were done in experimental colitis animal models induced by TNBS or DSS. These are severe models of inflammatory colitis, where lesions span the whole thickness of the gut wall, exhibit characteristics of chronic inflammation and causes irreversible losses of enteric neurons. However, in this study we wanted to investigate neuronal to non-neuronal cell communication adaptations carried out reversibly by purines and, therefore, we focused on the postinflammatory ileitis rat model induced by intraluminal instillation of TNBS in the rat 7 days before experimental protocols. Under these circumstances, the inflammatory reaction affects mainly the submucosa, with the presence of inflammatory infiltrates such as eosinophils, lymphocytes, macrophages, mononuclear and giant cells reaching myenteric

ganglia, without penetrating the boundaries of this structure. In this sense, ileitis caused by TNBS differs from other chronic inflammatory disease models because it lacks the chronic inflammatory phase (Moreels *et al.*, 2001b) that is usually accompanied by neuronal cell loss (Sanovic *et al.*, 1999; Linden *et al.*, 2005; Venkataramana *et al.*, 2015).

Postinflammatory ileitis increased the gastrointestinal transit measured by methylene blue gavage progression, while, paradoxically, we detected hypocontractility of the longitudinal muscle and a decrease in the frequency of spontaneous contractions that is compatible with the observed loss of pacemaker interstitial cells of Cajal. Several authors attributed muscle contraction changes to structural abnormalities of the smooth muscle layer due to oedema and smooth muscle hypertrophy. Structural modifications of the enteric wall may also reduce the lumen size of the gut resulting in faster progression of liquids (e.g. methylene blue), although one cannot exclude the existence of plastic changes in the neuronal network as a cause for acceleration in the gastrointestinal transit.

Data from this and other studies show that cholinergic neurotransmission is impaired in postinflammatory ileitis (see e.g. Poli *et al.*, 2001; Collins *et al.*, 1992a), without evidence of any modifications in the density and cellular distribution of VACHT-positive cholinergic neurons. The mechanisms underlying downregulation of ACh release in the inflamed ileum have been a matter of debate in the literature, but no unifying theory is available. Contradictory findings were produced when looking at the pathophysiological influence of immune cells and inflammatory mediators. Here, we put forward several hypotheses to explain inflammation-induced cholinergic hypoactivity based on the purinergic shift from a preferential adenosinergic tone under healthy conditions to a dominant ATP-mediated control of ACh release in TNBS-induced ileitis.

Regarding the adenosine neuromodulation deficit observed in postinflammatory ileitis, it may result, at least in part, from the partial loss of interstitial cells (ICC/FLC) population in the inflamed myenteric plexus. These cells are endowed with muscarinic M₃ receptors coupled to PLC/ IP₃ / PKC, whose

activation facilitates adenosine release from ENTs. This mechanism is significantly impaired after an inflammatory insult because the number of ICC/FLC decreases and ACh released from dysfunctional cholinergic nerve terminals might not reach high enough concentrations to stimulate M_3 receptors in these cells. In addition, we demonstrated that adenosine deficits may also result from deficient conversion of adenine nucleotides (AMP) into adenosine due to feed-forward inhibition of ecto-5'-nucleotidase/CD73 by high amounts of ATP and/or ADP generated in the inflamed myenteric synapse. The lack of adenosine release *per se* via equilibrative nucleotide transporters and/or its formation from released adenine nucleotides is affected further by the upregulation of ADA activity, which inactivates adenosine into inosine in the extracellular fluid (Vieira *et al.*, 2014). Interestingly, both membrane-bound and soluble forms of ecto-5'-nucleotidase/CD73 and ADA were detected in the inflamed myenteric plexus. Secretion of these enzymes is increased in TNBS-induced ileitis, thus contributing to unbalance the bulk production and/or inactivation of adenosine way from its location in the native tissue – the myenteric neuromuscular synapse. The result from these adaptations is more widespread organ affection. All these mechanisms ultimately contribute to brake of the cholinergic amplification loop operated by facilitatory muscarinic M_3 and adenosine A_{2A} receptors that is responsible for keeping the safety margin of myenteric neuromuscular transmission under stressful conditions.

Inflammation-induced glial cells proliferation significantly increases the amount of ATP in the myenteric synapse. This may be due to a vicious cycle that promotes the release of the nucleotide from these cells through a mechanism involving P2X7 receptors and pannexin-1 hemichannels. While ATP is critical for keeping the cholinergic tone above a minimum in postinflammatory ileitis, it upon hydrolysis into ADP by NTPDase2 may contribute to restrain nerve-evoked ACh release from inflamed cholinergic motor neurons. The major difference between these apparent contradictory actions of ATP is that the nucleotide can stimulate (via ionotropic P2X receptors) ACh release directly from cholinergic nerve terminals even when these cells are pathologically affected and unable to

generate normal action potentials, whereas activation of metabotropic P2Y₁ receptors requires nerve stimulation in order to downregulate ACh release.

Redistribution of NTPDase2, but not NTPDase3, from the ganglion region to the neuromuscular myenteric synapse leads to preferential ADP accumulation from released ATP, thus contributing to prolong inhibition of muscle-bound ecto-5'-nucleotidase/CD73, thereby delaying adenosine formation, and to promote activation of prejunctional inhibitory P2Y₁ receptors at the inflamed myenteric synapse.

Thus, this study provides a deeper understanding of the pathophysiological impact of purines in the communication between non-neuronal cells and neurons underlying dysregulation of cholinergic neurotransmission in postinflammatory ileitis. Data suggest that the purinergic cascade (e.g. ATP release mechanisms, ecto-nucleotidase enzymes and purinoceptors activation) may offer novel therapeutic targets to overcome long-term cholinergic neurotransmission deficits affecting motility in inflammatory enteric diseases.

VIII. REFERENCES

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