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REVIEW

Purinergic neuromuscular transmission in the gastrointestinal tract; functional basis for future clinical and pharmacological studies

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Nerve-mediated relaxation is necessary for the correct accomplishment of gastrointestinal (GI) motility. In the GI tract, NO and a purine are probably released by the same inhibitory motor neuron as inhibitory co-transmitters. The $P2Y_1$ receptor has been recently identified as the receptor responsible for purinergic smooth muscle hyperpolarization and relaxation in the human gut. This finding has been confirmed in $P2Y_1$-deficient mice where purinergic neurotransmission is absent and transit time impaired. However, the mechanisms responsible for nerve-mediated relaxation, including the identification of the purinergic neurotransmitter(s) itself, are still debatable. Possibly different mechanisms of nerve-mediated relaxation are present in the GI tract. Functional demonstration of purinergic neuromuscular transmission has not been correlated with structural studies. Labelling of purinergic neurons is still experimental and is not performed in routine pathology studies from human samples, even when possible neuromuscular impairment is suspected. Accordingly, the contribution of purinergic neurotransmission in neuromuscular diseases affecting GI motility is not known. In this review, we have focused on the physiological mechanisms responsible for nerve-mediated purinergic relaxation providing the functional basis for possible future clinical and pharmacological studies on GI motility targeting purine receptors.

Abbreviations

β-NAD, β-nicotinamide dinucleotide; EB, oesophageal body; EFS, electrical field stimulation; GI, gastrointestinal; ICC, interstitial cells of Cajal; IJP, inhibitory junction potential; IJPf, fast inhibitory junction potential; IJPs, slow inhibitory junction potential; IP_3, inositol 1,4,5-trisphosphate; KO, knockout; LES, lower oesophageal sphincter; L-NNA, N^{ω}-nitro-L-arginine; MRS2179, 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate; MRS2279, (1*R**,2*S**)-4-[2-chloro-6-(methylamino)-9*H*-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester; MRS2500, (1*R**,2*S**)-4-[2-iodo-6-(methylamino)-9*H*-purin-9-yl]-2 (phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; PDGFRα, platelet-derived growth factor receptor α; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; SK_{Ca}, small conductance calcium-activated potassium channel; $K_{Ca}2.3$ (SK3), small conductance calcium-activated potassium channel 3; TTX, tetrodotoxin; VIP, vasointestinal polypeptide; WT, wild type

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Table of Links

TARGETS	LIGANDS
$P2Y_1$ receptor	ATP
P2X receptors	Tetrodotoxin (TTX)
$K_{Ca}2.3$ channel	ODQ
SK_{Ca} channel	Apamin
SLC17A9	IP_3
5-HT_4 receptor	MRS2179
PDGFRα	MRS2500
	MRS2279
	PPADS
	Suramin

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a,b,c,d,e).

Introduction

Purine receptors are classified into two families: receptors for adenosine (P1 receptors) and receptors for ATP and ADP (P2 receptors). P2 receptors are separated into two groups based upon their transduction mechanism. P2X receptors are ligand-gated ion channels and P2Y receptors are GPCRs. At present, seven P2X (P2X1–7) and eight P2Y ($P2Y_{1\text{-}2\text{-}4\text{-}6\text{-}11\text{-}12\text{-}13\text{-}14}$) receptor subtypes have been identified. Previous data using non-selective purinergic antagonists such as pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) or suramin already demonstrated a role for purine receptors in several functions of the gastrointestinal (GI) tract, including synaptic, neuromuscular transmission and secretion. However, due to the lack of selectivity of these antagonists (Hoyle *et al.*, 1990; Vigne *et al.*, 1998; Xue *et al.*, 1999), it has, until recently, been impossible to identify the receptor(s) involved in purinergic neurotransmission. Newly developed antagonists of P2 receptors (Boyer *et al.*, 1996; Camaioni *et al.*, 1998; Cattaneo *et al.*, 2004) have become important pharmacological tools for investigating the role of purines in GI function. In the present review, we will focus on the $P2Y_1$ receptor, which is the receptor mainly involved in inhibitory neuromuscular transmission. The selectivity/potency of the pharmacological antagonists available might differ between species and it is noteworthy to point out important differences between frequently used laboratory animals and human tissue. Translational studies to move the research in purinergic neurotransmission from animal models to human samples have been a great part of the work of our laboratory for the last 10 years. Therefore, the aim of the present manuscript is to review the data available in the literature regarding the role of purine receptors and their pathways at the inhibitory neuromuscular junction.

Inhibitory junction potential (IJP)

In vitro, in intestinal preparations, electrical field stimulation (EFS) is usually employed to evoke tetrodotoxin (TTX)-sensitive action potentials in inhibitory motor neurons to release inhibitory neurotransmitters. EFS evokes an IJP in the smooth muscle cell, which is the electrophysiological basis for the mechanical relaxation or inhibition of the spontaneous contractions. It has been widely demonstrated that EFS induces the release of different neurotransmitters causing a fast IJP (IJPf) followed by a slow IJP (IJPs) (Crist *et al.*, 1992; He and Goyal, 1993). Maybe with the exception of the human oesophageal body (EB) and the lower oesophageal sphincter (LES) (Lecea *et al.*, 2011) (see below), this biphasic IJP is the most common electrophysiological response that can be recorded in different areas of the GI tract. Single pulses (or short trains of about 100 ms) induce an IJPf in human small intestine and colon (Figure 1) (Gallego *et al.*, 2006; 2014). In other species, such as rodents and guinea pigs, the same stimulus causes an IJPf followed by an IJPs. A biphasic IJP can be recorded in human tissue using long trains of stimulation with high frequencies (usually about 5 Hz) (Figure 2) (Keef *et al.*, 1993; Gallego *et al.*, 2008a). It has been functionally demonstrated that vasointestinal polypeptide (VIP) is released in the mouse internal anal sphincter after long trains of EFS, leading to an ultraslow hyperpolarization and relaxation (Keef *et al.*, 2013). It is not known whether VIP release can be measured in other areas of the GI tract under certain conditions of EFS. It is important to have all these differences in mind when muscle bath studies are performed, because different types of stimulation can cause the predominant release and/or post-junctional response of one or another neurotransmitter, whereas a different relative combination of neurotransmitters can be obtained by changing the frequencies of stimulation (Mañe *et al.*, 2014).

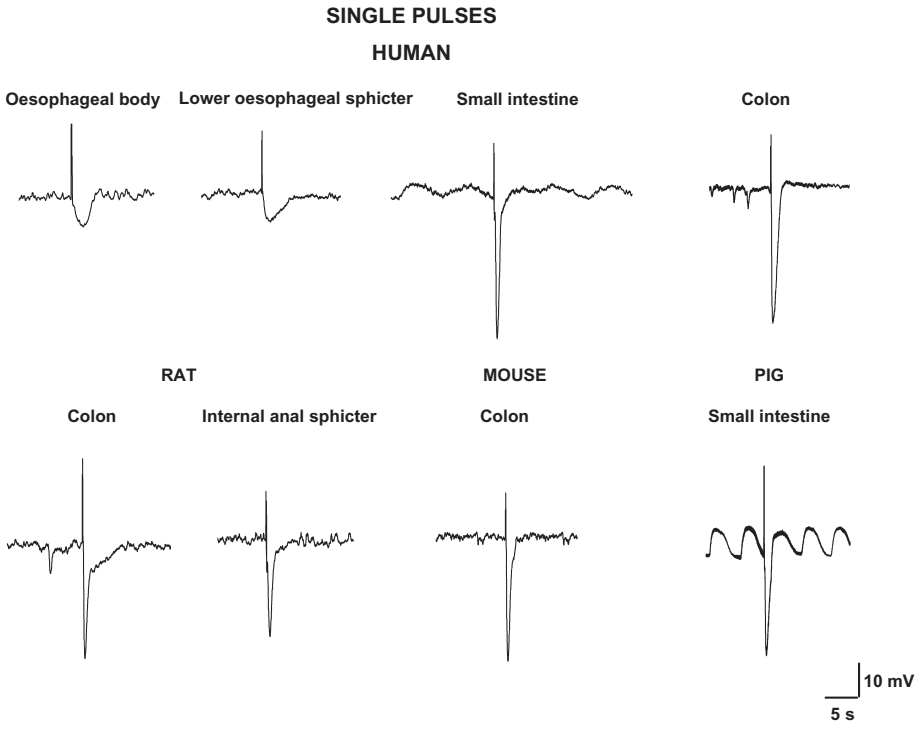

Figure 1

Single pulses or short trains elicit an IJPf in different areas of the GI tract. Note the absence of an IJPf in oesophageal tissues and the presence of spontaneous IJP in some tracings.

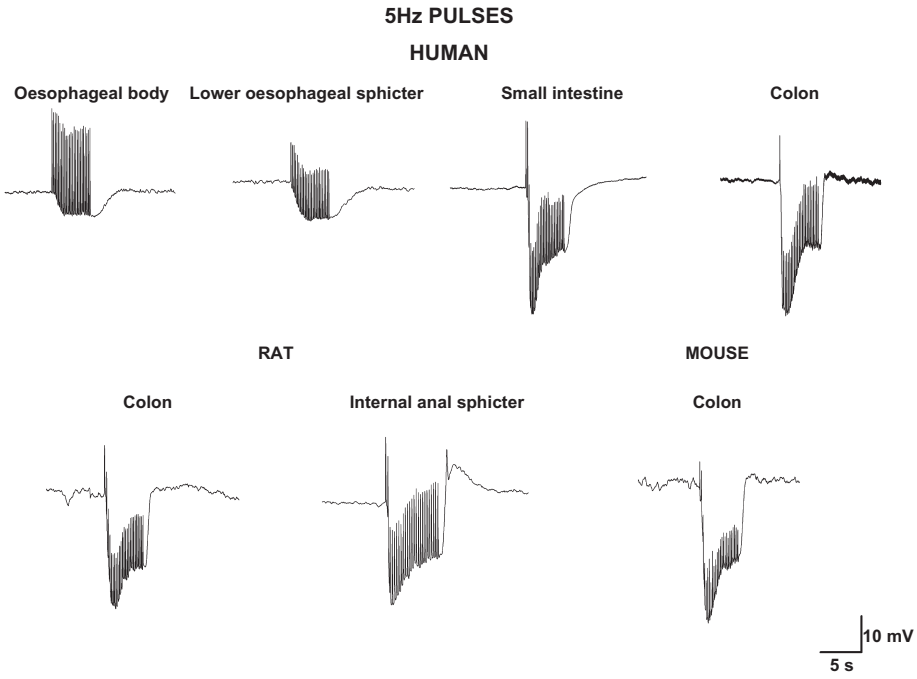

Figure 2

Pulses of 5 Hz for 5 s elicit a fast followed by a sustained hyperpolarization in different areas of the GI tract. Note the absence of an IJPf in the human oesophagus (oesophageal body and lower oesophageal sphincter).

Pharmacological evidence that the $P2Y_1$ receptor mediates the IJPf

In the vast majority of laboratory animals (mouse, rat and guinea pig) and in human GI tissue, the IJPf is largely insensitive to NOS inhibitors and partially sensitive to suramin and PPADs (Xue *et al.*, 1999). MRS2179, a selective $P2Y_1$ receptor antagonist (Boyer *et al.*, 1996; Gao *et al.*, 2006), has been used to study neuromuscular interaction in the GI tract. MRS2179 was effective at blocking both the IJPf (Figure 3) and the non-nitrergic mechanical relaxation in different tissues and areas of the GI tract, including the human small intestine and colon (Table 1). The potency of MRS2179 varies between species; the IC_{50} is usually 1 μM in guinea pig, pig and human tissue. However, higher concentrations of this antagonist are needed in rodents (data from colon and internal anal sphincter) to inhibit the IJPf and the purinergic mechanical relaxation (Table 1). Due to the lack of complete blockade in rodents, it was postulated that other P2Y receptor subtypes might participate in the purinergic inhibitory neurotransmission. The development of two new $P2Y_1$ antagonists, MRS2279 and MRS2500 (Cattaneo *et al.*, 2004), with higher selectivity and potency for the $P2Y_1$ receptor has opened up the possibility for further research. We have recently shown that the rank order of potency of $P2Y_1$ antagonists is MRS2500 > MRS2279 > MRS2179 both in rat and in human colonic tissue (Figure 3) (Grasa *et al.*, 2009; Gallego *et al.*, 2011). For example, 20 μM of MRS2179 is needed to inhibit about 50% of the IJPf in the rat colon, but 1 μM of MRS2500 completely blocks the IJPf in this tissue. Comparatively, the IC_{50} to inhibit the IJPf in the human colon is about 1 μM for MRS2179 and about 70 nM for MRS2500. It would be important to study these newly available antagonists in other tissues where MRS2179 was not able to completely block the IJPf or the non-nitrergic relaxation (Table 1). According to these pharmacological studies, it is reasonable to conclude that the P2 receptor responsible for the fast component of the IJP and the EFS-induced relaxation is the $P2Y_1$ receptor. An important exception is the dog's small intestine where the

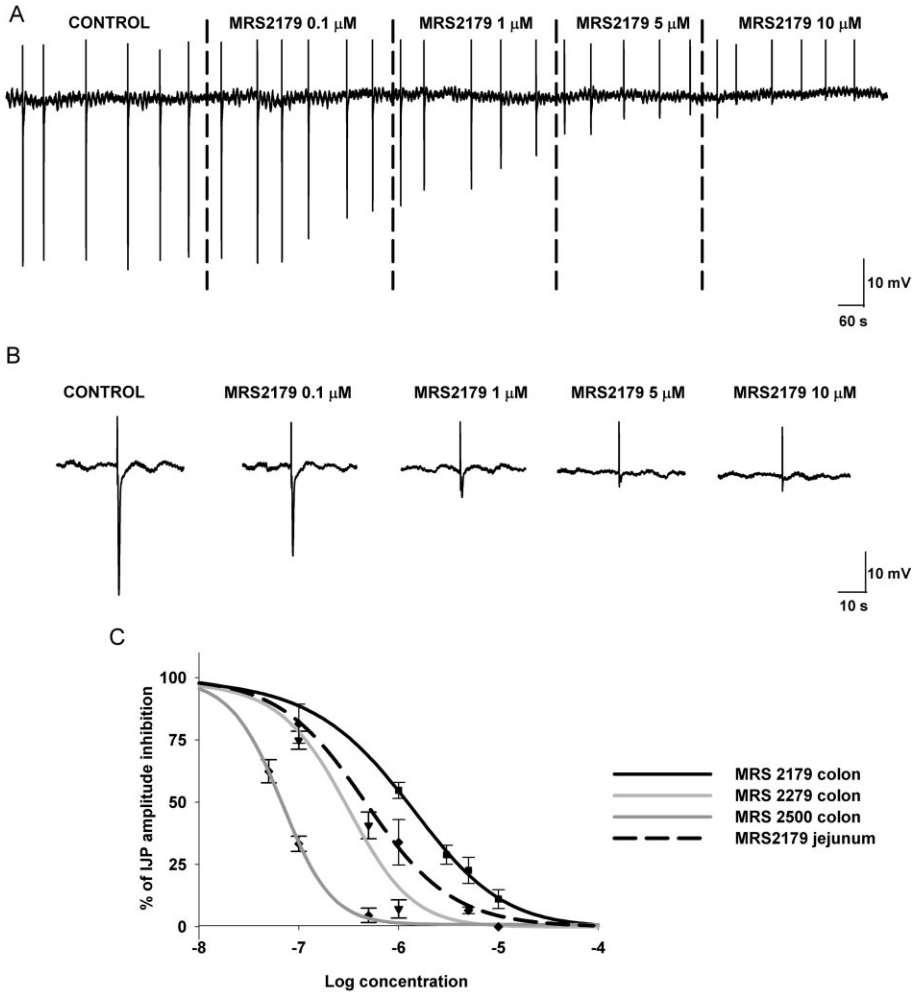

Figure 3

Inhibitory junction potentials are concentration-dependently inhibited by $P2Y_1$ receptor antagonists. Tracings are from human jejunum and data from human jejunum and colon.

Table 1

Effect of $P2Y_1$ antagonists on inhibitory junction potential and mechanical relaxation

Inhibitory junction potential				
Area of the GI tract	**Species**	**Drug**	**Inhibition**	**Reference**
Human tissue				
Colon		MRS2179		
Longitudinal			IC_{50}: 1.31 μM	Gallego *et al.* (2006)
Circular			IC_{50}: 1.21 μM	
Colon Circular		MRS2279 MRS2500	IC_{50}: 0.28 μM IC_{50}: 71 nM	Gallego *et al.* (2011)
Jejunum Circular		MRS2179	IC_{50}: 0.55 μM	Gallego *et al.* (2014)
Laboratory animals*				
Ileum (circular)	Guinea pig (FS)	MRS2179	IC_{50}: 0.2 μM	Wang *et al.* (2007)
Ileum (circular)	Pig (EFS)	MRS2179	IC_{50}: 0.7 μM	Gallego *et al.* (2008b)
Caecum	Mouse (EFS)	MRS2179	10 μM: about 25%	Zizzo *et al.* (2007)
Caecum	Mouse (EFS)	MRS2179 MRS2500	IC_{50}: 8.8 μM IC_{50}: 20.1 nM	Gil *et al.* (2013)
Colon	Mouse (EFS)	MRS2179	10 μM: about 80% inhibition	Zhang *et al.* (2010)
Colon	Rat (EFS)	MRS2179 MRS2279 MRS2500	IC_{50}: 13.1 μM IC_{50}: 17.8 nM IC_{50}: 14.0 nM	Grasa *et al.* (2009)
Internal anal sphincter	Mouse (EFS)	MRS2179	10 μM: about 50%	McDonnell *et al.* (2008)
Internal anal sphincter	Rat (EFS) and nicotinic-induced release	MRS2500	1 μM: 100%	Opazo *et al.* (2011)

Mechanical activity				
Area of the GI tract	**Species**	**Drug**	**Inhibition**	**Reference**
Human tissue				
Colon (circular)		MRS2179	IC_{50}: 0.87 μM	Gallego *et al.* (2006)
Colon (circular)		MRS2179	10 μM: 100% inhibition purinergic latency	Auli *et al.* (2008)
Ileum (longitudinal and circular)		MRS2179	10 μM: about 100% inhibition	Undi *et al.* (2009)
Colon (circular)		MRS2279 MRS2500	IC_{50}: 0.26 μM IC_{50}: 88 nM	Gallego *et al.* (2011)
Jejunum and ileum (circular)		MRS2179	10 μM: about 100% inhibition	Gallego *et al.* (2014)
Laboratory animals*				
Ileum (longitudinal)	Rat (mesenteric electrical stimulation)	MRS2179	10 μM: No effect	Kadowaki *et al.* (2003)
Jejunum (circular)	Mouse (EFS)	MRS2179	1 μM: From 100% to 60%	De Man *et al.* (2003)
Ileum (circular)	Pig (EFS)	MRS2179	10 μM: 60 to 80%	Gallego *et al.* (2008b)
Colon	Rat (EFS)	MRS2179 MRS2279 MRS2500	IC_{50}: 3.5 μM IC_{50}: 43.9 nM IC_{50}: 16.5 nM	Grasa *et al.* (2009)
Internal anal sphincter	Mouse (EFS: single stimuli)	MRS2179	10 μM: about 50% inhibition	McDonnell *et al.* (2008)
Internal anal sphincter	Rat (EFS) and nicotinic-induced release	MRS2500	1 μM: 100% inhibition	Opazo *et al.* (2011)
Internal anal sphincter	Sheep (EFS)	MRS2179	10 μM: about 35% inhibition	Acheson *et al.* (2009)

*Purinergic IJP and EFS-induced relaxation is absent in $P2Y_1$-deficient mice.
EFS, electrical field stimulation; FS, focal electrical stimulation (ganglia or interganglionic fibre tracts).

IJPf seems to be at least in part sensitive to NOS inhibitors and consequently is nitrergic (Christinck *et al.*, 1991a; Stark *et al.*, 1991). However, an interaction between purinergic and nitrergic neurotransmission has been postulated in this species (Xue *et al.*, 2000), but data using more selective $P2Y_1$ antagonists are not available. Differences in pharmacological potency between species and different mechanisms of inhibitory neurotransmission indicate the importance of the animal model when performing translational studies.

Purinergic and nitrergic co-transmission

There is no structural or functional evidence about the presence of two or more different types of inhibitory motor neurons in the enteric nervous system. The most probable mechanism is a co-transmission process, that is, the same neuron releases at least two transmitters (Burnstock, 1976). Therefore, it is assumed that a purine and NO are released from the same neuron. Inhibition of the IJPf by $P2Y_1$ receptor antagonists reveals the IJPs, which is then sensitive to NOS and GC inhibitors such as L-NNA and ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one) respectively (Gallego *et al.*, 2008a; Gil *et al.*, 2012). Accordingly, the IJPs is NO-mediated and its effect is due to the stimulation of soluble GC, which produces cGMP. These results suggest parallel pathways of co-transmission between purines and NO, although a prejunctional interaction between both pathways is not definitively discarded (Van Crombruggen *et al.*, 2007).

Lack of IJPf in $P2Y_1$ knockout (KO) mice

$P2Y_1$ KO mice are excellent biological tools to investigate the involvement of $P2Y_1$ receptors in purinergic neuromuscular transmission. Simultaneously, two groups published similar results showing that the IJPf is absent in the colon of $P2Y_1$ KO mice (Gallego *et al.*, 2012; Hwang *et al.*, 2012). Interestingly, experiments were independently performed and the concordance in the results was noteworthy (King, 2012). It is important to note that $P2Y_1$ KO mice exhibit preserved and functional nitrergic neurotransmission. The absence of IJPf in $P2Y_1$ KO mice is not restricted to the colon, it is also observed in other GI tissues such as the stomach and caecum (Figure 4) (Gil *et al.*, 2013). This experimental approach validates the pharmacological approach obtained in GI tissue from animals and humans and was considered substantial progress in the understanding of purinergic neuromuscular transmission in the gut (Goyal *et al.*, 2013).

Purinergic response rundown

Single or short train pulses elicit IJPf that shows a reduction in amplitude when a second pulse (test pulse) is applied at different short time intervals after the first conditioning pulse (Gallego *et al.*, 2008a). This mechanism has been previously denominated as IJP rundown in animal studies (King, 1994; Matsuyama *et al.*, 2002) and can be clearly visualized when a 1 Hz pulse is applied (Figure 5). In human intestinal tissues, IJP rundown occurs in both the colon and small intestine. In other species such as rodents, the IJP rundown is also present (Mañe *et al.*, 2014), but apparently less pronounced than in human tissue. The mechanism responsible for the IJP rundown is still not known and both pre- and post-junctional mechanisms might contribute to the decrease of the IJPf. In the hamster proximal colon, NO release might cause the IJPf rundown acting prejunctionally (Matsuyama *et al.*, 2002), but this is not the case in human colon as the IJP rundown is still present after NOS blockade (Gallego *et al.*, 2008a). It is possible that other purine receptors such as adenosine receptors might cause inhibition of purine release, but they are still not indentified. Post-junctional desensitization of the $P2Y_1$ receptor (see the section Intracellular pathways in smooth muscle cells) is another possibility to consider in future studies.

Inhibitory neural tone

EFS is the most common experimental procedure to induce *in vitro* neurotransmitter/s release. This is due to the fact that the electrical stimulus is repetitive, transient and usually independent of presynaptic inputs to inhibitory motor neurons. Interestingly, some tissues develop an inhibitory neural tone *in vitro* caused by 'spontaneous' release of inhibitory neurotransmitters not associated to classical EFS-induced junction potential. The neural tone is caused by action potentials in inhibitory neurons releasing both NO and a purine as co-transmitters. Accordingly, in tissues with endogenous neural activity, inhibitory neurotransmitters can be randomly released from nerve endings even in the absence of EFS. The post-junction electrophysiological consequences of an inhibitory neural tone are (i) neural-mediated hyperpolarized membrane potentials in smooth muscle cells and (ii) the appearance of spontaneous IJP (Figures 1 and 6). When the tissue is incubated with the neural blocker TTX, the membrane potential depolarizes, the tissue contracts and spontaneous IJP are inhibited (Gil *et al.*, 2010). Interestingly, spontaneous IJP are absolutely insensitive to L-NNA, they are apamin sensitive (Spencer *et al.*, 1998; Powell *et al.*, 2001) and are inhibited by $P2Y_1$ receptor antagonists (Gil *et al.*, 2010). It is also well known that smooth muscle cells depolarize and tone increases after incubation in L-NNA. These results could be explained by the process of co-transmission: NO being responsible for the level of the membrane potential in smooth muscle cells and a purine, through $P2Y_1$ receptors, for the spontaneous IJP. Thus, when an inhibitory neural tone is present, the muscular tone or spontaneous contractility of tissues incubated with L-NNA usually increases due to smooth muscle depolarization; whereas this does not occur after $P2Y_1$ receptor blockade. In fact, when $P2Y_1$ receptors are blocked, a decrease in spontaneous motility might occur (Gil *et al.*, 2010). Spontaneous IJP can be recorded in the colon of wild-type (WT) mice and are MRS2500-sensitive (mediated by $P2Y_1$ receptors). This pharmacological result is confirmed by the absence of spontaneous IJP in tracings obtained from $P2Y_1$ KO mice (Figure 6) that presented a preserved and functional nitrergic inhibitory neural tone (Gallego *et al.*, 2012).

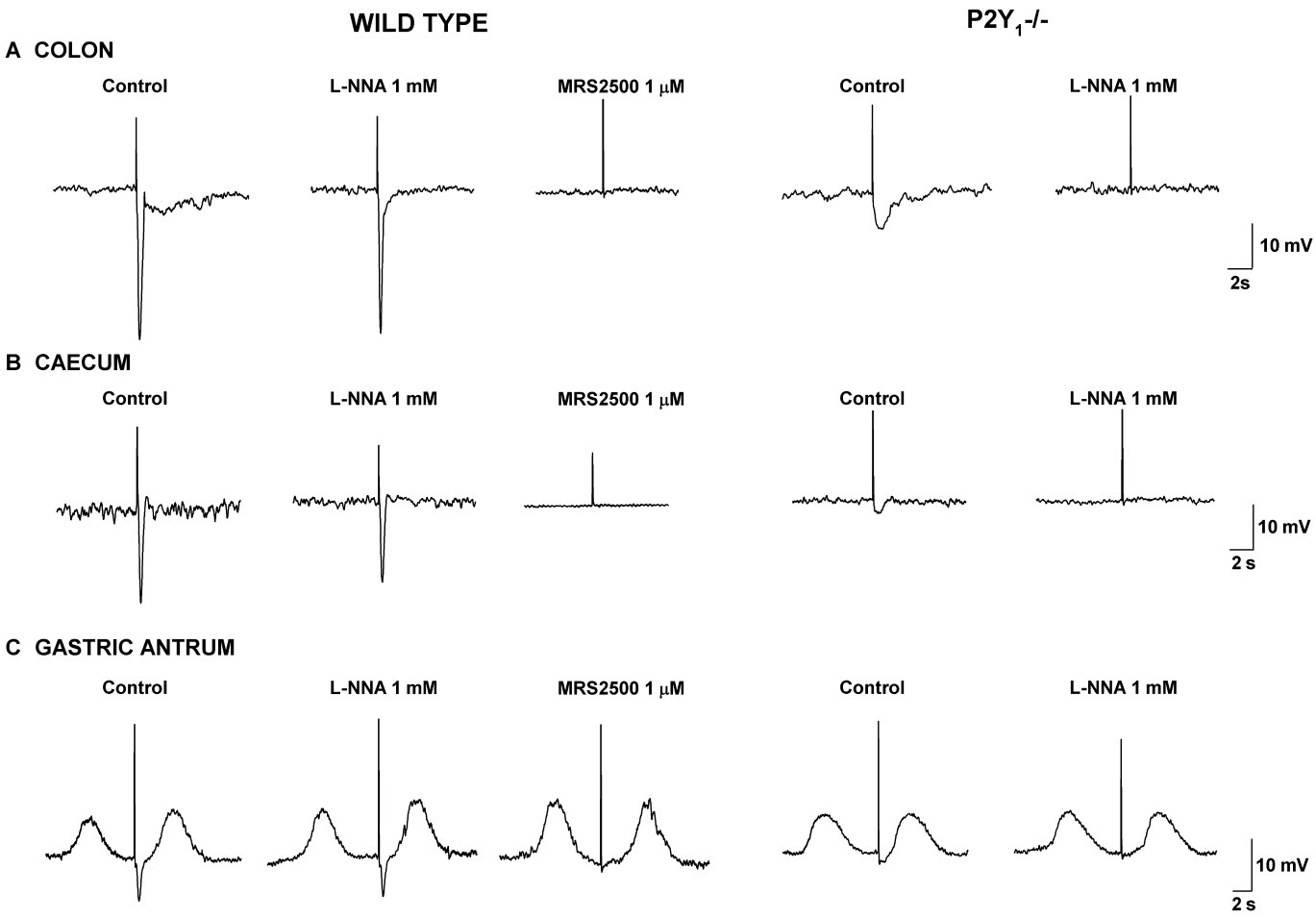

Figure 4

Representative tracings summarizing studies in knockout mice. MRS2500-sensitive IJPf recorded in the colon (A), caecum (B) and gastric antrum (C) in wild-type animals are absent in $P2Y_1$–/– mice. In $P2Y_1$–/– animals, the IJPs is totally L-NNA-sensitive.

Complementary roles for ATP and NO

Experimental data suggest that NO might mediate sustained inhibition and relaxation, whereas activation of $P2Y_1$ receptors probably causes phasic relaxation (Spencer *et al.*, 1998; Gil *et al.*, 2012). According to this hypothesis, (i) NO causes a sustained hyperpolarization, that is, the slow component of the IJP and continuous hyperpolarization when an inhibitory tone is present; (ii) no nitrergic desensitization occurs; otherwise, it would be impossible to constantly inhibit the motility; and (iii) NOS inhibitors cause a marked increase in tone and spontaneous motility when an inhibitory neural tone is present. In contrast, purinergic neurotransmission mediates phasic relaxation because (i) it causes a prominent but transient hyperpolarization; (ii) spontaneous IJP are recorded in a discontinuous manner; (iii) IJPf has a rundown; and (iv) blockade of $P2Y_1$ receptors does not increase spontaneous contractility. Therefore, both neurotransmitters have complementary physiological functions (Gallego *et al.*, 2008a; Gil *et al.*, 2012; Mañe *et al.*, 2014) (Table 2). The inhibitory electrophysiological and mechanical responses are only abolished when both pathways are inhibited, (Gallego *et al.*, 2006; 2014) (Figure 7).

Apamin versus $P2Y_1$ receptor antagonists

Apamin, a small conductance calcium-activated potassium channel (SK_{Ca}) blocker, is a pharmacological tool that has been used to distinguish between the IJPf and the IJPs. The terminology 'apamin sensitive *vs.* apamin resistant' is frequently used to distinguish both IJP components (Zhang *et al.*, 2010). sK_{Ca} currents are activated by P2Y receptor agonists and blocked by apamin in smooth muscle cells (Koh *et al.*, 1997; Vogalis and Goyal, 1997). Apamin usually reduces the IJPf, showing that SK_{Ca} channels are responsible for the fast hyperpolarization. Therefore, both $P2Y_1$ antagonist and apamin should have 'similar' effects. However, this is not totally true, and in some cases, important differences exist between $P2Y_1$ antagonists and apamin. The reduction in the IJPf amplitude caused by apamin varies depending on the species. Apamin

abolishes the IJP in the guinea pig and a major reduction is observed in other species such as rodents, pig or dog. In guinea pig, focal stimulation causes a 'pure' purinergic fast IJP, which is both MRS2179- and apamin-sensitive (Koh *et al.*, 1997; Wang *et al.*, 2007). These data show that in these species, smooth muscle hyperpolarization is largely mediated by SK_{Ca} activation. In contrast, in the mouse colon, apamin-sensitive and apamin-resistant IJPf (both of them are MRS2179-sensitive) have been recently reported. The difference between both IJPf might be the projection of the inhibitory motor neuron, that can be oral, aboral or circumferential (Zhang *et al.*, 2010). Interestingly, in the human small intestine and colon, the reduction obtained with apamin in the IJPf is only about 25–30%, suggesting that the majority of the response is independent of SK_{Ca} channels or, alternatively, the SK_{Ca} channels involved are apamin-insensitive (Xue *et al.*, 1999; Gallego *et al.*, 2006). Moreover, when the IJPf and the IJPs are recorded, apamin reduces both components in human colonic tissue (Keef *et al.*, 1993). Isolation of the nitrergic component with MRS2500 reveals an IJPs in the rat colon, which is nitrergic and partially inhibited by high concentrations of apamin (Gil *et al.*, 2012). Furthermore, apamin usually increases spontaneous motility in the colon and also depolarizes smooth muscle cells. These results suggest that apamin might not be an appropriate pharmacological tool to distinguish purinergic from nitrergic neurotransmission.

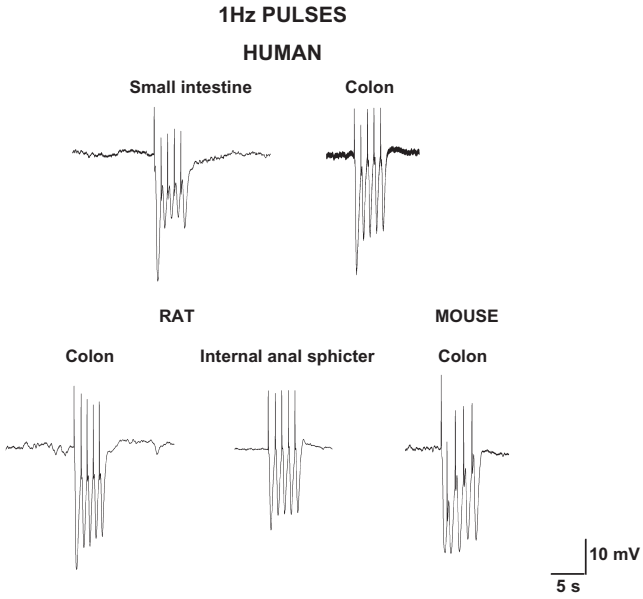

Figure 5
Pulse of 1 Hz for 5 s reveal the presence of an IJPf rundown. The first IJPf has a bigger amplitude compared with the following responses.

The oesophagus and LES: the exception

Due to anatomical similarities, the opossum has been an animal model to study neuromuscular transmission in the oesophagus. In this area of the GI tract, the IJP is largely apamin-insensitive (Cayabyab and Daniel, 1996; Jury *et al.*, 1996) and probably the contribution of SK_{Ca} channels is minor due to a major NO component (Christinck *et al.*, 1991b). Moreover, different innervations have been reported in clasp (biphasic IJP) and sling fibres (monophasic IJP) in the mouse LES with different sensitivities to apamin (Zhang *et al.*, 2008). In the pig, the IJP of the EB is NO-mediated (Lecea *et al.*, 2012). In the human EB and LES (both clasp and sling

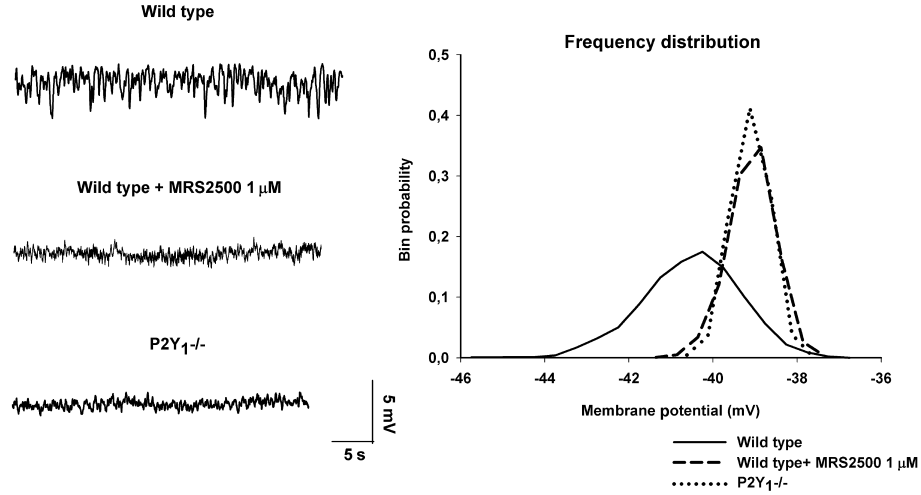

Figure 6
Spontaneous IJP are MRS2500 sensitive in wild-type mice and completely absent in $P2Y_1$ KO mice. Frequency distribution of the RMP fully supports these results. In the frequency distribution of recordings from wild-type animals, ongoing sIJP create a tail towards the most negative values. This tail does not appear in the frequency distribution obtained from tissue incubated with MRS2500 or from $P2Y_1$ KO mice. L-NNA-treated tissue (not shown) depolarizes smooth muscle cells without changing the internal frequency distribution, which is consistent with the presence of spontaneous IJP.

Table 2

Mechanical and electrophysiological responses of inhibition of nitrergic and purinergic pathways

	Inhibition of	
	Nitrergic neurotransmission	**Purinergic neurotransmission[1]**
Membrane potential[2]	Depolarization	No effect
Spontaneous motility[2]	Increase	No effect/Decrease[3]
Spontaneous IJP[2]	No effect	Inhibition
EFS-induced IJP	Inhibition of the slow component	Inhibition of the fast component
EFS-induced relaxation[4]	Partial reversion	No effect/Partial reversion

[1]Based upon previous data using inhibitors of $P2Y_1$ receptors (Gallego *et al.*, 2006; 2008a; 2011; Grasa *et al.*, 2009) and $P2Y_1$ KO mice (Gallego *et al.*, 2012).
[2]These criteria should be used if an inhibitory neural tone is present in the preparation (Gil *et al.*, 2010).
[3]A decrease in spontaneous motility might be expected if ATP is limiting pre-/post-junctional NO effect.
[4]EFS-induced relaxations might be reversed by $P2Y_1$ antagonists/NOS inhibitors depending upon the frequency of EFS.

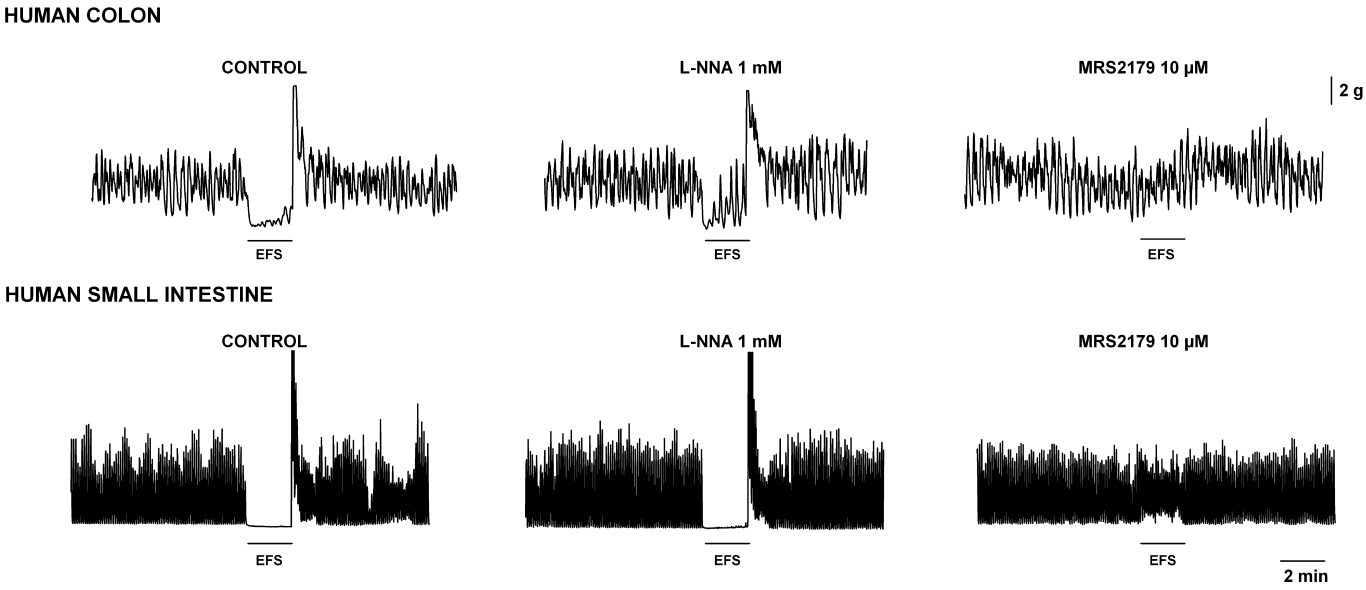

Figure 7

Both L-NNA and MRS2179 are necessary to inhibit (5 Hz, supramaximal voltage) EFS-induced relaxation in the human small and large intestine.

fibres), the IJP and the corresponding relaxation are monophasic and mainly nitrergic (Figures 1 and 2) (Lecea *et al.*, 2011).

Intracellular pathways in smooth muscle cells

$P2Y_1$ receptors are GPCRs that activate PLC. The second messenger, IP_3 (inositol 1,4,5-trisphosphate), causes the release of calcium from intracellular stores mainly located in the sarcoplasmic reticulum. This mechanism has been demonstrated in different subclasses of enteric neurons (Kimball *et al.*, 1996; Christofi *et al.*, 1997), enteric glial cells (Kimball and Mulholland, 1996) and smooth muscle of laboratory animals (Blottiere *et al.*, 1996; Pacaud *et al.*, 1996; Bayguinov *et al.*, 2000; Kong *et al.*, 2000). In colonic myocytes, a P2Y receptor agonist causes an increase in cytosolic 'calcium puffs/sparks' and increases spontaneous transient outward currents, which are both charybdotoxin and apamin sensitive (Bayguinov *et al.*, 2000; Kong *et al.*, 2000). Localized calcium release near the plasma membrane causes the electrical event responsible for purinergic hyperpolarization. Data from our laboratory show that ADPβS, a preferential $P2Y_{1/12/13}$ receptor agonist, causes calcium transients both in enteric neurons (Gallego *et al.*, 2008b) and in human cultured colonic smooth muscle cells (Martinez-Cutillas *et al.*, 2011). In both cell types, the calcium rise is blocked by MRS2179, showing that $P2Y_1$ receptors are specifically involved in the response. The

increase in the concentration of cytosolic calcium and/or DAG activates PKC, a kinase that has been reported to be responsible for $P2Y_1$ desensitization in platelets (Hardy *et al.*, 2005) and endothelial cells (Rodriguez-Rodriguez *et al.*, 2009). It is still not known if this pathway is responsible for the rundown of the IJPf. A recent paper suggests that the $P2Y_1$ receptor is a GPCR not linked to PLC. In this study, activation of $P2Y_1$ receptors in colonic myocytes causes a reduction in IP_3 and postulates a new mechanism of action for the receptor leading to smooth muscle hyperpolarization and relaxation (MacMillan *et al.*, 2012).

Role of $P2Y_1$ receptors in motility

$P2Y_1$ receptor antagonists have not been studied *in vivo*. In a set of experiments with anaesthetized rats where spontaneous motility was monitored with a strain gauge, NOS inhibition caused a dramatic and long-lasting increase in spontaneous motility, whereas MRS2500 induced a single but prominent contraction without a major effect on subsequent contractions (Gil *et al.*, 2010). These results further confirm the hypothesis that both inhibitory neurotransmitters have complementary physiological functions (Gil *et al.*, 2012). Studies performed to investigate colonic motility *in vitro* using transit of pellets showed that both incubation with MRS2500 (in WT animals) and depletion of $P2Y_1$ receptors (in KO mice) induced delayed colonic transit. These findings indicate that both nitrergic and purinergic inhibitory pathways are necessary to accomplish a proper motor function (Hwang *et al.*, 2012). Although complementary roles are suggested by these contractile and electrophysiological experiments, it is possible that one pathway might partially compensate for the other one when it is blocked pharmacologically or genetically removed in KO mice.

The 'intercalation' theory

Two theories are currently supported by different groups regarding the cell types involved in neuromuscular transmission:

1. The 'intercalation' theory suggests that a non-muscular cell type [interstitial cells of Cajal (ICC) or platelet-derived growth factor receptor α (PDGFRα+) cells] mediates neuromuscular transmission.
2. The direct theory suggests a direct contact between motor neurons and smooth muscle cells without any kind of intermediate cell.

This is a controversial issue, and contradictory experimental data supporting both theories have been published by outstanding groups in the field of neurogastroenterology. Conditionally, KO mice lacking GC in smooth muscle have functional nitrergic neurotransmission (Groneberg *et al.*, 2011). These experiments support the intercalation theory, suggesting that GC in ICC might transduce nitrergic inputs from inhibitory motor neurons to muscle (Burns *et al.*, 1996; Ward and Sanders, 2001; Suzuki *et al.*, 2003). However, nitrergic neuromuscular transmission is present in genetically modified animals where GC is removed from ICC, suggesting that both direct and indirect communications are possible (Groneberg *et al.*, 2013). Purinergic neurotransmission is largely independent of the ICC. Mutant animals with impaired development of ICC including Ws/Ws rats (Alberti *et al.*, 2007) and Wsh/Wsh mice (Figure 8) have intact purinergic neurotransmission. Recordings from colonic tissue display MRS2500-sensitive 'spontaneous' IJP and IJPf. Consequently, purinergic neurotransmission is independent of ICC. PDGFRα+ cells (fibroblast-like cells) can transduce purinergic signals and have the apparatus to do so (Cobine *et al.*, 2011; Kurahashi *et al.*, 2011; 2012) as shown by (i) the presence of $P2Y_1$ receptors in these cells (Kurahashi *et al.*, 2011); (ii) the abundance of $K_{Ca}2.3$ (previously known as SK3) channels (Vanderwinden *et al.*, 2002; Fujita *et al.*, 2003; Iino and Nojyo, 2009) that might contribute to the hyperpolarization; and (iii) the fact that potential agonists of $P2Y_1$ receptors activate large-amplitude apamin-sensitive currents that were blocked by MRS2500 (Fujita *et al.*, 2003; Kurahashi *et al.*, 2011). Accordingly, PDGFRα+ cells, as described previously for smooth muscle cells, have the potential/capacity to transduce purinergic inputs (Figure 9). Animals with a decreased number of PDGFRα+ cells will be important to investigate the relative contribution of each cell type to the intercalation hypothesis. One important issue that needs to be solved is how two different cell populations (ICC and PDGFRα+ cells) can transduce in parallel two neurotransmitters apparently co-transmitted from the same neuron. Another unknown mechanism is how signals are transduced from intercalated cells to smooth muscle (Figure 9). Finally, if the intercalation hypothesis is confirmed, then, as it was suggested long time ago by Ed Daniel for NO, exogenous addition of purines might not always exactly mimic (see below) the effect of endogenous release of neurotransmitters. 'If receptor to the same mediator on interstitial cells and on smooth muscle differ in the response they initiate, the actions of mediator added to the bath may not duplicate those of receptor mediate from nerves'. (Daniel and Posey-Daniel, 1984). With this sentence, Daniel already postulated that according to the intercalation hypothesis, exogenous addition of neurotransmitters might not exactly mimic the endogenous release if different receptors are located in smooth muscle and ICC. This might also be applicable for purinergic neurotransmission if the intercalation theory is validated (Figure 9).

Identification of the purinergic neurotransmitter

ATP was identified by Burnstock as the main purinergic inhibitory neurotransmitter in the GI tract (Burnstock *et al.*, 1970). ATP is rapidly hydrolysed by the activity of ectonucleotidases into ADP and adenosine that might be biologically active and contribute to smooth muscle hyperpolarization and relaxation. The work to demonstrate the relevance of purinergic neurotransmission has been long and difficult (Burnstock, 2008). ATP has been considered the main purinergic neurotransmitter in the human small and large intestine (Xue *et al.*, 1999; Gallego *et al.*, 2006). Recently,

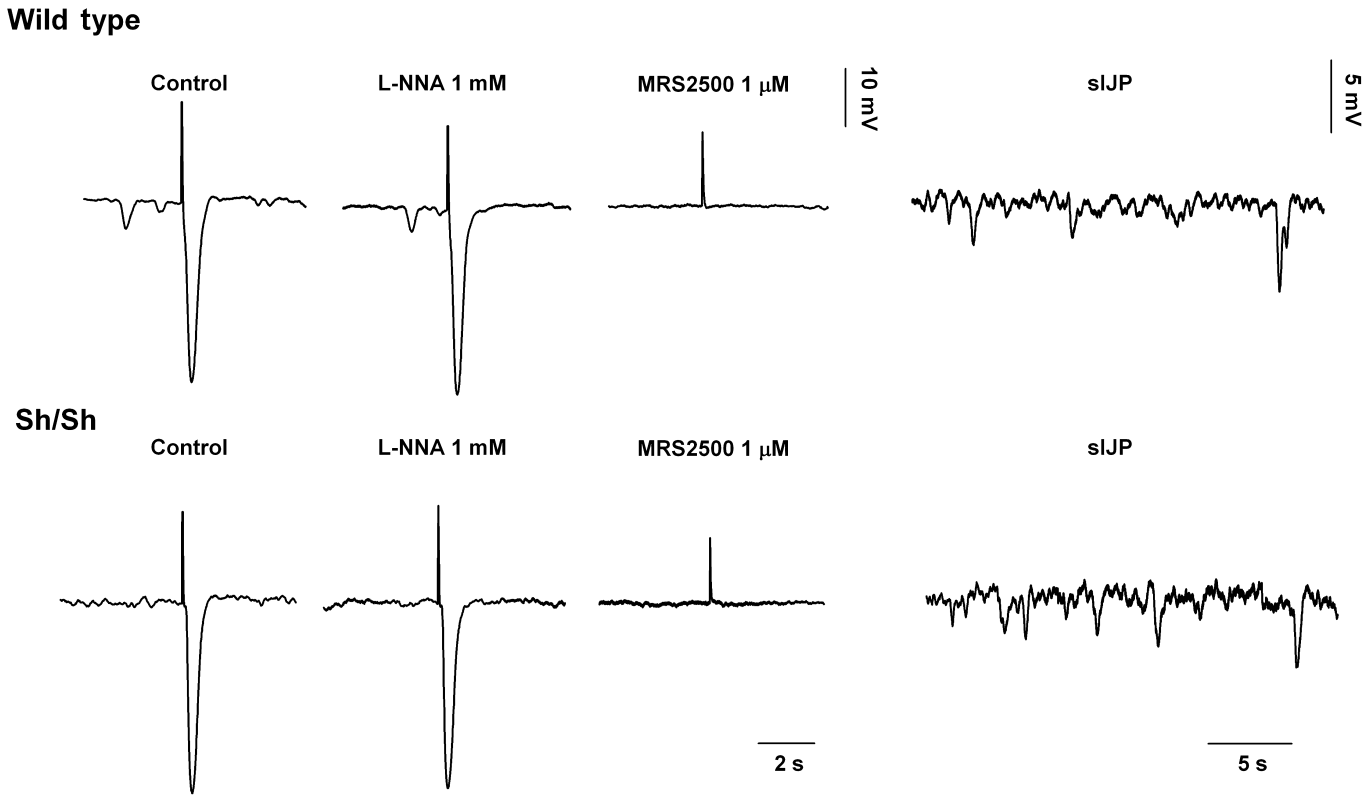

Figure 8
Purinergic fast and spontaneous IJP are recorded in deficient mouse ($W^{sh/sh}$) with impaired ICC development (unpublished data).

β-nicotinamide dinucleotide (β-NAD) and ADP-ribose (Mutafova-Yambolieva *et al.*, 2007; Durnin *et al.*, 2012) have been proposed to be the purinergic NANC inhibitory mediators in the GI tract. These two mediators bind to $P2Y_1$ receptors and cause apamin-sensitive and MRS2500-sensitive hyperpolarizations (Hwang *et al.*, 2011). However, in human tissues, high concentrations of β-NAD are needed to inhibit spontaneous contractility and the effect is not blocked by $P2Y_1$ receptor antagonists (Gallego *et al.*, 2011). Exogenously added β-NAD induces a small hyperpolarization in human tissue that does not mimic the IJPf (Gallego *et al.*, 2011). In the mice colon, β-NAD-induced hyperpolarization is partially blocked by MRS2500 and strongly reduced in $P2Y_1$ KO mice (Gallego *et al.*, 2012). However, in the caecum, β-NAD-induced hyperpolarization is insensitive to MRS2500 and still recorded in $P2Y_1$ KO mice (Gil *et al.*, 2013). β-NAD could bind to extrajunctional receptors, and consequently, it might not really mimic the endogenous release of the inhibitory neurotransmitter. However, this has still not been validated. Similarly, exogenously added ATP/ADP does not exactly mimic the endogenous neurotransmitter, for example, ATP-induced smooth muscle hyperpolarization in the human colon is insensitive to MRS2500 (Hwang *et al.*, 2011). ATP overflow measured after EFS is not blocked by TTX or ω-conotoxin (GVIA) (Durnin *et al.*, 2013). More than 40 years after the initial finding (Burnstock *et al.*, 1970; Burnstock, 2008), the nature of the purinergic neurotransmitter in the GI tract is still not known and is debatable (Goyal, 2011).

$P2Y_1$ receptors in other cell types

$P2Y_1$ receptors are located in different subclasses of enteric neurons including submucosal and myenteric neurons. It has been demonstrated that slow excitatory synaptic transmission is mediated by $P2Y_1$ receptors in guinea pigs (Hu *et al.*, 2003; Gao *et al.*, 2006; Gwynne and Bornstein, 2009). In this species, $P2Y_1$ receptors might also participate in neurogenic secretion (Fang *et al.*, 2006). $P2Y_1$ receptors also participate in the enterochromaffin neural secretomotor arch in the human small intestine. Using calcium imaging in human submucous neurons, stimulation of intermodal strands cause the release of purines that act on post-synaptic neurons causing $P2Y_1$/$G\alpha q$/$PLC/IP_3/Ca^{2+}$ signals. This effect is effectively blocked by the $P2Y_1$ antagonist MRS2179 (Wunderlich *et al.*, 2008). Recently, it has been demonstrated that neural purinergic release causes activation of glial cells and the response might be mediated by $P2Y_4$ and $P2Y_1$ receptors (Gomes *et al.*, 2009; Gulbransen and Sharkey, 2009). Altogether, these results demonstrate purinergic neural communication between enteric neurons and glial cells.

Translational studies

A problematic issue regarding purinergic neuromuscular transmission has been the difficulty in convincing clinicians

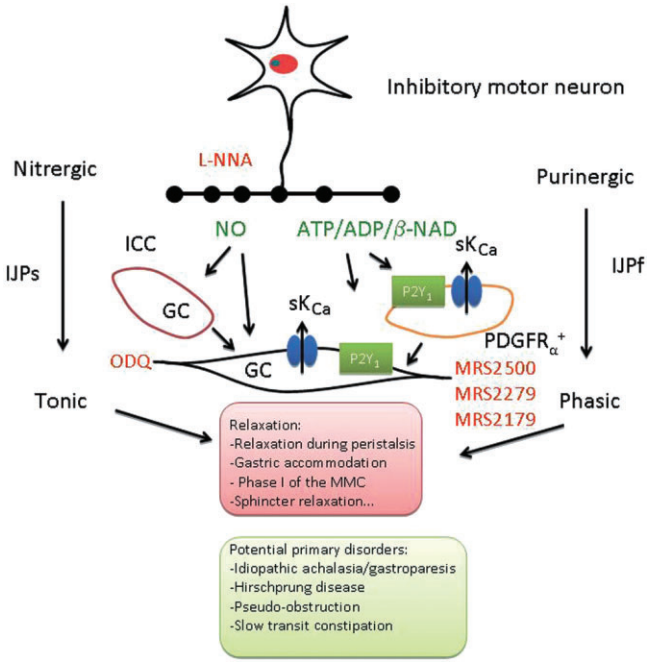

Figure 9

Smooth muscle relaxation is accomplished by enteric inhibitory motor neurons in the gastrointestinal tract. NO and a purine (ATP/ADP/β-NAD) are possibly co-released by inhibitory motor neurons. GC (ODQ sensitive) mediates the nitrergic slow component of the IJP. $P2Y_1$ receptors (MRS2179-, MRS2279- and MRS2500-sensitive) mediate the purinergic fast component of the IJP. Smooth muscle can transduce both nitrergic and purinergic signals through a direct communication. ICC and PDGFRα+ cells are potential intercalated cells that might transduce nitrergic and purinergic inputs to smooth muscle cells respectively. Due to the electrophysiological profile of the response, nitrergic IJP is tonic since it can be time-sustained, whereas purinergic IJP is phasic because the response runs down. The combination of both mechanisms is responsible for relaxation in different regions of the gastrointestinal tract that might be potentially impaired in primary and secondary disorders affecting the neuromuscular junction. [agonists (neurotransmitters) are depicted in green and antagonists (blocking these pathways at different levels) are depicted in red].

about the relevance of the mechanism in the human GI motility (Sanger *et al.*, 2013). Probably, the identification of NO early in the 1990s (Bult *et al.*, 1990; Christinck *et al.*, 1991a; Stark *et al.*, 1991; Boeckxstaens *et al.*, 1993; Keef *et al.*, 1993; Goyal and He, 1998) and the association of enteric pathologies with the lack of nitrergic neurons (Mearin *et al.*, 1993; Boeckxstaens *et al.*, 1994) was a strong argument to postulate that NO is the 'main' inhibitory mediator in the GI tract. Nowadays, gastroenterologists with great expertise in motility are usually not aware of purinergic nerve-mediated relaxation, probably due to the apparent lack of diseases associated with an impairment of purinergic neurotransmission. The general approach for clinicians to study neuromuscular diseases is pathological studies with tissue samples (Knowles *et al.*, 2010; 2011). Purinergic neurons are not routinely labelled with histopathological techniques. Only the quinacrine technique has been proposed as a potential marker of purinergic neurons (Olson *et al.*, 1976; Burnstock *et al.*, 1978). Recently, staining of the vesicular nucleotide transporter (V-NUT) SLC17A9 has been proposed as a marker for purinergic neurons (Chaudhury *et al.*, 2012) but the exclusiveness of the transporter in purinergic vesicles and not in other non-purinergic vesicles needs further evidence. Therefore, only experimental functional studies demonstrating purinergic neurotransmission in apparently healthy tissue have been demonstrated. Very few studies have been performed to investigate a possible impairment of purinergic neurotransmission in pathological conditions and most of them have been performed in animal models (Roberts *et al.*, 2012). In inflamed guinea pig distal colon, a marked decrease in the fast component of the IJP has been reported (Strong *et al.*, 2010). The reduction was attributed to an altered release or degradation of ATP acting on $P2Y_1$ receptors. Interestingly, the nitrergic component was not affected, suggesting a selective damage of the purinergic neurotransmission causing peristalsis impairment. A very interesting study has been recently published demonstrating selective impairment of purinergic release due to oxidative stress in two models of colonic inflammation (Roberts *et al.*, 2013). It is not known if purinergic neuromuscular transmission is impaired in inflamed samples from human tissue. Neuropharmacological studies on prokinetic drugs such as 5-HT_4 receptor agonists are usually focused on promoting excitatory neurotransmission and the general belief is that an increase in excitatory neurotransmission will promote transit. Unfortunately, it is usually not known if these drugs also promote inhibitory neurotransmission and no data are available about the effect of these drugs on purinergic neurotransmission, which, in turn, might also facilitate transit.

Conclusions

The research in purinergic neurotransmission in the GI tract started more than 40 years ago (Burnstock *et al.*, 1970). Recently, new important data have been generated using the newly developed selective $P2Y_1$ receptor antagonists and genetically modified animals that lack $P2Y_1$ receptors. According to these recent data, we have now strong reasons to believe that the receptor that contributes to purinergic smooth muscle relaxation has been identified. Inhibitory neuromuscular transmission in the GI tract therefore involves at least two inhibitory co-transmitters: a purine and NO (Figure 9). During the last 10 years, efforts have been made to demonstrate that this co-transmission is the general mechanism of neural-mediated relaxation in the human small and large intestine. It is feasible that different types of mechanical relaxation and consequently physiological roles can be ascribed to each inhibitory neurotransmitter (Figure 9). An effort should be made to further investigate possible purinergic neurotransmission involvement in neuromuscular diseases as $P2Y_1$ receptors are possible pharmacological/genetic targets to consider. The effect of drugs that modulate purinergic neuromuscular transmission should be also studied to find better treatments for GI motility disorders. Without this effort, purinergic neurotransmission will remain a crucial physiological finding without 'apparent' clinical or pharmacological relevance.

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Author contributions

M. J. wrote the manuscript. P. C. and A. A. provided human samples during the last years and contributed to the discussion of the manuscript. D. G. performed experiments presented as figures and contributed to the discussion, revision and editing of the manuscript.

Conflict of interest

The authors disclose no conflict of interest.

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