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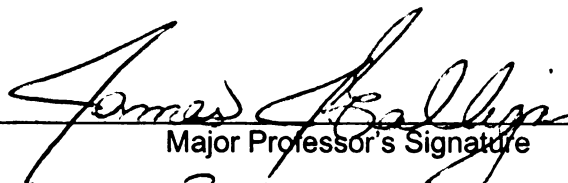
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**P2X RECEPTORS AND NEUROGENIC CONTROL OF MURINE COLONIC  
MOTILITY.**

**By**

**Matthew Peter DeVries**

**A THESIS**

**Submitted to  
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## **ABSTRACT**

### **P2X RECEPTORS AND NEUROGENIC CONTROL OF MURINE COLONIC MOTILITY.**

**By**

**Matthew Peter DeVries**

Excitatory and inhibitory motor input to mouse colonic longitudinal muscle were investigated. Isometric contractions/relaxations of mouse longitudinal muscle in perfused organ baths were measured to assess neuromuscular contractions. Measurement of colonic migrating motor complexes (MMCs) was used to assay reflex activity. Fecal output induced by bolus injection of 5-hydroxytryptophan was used as a measure of colonic motility in vivo. Responses in transgenic mice lacking either the P2X<sub>2</sub> or P2X<sub>3</sub> ATP receptor subunits were compared to control responses in C57B wild type (WT) mice. ATP-induced contractions that were TTX-sensitive and mediated by P2X<sub>3</sub> subunit containing receptors. Nitro L-arginine (NLA) and apamin treatment revealed TTX-insensitive contractions that were not affected by P2X<sub>2</sub> or P2X<sub>3</sub> receptor subunit knockout. Nicotine induced contractions in all mice that that was blocked by NLA and apamin. NLA and apamin did not reveal a nicotine induced contraction. Frequency and amplitude of MMCs were not affected by P2X<sub>2</sub> or P2X<sub>3</sub> receptor deletion, nor was 5-hydroxytryptophan induced fecal output. I conclude that ATP stimulates an excitatory motor input to mouse colonic longitudinal muscle via P2X<sub>3</sub> subunit containing receptors on neuron cell bodies.

**This is dedicated to my parents Shaun and Christine DeVries and my siblings  
whose personal sacrifice made this degree possible**

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## **Introduction**

### **ATP as a neurotransmitter**

By 1970, adenosine 5'-triphosphate (ATP) was already considered one of the most biologically important molecules due to its role in oxidative phosphorylation, respiration, and as a precursor nucleotide in DNA and RNA synthesis (49, 92). In that year, the laboratory of Geoffrey Burnstock published a landmark study illustrating another important role for ATP (26). Using the criteria set forth by Sir John Eccles in 1964, Burnstock was the first to put forward ATP as a neurotransmitter (26, 45).

Today ATP and its receptors are recognized as important mediators of neurotransmission in the central and peripheral nervous systems (23). ATP can be released via constitutively active slow mechanisms such as ATP binding cassette protein transporters, connexin hemi-channels, and mitochondrial porins (50, 74, 89). ATP release can also occur in a quantal,  $\text{Ca}^{2+}$ -dependent manner (22, 50, 65, 72). Co-release of ATP with other neurotransmitters such as acetylcholine, catecholamines, and  $\gamma$ -amino butyric acid has been demonstrated *in vitro* from motor neurons and in expression system models of synaptic release (22, 50, 89, 123). ATP is metabolized by ectonucleotidases in the extracellular space is metabolized to ADP, AMP, and adenosine all of which can act as signalling molecules themselves (88, 110, 143). ATP and its metabolites have the ability to modulate the activity of many biological signalling proteins including ATP gated potassium channels, neuronal nicotinic acetylcholine receptors, and

ectonucleotidases, as well as affect the expression of acetylcholinesterases and neuromuscular nicotinic acetylcholine receptors (32, 40, 46, 74, 88, 111, 122, 143). ATP directly activates two classes of receptors, the G-protein coupled metabotropic P2Y and the ionotropic P2X receptors (1, 27, 110). The actions of ATP are complex and domain specific with the magnitude, duration, and direction of ATP mediated effects being dependent on the extracellular concentration of ATP, amount of ATP metabolism, and concentration of ATP sensitive receptors in a given tissue at any given time. The aim of these studies was to characterize the role of excitatory ATP signalling in control of colonic motility.

### **Innervation of the GI tract**

The function of the gastrointestinal tract is under the control of the enteric nervous system (ENS), a division of the autonomic nervous system. The ENS is comprised of the neurons housed in the wall of the gastrointestinal tract. The term intrinsic is used to describe these neurons because their cell somas remain within the gastrointestinal tract.

Sympathetic and Parasympathetic divisions of the nervous system also control the gastrointestinal tract. Sensory afferent neurons whose cell bodies are in the dorsal root and nodose ganglia also supply the gastrointestinal tract. These neurons carry information about chemical and mechanical activity in the gastrointestinal tract to the central nervous system. These sensory outputs along with the parasympathetic and sympathetic input are termed extrinsic nerves because their cell bodies are outside the gastrointestinal tract.

## ***Sympathetic***

### ***Sympathetic – origin***

The celiac, superior mesenteric, and the inferior mesenteric are the sympathetic prevertebral ganglia that house the sympathetic neurons that innervate the GI tract (77). The postganglionic sympathetic nerves emerge from the mesenteric nerves that travel parallel to the mesenteric arteries and veins (77). The neurons in the ganglia receive convergent input from sympathetic preganglionic nerves (77). The presynaptic nerves synapse on the dendrites of the ganglion cells, the synapses are cholinergic (77). The celiac ganglion receives its presynaptic input via the greater splanchnic nerve (77). The lesser splanchnic innervates the superior mesenteric ganglia and the lumbar splanchnic innervates the inferior mesenteric ganglia (77). Prevertebral ganglia also receive adrenergic input from adjacent prevertebral ganglia (77). Visceral sensory afferent neurons from the wall of the gut synapse on prevertebral cells as well (77). The neurons in the splanchnic nerves project from sympathetic nuclei in the intermediolateral of the spinal column (at the T-4, L-3, or L-4 level in humans), leaving via the ventral root, then traveling in the white rami through the paravertebral ganglia without synapsing, emerging to form the splanchnic nerves (77). Projections from the lumbar ganglia form the lumbar colonic nerve that innervates the colon (134).

### ***Sympathetic – termination***

Sympathetic nerves innervate the blood vessels, sphincters, mucosa, and myenteric and submucosal plexuses, but not intestinal muscle (77). While sympathetic axons pass through the myenteric and submucosal plexuses there is no ultrastructural evidence of somatic sympathetic synapses in the myenteric plexus and only a few in the submucosal ganglia (94). Cells in the celiac and superior mesenteric ganglia provide most of the sympathetic innervation to the stomach and small intestine and the inferior mesenteric ganglia innervates the large intestine (77). There are areas of the small intestine that receive overlapping sympathetic innervation from adjacent prevertebral ganglia (77). Outputs from the lumbar colonic nerve innervate the anal sphincter and distal colon (134).

### ***Sympathetic – neurotransmitters and function***

While the majority of postsynaptic cell bodies in the prevertebral ganglia are adrenergic, a percentage show immunoreactivity for somatostatin and/or neuropeptide Y (77). A broad description of the principal effects of sympathetic outflow on the GI tract is to decrease motility, increase sphincter tone, shift from secretion to absorption, and increase vasoconstriction in the mucosa (77). The lumbar colonic sympathetic nerve provides a tonic inhibition of sacral parasympathetic neurons, inhibiting the defecation reflex (134).

## ***Parasympathetic***

### ***Parasympathetic – origin***

Parasympathetic efferent innervation of the gut arises in the dorsal motor nucleus of the vagus in the medulla (77). These long preganglionic cholinergic fibers travel via the vagus nerve (cranial nerve X) (77). Neurons from the sacral parasympathetic ganglia of the spinal cord provide parasympathetic preganglionic input to the distal gastrointestinal tract (31, 134).

### ***Parasympathetic – termination***

Anterograde tracing studies of neurons in the dorsal motor nucleus reveal terminals of vagal efferents in the myenteric ganglia (77). There are no terminals of vagal efferent innervation in submucosal ganglia (77). Vagal innervation is most conspicuous in the antrum of the stomach and the density of vagal efferent terminals decreases steadily along the length of the GI tract and vagal efferent terminals are virtually absent by the ileocecal-junction (77). The colon has very sparse vagal efferent supply (31). The distal colon and anal sphincter receive parasympathetic input via projections from sacral parasympathetic ganglia forming the pelvic nerves (31, 134).

### ***Parasympathetic – neurotransmitters and function***

Acetylcholine is the major parasympathetic neurotransmitter mediating excitation (31). Substance P is also an excitatory parasympathetic neurotransmitter in the gastrointestinal tract (31). Inhibition mediated by vagal

preganglionic parasympathetic fibers is mediated by ATP, nitric oxide (NO), and vasoactive inhibitory peptide (VIP) (31). Parasympathetic output acts to increase motility and secretion by the gastrointestinal tract, thus parasympathetic activation increases activity of secretomotor neurons while the tone of sphincters is inhibited (31). Retrograde labeling studies show that efferent vagal nerve endings are not found in the submucosal ganglia (7). Vagally stimulated secretion at the mucosa must be mediated by vagal stimulation of myenteric neurons which in turn synapse onto submucosal secretomotor neurons. In the distal colon parasympathetic output from the sacral ganglia is excitatory (31). Parasympathetic activation is inhibitory to the anal sphincter; this with the parasympathetic excitation in the distal colon mediates the involuntary part of the defecation behavior (31, 134). Anatomical investigations of whether sacral efferent nerve endings terminate in the myenteric ganglia or directly on the colonic smooth muscle to date have not been done.

## ***Sensory***

### ***Sensory – origin***

There are three main sources of sensory nerves supplying the gastrointestinal tract. The sensory nerves travel in the vagus nerve, the splanchnic nerve, and the pelvic nerves. The cell bodies of vagal afferents are located in the vagus and project centrally to the nucleus tractus solitarius (6). The great majority of fibers in the vagus nerve (up to 90%) are sensory afferents that supply the gut traveling to the nodose and jugular ganglia of the vagus nucleus (77). The nerve cell bodies of splanchnic afferents are located in the

dorsal root ganglia of thoracolumbar spinal region (6). The central projections of terminate on second order neurons in the dorsal horn of the spinal cord (6).

Gastrointestinal sensory information is processed in thalamic nuclei and higher sites throughout the central nervous system (6). Pelvic afferent neuron cell bodies are in the dorsal root ganglia of the sacral region spinal cord with central projections similar to those of splanchnic afferent neurons (6).

### ***Sensory – termination***

There are three functional types of nerve endings from afferent sensory neurons that project to the gastrointestinal tract (6). The first is responsive to input from the serosa and mesenteric blood vessels, the second lies in the circular and longitudinal muscle layers and the myenteric plexus (see below) and the third reacts to input at the mucosal epithelium (6). Endings at the serosa and mesenteric blood vessels respond to distension, these terminals do not respond unless the distention is sufficient to distort the serosa (6). Sensory endings in the myenteric plexuses are intraganglionic laminar endings (IGLEs) that contact connective tissue and glial cells that encapsulate the myenteric ganglia (see below) (6). IGLEs also respond to tension but have a much lower threshold than serosal endings (6). It has been proposed that IGLEs sense shearing forces between the circular and longitudinal muscle layers (6). Sensation in the muscle layers comes from intramuscular arrays (IMAs) (6). IMAs are axons in parallel with the muscle cells in the layer acting as “in series tension receptor endings” (6). Mucosal endings respond to mechanical perturbation rather than tension (6). There is evidence that all three types of endings respond to chemical stimulation

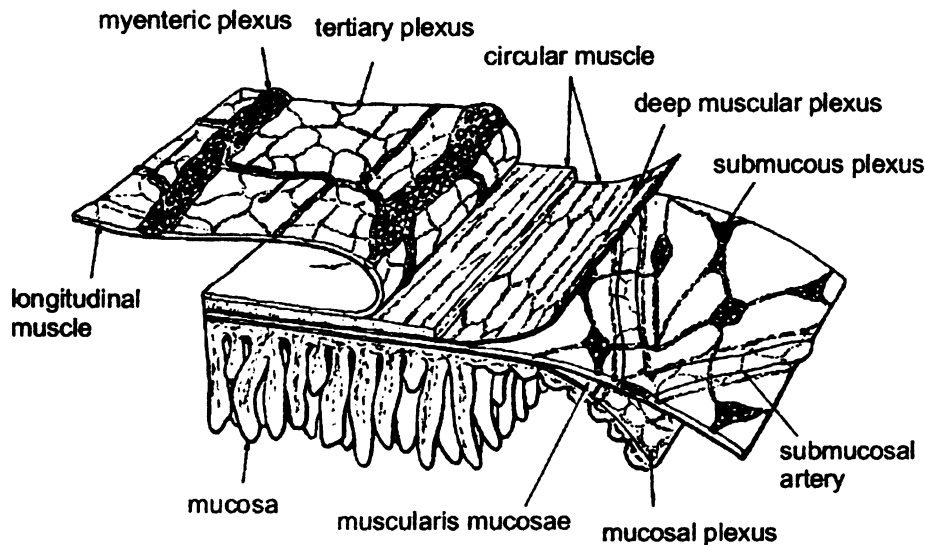
as well (6). Vagal afferent neurons primarily innervate the upper gastrointestinal tract and pelvic afferent neurons innervate the colorectal region of the gastrointestinal tract (6). Splanchnic afferents relay sensory information from the entire gastrointestinal tract (6).

### ***Sensory – neurotransmitters and function***

Afferent terminals are reactive to 5-HT, ATP, acetylcholine and histamine, as well as various gastrointestinal peptide hormones (6, 8, 107). The centrally projecting processes of gastrointestinal spinal and vagal are primarily glutamatergic (6, 8, 107). Afferent innervation conveys sensory information from the gastrointestinal tract to the central nervous system allowing for the coordination of gastrointestinal reflexes and the integration of gastrointestinal information with behavioral responses such as food intake (6). Afferent innervation also mediates sensation from the gut allowing perception of normal gastrointestinal stimuli (distension for example) and also noxious, and nociceptive stimuli (6).

### ***Intrinsic Nerves***

The intestine is composed of multiple concentric layers (**Figure 1**). The innermost layer is the mucosa; it is composed of many different cells which specialize in secretion, absorption, sensation, and immunity. The next layer is the submucosal layer where the submucosal plexus lies. The submucosal plexus is composed of ganglia and the axons of nerve cell bodies that comprise the ganglia. Submucosal neurons project to the mucosal layer and it is these



**Figure 1 – Layers of the intestine.** Adapted from Furness and Costa 1987 (54). Take special note of the myenteric and submucosal plexus that contain the cell bodies of the neurons that make up the intrinsic innervation of the GI tract.

neurons that modulate secretion and absorption. The next layer is the circular muscle layer. These muscles wrap circumferentially around the lumen of the intestine and they control the caliber of the organ. Lying on the outermost layer is the longitudinal muscle which controls the lengthening and shortening of the intestine. Sandwiched between these two muscle layers is the myenteric plexus composed of the myenteric ganglia and the axons of the neurons in those ganglia. The myenteric plexus controls motility.

Bayliss and Starling were the first to propose that the gut possessed an intrinsic circuitry of neurons that controlled gut motility. Their work on anesthetized dogs showed that pressure applied to the intestinal lumen caused a contraction oral to the point of stimuli and a relaxation anal to the point of stimulation, a phenomenon which persisted after complete vagotomy. They referred to this reflex as the “law of the intestine” which today is called the

peristaltic reflex. Work by Paul Trendelenburg at the beginning of the last century confirmed what Bayliss and Starling postulated when he was able to measure intestinal reflexes in vitro. In his 1921 book *The Autonomic Nervous System*, John N. Langley was the first to classify the autonomic nervous system in three divisions, the sympathetic, the parasympathetic, and enteric divisions (87). Langley separated the enteric division from the sympathetic and parasympathetic divisions because enteric neurons had a different embryonic origin, enteric nerves exhibited significant functional autonomy and because the efferent nervous supply of the intestine was too sparse to produce effective control of gut function (87).

### ***Submucosal Neurons***

#### ***Anatomy***

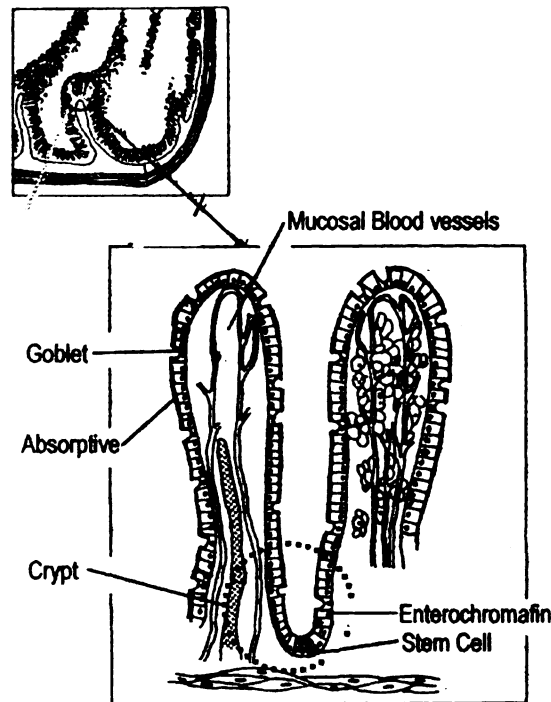
Cell bodies of two primary functional cell types are found in the submucosal ganglia, secretomotor neurons and intrinsic primary afferent neurons (IPAN) (138). IPANs project and couple to receptor cells at the intestinal mucosa such as enterochromaffin and immune cells allowing for the transduction of mechanical and chemical signals from intestinal contents to the submucosal ganglia (138). IPANs synapse onto secretomotor cells in the submucosal ganglia which in turn project to enterocytes at the mucosa to modulate and mediate secretory reflexes (138). IPANs and secretomotor neurons of submucosal ganglia share similar morphology to the IPANs and motor neurons of the myenteric ganglia discussed below in detail.

### ***Submucosal neurotransmitters and reflexes***

Mechanical distortion of the mucosal surface initiates the release of 5-HT from enterochromaffin cells activating 5-HT receptors on submucosal IPANs (138). IPANs release acetylcholine and substance P to activate secretomotor and interneurons (138). Submucosal neurons also receive input from neurons in the myenteric plexus (see below) allowing for integration of mucosal secretory and motility reflexes (138). Extrinsic sensory afferents that also project to myenteric neurons via terminals on axon collaterals release substance P initiating mucosal secretion (138). In the colon excitatory processes of IPANs project from the cell soma directly to enterocytes to mediate  $\text{Cl}^-$  secretion (39, 138). Submucosal reflexes show polarity and are oriented anally (99, 138).

### ***Secretion and absorption by the intestine***

Submucosal neurons control and coordinate secretion of enzymes and mucous, as well as the absorption of nutrients in response to stimuli by mucosal contents. The GI tract has direct secretory connections for digestive accessory organs like the pancreas, liver, and gallbladder. In addition the intestinal mucosa secretes enzymes, mucous, and hormones to aid in digestion, protect the mucosa protection, and regulate GI motility. Absorption of nutrients also occurs here. The intestinal mucosa is composed of villi, which increase the secretive and absorptive surface area of the intestine (**Figure 2**) (118). The upper third of each villus consists mainly of absorptive cells whose apexes have further projections called microvilli comprising the brushborder where most nutrient



**Figure 2 – Mucosal cells of the intestinal tract.** Adapted from Saunders 2004 (118). The secretory and absorptive activity of the cells here are under the direct control of the submucosal plexus.

absorption takes place. It also here that much of the intestinal secretion occurs, enzymes are secreted to complete breakdown of macromolecules to basic nutrients (77). At the base of the villi are the intestinal crypts where stem-cells proliferate and differentiate into new absorptive cells, goblet cells, endocrine cells and other types of cells (118). The intestinal mucosa also has goblet cells which secrete mucous for lubrication (118). Chemosensory and mechanosensory signal transduction from the mucosal content to the myenteric layer has been demonstrated, though the receptors are yet to be found. One of the proposed intermediates that may be acting here are the enterochromaffin cells through secretion of 5-hydroxytryptamine (5-HT), in a paracrine fashion (55). In the colon secretion and absorption of water is regulated with the secretion of water being

coupled to overall  $\text{Cl}^-$  secretion through a variety of specialized  $\text{Cl}^-$  channels on the luminal apex of colonic enterocytes (86).

### ***Myenteric Plexus***

The myenteric plexus is composed of the myenteric ganglia, the interganglionic nerve strands that connect them and secondary nerve strands that run away from the ganglia splitting into tertiary connectives that branch along the muscle layers (77). The ganglia themselves are evenly spaced around the circumference of the gut. Whereas in the rest of the peripheral nervous system, nerves are suspended and stabilized on networks of collagen webbing, myenteric ganglia are sheathed within glial cells that are similar to astrocytes in the CNS (77). The number of neurons within each ganglion varies greatly between mammal species. Guinea pig myenteric ganglia typically contain about 100 neurons while the mouse has closer to 40 (57). The cell bodies within these ganglia consist of a variety of types depending on the classification criteria. These neurons can be classified by: morphology, protein expression, electrophysiology, and function.

### ***Myenteric electrophysiology***

Intracellular recordings of membrane potential were used to characterize the action potentials from two types of myenteric neuron in the guinea pig intestine (71, 102). In the first type of cell, the action potential had a fast rising phase mediated entirely by  $\text{Na}^+$  and a fast falling phase caused by  $\text{K}^+$  efflux that occurs with a single decay constant (71, 102). These were called S-neurons for

“synaptic”, because of the prominent fast excitatory postsynaptic potentials (fEPSP) recorded from these neurons (77). The fEPSP is nerve mediated as it is blocked by the  $\text{Na}^+$  channel antagonist tetrodotoxin (TTX) (71, 102). All fEPSPs are at least partly mediated by acetylcholine because antagonists of nicotinic cholinergic receptors inhibit the fEPSP (61, 71, 102). Some fEPSPs show mixed pharmacology being partly mediated by ATP and 5-HT (91).

A second type of enteric neuron are AH neurons as the action potential is followed by a pronounced “after hyperpolarization” (71). The action potential in the AH neuron has hump on the falling phase that is caused by the opening of N-type voltage-operated  $\text{Ca}^{2+}$  channels (114). A train of high frequency stimulation of presynaptic nerve fibers causes a slow excitatory post synaptic potential (sEPSP) that is seconds to onset and can last up to a few minutes (10). The sEPSP is mediated by substance P and neurokinin A (9). These neurokinins bind to NK-3 receptors on the AH neurons resulting in a decreased  $\text{gK}^+$  and  $\text{gK}^+_{\text{Ca}}$  and an increased  $\text{gCl}^-$  (10). NK-3 receptors couple to a pertussis toxin insensitive G-protein that stimulates the activation of protein kinase C and adenylate cyclase (10). This results in activation of protein kinases that inhibit  $\text{gK}^+$ , or  $\text{gK}^+_{\text{Ca}}$ , alters  $\text{Ca}^{2+}$  buffering mechanisms, activates  $\text{gCl}^-$ , or any combination of these (10).

### ***Myenteric morphology***

Neurons in the myenteric plexus fall into two broad morphological types, termed Dogiel Type I and Dogiel Type II.

The primary feature of Type I neurons is their “club like” or “paddle like” projections from the cell soma (18, 54, 77). These dendrites extend in the plane of the myenteric ganglia (77). Type I neurons also have a single long axon (18). Neurons of this type project both orally and anally, remaining within the ganglia as well as projecting to other ganglia (77). The axons of some Type I neurons project to the muscle layers of the gut (77). Myenteric neurons that show S-type electrophysiology also have Dogiel type I morphology (18, 54, 71, 77, 102).

Dogiel Type II neurons have a smooth contoured outline (18). Type II neurons also have multiple long smooth axons (18). The axons of Type II neurons project to the mucosa, and sometimes to the submucosa, as well as projecting anally (18). Type II neurons synapse onto other Type II neurons (18). Type II have AH-type electrophysiology (18, 54, 71, 77, 102).

### ***Myenteric neurochemistry***

In guinea pig, calbindin positive neurons make up a quarter (24.6%) of all neurons in the myenteric ganglia (41). Calbindin is a  $\text{Ca}^{2+}$  binding protein involved in the regulation of  $\text{Ca}^{2+}$  concentration that is found in a subset of myenteric neurons that project to the intestinal mucosa (56, 126). In further studies correlating electrophysiology, and morphology with this chemical marker, it was found that calbindin exclusively labels neurons with Type II morphology and AH type electrophysiology (30, 34, 41, 52, 73, 75, 82, 83, 100, 105, 142). Between 84 and 87% of all neurons of this type are positive for calbindin (41, 53). This makes calbindin an effective marker for Dogiel Type II, AH-type neurons.

The  $\text{Ca}^{2+}$  binding protein calretinin labels 24% of neurons in the myenteric plexus.(41, 53) Furthermore, calretinin and calbindin staining is mutually exclusive.(41, 53) By this it can be deduced that calretinin positive cells are of the Dogiel Type I, S-type classification. A great majority of these neurons would go unlabeled because calretinin only labels a subset, approximately 50% of S-type neurons.(41, 53) When other peptides, chemicals, and proteins are explored however it becomes possible to distinguish subpopulations of S-neurons based on chemical “coding” of 2 or more markers that correspond closely not only to Dogiel Type I/S-type morphology and electrophysiology but also to neuron function (see below).

### ***Myenteric motor neurons***

Myenteric motor neurons innervate enteric smooth muscle. In the myenteric plexus four different subtypes of motor neuron have been identified (53). All myenteric motor neurons are S-type/Dogiel Type I neurons (53). The four subtypes are longitudinal muscle excitatory, longitudinal muscle inhibitory, circular muscle excitatory, and circular muscle inhibitory (53). Both populations of excitatory neurons primarily secrete acetylcholine onto muscarinic receptors on the smooth muscle and are immunoreactive for choline acetyl transferase (ChAT), the enzyme that synthesizes acetylcholine (53). In addition to ChAT, motor neurons projecting to the longitudinal muscle also express calretinin (53). Both classes of inhibitory neuron express nitric oxide synthase (NOS), the synthetic enzyme of nitric oxide, which is the primary inhibitory neurotransmitter

(53). Inhibitory neurons projecting to the circular muscle express the opioid enkephalin and the inhibitory transmitter vasoactive intestinal peptide (VIP) (53).

### ***Myenteric interneurons***

Four subtypes of interneurons have been described in the myenteric plexus, three projecting anally (descending) and one that projects orally (ascending).(53) The ascending interneurons are cholinergic and form a chain that projects up the length of the gut.(53) The descending interneurons are chemically coded as ChAT/NOS/VIP (with a variety of peptide transmitters in with variable prominence), ChAT/Somatostatin, and ChAT/5-HT.(53) Electrophysiological studies have shown the ChAT/NOS/VIP interneurons participate in local motility reflexes while the ChAT/SOM interneurons work in long distance motility reflexes.(53) The ChAT/5-HT interneurons appear to act in secretomotor responses and not motility directly.(53) All myenteric interneurons are S-type/Dogiel Type I class neurons.(53)

### ***Myenteric sensory***

Studies showed however that intestinal motor reflexes remain intact when the extrinsic nerve supply to the intestines had been cut and after allowing their axons to degenerate.(55) Subsequent electrophysiological studies have shown that some neurons in the myenteric plexus react to direct chemical and mechanical stimuli of the mucosa.(11, 53, 55, 85) These first neurons in reflex responses were termed intrinsic primary afferent neurons (IPANs) of the enteric nervous system.(11, 53, 55, 85) IPANs are all AH-type/Dogiel type II neurons.

## ***Neural control of colonic motility***

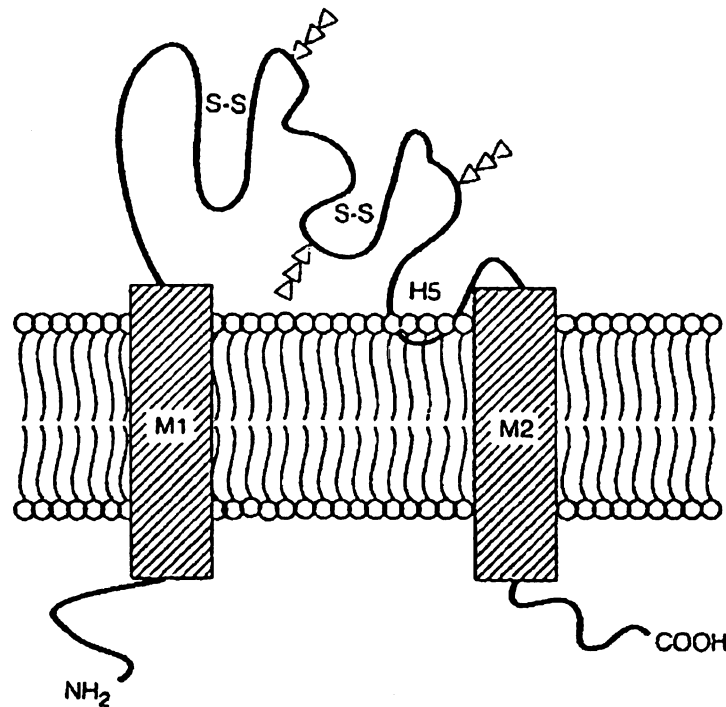
### ***Purinergic***

In a 1963 publication, Burnstock et al made the first reports on neurogenic inhibition after measuring inhibitory potentials elicited by stretch and nerve stimulation in guinea pig taenia coli (24). They later showed that inhibitory neurotransmission in the colon was intrinsic to the enteric nervous system (ENS) and after blocking cholinergic and adrenergic neurotransmission they concluded that the intrinsic inhibition was non-cholinergic and non-adrenergic (24, 25). Utilizing the Eccles criteria for classifying a substance as a neurotransmitter, Burnstock et al showed that: 1) ATP and its biosynthetic machinery were present in the inhibitory neurons 2) ATP was released when nerves were activated 3) ATP and the unknown inhibitory substance had the same effect on smooth muscle 4) ATP degrading enzymes were found in the same cells that released it and 5) Drugs that altered neurogenic inhibition also altered the effect of exogenous ATP (26, 45). Based on these observations Burnstock et al concluded that ATP was an inhibitory neurotransmitter in the ENS.

### ***ATP Receptors***

Multiple purinergic receptors were first proposed to exist by Burnstock after noting the different rank order potency of ATP and its break down products ADP, AMP, and adenosine, and structural analogues and methyl-xanthene antagonism in several different bioassays (27, 110, 133). On this basis purinergic receptors were first divided into those being most potently activated by

adenosine or by ATP (27, 110, 133). Those receptors that were most potently activated by adenosine and antagonized by methyl-xanthenes were termed P1 receptors while those activated most potently by ATP were called P2 receptors (27, 110, 133). P2 receptors were further subdivided when it was found that some P2 receptors were ionotropic channels while others were G-protein coupled metabotropic receptors (5, 44). The ionotropic P2 receptors were termed P2X receptors while the metabotropic receptors were named P2Y receptors (1).



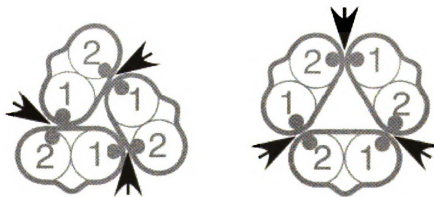
**Figure 3 – P2X receptor subunit structure.** Adapted from Ralevic and Burnstock 1998 (110).

### ***P2X receptors***

P2X receptors are a non-specific cation channels (17). P2X receptors are trimers comprised of one or more of seven separate subunits(3, 76, 103, 135).

P2X receptor subunits consist of cytosolic N and C termini with 2 hydrophobic membrane spanning regions (M1 and M2) (Figure 3) (3). Between M1 and M2 lies a large extracellular loop which contains the ATP binding site (3, 76, 135).

Amino acids in both the M1 and M2 determine cation permeability (135). The membrane spanning M2 region dictates the assembly of the functional receptor (76). Cross linking studies using P2X receptors heterologously expressed in xenopus oocytes were the first to show that the trimer was the functional configuration of the P2X receptor (101). A later study using heterologous expression of mutated receptors expressed in human embryonic kidney cells showed that P2X receptor subunits assembled in a “head to tail” type configuration joining the M1 region of one subunit to the M2 region of the next (76) (Figure 4).



**Figure 4 – Functional P2X receptor quaternary structure.** Adapted from Jiang et al 2003 (76). This illustrates how the subunits of assembled trimer would connect and also shows how ATP binding would alter the tertiary structure of the subunits allowing the receptor to go from a non-conducting to a conducting state. Arrows indicate areas where disulfide bonds form between amino acids containing sulfate groups.

### ***P2X receptor subtypes***

Seven P2X receptor subunits and a number of splice variants have been identified and cloned (P2X1-7) (103). P2X receptors are found most physiological systems with receptors comprised of the seven subunits localizing to specific tissues. P2X<sub>1</sub> receptors are found non-neuronally in vas deferens, urinary bladder, lung, spleen, as well as arteries and arterioles (33, 110, 136, 137). In neuronal tissue, P2X<sub>1</sub> receptors are localized to dorsal root, trigeminal, and celiac ganglia; spinal cord and brain (38, 110, 136). P2X<sub>4</sub> receptors are found in dorsal root ganglia, spinal cord, sensory ganglia, superior cervical ganglion, lung, bronchial epithelium, thymus, bladder, adrenal gland, testis, vas deferens and brain, as well as being the only P2X receptor found in acinar cells of the salivary gland (14, 21, 63, 110, 127, 128). With the exception of the mesencephalic nucleus of the trigeminal nerve, P2X<sub>5</sub> receptors are found on neuronal tissue outside the CNS including the spinal cord, trigeminal and dorsal root ganglia (38, 110). P2X<sub>6</sub> receptors are expressed heavily in the CNS, especially in Purkinje cells and the ependyma as well as peripherally in celiac, trigeminal, and dorsal root ganglia and in endocrine cells of the uterus, ovary, and bronchial epithelium (38, 110). P2X<sub>7</sub> receptors are distributed primarily in cells of hemopoietic origin including bone marrow, mast cells, granulocytes, lymphocytes, and macrophages (37, 103, 110). More recent studies have also found P2X<sub>7</sub> receptors in the brain and peripheral nervous tissue (84, 96, 124). In regards to the present study (see *use of transgenic mice* and *rationale* below) P2X<sub>2</sub> and P2X<sub>3</sub> receptors, in addition to other tissues, have been localized to the

enteric nervous system (12, 62, 108, 110, 112). P2X<sub>2</sub> receptors are also found in bladder, brain, spinal cord, superior cervical ganglia, adrenal medulla, vas deferens, and pituitary gland (110). Early studies using *in situ* hybridization had concluded that P2X<sub>3</sub> receptors were found only in sensory ganglia mediating nociceptive transmission (14, 64, 93, 101, 110). More recent studies using immunohistochemistry and pharmacological profiling have shown both anatomically and functionally that P2X<sub>3</sub> receptors are also prevalent in the enteric nervous system (12, 42, 108, 112).

P2X receptors assemble as heteromeric or homomeric functional channels, whose subunit composition determines the pharmacological and functional properties of the receptor (3, 76, 103, 135). All P2X receptors are permeable to Ca<sup>2+</sup>, with the P2X<sub>2</sub> receptor being considered most Ca<sup>2+</sup> permeable (110). P2X<sub>1</sub> and P2X<sub>3</sub> rapidly desensitize to endogenous ATP exposure as well exogenous agonist application (110).

### ***Pharmacology of P2X receptors***

Drugs that activate P2X receptors are ATP, ADP, and various ATP analogues (103, 110). Stable ATP analogues like  $\alpha\beta$ -methylene ATP ( $\alpha\beta$ MeATP) and  $\beta\gamma$ -methylene ATP ( $\beta\gamma$ MeATP) are selective for the P2X<sub>1</sub> and P2X<sub>3</sub> receptors, showing less potent activation of other receptor subtypes (110). ATP and some of its analogues are degraded by endonucleotidases and the stability of an analogue in the presence of these enzymes is a key factor in determining an agonist potency (110). 2',3'-O-(4-benzoylbenzoyl)ATP (BzATP) is 300% more potent than ATP at P2X<sub>7</sub> receptors, while it elicits only a fraction of

ATP-induced effects in other receptor subtypes (103, 110). The trypanoside, suramin, is an antagonist at all P2X receptors except those containing P2X<sub>4</sub> and P2X<sub>6</sub> subunits (103, 110). NF023 is a more potent antagonist at P2X<sub>1</sub> and P2X<sub>2</sub> receptors (103, 110). Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) was originally thought to be a P2X selective antagonist, but is now known that it blocks P2Y<sub>1</sub> receptors (103, 110). 2',3'-O-(2',4',6')-trinitrophenyl-ATP (TNPATP) is most potent at P2X<sub>1</sub> and P2X<sub>3</sub> receptors, but at high enough concentrations it will block all P2X subunit containing receptors (103, 110).

While constructing agonist-antagonist rank order potency profiles is a useful tool in identifying P2X receptors in heterologous expression systems, and tissues that express a single P2X receptor subunit, these conditions are not met in many tissues, including the gut (30, 66, 81, 108, 113, 139). Also confounding this issue is the fact that functional P2X receptors can be heteromeric consisting of 2 different subtypes. Further more, all the drugs discussed here will also have some action at P2Y receptors (see below). Pharmacological identification of P2X receptors in tissues is problematic as there are few drugs that can reliably discriminate among the P2X receptor subtypes.

### ***Use of transgenic mice to study the function of P2 receptors in the gastrointestinal tract***

Transgenic technology allows genes encoding specific proteins to be removed or added to the genome of a mouse. This is especially valuable in systems where pharmacological tools are poor. When used to delete the gene encoding a receptor, comparisons of responses obtained in wild type (WT) and

transgenic mice can be utilized as though those responses were obtained in the presence (WT) and absence (KO) of highly specific antagonists. This study used two types of transgenic mice. One type of mouse has had the P2X<sub>2</sub> receptor subunit deleted from its genome while the other type of mouse had the P2X<sub>3</sub> receptor subunit deleted (12, 35, 36, 112).

Previous studies using these animals have shown that deletion of the P2X<sub>3</sub> receptor subunit causes impaired distention-induced peristalsis in the ileum (12). AH neurons in P2X<sub>3</sub> knockout mice (P2X<sub>3</sub><sup>-/-</sup>) did not respond to  $\alpha\beta$ MeATP induced excitation, an effect that is present in WT controls (12). P2X<sub>2</sub> subunit deletion eliminates the purinergic component of fast excitatory post synaptic potentials recorded from S neurons in the mouse ileum as well as abolishes the ATP sensitivity of these neurons (112). Distention-evoked peristalsis in the ileum is also impaired in the small intestine of P2X<sub>2</sub> knockout mice (P2X<sub>2</sub><sup>-/-</sup>) as compared to WT controls (112).

### ***P2Y receptors***

P2Y receptors are metabotropic G-protein coupled receptors (66). Cloned P2Y receptors include the P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub> and P2Y<sub>11</sub> (110). P2Y receptors can couple to excitatory mechanisms, in the case of the neuronal P2Y<sub>1</sub> receptor mediating the release of nitric oxide, an inhibitor of smooth muscle tone, in the mouse colon (66). P2Y receptors can also couple to inhibitory mechanisms, for example the P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors on colonic longitudinal muscle mediate relaxation (66). Most P2Y receptors couple to phospholipase C (PLC), increasing intracellular IP<sub>3</sub>, mobilizing intracellular Ca<sup>2+</sup>, however P2Y

coupling to the inhibition of adenylate cyclase has also been described rat brain capillary endothelium (110). P2Y receptors are more sensitive to ADP and ADP analogues like ADP $\beta$ S, ADP $\beta$ F, and 3'-deoxyATP $\alpha$ S (110).  $\alpha\beta$ MeATP has no activity at P2Y receptors. Other than  $\alpha\beta$ MeATP, drugs that activate P2X receptors will have some effect on P2Y receptors, making differentiation of their action difficult in vitro (66, 110). Antagonists of terminal effectors of P2Y activation such as apamin, which blocks the small conductance Ca<sup>2+</sup> gated K<sup>+</sup> channel, are useful but this also blocks the actions of other G-protein coupled receptors that may also converge on those ion channels.

### ***Cholinergic***

Acetylcholine is an important neurotransmitter in the ENS where it contributes to neural mechanisms controlling all reflex pathways. Acetylcholine activates two main classes of receptor. Muscarinic receptors are G-protein coupled metabotropic receptors mediating slow excitatory or inhibitory responses. Nicotinic receptors are acetylcholine gated ionotropic channels mediating rapid excitatory synaptic responses.

### ***Muscarinic***

In the gut, muscarinic receptors are found on the cell body and nerve terminals of enteric neurons (68). Muscarinic receptors are also on gastrointestinal smooth muscle and mucosal enterocytes (68). M1 muscarinic receptors on enteric nerve cell bodies mediate excitation while M2 receptors on nerve endings are inhibitory autoreceptors (68, 95). M2 muscarinic receptors on

smooth muscle and secretory enterocytes are excitatory (68). M3 receptors mediate colonic contraction in multiple species (80, 119, 140). Antagonists such as atropine and scopolamine partly inhibit colonic migrating motor complexes (MMCs – see below) and these drugs abolish components of MMCs that are resistant to non-cholinergic antagonists (20, 29). The presences of muscarinic receptors have also been demonstrated electrophysiologically on colonic smooth muscle cells, suggesting acetylcholine mediates excitatory input to colonic smooth muscle (97).

### ***Nicotinic***

Nicotinic receptors have never been shown on intestinal smooth muscle and appear to mediate only neuronal communication. Nicotinic receptors are pentameric and comprised of combinations of  $\alpha$  and  $\beta$  subunits (62). There are eight  $\alpha$  ( $\alpha 2$ - $\alpha 9$ ) and three  $\beta$  ( $\beta 2$ - $\beta 4$ ) subunits, and the combination of subunits determines the permeability, kinetics, and pharmacology of receptors (62). Immunohistochemical, electrophysiological, and functional studies in the gastrointestinal tract have identified  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha 7$ , and  $\beta 2$  in the myenteric and submucosal ganglia (58, 59, 62, 90, 121). Nicotinic receptors have been demonstrated on interneurons mediating inhibitory transmission in rat colon (13). Antagonists of nicotinic receptors abolish MMCs in the mouse colon (51). Hexamethonium abolishes neurogenic phasic contractions in mouse colonic longitudinal smooth muscle (109). Nicotinic receptor antagonism also blocks excitatory and inhibitory junction potentials elicited by mechanical stimulation in guinea pig colon (130). In this study, nicotinic receptor agonists were used to

activate neurogenic responses to assess P2X receptor mediated responses that may be activated by nicotinic receptors on enteric neurons.

## **Colonic Motility**

### ***MMC***

Colonic MMC's require the action of neurally released acetylcholine, serotonin, nitric oxide, ATP, tachykinins, and other neuropeptides(20). The colonic MMC is a rhythmic motility pattern characterized by long duration contractions that move down the colon (51). It is thought that the MMC provides contractions required to mix and knead the solid and semi-solid contents of the colon (51). The MMC is a neurally dependent motility pattern that has been studied extensively in recent literature (20, 28, 29, 51). The present work utilizes P2X<sub>2</sub> and P2X<sub>3</sub> gene-deleted mice to probe the specific role ATP mediated excitation plays in mediating this in vitro reflex.

### ***Defecation***

Voluntary defecation is mediated by skeletal muscle under control of somatosensory nerves. However voluntary defecation can be overridden by autonomic mechanisms activated pathologically or pharmacologically resulting in increased secretion and motility and consequently feces excretion (4, 16, 117). Increases in gastrointestinal 5-HT levels caused by 5-hydroxytryptophan (5-HTP) treatment of rats or mice induces diarrhea (4, 117). This response is mediated by activation of 5-HT<sub>4</sub> receptors, to cause an increase propulsive motility and colonic secretion (4). This conclusion is based on the observation that 5-HT<sub>4</sub>

receptor antagonists block 5-HTP-induced defecation (4, 117). However, 5-HT<sub>4</sub> receptor antagonists do not affect normal defecation and they do not induce constipation (4, 117). This suggests that the 5-HT<sub>4</sub> receptors in animal models contribute only to pathophysiological changes leading to diarrhea (4, 117). Studies also indicate that the 5-HT<sub>1</sub>, 5-HT<sub>2</sub>, and 5-HT<sub>3</sub> receptors may contribute to diarrheal states (4). While the role of 5-HT receptors in initiating this behavior is well characterized, the neural circuits mediating this response have not been identified. In the present study the potential role of P2X<sub>2</sub> and P2X<sub>3</sub> in mediating the MMC will be probed using transgenic mice. Treatment with 5-HTP induces defecation in conscious fed mice (16, 70, 116, 117). 5-HTP is a precursor to the 5-HT. In the stomach 5-HT treatment *in vivo* produces gastric spasm by action directly on gastric smooth muscle, while 5-HTP treatment increases cholinergic function and gastric motility (116). Atropine, a muscarinic cholinergic antagonist prevented prolonged increases of intestinal contractility induced by i.v. injection of 5-HTP while having no effect on the short lived contractility increases seen with 5-HT treatment (69). 5-HT treatment acts to directly cause contraction of enteric smooth muscle. However, because 5-HTP must be taken up by neurons or enterochromaffin cells and converted to 5-HT before release, 5-HTP mediates a neurogenic sensitization of colonic reflexes, resulting in increased motility as well as chloride and mucous secretion (15, 69, 70, 79, 98, 116) Treatment with equivalent volumes of vehicle (0.9% saline) has been shown not to increase fecal output as compared to untreated controls (4).

### ***Longitudinal Muscle***

The longitudinal muscle is innervated by excitatory and inhibitory myenteric neurons (106). Longitudinal muscle contractions are elicited by multiple mechanisms. For example, functional studies have shown that 5-HT mediates contractions in guinea pig longitudinal muscle via an action at 5-HT<sub>2A</sub>, 5-HT<sub>3</sub>, and 5-HT<sub>4</sub> receptors (19, 48, 109). Longitudinal muscle in the guinea pig shows periodic phasic contractions that are scopolamine-resistant but blocked by the nicotinic receptor antagonist hexamethonium and by the sodium channel blocker tetrodotoxin (TTX) (109). This shows that these contractions are neurogenic and the neuronal communication is cholinergic, leading to the release of a non-cholinergic transmitter to mediating the contraction. Phasic contractions of colonic longitudinal muscle are abolished by the 5-HT<sub>3</sub> antagonist ondansetron showing that serotonin mediates excitation in longitudinal muscle reflex pathways (109). ATP and adenosine relax pre-contracted colon longitudinal muscle from mouse (66). While ATP can relax pre-contracted tissue, ATP causes a concentration-dependent contraction of resting colon longitudinal muscle (66). Mechanical stimulation of the mucosa of the colon initiates a coordinated contraction both orally and anally that is blocked by TTX, partially abolished by hexamethonium (132). The hexamethonium resistant component is blocked by the P<sub>2</sub> receptor antagonist PPADS (132). All these mechanisms of longitudinal muscle contraction and relaxation are complex and are transduced through multiple neurons and neurotransmitters; in the present study P<sub>2</sub>X<sub>2</sub> and P<sub>2</sub>X<sub>3</sub> knockout mice will be used to explore mechanisms of longitudinal muscle

contractions to assess what role purinergic excitation plays in neurogenic contractions of mouse colonic longitudinal muscle.

### ***Circular Muscle***

The majority of what is known about colonic circular smooth muscle contractions in mouse has been gathered by studying of colonic MMCs and the ability of drugs to potentiate or inhibit MMC frequency and amplitude (20, 28, 29, 47, 51, 67, 109). Alosetron and ondansetron, 5-HT<sub>3</sub> antagonists, inhibit both frequency and amplitude of MMCs (28). Hexamethonium and TTX block MMCs, with TTX increasing the resting the tone of the tissue suggesting there is a tonic, neurally mediated inhibitory tone in the mouse colon (51). Nitric oxide (NO) plays a role in the MMC as nitro L-arginine increased resting tone and increased the frequency of MMCs by 80% (51). MMC's are mediated by both acetylcholine and substance P as they are blocked by neurokinin-1 (NK-1) and NK-2 antagonists and atropine (20). ATP also plays a role in the MMC as the P<sub>2</sub> receptor antagonist suramin increases the amplitude of MMCs (20). Electrophysiological studies of circular muscle during stretch activated reflexes show that excitatory junction potentials (EJPs) and inhibitory junction potentials (IJPs) persisted in the presence of the P<sub>2</sub> receptor antagonist PPADS.

### **Rationale**

Purinergic receptors are potential therapeutic targets for treatments of heart attack, stroke, cancer, erectile dysfunction, bladder hyperactivity, and intestinal motility disorders (23). Gastrointestinal motility disorders, including

inflammatory bowel disease and irritable bowel syndrome require greater understanding of neuronal mechanisms controlling motility including a more complete understanding of the role of ATP signaling in controlling motility.

The goal of this study is to further the understanding of the contribution of purinergic mechanisms in controlling colonic motility. To this end pharmacological manipulations will be used to identify mechanisms that mediate neurogenic and non-neurogenic smooth muscle contractions and relaxations. With regards to purinergic signaling, pharmacological tools available for studies of P2 receptors are inadequate for identification of specific purinergic receptor subtypes. Transgenic P2X<sub>2</sub> and P2X<sub>3</sub> receptor knockout mice will be utilized as would a highly specific antagonist in normal animals to probe the roles these receptors may play in excitatory and inhibitory signaling in the mouse colon.

Cholinergic excitation in the colon via muscarinic receptors is well documented in studies of spontaneous colonic contractions (20, 29, 129). However the source of endogenously released acetylcholine isn't known. Acetylcholine is potentially released from excitatory motor neurons in the myenteric plexus, or from parasympathetic nerve endings. Inhibitors of nerve activity such as TTX increase the resting tone between MMC contractions, suggesting an overall nerve-mediated inhibitory tone in the colon. However, how these nerves are activated, and what mediators they release to cause relaxation are unknown. ATP has an excitatory effect on mouse colonic longitudinal muscle *in vitro* (66). Receptors mediating that contraction and how those receptors couple to contractions need to be identified (66). Purinergic signaling contributes

to the MMC as P2 antagonists block the non-cholinergic excitation that contributes to the MMC, but the specific receptors mediating P2 contribution to the MMC are unidentified (20). 5-HTP induced fecal output is a complex assay of colonic motility *in vivo*. In the present study this assay will be used to probe the contribution of P2X receptors in controlling colonic motility *in vivo*.

## **Specific Aims**

### **Specific Aim 1 – Characterize the excitatory and inhibitory motor input to smooth muscle in the mouse colon**

#### ***Specific Aim 1a – Inhibitory input***

The longitudinal muscle shortens the colon during propulsion of colonic contents. Nicotinic acetylcholine receptor antagonists block neurogenic phasic contractions in the colon (66). Myenteric motor neurons projecting to the longitudinal muscle have nicotinic receptors and blocking these receptors prevents inhibitory signals to the muscle (104, 106, 131). ATP relaxes pre-contracted colon longitudinal muscle (66). However, a model describing integrated excitatory and inhibitory motor input has yet to be developed. The aim of these studies is to identify the neurogenic inhibitory motor mechanisms in the colon.

#### ***Specific Aim 1b – Excitatory input***

ATP contracts mouse colonic longitudinal muscle but the sites and mechanisms of action are unknown (66). There is overall nerve mediated relaxation observed in the colon. Neurotransmitters mediating that relaxation may also mediate excitatory communication. The aim of these experiments will be to characterize the neurogenic excitatory motor input to the colonic smooth muscle under conditions when inhibitory input has been blocked.

### **Specific Aim 2 – Effects of P2X receptor deletion on colonic motility in vitro**

Mouse colonic MMCs provide a useful model for studies of the neural circuitry controlling propulsive motility (20, 28, 29, 51, 109). Nitric oxide synthase inhibitors and inhibitors of P2 receptor signaling potentiate MMCs while muscarinic antagonists inhibit but do not abolish MMCs in the colon (20, 29, 51, 109). The aim of these studies will be to assess the role that P2X receptors play in the MMC.

### **Specific Aim 3 – Characterize the contribution of P2X receptors to 5-HTP stimulated fecal output**

5-HTP, a precursor of 5-HT, causes diarrhea in mice (4, 16, 117). The stimulation of colonic motility and secretion occurs through actions of 5-HT on 5-HT receptors (4, 117). The role other neurotransmitters may play in 5-HTP-induced diarrhea is unknown. These studies will probe the role excitatory purinergic signaling may play in this reflex by using transgenic mice that lack P2X<sub>2</sub> and P2X<sub>3</sub> receptor subunits.

## **Methods**

### **Animal model**

P2X<sub>2</sub><sup>-/-</sup> and P2X<sub>3</sub><sup>-/-</sup> were derived as previously described (35, 36). All mice in this study have the genetic background 129Ola x C57BL/6 (Harlan), and were derived from homozygous F2 crosses maintained at Roche Palo Alto (Roche Pharmaceuticals inc. Palo Alto CA., USA.). Genotype confirmation of all animals was carried out by Southern blot and real time polymerase chain reaction (RTPCR) analysis as previously described (12, 36, 112).

### **Statistics**

Data are expressed as means ± SEM. *n* refers to number of animals from which tissue was obtained. Statistical analysis was performed using Student's *t* test for unpaired data when comparing 2 groups or a 2-way analysis of variance when simultaneously comparing species, drug, and time or concentration. A P value less than 0.05 was considered significant. When 2-way ANOVA indicated a significant difference, the Bonferroni correction was used as a *post hoc* test to identify significant differences. All data was plotted and analyzed using Prism 4.0 (Graphpad Software, San Diego, CA, USA).

### **Specific Aim 1**

#### ***Isometric contractions of colonic smooth muscle***

For longitudinal smooth muscle preparations, 2 cm segments of distal colon were suspended in the longitudinal plane between stationary hooks and

isometric force transducers (Grass Instruments FT03C, Grass-Telefactor Co. West Warwick, RI. USA or Radnoti 159901A, Radnoti Glass Technology, Inc., Monrovia, CA. USA) in 20 ml jacketed baths containing 37°C Krebs' solution. Tissues were placed under 1g initial tension and allowed 30 minutes equilibration time before experiments. Mechanical activity of the colonic segments was recorded using either a Grass Chart Recorder (Model 7D, Grass-Telefactor Co. West Warwick, RI. USA) or an iWorx digital acquisition system (iWorx188 – iWorx inc. Dover, NH., USA) and a personal computer.

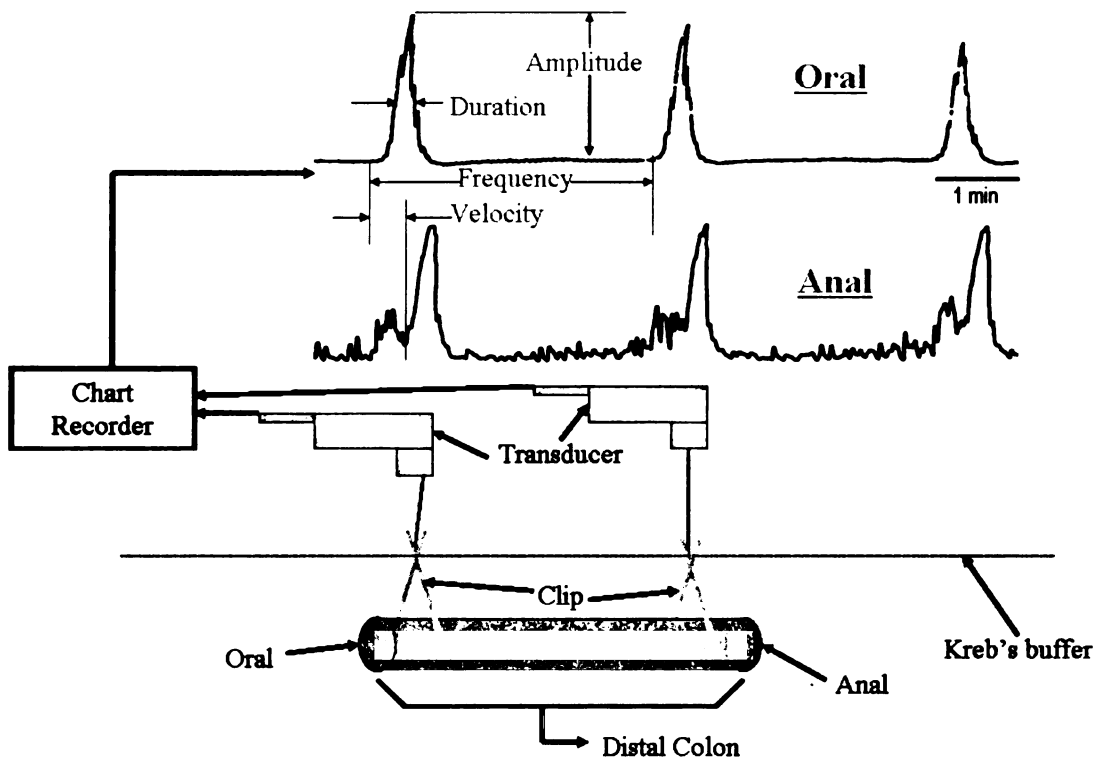
Drugs were added to the baths in volumes of 2-20µl. Agonist concentration-response curves were constructed in a non-cumulative manner with 15 minute intervals between agonist doses. Two preparations were obtained from each animal. One served as a control agonist response while the other was treated continuously with various drugs.

## **Specific Aim 2**

### ***Migrating motor complex characterization***

Methods for assay of colonic MMCs were adapted from those previously demonstrated in the literature (20, 28, 29, 51) (**Figure 5**). The colon was removed from the caecum to the rectum, fecal contents were gently flushed away with Krebs' solution and the mesentery dissected away. An 18 gauge stainless steel rod was inserted into the lumen and then secured at the bottom of a lexan chamber filled with Krebs' solution maintained at 37°C. A microserfine clip was placed at the end of the proximal colon, 5mm anal to the end of the haustra. A second clip was placed 2cm distal to the first. The clips were connected to

isometric force transducers (FT03C; Grass Instruments) with surgical silk. The tissue was allowed 60 minutes to equilibrate. The MMC frequency, oral amplitude, anal amplitude, oral duration, anal duration, and velocity of transmission from oral to anal clip were measured (28).



**Figure 5 – Migrating motor complex measurement.** This apparatus allows for the measure of colonic MMCs and the quantification of MMC amplitude, frequency, duration and velocity.

### Specific Aim 3

#### *Fecal output assay*

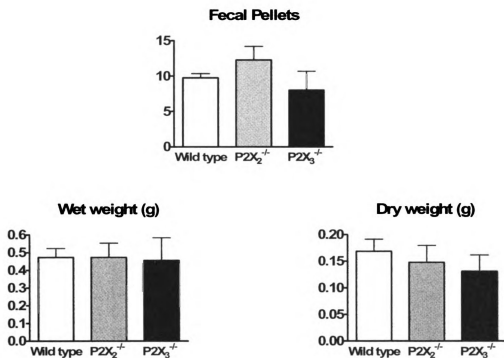
In the present study  $P2X_2^{-/-}$  and  $P2X_3^{-/-}$  and WT control mice were injected with 5-hydroxytryptophan (10 mg/kg) i.p. to induce defecation (16). Fecal output was assayed by counting the number of fecal pellets produced in the 60

minutes post injection. The fecal wet and dry weight over that time was also obtained.

## Results

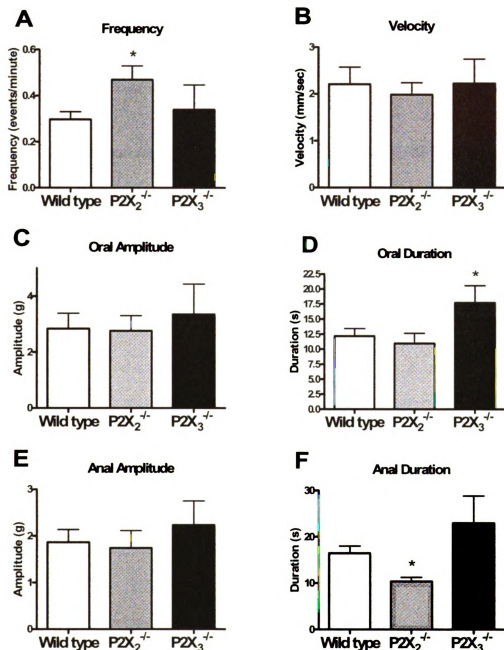
### Fecal output assay

5-hydroxytryptophan was used to induce defecation in WT,  $P2X_2^{-/-}$  and  $P2X_3^{-/-}$  mice. The literature shows that saline injection over the same time course does not induce defecation, with average fecal pellet output being less than 1 (4). Fecal output was assessed as both number of fecal pellets expelled and total fecal mass over 1 hour. Neither  $P2X_2$  nor  $P2X_3$  gene deletion altered the fecal output in terms of fecal pellets expelled or the weight of the fecal mass (**Figure 6**).



**Figure 6 – Fecal output assay.** 5-hydroxytryptophan was used to induce defecation. Fecal output was unaffected by P2X receptor subunit deletion as compared to wild type controls.

## Migrating motor complexes

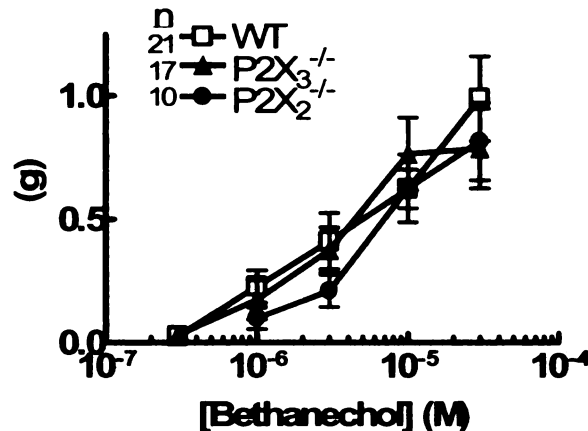


**Figure 7 – Colonic migrating motor complexes.** A colonic reflex is measured and quantified as described above (Migrating motor complexes).

In this experiment, six parameters were measured to determine the consequences of P2X<sub>2</sub> and P2X<sub>3</sub> gene deletion on colonic MMCs. The mean

values of these parameters were measured over 30 minutes following a 30 minute equilibration period in 20 WT and 9 each of  $P2X_2^{-/-}$  and  $P2X_3^{-/-}$  mice. While neither receptor deletion caused a general potentiation or attenuation of the MMC there were several differences associated with P2X subunit deletion. The frequency of MMCs in the  $P2X_2^{-/-}$  mice was increase by ~57% ( $0.3 \pm 0.03/\text{minute}$  vs.  $0.47/\text{minute} \pm 0.06$ ,  $p < 0.05$ ) over WT controls and the duration of the anal contractions were 37% shorter ( $16.46 \pm 1.5\text{s}$  vs.  $10.36 \pm 0.92\text{s}$ ,  $p < 0.05$ ).  $P2X_3$  subunit deletion increased the duration of the oral contractions by 46% ( $12.16 \pm 1.3\text{s}$  vs.  $17.71 \pm 2.9\text{s}$ ,  $p < 0.05$ ). These data indicate that while  $P2X_2$  and  $P2X_3$  receptors may play a role in modulating some components of the MMC, these receptors are not essential for the motor pattern.

## P2X gene deletion does not affect cholinergic contractions of longitudinal smooth muscle



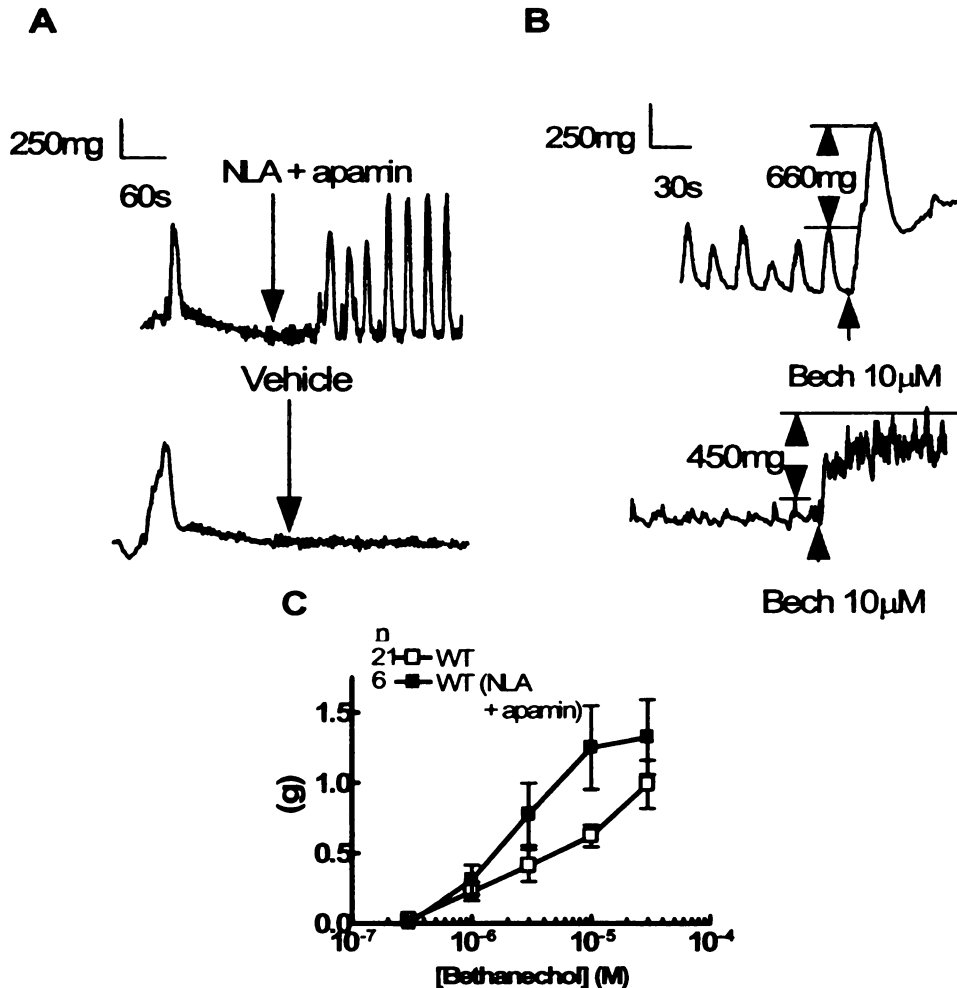
**Figure 8 – Bethanechol concentration-response curve.** Isometric contractions of mouse colonic longitudinal muscle. Open squares are wild type mice, Triangles are P2X<sub>3</sub> and circles are P2X<sub>2</sub> gene knockout respectively. All symbols represent the mean of the absolute value of the contractions  $\pm$  SEM. n represents number of replicates at EC<sub>50</sub>. Significance was tested using 2-way ANOVA. These data suggest that P2X<sub>2</sub> and P2X<sub>3</sub> receptor subunit deletion does not alter bethanechol reactivity of the smooth muscle.

Bethanechol is a selective agonist for muscarinic cholinergic receptors. It was used to probe baseline contractility of colonic longitudinal smooth muscle in the WT control mice and to determine if there were any changes in longitudinal muscle contractility in P2X<sub>2</sub><sup>-/-</sup> and P2X<sub>3</sub><sup>-/-</sup> mice.

Bethanechol (0.3 – 30  $\mu$ M) induced a concentration-dependent contraction of colonic longitudinal muscle in tissues from WT and P2X<sub>2</sub><sup>-/-</sup> and P2X<sub>3</sub><sup>-/-</sup> mice (**Figure 8**). P2X gene deletion had no effect on bethanechol-induced contractions at any concentration (**Figure 8**) (**Table 1**) ( $p > 0.05$ ). The muscarinic receptor antagonist scopolamine (1  $\mu$ M) blocked all bethanechol induced contractions (curve not shown) (**Table 1**). TTX did not affect bethanechol-induced contractions ( $p > 0.05$ ) at any concentration (curve not shown) (**Table 1**).

P2X gene deletion did not reveal any TTX sensitive components of muscarinic receptor mediated contractions (**Table 1**).

***Nitro-L-Arginine (NLA) and apamin reveals constitutive relaxation in mouse colonic smooth muscle***



**Figure 9 – Bethanechol with and without NLA (100µM) + Apamin(100nM).** A. application of NLA and apamin increased the frequency of spontaneous phasic contractions (top) as compared to vehicle treated controls. B. contractions in the middle of the concentration response curve where higher amplitude in NLA and apamin treated tissue (top) than those that were treated with vehicle. C. Isometric contractions of mouse colonic longitudinal muscle. Open squares are wild type mice, Triangles are P2X<sub>3</sub> and circles are P2X<sub>2</sub> gene knockout respectively. All symbols represent the mean of the absolute value of the contractions  $\pm$  SEM. n represents number of actions in the colon, a conicance was tested using 2-way ANOVA. These data suggest that NO and SK channels mediate a constitutive relaxation present in colonic longitudinal muscle.

NO mediated activation of guanylate cyclase and activation of small conductance Ca<sup>2+</sup> activated K<sup>+</sup> channels (SK) are mechanisms of neurogenic relaxations in mouse colon smooth muscle (66). NLA is an antagonist of nitric

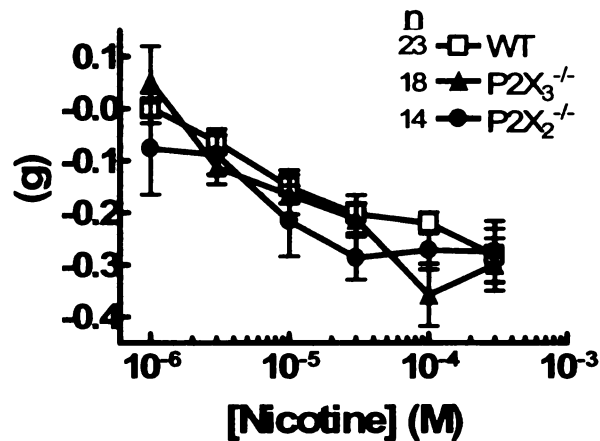
oxide synthase and apamin is a selective antagonist of SK channels. NLA (100  $\mu$ M) and apamin (100 nM) were used to block the effects of constitutive NO release and activation of SK channels.

NLA and apamin increased the bethanechol-induced contractions in WT longitudinal muscle. NLA and apamin increased the frequency of phasic neurogenic contractions (**Figure 9A**). Comparison of control contractions with those occurring after NLA and apamin treatment shows a significant difference along the linear part of the curve (**Figure 9C** - summarized) ( $p < 0.05$ ). This was not affected by P2X<sub>2</sub> or P2X<sub>3</sub> gene deletion (data not shown).

|                        | WT        | P2X <sub>2</sub> <sup>-/-</sup> | P2X <sub>3</sub> <sup>-/-</sup> |
|------------------------|-----------|---------------------------------|---------------------------------|
| <b>Bethanechol</b>     | 989 ± 171 | 815±157                         | 786±162                         |
| <b>+TTX</b>            | 768±187   | 671±145                         | 1065±146                        |
| <b>+Scopolamine</b>    | 35±17     | 0±0                             | 17±11                           |
| <b>+NLA and Apamin</b> | 1325±266  | -                               | -                               |

**Table 1** – Contraction force (mg) at maximum bethanechol concentration (30  $\mu$ M), with TTX (300 nM), Scopolamine (10  $\mu$ M), and NLA (100  $\mu$ M) and apamin (100 nM)

## Neurogenic relaxations of longitudinal muscle

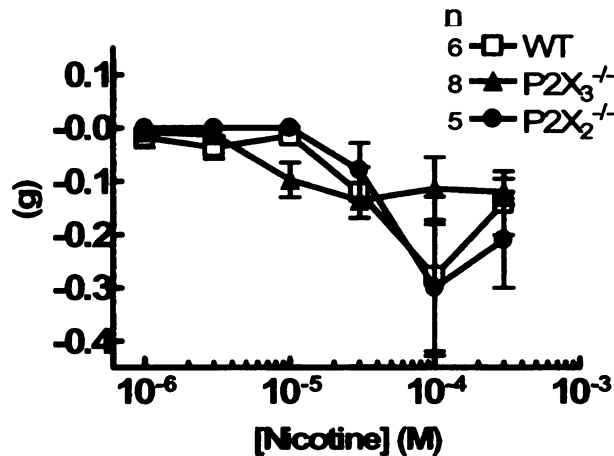


**Figure 10 – Nicotine concentration response curve.** Isometric relaxations of mouse colonic longitudinal muscle. Open squares are wild type mice, Triangles are P2X<sub>3</sub> and circles are P2X<sub>2</sub> gene knockout respectively. All symbols represent the mean of the absolute value of the relaxation  $\pm$  SEM. n represents number of replicates at EC50. Significance was tested using 2-way ANOVA. These data show that predominant effect of nicotinic receptor activation in mouse colon is a relaxation.

Nicotinic receptors are present on motor neurons in mouse and rat colon (13, 104, 106). Nicotine was used to activate these receptors in an effort to characterize the role of nicotinic receptors on mouse colonic longitudinal muscles as well as what role these receptors may play in P2X receptor-mediated responses.

Nicotine (1-300  $\mu$ M) caused a concentration-dependent relaxation of colonic longitudinal muscle in WT mice (**Figure 11**). This relaxation was unaffected by P2X<sub>2</sub> or P2X<sub>3</sub> gene deletion (**Figure 11**).

**Nicotinic receptor mediated relaxations are TTX resistant a response that was unaffected by P2X<sub>2</sub> or P2X<sub>3</sub> gene deletion**

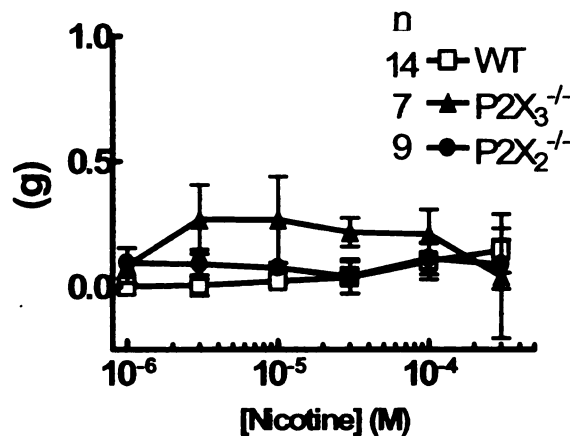


**Figure 11 – Nicotine concentration-response curve with TTX (300 nM).** Isometric relaxations of mouse colonic longitudinal muscle. Open squares are wild type mice, Triangles are P2X<sub>3</sub> and circles are P2X<sub>2</sub> gene knockout respectively. All symbols represent the mean of the absolute value of the relaxation  $\pm$  SEM. n represents number of replicates at EC<sub>50</sub>. Significance was tested using 2-way ANOVA. The TTX insensitivity of nicotine induced relaxations illustrates the presence of a prejunctional nicotinic receptor that mediates relaxations.

Relaxations of colonic longitudinal muscle caused by nicotine were not blocked by TTX in WT, P2X<sub>2</sub>, or P2X<sub>3</sub> gene deletion mice (**Figure 11**) (**Table 2**).

These data suggest that the nicotinic receptors that mediate relaxations in mouse colon are localized to the terminals of inhibitory motor neurons.

## Nicotinic receptors are not on excitatory motor neurons in the mouse colon



**Figure 12 – Nicotine concentration-response curve with NLA (10  $\mu$ M) and apamin (100nM).** Isometric relaxations of mouse colonic longitudinal muscle. Open squares are wild type mice, Triangles are P2X<sub>3</sub> and circles are P2X<sub>2</sub> gene knockout respectively. All symbols represent the mean of the absolute value of the relaxation  $\pm$  SEM. n represents number of replicates at EC50. Significance was tested using 2-way ANOVA. These data show that nicotine-induced relaxations are mediated by neurogenic NLA and apamin dependent mechanisms and that activation of nicotinic receptors does not cause a contraction that is hidden by the predominant relaxation.

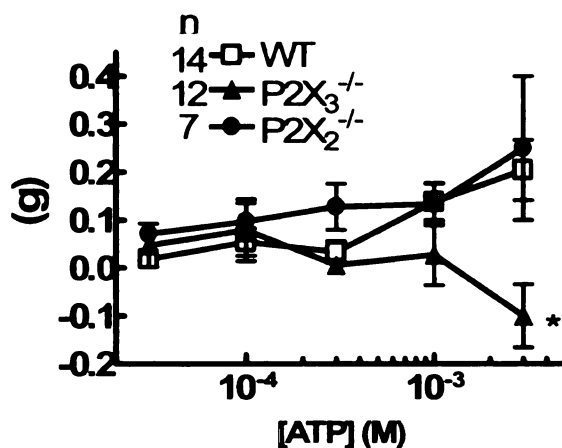
Relaxations caused by nicotine in mouse colonic smooth muscle where eliminated by treatment with NLA and apamin (**Figure 12**) (**Table 2**)

While relaxation is clearly the predominant effect of nicotine in these tissues, treatment with NLA and apamin would have revealed any excitatory/contractile effects mediated by nicotine, if they were present. Furthermore these data also address the question of whether nicotine may be acting directly on the smooth muscle in this tissue, because the relaxation mediated by nicotine is clearly dependent on mechanisms blocked by NLA and apamin.

|                        | WT      | P2X <sub>2</sub> <sup>-/-</sup> | P2X <sub>3</sub> <sup>-/-</sup> |
|------------------------|---------|---------------------------------|---------------------------------|
| <b>Nicotine</b>        | -282±51 | -275±59                         | -300±50                         |
| <b>+TTX</b>            | -141±60 | -210±90                         | -120±24                         |
| <b>+Scopolamine</b>    | -207±88 | -139±35                         | -267±109                        |
| <b>+NLA and Apamin</b> | 146±88* | 90±98*                          | 43±247*                         |

**Table 2** – Relaxation force (mg) at maximum nicotine concentration (300  $\mu$ M), with TTX (300 nM), Scopolamine (10  $\mu$ M), and NLA (100  $\mu$ M) and apamin (100 nM). (\*) = ( $p < 0.05$ ) different from same species control.

### ATP induced contractions in mouse colon

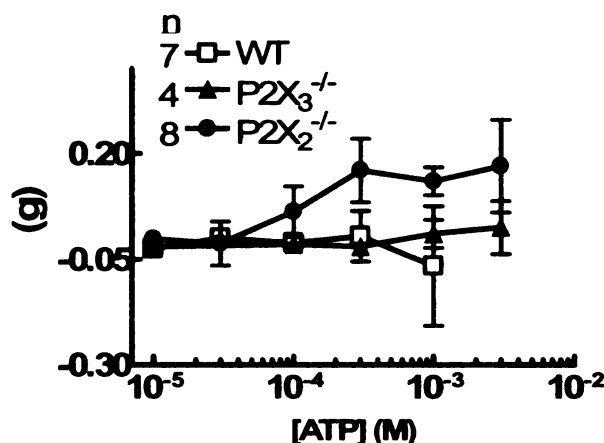


**Figure 13** – ATP concentration-response curve. Isometric contractions of mouse colonic longitudinal muscle. Open squares are wild type mice, Triangles are P2X<sub>3</sub> and circles are P2X<sub>2</sub> gene knockout respectively. All symbols represent the mean of the absolute value of the contractions  $\pm$  SEM. n represents number of replicates at EC<sub>50</sub>. Significance was tested using 2-way ANOVA. \* =  $p < 0.05$ . ATP mediates contraction of longitudinal muscle in the mouse colon by activating P2X<sub>3</sub> and not P2X<sub>2</sub> receptors.

ATP caused concentration-dependent (0.01 – 3 mM) contractions of colonic longitudinal muscle in WT and P2X<sub>2</sub> gene deletion mice (**Figure 13**) (**Table 3**). P2X<sub>3</sub><sup>-/-</sup> mice did not exhibit contractions to ATP at any concentration (**Figure 13**) (**Table 3**). These data indicate that ATP mediates a contraction in

the longitudinal muscle of mouse colon and that these contractions are mediated by P2X receptors containing the P2X<sub>3</sub> subunit.

#### ATP-induced contractions are TTX sensitive

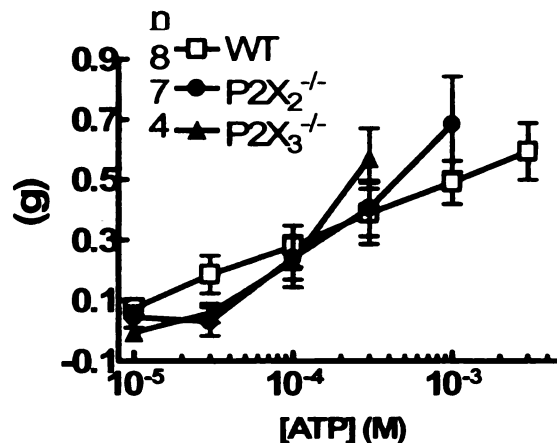


**Figure 14 – ATP concentration-response curve with TTX (300 nM).** Isometric contractions of mouse colonic longitudinal muscle. Open squares are WT mice, Triangles are P2X<sub>3</sub> and circles are P2X<sub>2</sub><sup>-/-</sup> gene knockout respectively. All symbols represent the mean of the absolute value of the contractions  $\pm$  SEM. n represents number of replicates at EC<sub>50</sub>. Significance was tested using 2-way ANOVA. These data show that activation of P2X<sub>3</sub> receptors mediates contractions in a neurogenic manner.

Contractions caused by ATP were blocked by TTX in all mice (**Figure 14**) (**Table 3**). This suggests that P2X<sub>3</sub> receptors are on the soma of excitatory motor neurons in the mouse colon.

The contractions caused by ATP were not blocked by the muscarinic cholinergic antagonist scopolamine (**Table 3**) showing that P2X<sub>3</sub> mediated contractions are not acetylcholine mediated but rather they are caused by the release of a non-cholinergic excitatory neurotransmitter.

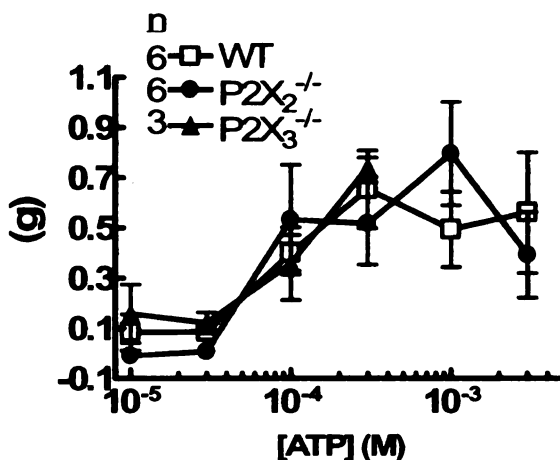
## ATP induces relaxations in mouse colon that are NLA and apamin sensitive



**Figure 15 – ATP concentration-response curve with NLA (10  $\mu$ M) and apamin (100nM).** Isometric contractions of mouse colonic longitudinal muscle. Open squares are wild type mice, Triangles are P2X<sub>3</sub><sup>-/-</sup> and circles are P2X<sub>2</sub><sup>-/-</sup> respectively. All symbols represent the mean of the absolute value of the contractions  $\pm$  SEM. n represents number of replicates at EC50. Significance was tested using 2-way ANOVA. The potentiation of ATP-induced contractions in wild type and P2X<sub>2</sub><sup>-/-</sup>, and the appearance of an ATP-induced contraction show that ATP also mediates an NLA and apamin dependent relaxation of mouse colonic longitudinal muscle.

Contractions caused by ATP were increased by ~40% at the highest ATP concentrations in control mice (**Figure 15**) (**Table 3**) ( $p < 0.05$ ). Comparable increases in contraction force were also seen P2X<sub>2</sub><sup>-/-</sup> mice (**Figure 15**) (**Table 2**). NLA and apamin treatment revealed a contraction in P2X<sub>3</sub><sup>-/-</sup> mice, which showed no contractile response to ATP in the absence of NLA and apamin (**Figure 15**) (**Table 3**). These data suggest that ATP in addition to causing contractions via an action on P2X<sub>3</sub> subunit containing receptors on the soma of excitatory motor neurons also causes a relaxation that is sensitive to NLA and apamin. Also revealed by this experiment is a contraction that is not mediated by P2X<sub>3</sub> subunit containing receptors.

**TTX-insensitive ATP contractions are mediated by non P2X<sub>2</sub> or P2X<sub>3</sub> receptor**



**Figure 16 – ATP contractions with NLA (10  $\mu$ M) and apamin (100 nM) and TTX(300 nM).** Isometric contractions of mouse colonic longitudinal muscle. Open squares are C57B wild type mice, Triangles are P2X<sub>3</sub><sup>-/-</sup> and circles are P2X<sub>2</sub><sup>-/-</sup> respectively. All symbols represent the mean of the absolute value of the contractions  $\pm$  SEM. n represents number of replicates at EC50. Significance was tested using 2-way ANOVA. The persistence of ATP-induced contractions in the presence of TTX shows that ATP is mediating a contraction via P2X receptor that is either prejunctional or directly on the longitudinal smooth muscle. This receptor does not contain the P2X<sub>2</sub> or P2X<sub>3</sub> subunit.

Using NLA and apamin to eliminate inhibitory synaptic input and TTX to block neuronal impulses, ATP caused a concentration-dependent contraction in WT mice (**Figure 2**) (**Table 3**). These data suggest there are prejunctional P2X receptors coupled to the release of excitatory mediators from terminals of neurons that supply longitudinal muscle. TTX resistant ATP-induced contractions in the presence of NLA and apamin were not affected by either P2X<sub>2</sub> or P2X<sub>3</sub> gene deletion (**Figure 16**).

This study shows that ATP mediates a TTX-insensitive contraction of mouse longitudinal smooth muscle that is revealed after NLA and apamin treatment, indicating the presence of a prejunctional P2X receptor. Deletion of

P2X<sub>2</sub> or P2X<sub>3</sub> subunits does not block this contraction, showing that P2X receptors containing this subunit do not mediate this contraction.

|                                     | <b>WT</b>             | <b>P2X<sub>2</sub><sup>-/-</sup></b> | <b>P2X<sub>3</sub><sup>-/-</sup></b> |
|-------------------------------------|-----------------------|--------------------------------------|--------------------------------------|
| <b>ATP</b>                          | 204±63                | 250±150                              | -100±66                              |
| <b>+TTX</b>                         | -66±142* (@ 1 mM ATP) | 170±109                              | 25±63                                |
| <b>+scopolamine</b>                 | 213±297               | 150±50                               | 200±153                              |
| <b>+NLA and apamin</b>              | 593±94 *              | 683±159 * (@ 1 mM ATP)               | 570±100                              |
| <b>+NLA, apamin and TTX</b>         | 560±240               | -                                    | -                                    |
| <b>+NLA, apamin and scopolamine</b> | 438±98(@ 1 mM ATP)    | -                                    | -                                    |

**Table 3** – Contraction (mg) at maximum ATP concentration (3 mM), with TTX (300 nM), Scopolamine (10 µM), and NLA (100 µM) and apamin (100 nM). (\*) = (p < 0.05) different from control.

## **Discussion**

### **P2X<sub>2</sub> and P2X<sub>3</sub> receptor subunits do not mediate 5-HTP-induced defecation in vivo**

Deletion of the P2X<sub>2</sub> and the P2X<sub>3</sub> receptor subunits did not affect fecal pellet output or fecal weight (wet and dry) as compared to that occurring in WT mice. These data suggest that P2X<sub>2</sub> and P2X<sub>3</sub> do not contribute to 5-HTP induced defecation, or that the loss of these receptors has been compensated for by replacement with other neural signalling mechanisms in development. The lack of difference is not entirely surprising because the transgenic animals were not phenotypically different from the WT mice (12, 112). P2X<sub>2</sub><sup>-/-</sup> and P2X<sub>3</sub><sup>-/-</sup> mice had similar body weight to the WT mice and did not show any overt signs of abnormality (12, 112). Potentially, 5-HTP may act through a mechanism that overrides any activity that is mediated by P2X<sub>2</sub> or P2X<sub>3</sub> receptors. Another possibility is that the 5-HTP induced complete colonic evacuation in all mice, and thus over the hour all animals simply produced 100% of their available fecal mass, which would be similar in animals with similar body weight. A difference between WT and P2X knockout mice would only be seen if the loss of P2X<sub>2</sub> or P2X<sub>3</sub> receptors caused a delay in colonic propulsion. If the loss of these subunits caused an increase in colonic propulsion, the 5-HTP assay would not detect this increase.

## **P2X<sub>2</sub> and P2X<sub>3</sub> subunit containing receptors are not essential in the colonic MMC.**

While P2X<sub>2</sub><sup>-/-</sup> animals showed changes in 2 parameters (increased frequency and decreased anal duration) of the MMC and P2X<sub>3</sub><sup>-/-</sup> animals were different in a single parameter (lengthened oral duration), neither knockout mouse showed any general change in the MMC. If P2X<sub>2</sub> or P2X<sub>3</sub> receptors were major contributors to the MMC, it would be expected that all of the parameters would be different, and in the same direction.

It is not surprising that P2X receptor gene deletion did not cause global changes in colonic MMCs because, as previously reported, these animals were identical in weight to control animals and had no outward deleterious consequences of the gene deletion (12). P2X<sub>2</sub> and P2X<sub>3</sub> receptor knockout has previously been shown to impair *in vitro* peristalsis in mouse ileum (12, 112). An electrophysiological survey of purinergic fEPSPs along the length of the guinea pig gastrointestinal tract showed that while purinergic fEPSPs were common in myenteric neurons in the ileum, they were less prevalent in colon (91). These studies along with the data presented in this work would support a conclusion that P2X communication is more important in the ileum than in the colon. However, it is not unreasonable to suggest that P2X communication may be important in the colon and there are redundant mechanisms in the gut compensating for the loss of the P2X receptors as the ENS is capable of marked plasticity. Perhaps challenging these animals by removing more of the available excitatory neurotransmitters would reveal a decrement in the MMC of knockout

animals. It is difficult to relate effects seen in these studies to those observed in isometric contractions of longitudinal muscle because as shown in **Figure 5** this preparation is looking at contractions of circular muscle, which has its own population of inhibitory and excitatory motor neurons. The circular muscle motor neurons may have different mechanisms through which they mediate contractions and relaxations. In a study measuring synchronous contractility from both circular and longitudinal muscle in guinea pig colon showed that antagonists which interrupted reflexes in longitudinal muscle also interrupted reflexes in circular muscle (125). In cat colon, atropine attenuates the amplitude of contractions in both longitudinal and circular muscle preparations (2). Muscarinic agonists and antagonists had similar efficacy on both longitudinal and circular muscle in human colon (80). Discrete measure of circular muscle contractions is yet to be done in mouse; however this historical work suggests that similar neurotransmitters mediate contractions in both longitudinal and circular muscle layers. While these studies do not address the specific nerves or specific synaptic mechanisms involved, there is no evidence available that indicates that they are different.

#### **P2X receptor subunit deletion does not alter the longitudinal muscle contractility coupled to muscarinic receptor activation**

The aim of these studies was to use pharmacological methods and transgenic manipulations to characterize the contribution of P2X receptors to neural and neuromuscular control of the longitudinal muscle layer in the mouse colon.

The muscarinic agonist, bethanechol, causes a concentration-dependent contraction of mouse colonic longitudinal muscle. Removal of tonic inhibition with NLA and apamin potentiated bethanechol-induced contractions. Bethanechol contractions were insensitive to TTX. Neither P2X<sub>2</sub> nor P2X<sub>3</sub> subunit deletion affected bethanechol contractions. These data suggest that bethanechol acts on muscarinic receptors directly on the colonic smooth muscle to stimulate contractions. The fact that TTX did not affect bethanechol-induced contractions argues that muscarinic receptors are on the smooth muscle and not on the soma of the motor neurons synapsing on the smooth muscle. This is supported by previous studies showing that M2 and M3 type muscarinic receptors are on gastrointestinal smooth muscle (115).

Data from the present study shows that treatment with NLA and apamin increases the frequency of phasic contractions of mouse colonic longitudinal muscle. Previous studies have shown that inhibition of NO production and apamin will increase the amplitude of MMCs measured from colonic circular muscle in mice (20). The present study also shows that NLA and apamin treatment will potentiate bethanechol induced contractions. These data indicate the presence of constitutive relaxation in the mouse colon that is mediated by NO and SK channel activation.

### **Nicotinic receptors in the colon**

Electrophysiological studies in mouse colon showed that the nicotinic receptor antagonist hexamethonium blocks inhibitory junction potentials in guinea pig colonic circular muscle (13, 130). Spencer et al also found that

hexamethonium blocked ascending excitatory junction potentials (EJP) in guinea pig colon (130). Bian et al concluded that acetylcholine acting via nicotinic receptors is the major neurotransmitter from sensory neurons to inhibitory motor neurons in the rat colon (13). The present study supports the conclusion that nicotinic receptors are localized to inhibitory motor neurons supplying the longitudinal muscle layer in the mouse colon. Blockade of relaxations with NLA and apamin abolished nicotine-induced relaxations and furthermore failed to reveal a nicotine mediated contraction. The Spencer et al study showed EJPs in guinea pig smooth muscle were hexamethonium sensitive, suggesting that nicotinic receptors are not exclusive to inhibitory motor neurons, however this effect may be due to nicotinic receptors on interneurons activating non-cholinergic excitatory motor neurons rather than directly on the excitatory motor neurons (130). Another possible interpretation is that there is a key difference between the excitatory neural input to the longitudinal muscle in murine and guinea pig colon. The longitudinal muscle in the mouse colon may lack excitatory input that is present in guinea pig. Though nicotinic receptors have never been localized to intestinal smooth muscle, nicotinic receptor gated  $\text{Ca}^{2+}$  entry, activating an apamin sensitive mechanism on the smooth muscle remains a possibility.

Nicotine-induced relaxations were TTX-insensitive and  $\text{P2X}_2$  and  $\text{P2X}_3$  gene deletion did not alter nicotine-induced relaxations in the presence or absence of TTX. These experiments show that nicotinic receptors are on prejunctional nerve endings of inhibitory motor neurons. These data also show

that nicotinic receptors are not on excitatory motor neurons in the mouse colon. Presynaptic nicotinic receptors have been previously described in the myenteric plexus of the guinea pig ileum (120). Prejunctional nicotinic receptors on nerve endings have been shown to cause non-cholinergic contractions in guinea pig ileum through the release of peptides (59). Prejunctional nicotinic receptors in the myenteric plexus have been shown to mediate TTX-insensitive slow synaptic events recorded from AH-neurons (120). While the literature and these data support the conclusion that prejunctional nicotinic receptors are found on inhibitory neurons synapsing onto longitudinal muscle, somatic nicotinic receptors may also exist. It can also be concluded that P2X<sub>2</sub> and P2X<sub>3</sub> subunit containing receptors do not play a role in nicotinic receptor mediated relaxations in the mouse colon.

It was surprising to find that nicotine did not cause contractions in the mouse colon as nicotine in other regions of the gut mediates contraction via activation of excitatory motor neurons. Nicotinic receptors mediate activation of excitatory motor neurons in guinea pig and mouse ileum longitudinal smooth muscle (12, 59, 112). In the present study, muscarinic receptor activation produced a TTX-resistant contraction, illustrating that muscarinic receptors are on colonic longitudinal smooth muscle. Prejunctional muscarinic receptors have been described previously on motor nerve endings in the gastrointestinal tract. However, the muscarinic receptors mediate inhibition of transmitter release (68). The colon is innervated by parasympathetic efferent neurons from sacral spinal

ganglia, which would be potential source of endogenous acetylcholine to activate muscarinic receptors on longitudinal muscle (134).

### **P2X receptors in the colon**

Electrophysiological studies in the myenteric plexus have shown that ATP acting via P2X receptors mediates excitation in myenteric neurons in guinea pig ileum and colon as well as in mouse ileum (12, 60, 78, 91, 104, 112). P2X<sub>2</sub> subunit immunoreactivity is on neurons in the mouse colon (66). In guinea pig P2X<sub>2</sub> and P2X<sub>3</sub> subunits have been found via immunohistochemistry in colonic neurons (30, 108). P2X<sub>2</sub> receptors localize to inhibitory motor neurons and IPANs of the myenteric plexus as well as IPANS in the submucosal plexus (30). P2X<sub>3</sub> receptors show a degree of overlap with P2X<sub>2</sub> receptors, however they are absent from IPANs (108). In the colon P2X<sub>3</sub> receptors are seen on all cholinergic secretomotor neurons in the submucosal plexus (108). P2X<sub>3</sub> receptors have also been localized to myenteric neurons in the human colon (141).

Currently pharmacological investigations into excitatory purinergic synaptic and neuromuscular transmission is slowed by the lack of selective drugs capable of definitively attributing effects to receptors composed of specific P2X subunits (103). Through recent advances in transgenic manipulations of mice, genes encoding specific P2X receptors subtypes can be deleted, resulting in de facto subunit specific antagonism.

Work by Giaroni et al (2002) showed that ATP caused contractions of longitudinal muscle in mouse colon (66). We have confirmed these findings, however our new data shows that P2X<sub>3</sub> but not P2X<sub>2</sub> gene deletion abolished

these ATP induced contractions. The ATP-induced contractions were blocked by TTX but not scopolamine. Blockade of relaxation with NLA and apamin significantly potentiated ATP induced contractions. NLA and apamin treatment also revealed TTX-resistant ATP mediated contractions in all mice. From these studies we conclude that excitatory motor neurons in mouse colon can be activated by ATP acting via P2X<sub>3</sub> receptors to cause the release of a non-cholinergic excitatory neurotransmitter. Giaroni et al observed that  $\alpha\beta$ MeATP, which has no activity at P2X<sub>2</sub> receptors, was a potent agonist in eliciting contractions in mouse colonic longitudinal muscle (66). From this they concluded that P2X<sub>3</sub> receptors must mediate contractions in the colon, a conclusion that is supported by the present study (66). Giaroni et al reported ATP induced colonic contractions were TTX insensitive, while in the present study, TTX insensitive contractions were only revealed in the presence of NLA and apamin (66). This discrepancy could possibly be explained by different levels of inhibitory tone between the mice used Giaroni et al and the mice used in the present study.

Based on the fact that ATP and 2MeSATP induced contractions did not desensitize, Giaroni also concluded that P2X<sub>2</sub> (in addition to the previously mentioned P2X<sub>3</sub> mediated effect) receptors on the smooth muscle were responsible for TTX resistant contractions (66). In the present study, TTX resistant contractions in the presence of NLA and apamin were still present P2X<sub>2</sub> knockout animals, data which refutes the previously mentioned conclusion (66). It is possible that P2X<sub>2</sub> receptor subunits are forming a heteromeric receptor on the smooth muscle which in the face of P2X<sub>2</sub> subunit deletion converts to a

homomeric receptor, allowing contractions to be maintained. Another possibility is TTX resistant contractions are mediated by a prejunctional P2X receptor that lacks either the P2X<sub>2</sub> or P2X<sub>3</sub> subunit. In previous studies, NK-1 and NK-2 antagonists attenuated the amplitude and duration of mouse colonic MMCs (20). Neutral endopeptidase inhibitors which prevent the degradation and deactivation of substance P, also potentiate contractions in rat and ferret ileum and duodenum (43). Substance P release could be coupled to the afore mentioned prejunctional non P2X<sub>2</sub>, P2X<sub>3</sub> subunit containing P2X receptor. P2 receptor immunoreactivity has been demonstrated on colonic smooth muscle(66). A recent anatomical survey found P2X<sub>5</sub> immunoreactivity on nerve endings in the mouse, however this study only looked at nerve endings in the myenteric plexus and not motor neuron-smooth muscle junctions (113).

### **Summary and Conclusions**

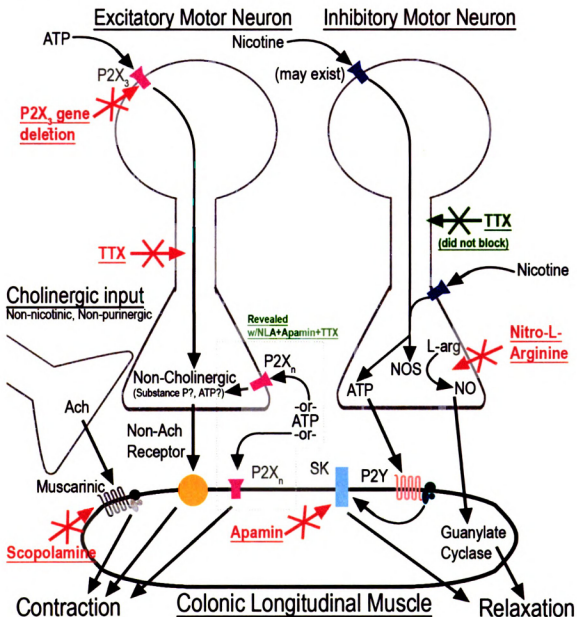
Cholinergic contractions of mouse colonic longitudinal muscle are not mediated by nicotinic receptors on excitatory motor neurons but possibly by parasympathetic nerve endings or by excitatory motor neurons that lack nicotinic receptors. Nicotinic receptors, perhaps on inhibitory motor neuron soma and/or terminals, mediate an NLA and apamin sensitive relaxation of mouse colonic longitudinal muscle. ATP mediates TTX sensitive contractions in mouse colonic longitudinal muscle dependent on P2X<sub>3</sub> subunit containing receptors on excitatory motor neuron soma. ATP mediates a TTX resistant contraction in mouse colonic longitudinal muscle via presynaptic non-P2X<sub>2/3</sub> subunit containing

P2X receptors mediating the release of excitatory transmitters (or acting on the smooth muscle directly).

**Figure 17** illustrates a proposed model of motor innervation to mouse colonic smooth muscle based on the data presented here. ATP activates excitatory motor input via a somatic P2X<sub>3</sub> subunit containing receptor as evidenced by the lack of an ATP mediated contraction in P2X<sub>3</sub><sup>-/-</sup> mice and the TTX sensitivity of ATP induced contractions in WT mice. ATP also activates a non-P2X<sub>2</sub>, P2X<sub>3</sub> subunit-containing P2X receptor to mediate TTX-resistant contractions that are revealed by NLA and apamin treatment. Bethanechol activates muscarinic cholinergic receptors on smooth muscle cells to mediate contraction; however the endogenous source of the acetylcholine receptor to activate that receptor is at this time unknown. Nicotine activates inhibitory motor neurons via a prejunctional nicotinic cholinergic receptor illustrated by TTX-resistant nicotine-induced relaxations in all mouse strains. Nicotine mediated activation of inhibitory motor neurons mediate relaxation via release of NO and ATP as NLA and apamin block nicotine-mediated contractions.

## Proposed Model

Figure 17 – Proposed Model



Images in this thesis are presented in color.

## **LITERATURE CITED**

1. **Abbracchio MP and Burnstock G.** Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol Ther* 64: 445-475, 1994.
2. **Anuras S and Christensen J.** Effects of autonomic drugs on cat colonic muscle. *Am J Physiol* 240: G361-364, 1981.
3. **Aschrafi A, Sadtler S, Niculescu C, Rettinger J, and Schmalzing G.** Trimeric architecture of homomeric P2X2 and heteromeric P2X1+2 receptor subtypes. *J Mol Biol* 342: 333-343, 2004.
4. **Banner SE, Smith MI, Bywater D, Gaster LM, and Sanger GJ.** Increased defaecation caused by 5-HT4 receptor activation in the mouse. *Eur J Pharmacol* 308: 181-186, 1996.
5. **Benham CD and Tsien RW.** A novel receptor-operated Ca<sup>2+</sup>-permeable channel activated by ATP in smooth muscle. *Nature* 328: 275-278, 1987.
6. **Berthoud HR, Blackshaw LA, Brookes SJ, and Grundy D.** Neuroanatomy of extrinsic afferents supplying the gastrointestinal tract. *Neurogastroenterol Motil* 16 Suppl 1: 28-33, 2004.
7. **Berthoud HR, Jedrzejewska A, and Powley TL.** Simultaneous labeling of vagal innervation of the gut and afferent projections from the visceral forebrain with dil injected into the dorsal vagal complex in the rat. *J Comp Neurol* 301: 65-79, 1990.
8. **Berthoud HR and Neuhuber WL.** Functional and chemical anatomy of the afferent vagal system. *Auton Neurosci* 85: 1-17, 2000.
9. **Bertrand PP and Galligan JJ.** Contribution of chloride conductance increase to slow EPSC and tachykinin current in guinea-pig myenteric neurones. 481 ( Pt 1): 47-60, 1994.
10. **Bertrand PP and Galligan JJ.** Signal-transduction pathways causing slow synaptic excitation in guinea pig myenteric AH neurons. *Am J Physiol* 269: G710-720, 1995.
11. **Bertrand PP, Kunze WA, Bornstein JC, Furness JB, and Smith ML.** Analysis of the responses of myenteric neurons in the small intestine to chemical stimulation of the mucosa. *Am J Physiol* 273: G422-435, 1997.
12. **Bian X, Ren J, DeVries M, Schnegelsberg B, Cockayne DA, Ford AP, and Galligan JJ.** Peristalsis is impaired in the small intestine of mice lacking the P2X3 subunit. 551: 309-322, 2003.
13. **Bian XC, Bornstein JC, and Bertrand PP.** Nicotinic transmission at functionally distinct synapses in descending reflex pathways of the rat colon. *Neurogastroenterol Motil* 15: 161-171, 2003.
14. **Bo X, Zhang Y, Nassar M, Burnstock G, and Schoepfer R.** A P2X purinoceptor cDNA conferring a novel pharmacological profile. *FEBS Lett* 375: 129-133, 1995.
15. **Borman RA and Burleigh DE.** Evidence for the involvement of a 5-HT4 receptor in the secretory response of human small intestine to 5-HT. *Br J Pharmacol* 110: 927-928, 1993.

16. **Bourin M, Hascoet M, and Deguiral P.** 5-HTP induced diarrhea as a carcinoid syndrome model in mice? *Fundam Clin Pharmacol* 10: 450-457, 1996.
17. **Brake AJ, Wagenbach MJ, and Julius D.** New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature* 371: 519-523, 1994.
18. **Brehmer A, SchrodL F, and Neuhuber W.** Morphological classifications of enteric neurons--100 years after Dogiel. *Anat Embryol (Berl)* 200: 125-135, 1999.
19. **Briejer MR, Akkermans LM, and Schuurkes JA.** Interactions of serotonin with multiple receptors and neurotransmitters in the guinea-pig isolated colon. *Arch Int Pharmacodyn Ther* 329: 121-133, 1995.
20. **Brierley SM, Nichols K, Grasby DJ, and Waterman SA.** Neural mechanisms underlying migrating motor complex formation in mouse isolated colon. *British Journal of Pharmacology* 132: 507-517, 2001.
21. **Buell G, Michel AD, Lewis C, Collo G, Humphrey PP, and Surprenant A.** P2X1 receptor activation in HL60 cells. *Blood* 87: 2659-2664, 1996.
22. **Burnstock G.** Cotransmission. *Curr Opin Pharmacol* 4: 47-52, 2004.
23. **Burnstock G.** Potential therapeutic targets in the rapidly expanding field of purinergic signalling. *Clin Med* 2: 45-53, 2002.
24. **Burnstock G, Campbell G, Bennett M, and Holman ME.** Inhibition of the Smooth Muscle on the Taenia Coli. *Nature* 200: 581-582, 1963.
25. **Burnstock G, Campbell G, and Rand MJ.** The inhibitory innervation of the taenia of the guinea-pig caecum. *J Physiol* 182: 504-526, 1966.
26. **Burnstock G, Campbell G, Satchell D, and Smythe A.** Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br J Pharmacol* 40: 668-688, 1970.
27. **Burnstock G and Kennedy C.** Is there a basis for distinguishing two types of P2-purinoceptor? *Gen Pharmacol* 16: 433-440, 1985.
28. **Bush TG, Spencer NJ, Watters N, Sanders KM, and Smith TK.** Effects of alosetron on spontaneous migrating motor complexes in murine small and large bowel in vitro. *American Journal of Physiology Gastrointestinal and Liver Physiology* 281: G974-983, 2001.
29. **Bush TG, Spencer NJ, Watters N, Sanders KM, and Smith TK.** Spontaneous migrating motor complexes occur in both the terminal ileum and colon of the C57BL/6 mouse in vitro. *Autonomic Neuroscience* 84: 162-168, 2000.
30. **Castelucci P, Robbins HL, Poole DP, and Furness JB.** The distribution of purine P2X(2) receptors in the guinea-pig enteric nervous system. *Histochem Cell Biol* 117: 415-422, 2002.
31. **Chang HY, Mashimo H, and Goyal RK.** Musings on the wanderer: what's new in our understanding of vago-vagal reflex? IV. Current concepts of vagal efferent projections to the gut. *Am J Physiol Gastrointest Liver Physiol* 284: G357-366, 2003.

32. **Choi RC, Man ML, Ling KK, Ip NY, Simon J, Barnard EA, and Tsim KW.** Expression of the P2Y1 nucleotide receptor in chick muscle: its functional role in the regulation of acetylcholinesterase and acetylcholine receptor. *J Neurosci* 21: 9224-9234, 2001.
33. **Chvatchko Y, Valera S, Aubry JP, Renno T, Buell G, and Bonnefoy JY.** The involvement of an ATP-gated ion channel, P(2X1), in thymocyte apoptosis. *Immunity* 5: 275-283, 1996.
34. **Clerc N, Furness JB, Li ZS, Bornstein JC, and Kunze WA.** Morphological and immunohistochemical identification of neurons and their targets in the guinea-pig duodenum. *Neuroscience* 86: 679-694, 1998.
35. **Cockayne DA, Dunn PM, Burnstock G, and A.P.D. F.** Generation and electrophysiological characterization of P2X2 and P2X2/3 knockout mice. *Society for Neuroscience*, 2002, p. 12.
36. **Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic S, Malmberg AB, Cain G, Berson A, Kassotakis L, Hedley L, Lachnit WG, Burnstock G, McMahon SB, and Ford AP.** Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature* 407: 1011-1015., 2000.
37. **Collo G, Neidhart S, Kawashima E, Kosco-Vilbois M, North RA, and Buell G.** Tissue distribution of the P2X7 receptor. *Neuropharmacology* 36: 1277-1283, 1997.
38. **Collo G, North R, Kawashima E, Merlo-Pich E, Neidhart S, Surprenant A, and Buell G.** Cloning OF P2X5 and P2X6 receptors and the distribution and properties of an extended family of ATP-gated ion channels. *J Neurosci* 16: 2495-2507, 1996.
39. **Cooke HJ, Sidhu M, Fox P, Wang YZ, and Zimmermann EM.** Substance P as a mediator of colonic secretory reflexes. *Am J Physiol* 272: G238-245, 1997.
40. **Cooper EC and Jan LY.** Ion channel genes and human neurological disease: recent progress, prospects, and challenges. *Proc Natl Acad Sci U S A* 96: 4759-4766, 1999.
41. **Costa M, Brookes SJ, Steele PA, Gibbins I, Burcher E, and Kandiah CJ.** Neurochemical classification of myenteric neurons in the guinea-pig ileum. *Neuroscience* 75: 949-967, 1996.
42. **De Man JG, De Winter BY, Seerden TC, De Schepper HU, Herman AG, and Pelckmans PA.** Functional evidence that ATP or a related purine is an inhibitory NANC neurotransmitter in the mouse jejunum: study on the identity of P2X and P2Y purinoceptors involved. *Br J Pharmacol* 140: 1108-1116, 2003.
43. **Djokic TD, Sekizawa K, Borson DB, and Nadel JA.** Neutral endopeptidase inhibitors potentiate substance P-induced contraction in gut smooth muscle. *Am J Physiol* 256: G39-43, 1989.
44. **Dubyak GR.** Signal transduction by P2-purinergic receptors for extracellular ATP. *Am J Respir Cell Mol Biol* 4: 295-300, 1991.
45. **Eccles JC.** *The physiology of synapses*. Berlin,: Springer, 1964.
46. **Edwards G and Weston AH.** The pharmacology of ATP-sensitive potassium channels. *Annu Rev Pharmacol Toxicol* 33: 597-637, 1993.

47. **Ehrstrom M, Naslund E, Ma J, Kirchgessner AL, and Hellstrom PM.** Physiological regulation and NO-dependent inhibition of migrating myoelectric complex in the rat small bowel by OXA. *Am J Physiol Gastrointest Liver Physiol* 285: G688-695, 2003.
48. **Elswood CJ, Bunce KT, and Humphrey PP.** Identification of putative 5-HT<sub>4</sub> receptors in guinea-pig ascending colon. *Eur J Pharmacol* 196: 149-155, 1991.
49. **Engelhardt WA.** Life and Science. *Annual Review of Biochemistry* 51: 1-20, 1982.
50. **Fabbro A, Skorinkin A, Grandolfo M, Nistri A, and Giniatullin R.** Quantal release of ATP from clusters of PC12 cells. *J Physiol* 560: 505-517, 2004.
51. **Fida R, Lyster DJ, Bywater RA, and Taylor GS.** Colonic migrating motor complexes (CMMCs) in the isolated mouse colon. *Neurogastroenterol Motil* 9: 99-107, 1997.
52. **Fletcher EL, Clark MJ, Senior P, and Furness JB.** Gene expression and localization of GABA(C) receptors in neurons of the rat gastrointestinal tract. *Neuroscience* 107: 181-189, 2001.
53. **Furness JB.** Types of neurons in the enteric nervous system. *J Auton Nerv Syst* 81: 87-96, 2000.
54. **Furness JB and Costa M.** *The enteric nervous system*. Edinburgh ; New York: Churchill Livingstone, 1987.
55. **Furness JB, Kunze WA, Bertrand PP, Clerc N, and Bornstein JC.** Intrinsic primary afferent neurons of the intestine. *Prog Neurobiol* 54: 1-18, 1998.
56. **Furness JB, Trussell DC, Pompolo S, Bornstein JC, and Smith TK.** Calbindin neurons of the guinea-pig small intestine: quantitative analysis of their numbers and projections. *Cell Tissue Res* 260: 261-272, 1990.
57. **Furukawa K, Taylor GS, and Bywater RA.** An intracellular study of myenteric neurons in the mouse colon. *J Neurophysiol* 55: 1395-1406, 1986.
58. **Galligan JJ.** Mechanisms of excitatory synaptic transmission in the enteric nervous system. *The Tokai Journal of Experimental and Clinical Medicine* 23: 129-136, 1998.
59. **Galligan JJ.** Nerve terminal nicotinic cholinergic receptors on excitatory motoneurons in the myenteric plexus of guinea pig intestine. *J Pharmacol Exp Ther* 291: 92-98, 1999.
60. **Galligan JJ and Bertrand PP.** ATP mediates fast synaptic potentials in enteric neurons. *J Neurosci* 14: 7563-7571, 1994.
61. **Galligan JJ, LePard KJ, Schneider DA, and Zhou X.** Multiple mechanisms of fast excitatory synaptic transmission in the enteric nervous system. *J Auton Nerv Syst* 81: 97-103, 2000.
62. **Galligan JJ and North RA.** Pharmacology and function of nicotinic acetylcholine and P2X receptors in the enteric nervous system. *Neurogastroenterol Motil* 16: 64-70, 2004.
63. **Garcia-Guzman M, Soto F, Gomez-Hernandez JM, Lund PE, and Stuhmer W.** Characterization of recombinant human P2X<sub>4</sub> receptor reveals pharmacological differences to the rat homologue. *Mol Pharmacol* 51: 109-118, 1997.

64. **Garcia-Guzman M, Stuhmer W, and Soto F.** Molecular characterization and pharmacological properties of the human P2X3 purinoceptor. *Brain Res Mol Brain Res* 47: 59-66, 1997.
65. **Gatof D, Kilic G, and Fitz JG.** Vesicular exocytosis contributes to volume-sensitive ATP release in biliary cells. *Am J Physiol Gastrointest Liver Physiol* 286: G538-546, 2004.
66. **Giaroni C, Knight GE, Ruan HZ, Glass R, Bardini M, Lecchini S, Frigo G, and Burnstock G.** P2 receptors in the murine gastrointestinal tract. *Neuropharmacology* 43: 1313-1323, 2002.
67. **Glasgow I, Mattar K, and Krantis A.** Rat gastroduodenal motility in vivo: involvement of NO and ATP in spontaneous motor activity. *Am J Physiol* 275: G889-896, 1998.
68. **Goyal RK.** Identification, localization and classification of muscarinic receptor subtypes in the gut. *Life Sci* 43: 2209-2220, 1988.
69. **Haverback BJ and Davidson JD.** Serotonin and the gastrointestinal tract. *Gastroenterology* 35: 570-578, 1958.
70. **Hedge SS, Moy TM, Perry MR, Loeb M, and Eglen RM.** Evidence for the involvement of 5-hydroxytryptamine 4 receptors in 5-hydroxytryptophan-induced diarrhea in mice. *J Pharmacol Exp Ther* 271: 741-747, 1994.
71. **Hirst GDS, Holman, M.E. & Spence, I.** Two types of neurones in the myenteric plexus of duodenum in the guinea-pig. *Journal of Physiology* 236: 303-326, 1974.
72. **Hollins B and Ikeda SR.** Heterologous expression of a P2x-purinoceptor in rat chromaffin cells detects vesicular ATP release. *J Neurophysiol* 78: 3069-3076, 1997.
73. **Hu HZ, Liu S, Gao N, Xia Y, Mostafa R, Ren J, Zafirov DH, and Wood JD.** Actions of bradykinin on electrical and synaptic behavior of neurones in the myenteric plexus of guinea-pig small intestine. *Br J Pharmacol* 138: 1221-1232, 2003.
74. **Jacobson KA, Jarvis MF, and Williams M.** Purine and pyrimidine (P2) receptors as drug targets. *J Med Chem* 45: 4057-4093, 2002.
75. **Jenkinson KM, Morgan JM, Furness JB, and Southwell BR.** Neurons bearing NK(3) tachykinin receptors in the guinea-pig ileum revealed by specific binding of fluorescently labelled agonists. *Histochem Cell Biol* 112: 233-246, 1999.
76. **Jiang LH, Kim M, Spelta V, Bo X, Surprenant A, and North RA.** Subunit arrangement in P2X receptors. *J Neurosci* 23: 8903-8910, 2003.
77. **Johnson LR.** *Physiology of the gastrointestinal tract.* New York: Raven Press, 1994.
78. **Johnson PJ, Shum OR, Thornton PD, and Bornstein JC.** Evidence that inhibitory motor neurons of the guinea-pig small intestine exhibit fast excitatory synaptic potentials mediated via P2X receptors. *Neurosci Lett* 266: 169-172, 1999.
79. **Kellum JM, Budhoo MR, Siriwardena AK, Smith EP, and Jebraili SA.** Serotonin induces Cl<sup>-</sup> secretion in human jejunal mucosa in vitro via a nonneural pathway at a 5-HT<sub>4</sub> receptor. *Am J Physiol* 267: G357-363, 1994.

80. **Kerr PM, Hillier K, Wallis RM, and Garland CJ.** Characterization of muscarinic receptors mediating contractions of circular and longitudinal muscle of human isolated colon. *Br J Pharmacol* 115: 1518-1524, 1995.
81. **Kimball BC and Mulholland MW.** Enteric glia exhibit P2U receptors that increase cytosolic calcium by a phospholipase C-dependent mechanism. *J Neurochem* 66: 604-612, 1996.
82. **Kirchgessner AL and Liu MT.** Differential localization of Ca<sup>2+</sup> channel  $\alpha$ 1 subunits in the enteric nervous system: presence of  $\alpha$ 1B channel-like immunoreactivity in intrinsic primary afferent neurons. *J Comp Neurol* 409: 85-104, 1999.
83. **Kirchgessner AL and Liu MT.** Immunohistochemical localization of nicotinic acetylcholine receptors in the guinea pig bowel and pancreas. *J Comp Neurol* 390: 497-514, 1998.
84. **Kobayashi K, Fukuoka T, Yamanaka H, Dai Y, Obata K, Tokunaga A, and Noguchi K.** Differential expression patterns of mRNAs for P2X receptor subunits in neurochemically characterized dorsal root ganglion neurons in the rat. *J Comp Neurol* 481: 377-390, 2005.
85. **Kunze WA, Furness JB, Bertrand PP, and Bornstein JC.** Intracellular recording from myenteric neurons of the guinea-pig ileum that respond to stretch. *J Physiol* 506 ( Pt 3): 827-842, 1998.
86. **Kunzelmann K and Mall M.** Electrolyte transport in the mammalian colon: mechanisms and implications for disease. *Physiol Rev* 82: 245-289, 2002.
87. **Langley JN.** *The autonomic nervous system.* Cambridge,: W. Heffer & sons, 1921.
88. **Lazarowski ER, Boucher RC, and Harden TK.** Constitutive release of ATP and evidence for major contribution of ecto-nucleotide pyrophosphatase and nucleoside diphosphokinase to extracellular nucleotide concentrations. *J Biol Chem* 275: 31061-31068, 2000.
89. **Lazarowski ER, Boucher RC, and Harden TK.** Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. *Mol Pharmacol* 64: 785-795, 2003.
90. **LePard KJ and Galligan JJ.** Analysis of fast synaptic pathways in myenteric plexus of guinea pig ileum. *Am J Physiol* 276: G529-538, 1999.
91. **LePard KJ, Messori E, and Galligan JJ.** Purinergic fast excitatory postsynaptic potentials in myenteric neurons of guinea pig: distribution and pharmacology. *Gastroenterology* 113: 1522-1534, 1997.
92. **Lewin B.** *Genes VII.* Oxford ; New York: Oxford University Press, 2000.
93. **Lewis C, Neidhart S, Holy C, North RA, Buell G, and Surprenant A.** Coexpression of P2X<sub>2</sub> and P2X<sub>3</sub> receptor subunits can account for ATP-gated currents in sensory neurons. *Nature* 377: 432-435, 1995.
94. **Llewellyn-Smith IJ, Wilson AJ, Furness JB, Costa M, and Rush RA.** Ultrastructural identification of noradrenergic axons and their distribution within the enteric plexuses of the guinea-pig small intestine. *J Neurocytol* 10: 331-352, 1981.

95. **Matsui M, Motomura D, Fujikawa T, Jiang J, Takahashi S, Manabe T, and Taketo MM.** Mice lacking M2 and M3 muscarinic acetylcholine receptors are devoid of cholinergic smooth muscle contractions but still viable. *J Neurosci* 22: 10627-10632, 2002.
96. **Miras-Portugal MT, Diaz-Hernandez M, Giraldez L, Hervas C, Gomez-Villafuertes R, Sen RP, Gualix J, and Pintor J.** P2X7 receptors in rat brain: presence in synaptic terminals and granule cells. *Neurochem Res* 28: 1597-1605, 2003.
97. **Molleman A, Liu LW, and Huizinga JD.** Muscarinic activation of transient inward current and contraction in canine colon circular smooth muscle cells. *Can J Physiol Pharmacol* 79: 34-42, 2001.
98. **Moore BA, Sharkey KA, and Mantle M.** Role of 5-HT in cholera toxin-induced mucin secretion in the rat small intestine. *Am J Physiol* 270: G1001-1009, 1996.
99. **Neunlist M, Frieling T, Rupprecht C, and Schemann M.** Polarized enteric submucosal circuits involved in secretory responses of the guinea-pig proximal colon. *J Physiol* 506 ( Pt 2): 539-550, 1998.
100. **Neunlist M and Schemann M.** Projections and neurochemical coding of myenteric neurons innervating the mucosa of the guinea pig proximal colon. *Cell Tissue Res* 287: 119-125, 1997.
101. **Nicke A, Baumert HG, Rettinger J, Eichele A, Lambrecht G, Mutschler E, and Schmalzing G.** P2X1 and P2X3 receptors form stable trimers: a novel structural motif of ligand-gated ion channels. *Embo J* 17: 3016-3028, 1998.
102. **Nishi S and North RA.** Intracellular recording from the myenteric plexus of the guinea-pig ileum. *J Physiol* 231: 471-491, 1973.
103. **North RA and Surprenant A.** Pharmacology of cloned P2X receptors. *Annual Review of Pharmacology and Toxicology* 40: 563-580, 2000.
104. **Nurgali K, Furness JB, and Stebbing MJ.** Analysis of purinergic and cholinergic fast synaptic transmission to identified myenteric neurons. *Neuroscience* 116: 335-347, 2003.
105. **Nurgali K, Furness JB, and Stebbing MJ.** Correlation of electrophysiology, shape and synaptic properties of myenteric AH neurons of the guinea pig distal colon. *Autonomic Neuroscience* 103: 50-64, 2003.
106. **Nurgali K, Stebbing MJ, and Furness JB.** Correlation of electrophysiological and morphological characteristics of enteric neurons in the mouse colon. *J Comp Neurol* 468: 112-124, 2004.
107. **Olsson C, Costa M, and Brookes SJ.** Neurochemical characterization of extrinsic innervation of the guinea pig rectum. *J Comp Neurol* 470: 357-371, 2004.
108. **Poole DP, Castellucci P, Robbins HL, Chiocchetti R, and Furness JB.** The distribution of P2X3 purine receptor subunits in the guinea pig enteric nervous system. *Autonomic Neuroscience* 101: 39-47, 2002.
109. **Powell AK and Bywater RA.** Murine intestinal migrating motor complexes: longitudinal components. *Neurogastroenterol Motil* 15: 245-256, 2003.

110. **Ralevic V and Burnstock G.** Receptors for purines and pyrimidines. *Pharmacol Rev* 50: 413-492, 1998.
111. **Redegeld FA, Caldwell CC, and Sitkovsky MV.** Ecto-protein kinases: ecto-domain phosphorylation as a novel target for pharmacological manipulation? *Trends Pharmacol Sci* 20: 453-459, 1999.
112. **Ren J, Bian X, DeVries M, Schnegelsberg B, Cockayne DA, Ford AP, and Galligan JJ.** P2X2 subunits contribute to fast synaptic excitation in myenteric neurons of the mouse small intestine. *J Physiol* 552: 809-821, 2003.
113. **Ruan HZ and Burnstock G.** The distribution of P2X(5) purinergic receptors in the enteric nervous system of mouse. *Cell Tissue Res*, 2004.
114. **Rugiero F, Gola M, Kunze WA, Reynaud JC, Furness JB, and Clerc N.** Analysis of whole-cell currents by patch clamp of guinea-pig myenteric neurones in intact ganglia. *J Physiol* 538: 447-463, 2002.
115. **Sanders KM.** G protein-coupled receptors in gastrointestinal physiology. IV. Neural regulation of gastrointestinal smooth muscle. *Am J Physiol* 275: G1-7, 1998.
116. **Sanger GJ and McClelland CM.** Increased gastric cholinergic activity evoked by 5-hydroxy-L-tryptophan in the rat. *Eur J Pharmacol* 127: 179-185, 1986.
117. **Sanger GJ, Yoshida M, Yahyah M, and Kitazumi K.** Increased defecation during stress or after 5-hydroxytryptophan: selective inhibition by the 5-HT(4) receptor antagonist, SB-207266. *British Journal of Pharmacology* 130: 706-712, 2000.
118. **Saunders DE, Rubin CE, and Ostrow JD.** Small Bowel. In: *University of Washington Division of Gastroenterology HuBio551 Course* (35 ed.). 1959 N.E. Pacific, Seattle, Washington 98195: University of Washington, 2004.
119. **Sawyer GW and Ehlert FJ.** Contractile roles of the M2 and M3 muscarinic receptors in the guinea pig colon. *J Pharmacol Exp Ther* 284: 269-277, 1998.
120. **Schneider DA and Galligan JJ.** Presynaptic nicotinic acetylcholine receptors in the myenteric plexus of guinea pig intestine. *American Journal of Physiology Gastrointestinal and Liver Physiology* 279: G528-535, 2000.
121. **Schneider DA, Perrone M, and Galligan JJ.** Nicotinic acetylcholine receptors at sites of neurotransmitter release to the guinea pig intestinal circular muscle. *J Pharmacol Exp Ther* 294: 363-369, 2000.
122. **Searl TJ, Redman RS, and Silinsky EM.** Mutual occlusion of P2X ATP receptors and nicotinic receptors on sympathetic neurons of the guinea-pig. *J Physiol* 510 ( Pt 3): 783-791, 1998.
123. **Silinsky EM and Redman RS.** Synchronous release of ATP and neurotransmitter within milliseconds of a motor nerve impulse in the frog. *J Physiol* 492 ( Pt 3): 815-822, 1996.
124. **Sim JA, Young MT, Sung HY, North RA, and Surprenant A.** Reanalysis of P2X7 receptor expression in rodent brain. *J Neurosci* 24: 6307-6314, 2004.

125. **Smith TK and Robertson WJ.** Synchronous movements of the longitudinal and circular muscle during peristalsis in the isolated guinea-pig distal colon. *J Physiol* 506 ( Pt 2): 563-577, 1998.
126. **Song ZM, Brookes SJ, and Costa M.** All calbindin-immunoreactive myenteric neurons project to the mucosa of the guinea-pig small intestine. *Neurosci Lett* 180: 219-222, 1994.
127. **Soto F, Garcia-Guzman M, Gomez-Hernandez JM, Hollmann M, Karschin C, and Stuhmer W.** P2X4: an ATP-activated ionotropic receptor cloned from rat brain. *Proc Natl Acad Sci U S A* 93: 3684-3688, 1996.
128. **Soto F, Garcia-Guzman M, and Stuhmer W.** Cloned ligand-gated channels activated by extracellular ATP (P2X receptors). *J Membr Biol* 160: 91-100, 1997.
129. **Spencer NJ, Hennig GW, and Smith TK.** Electrical rhythmicity and spread of action potentials in longitudinal muscle of guinea pig distal colon. *American Journal of Physiology Gastrointestinal and Liver Physiology* 282: G904-917, 2002.
130. **Spencer NJ, Hennig GW, and Smith TK.** A rhythmic motor pattern activated by circumferential stretch in guinea-pig distal colon. *J Physiol* 545: 629-648, 2002.
131. **Spencer NJ, Hennig GW, and Smith TK.** Stretch-activated neuronal pathways to longitudinal and circular muscle in guinea pig distal colon. *American Journal of Physiology Gastrointestinal and Liver Physiology* 284: G231-241, 2003.
132. **Spencer NJ, Walsh M, and Smith TK.** Purinergic and cholinergic neuro-neuronal transmission underlying reflexes activated by mucosal stimulation in the isolated guinea-pig ileum. 522 Pt 2: 321-331, 2000.
133. **Straub RW and Bolis L.** *Cell membrane receptors for drugs and hormones : a multidisciplinary approach.* New York: Raven Press, 1978.
134. **Tai C, Booth AM, de Groat WC, and Roppolo JR.** Colon and anal sphincter contractions evoked by microstimulation of the sacral spinal cord in cats. *Brain Res* 889: 38-48, 2001.
135. **Torres GE, Egan TM, and Voigt MM.** Identification of a domain involved in ATP-gated ionotropic receptor subunit assembly. *J Biol Chem* 274: 22359-22365, 1999.
136. **Valera S, Hussy N, Evans RJ, Adami N, North RA, Surprenant A, and Buell G.** A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP. *Nature* 371: 516-519, 1994.
137. **Valera S, Talabot F, Evans RJ, Gos A, Antonarakis SE, Morris MA, and Buell GN.** Characterization and chromosomal localization of a human P2X receptor from the urinary bladder. *Receptors Channels* 3: 283-289, 1995.
138. **Vanner S and Macnaughton WK.** Submucosal secretomotor and vasodilator reflexes. *Neurogastroenterol Motil* 16: 39-43, 2004.
139. **Vial C and Evans RJ.** Smooth muscles does not have a common P2x receptor phenotype: expression, ontogeny and function of P2x1 receptors in mouse ileum, bladder and reproductive systems. *Autonomic Neuroscience* 92: 56-64, 2001.

140. **Xie DP, Chen LB, Liu CY, Zhang CL, Liu KJ, and Wang PS.** Arecoline excites the colonic smooth muscle motility via M3 receptor in rabbits. *Chin J Physiol* 47: 89-94, 2004.
141. **Yiangou Y, Facer P, Baecker PA, Ford AP, Knowles CH, Chan CL, Williams NS, and Anand P.** ATP-gated ion channel P2X(3) is increased in human inflammatory bowel disease. *Neurogastroenterol Motil* 13: 365-369, 2001.
142. **Young HM, Kunze WA, Pompolo S, Furness JB, and Bornstein JC.** Combined intracellular injection of Neurobiotin and pre-embedding immunocytochemistry using silver-intensified gold probes in myenteric neurons. *J Neurosci Methods* 51: 39-45, 1994.
143. **Zimmermann H.** Two novel families of ectonucleotidases: molecular structures, catalytic properties and a search for function. *Trends Pharmacol Sci* 20: 231-236, 1999.

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