GHENT UNIVERSITY

FACULTY OF MEDICINE AND HEALTH SCIENCES

Ghent University
Faculty of Medicine and Health Sciences
Department of Pharmacology – Heymans Institute

PHOSPHODIESTERASE-MEDIATED REGULATION OF 5-HT$_4$ RECEPTORS, FACILITATING CHOLINERGIC NEUROTRANSMISSION, IN THE GASTROINTESTINAL TRACT

Vicky Pauwelyn

Promotor: Prof. dr. Romain A. Lefebvre

Dissertation submitted to Ghent University in fulfilment of the requirements for the degree of Doctor in Health Sciences

2018

GHENT UNIVERSITY

FACULTY OF MEDICINE AND HEALTH SCIENCES

Ghent University
Faculty of Medicine and Health Sciences
Department of Pharmacology – Heymans Institute

PHOSPHODIESTERASE-MEDIATED REGULATION OF 5-HT$_4$ RECEPTORS, FACILITATING CHOLINERGIC NEUROTRANSMISSION, IN THE GASTROINTESTINAL TRACT

Vicky Pauwelyn

Promotor: Prof. dr. Romain A. Lefebvre

Dissertation submitted to Ghent University in fulfilment of the requirements for the degree of Doctor in Health Sciences

2018

PROMOTOR

Prof. dr. Romain A. Lefebvre
Department of Pharmacology – Heymans Institute
Faculty of Medicine and Health Sciences
Ghent University, Belgium

READING AND EXAMINATION COMMITTEE

Prof. dr. Ernst Rietzschel (president)
Biobanking & Cardiovascular Prevention
Department of Internal medicine
University Hospital Ghent, Belgium

Prof. dr. Johan Van de Voorde
Vascular Research Unit
Department of Pharmacology – Heymans Institute
Faculty of Medicine and Health Sciences
Ghent University, Belgium

Prof. dr. Danny De Looze
Department of Gastroenterology
University Hospital Ghent, Belgium

Prof. dr. Ken Bracke
Laboratory for Translational Research in Obstructive Pulmonary Diseases
Department of Respiratory Medicine
University Hospital Ghent, Belgium

Dr. Sarah Cosyns
Laboratory for Experimental Surgery
Department of Surgery
Faculty of Medicine and Health Sciences
Ghent University, Belgium

Prof. dr. Inge Depoortere
Translational Research Center for Gastrointestinal Disorders (TARGID)
Gut Peptide Research Lab
Department of Chronic Diseases, Metabolism and Ageing
Faculty of Medicine
University of Leuven, Belgium

Prof. dr. Gareth Sanger
National Bowel Research Centre
Blizard Institute
Queen Mary University of London, United Kingdom

Table of contents

List of abbreviations

	5'-AMP	5'-adenosine monophosphate
	5'-GMP	5'-guanosine monophosphate
	5-HIAA	5-hydroxyindoleacetic acid
	5-HT	5-hydroxytryptamine; serotonin
	5-HT_4 receptor	5-hydroxytryptamine 4 receptor
A	AC	adenylyl cyclase
	ACh	acetylcholine
	ANOVA	analysis of variance
	ATP	adenosine triphosphate
	AUC	area under the curve
C	cAMP	cyclic adenosine monophosphate; adenosine-3',5'-cyclic monophosphate
	cGMP	cyclic guanosine monophosphate; guanosine-3',5'-cyclic monophosphate
	cilo	cilostamide
	CNS	central nervous system
	COPD	chronic obstructive pulmonary disease
D	dpm	disintegrations per minute
	DMSO	dimethyl sulfoxide
E	EC	enterochromaffin
	EFS	electrical field stimulation
	EHNA	erythro-9-(2-hydroxy-3-nonyl)adenine
	ENS	enteric nervous system
G	GDP	guanosine diphosphate
	GI	gastrointestinal
	GPCR	G protein-coupled receptor
	GTP	guanosine triphosphate
H	hERG	human ether-a-go-go-related gene
I	IBMX	3-isobutyl-1-methylxanthine
	IBS(-C)	irritable bowel syndrome (with constipation)
	IC_{50}	half maximal inhibitory concentration
	ICC	interstitial cell of Cajal

IPAN ..... intrinsic primary afferent neuron

K K_i ..... inhibitory constant

L L-NAME ..... N_ω-nitro-L-arginine methyl ester hydrochloride

M MAO ..... monoamine oxidase
MMC ..... migrating motor complex

M ND ..... not determined
NO ..... nitric oxide
ns ..... not significant

P PDE ..... phosphodiesterase; cyclic nucleotide phosphodiesterase
PKA ..... protein kinase A
PKC ..... protein kinase C
PKG ..... protein kinase G
pru ..... prucalopride

R roflu ..... roflumilast
roli ..... rolipram

S S_1 ..... first application of EFS at the 13th min (sample 5)
S_2 ..... second application of EFS at the 73rd min (sample 25)
S_2/S_1 ..... ratio of the tritium release by S_2 compared to the release by S_1
S.E.M ..... standard error of the mean
SERT ..... serotonin transporter
SOM ..... somatostatin

T TPH ..... tryptophan hydroxylase
TK ..... tachykinins
TTX ..... tetrodotoxin

V V_{max} ..... supramaximal voltage
$V_{50\%}$ ..... submaximal voltage inducing contractions with an amplitude of approximately 50% of that obtained at V_{max}
velu ..... velusetrag
vinpo ..... vinpocetine
VIP ..... vasoactive intestinal peptide

CHAPTER I

Literature survey

CHAPTER I - Literature survey

I.1 Gastrointestinal motility

I.1.1 Anatomy of the digestive system

I.1.1.1 Macroscopic

The human digestive system includes two main parts: the gastrointestinal (GI) tract and the accessory digestive organs which lie externally to the GI tract (Figure I.1). The GI tract is a 6 to 9 m long canal that stretches from mouth, over the pharynx, esophagus, stomach, small and large intestine, to the anus ending with the inner and outer anal sphincters (Boron and Boulpaep, 2009). These segments are separated by sphincters: upper and lower esophageal sphincter, pylorus and ileocecal valve. The stomach, small and large intestine are further subdivided in regions (Figure I.1). The teeth, tongue, gallbladder and digestive glands (salivary glands, liver and exocrine pancreas) are the accessory digestive organs; the digestive glands are connected to the GI tract by ducts and empty their content into the GI tract.

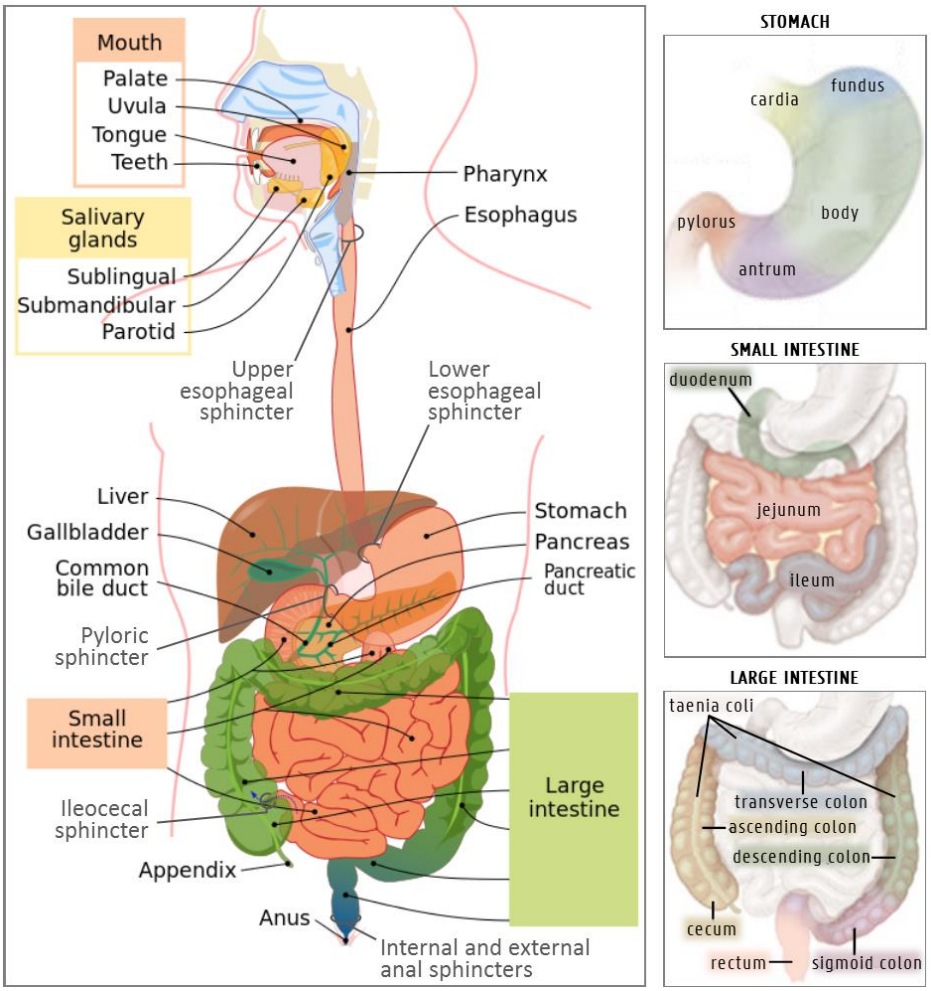


Figure I.1 – Schematic overview of the macroscopic anatomy of the human digestive tract consisting of the GI tract and the accessory digestive organs (left; adapted from Villarreal, 2016). Subdivision of the stomach, small and large intestine in regions (right; adapted from Hightower *et al.*, 2003).

I.1.1.2 Microscopic

The structure of the wall of the GI tract varies along its length, but has a quite similar characteristic organization with four concentric layers in common (Figure I.2): mucosa, submucosa, muscularis and serosa (Boron and Boulpaep, 2009; Barrett *et al.*, 2010).

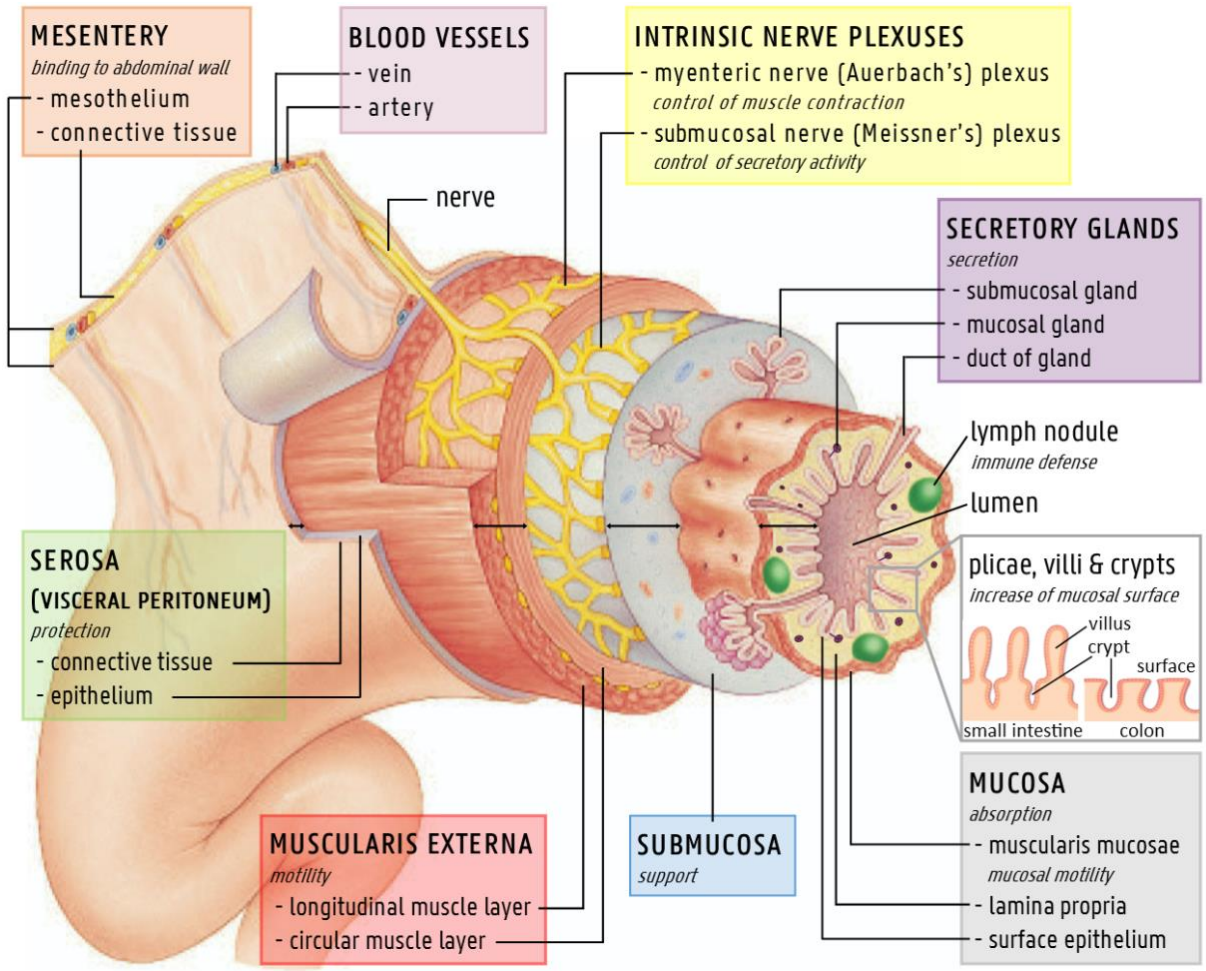


Figure I.2 – Cross section of the GI tract: microscopic layers and their main function (in italic). Adapted from Seeley *et al.*, 2004; Koeppen and Stanton, 2008.

The innermost layer enclosing the intestinal lumen is the **mucosa** and consists of three sublayers (Boron and Boulpaep, 2009; Barrett *et al.*, 2010). The first cell layer is the columnar epithelium, this is the zone where the enterochromaffin (EC) cells are located (enteroendocrine cells synthetizing serotonin [5-hydrotryptamine; 5-HT] and functioning as sensory transducers that respond to chemical and mechanical intraluminal stimuli (Mawe and Hoffman, 2013); see I.2.1). An underlying loose connective tissue layer, known as the lamina propria, contains mucosal capillaries, enteric nerve endings and immune cells. The third sublayer of the mucosa, called the muscularis mucosae, is a thin smooth muscle layer which produces local movement and folding of the mucosa. In the small intestine, the surface area of the mucosa is amplified by macroscopic organization into large folds or plicae, by folding in and out of the epithelial layer forming villi and crypts, and by microscopic folds on the apical surfaces of the epithelial cells called microvilli. In the mucosa some variations are found along the different regions in

the GI tract with distinct cell types in the epithelium (amongst others more than 15 types of enteroendocrine cells have been identified; Barrett *et al.*, 2010) and variation in the realization of the mucosal surface enlargement. The transition of the mucosa from one form to another is abrupt at four points along the GI tract: the gastro-esophageal, gastroduodenal, ileocecal and recto-anal junction. For example, villi are present in the small intestine but not in the large intestine.

The mucosa is surrounded by a second layer, namely the **submucosa**. It consists of loose connective tissue with larger blood vessels, lymphatic components, the submucosal nervous plexus or Meissner's plexus and secretory glands.

The third layer is the **muscularis externa**, consisting of an inner circular and an outer longitudinal muscle layer (Boron and Boulpaep, 2009; Barrett *et al.*, 2010). They are involved in GI motility (see I.1.4) and are controlled by the myenteric nervous plexus or Auerbach's plexus (see I.1.3.1), a dense network of neurons and neuronal fibers in between the circular and longitudinal muscle layers.

The outer layer, the adventitia is a thin connective tissue layer covered with squamous epithelial cells surrounding the GI tract (Boron and Boulpaep, 2009; Barrett *et al.*, 2010). It conducts the major vessels and nerves. Where the gut lies within the abdominal cavity, the adventitia is referred to as the **serosa** or visceral peritoneum.

I.1.2 Physiological processes in the GI tract

Beside the vital role in immune defense, the main function of the digestive system is to efficiently digest food allowing absorption of sufficient nutrients, to fulfil the daily caloric need of approximately 30 kcal/kg body weight (in sedentary conditions), and to eliminate waste (Boron and Boulpaep, 2009). This is fulfilled by several major physiologic processes (Figure I.3): motility providing propulsion and mechanical digestion, secretion for chemical digestion, absorption and defecation. Each part of the GI tract has evolved to contribute in a specialized way to these processes.

In the mouth ingested food is mechanically disrupted by chewing (Boron and Boulpaep, 2009). The salivary and lingual enzymes, amylase for carbohydrates and lipase for lipids, initiate carbohydrate and fat digestion. Food is propelled from the mouth via the oropharynx into the esophagus during swallowing and transported along the esophagus to the stomach.

The stomach temporarily stores the food, further mechanically disrupts it, initiates protein enzymatic digestion by secreting pepsin, sterilizes the meal by acid secretion, continues lipid digestion by secretion of gastric lipase, and allows passage of the gastric content through the pylorus into the duodenum (Boron and Boulpaep, 2009).

The pancreas secretes HCO_3^- to neutralize gastric acid and digestive enzymes (lipase, amylase, trypsin, chymotrypsin, elastase and carboxypeptidase A and B) into the small intestine where lipid, protein and carbohydrate digestion is continued and completed by these pancreatic enzymes and by the enzymes at the brush border of the small intestine (Boron and Boulpaep, 2009; Barrett *et al.*, 2010). The gallbladder stores bile containing bile acids, which are secreted by the liver, and delivers the bile to the small intestine during a meal where it plays a key role in lipid digestion. The small intestine not only digests food, it is also the primary site for the absorption of water, electrolytes and nutrients (lipids,

carbohydrates and amino acids) into blood and lymph capillaries in the wall of the GI tract. The large intestine further reabsorbs fluids and electrolytes and also stores the fecal matter before it leaves the body by the defecation process.

From mouth to anus, progression of the intestinal content throughout the GI tract is ensured by the so called propulsion provided by the muscular layer of the GI wall. In addition the flow of the food bolus is restricted ante- and retrogradely by the upper and lower esophageal sphincter, the pylorus, the ileocecal sphincter, the inner and outer anal sphincters (Figure I.1) (Boron and Boulpaep, 2009). Propulsion and mechanical digestion are the two motility processes in the GI tract as further discussed in I.1.4.

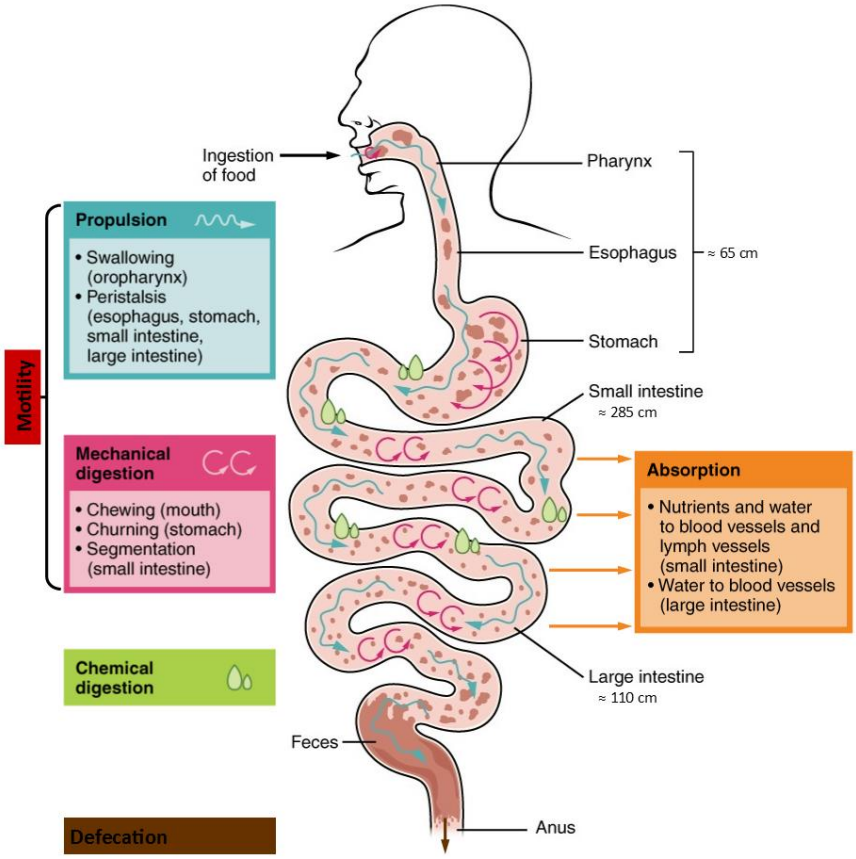


Figure I.3 – Schematic summary of the physiological processes that take place in the GI tract. Adapted from OpenStax - Rice University, 2013.

To allow a continuous optimal adjustment in secretion, motility and absorption in response to a meal the physiological processes in the GI tract are well coordinated by the interplay of (Boron and Boulpaep, 2009):

- Neuronal control by extrinsic and intrinsic nerves; this is further discussed in I.1.3.
- Hormonal control by GI hormones (e.g. gastrin and secretin) produced by specialized epithelial cells, called enteroendocrine cells, located in the mucosal epithelium of the stomach and small intestine. These hormones reach their targets via the bloodstream.
- Local factors such as nutrients and pH of the luminal contents.

- The immune system through mast cells in the lamina propria, which are sensitive to neurotransmitters and are capable to release chemical mediators (e.g. histamine) directly influencing smooth muscle and epithelial cells.

I.1.3 Neuronal control of the GI tract

The neuronal control of the GI tract consists of intrinsic nerves located within the GI tract, the so-called enteric nervous system (ENS), and by extrinsic nerves localized outside the GI tract (Boron and Boulpaep, 2009; Barrett *et al.*, 2010).

I.1.3.1 Intrinsic nerves: the ENS

The ENS is a complex reflex circuit consisting of approximately 100 million neurons which is located solely within the GI tract (Boron and Boulpaep, 2009; Barrett *et al.*, 2010). The ENS can operate totally independently within the GI tract, without participation of the extrinsic pathways, but can be modulated by input from them.

The neurons of the ENS are primarily, but not exclusively, clustered in two ganglionic plexuses (Figure I.2): the submucosal or Meissner's plexus and the myenteric or Auerbach's plexus (Boron and Boulpaep, 2009). The submucosal plexus is located in the submucosa of man in the stomach, small and large intestine. In experimental animals (mouse, rat and guinea pig) it is only prominent in the small and large intestine; some isolated ganglia can be found in the esophagus and stomach. The submucosal plexus innervates glandular epithelium, intestinal endocrine cells and submucosal blood vessels and is primarily involved in the control of intestinal secretion (Barrett *et al.*, 2010). The myenteric plexus lies between the circular and longitudinal muscle layers throughout the GI tract from the proximal end of the esophagus to the rectum. This plexus controls GI smooth muscle contraction and relaxation and is thus primarily involved in GI motility (Barrett *et al.*, 2010).

Three neuron types are functionally characterized in the ENS forming interneuronal connections, sensory and (secreto)motor circuits (Boron and Boulpaep, 2009):

- Sensory or afferent neurons: monitor changes in luminal activity, including mucosal chemical (e.g. pH, osmolality, nutrients) and/or mechanical stimulation (wall distension). These stimuli or 5-HT released from EC cells stimulate the sensory neurons.
- Interneurons: are activated by sensory neurons and activate the efferent (secreto)motor neurons.
- Efferent (secreto)motor neurons: stimulate or inhibit the effector cells, namely smooth muscle, epithelial cells, enteric endocrine cells and submucosal blood vessels.

I.1.3.2 Extrinsic nerves

Although the ENS can operate independently of the central nervous system (CNS), the neuronal control of GI function is also modified by extrinsic pathways that receive input from the CNS and thus forms a bidirectional communication between the ENS and CNS (brain-gut axis). The extrinsic nervous system can be subdivided in the para- and orthosympathetic nervous system. It innervates the GI tract and contributes to the control of motility, secretion and blood flow (Spiller *et al.*, 2004). Extrinsic nerves are

able to modulate, but not entirely control, the motor activity. Via afferent fibers information from chemoreceptors, osmoreceptors and mechanical receptors is carried to autonomic centers, and an effect is produced by efferent fibers. Parasympathetic stimulation results in increased secretion and motility, and sphincter relaxation; orthosympathetic stimulation has the opposite effect.

The **parasympathetic** nervous system innervating the GI tract is supplied by the vagus nerve, innervating the esophagus, stomach, gallbladder, pancreas, small intestine, cecum and proximal part of the colon, and the pelvic nerves, innervating the distal part of the colon, rectum and anal canal. Parasympathetic afferent fibers transmit information from sensory nerve endings in the gut to the CNS through cell bodies in the nodose ganglia projecting to the brainstem (vagus nerve) or in the dorsal root ganglia (pelvic nerves). Preganglionic efferent parasympathetic fibers, originating from the brainstem (vagus nerve) or sacral spinal cord (pelvic nerves), run to the gut wall where they synapse with postganglionic excitatory or inhibitory enteric neurons of the ENS.

Afferent **orthosympathetic** fibers transfer information from the gut to the CNS by sensory neurons that have their endings in the gut wall and cell bodies in the dorsal root ganglia. Preganglionic efferent orthosympathetic fibers, which are cholinergic, arise within the thoracolumbar part of the spinal cord and make nicotinic synapses in the para- and/or prevertebral ganglia with postganglionic noradrenergic neurons that innervate primarily the ENS or directly end on secretory, absorptive or muscle cells.

Although GI functions are controlled by the autonomic nervous system and occur mainly independent of conscious perception, autonomic nerves innervating the GI tract communicate with higher CNS centers that influence the GI tract as well as cognitive and behavioral functions (Browning and Travagli, 2014). For example, the sight and smell of food increase gastric acid secretion through vagal fibers.

I.1.4 GI motility

Beside the segment specific patterns, such as chewing, swallowing, vomiting, gastric emptying and defecation, GI motility includes some general patterns, namely segmentation (retardation and mixing), peristalsis (propulsion) and migrating motor complex (MMC) (Barrett *et al.*, 2010).

MMCs are cycles of motor activity that migrate from the stomach to the distal ileum at a rate of 5 cm/min (Barrett *et al.*, 2010). They occur during fasting between periods of digestion at intervals of approximately 90 min and are immediately stopped by ingestion of food, with a return to the GI motility pattern with peristalsis and segmentation. MMCs likely serve to clear the stomach and small intestine of luminal contents in preparation for the next meal. At the level of the stomach MMCs are initiated by motilin and stopped by ingestion of food, which suppresses motilin release.

Peristalsis (Figure I.4 A) is the result of a series of local reflexes initiated by an intraluminal stimulus and resulting in muscle contraction above and relaxation below the intraluminal stimulus (Barrett *et al.*, 2010); this is further discussed in I.1.5. The wave of contraction/relaxation moves from oral to caudal and propels the food bolus forward at a rate varying from 2 to 25 cm/s. Peristaltic activity occurs from the esophagus to the rectum and its occurrence is regulated by the ENS, independently of extrinsic innervation, but can be influenced by autonomic input.

Segmentation (Figure I.4 B) is related to peristalsis, but is designed on the one hand to retard the movement of the intestinal contents along the GI tract to provide time for digestion and absorption (Barrett *et al.*, 2010). On the other hand it mixes the intestinal content with digestive secretions promoting chemical digestion. Segmentation is achieved by contraction at both ends of a bowel segment followed by a second contraction in the center of the segment forcing the contents partly backward and partly forward.

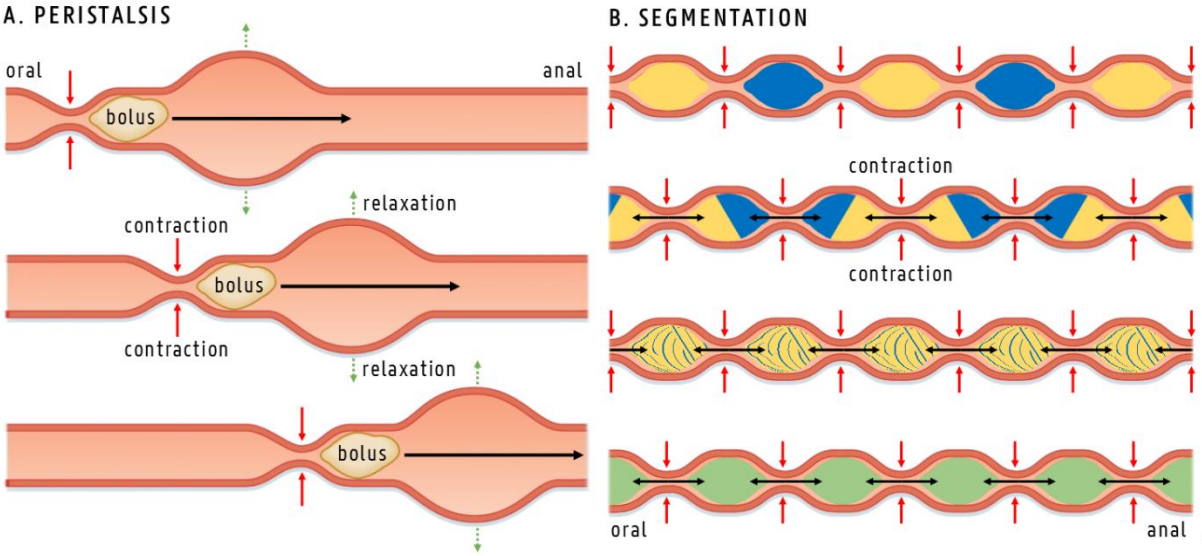


Figure I.4 - Schematic representation of peristalsis (A) and segmentation (B). Contractions and relaxations of the smooth muscle are indicated with red and green dotted arrows respectively. Based upon Koeppen and Stanton, 2008.

I.1.5 Neuronal regulation of peristalsis: intrinsic reflex circuits

A unique feature of the GI tract is the existence of intrinsic reflex circuits consisting of neurons of the ENS that regulate epithelial secretion, vasodilation and motility. Peristalsis is the result of a series of local reflexes consisting of a muscle contraction above and relaxation below the intraluminal stimulus (Figure I.5). Mucosal chemical (e.g. pH, osmolality, nutrients) and/or mechanical stimulation (wall distension) stimulate sensory or intrinsic primary afferent neurons (IPANs) directly or via 5-HT released from EC cells (De Ponti, 2004). IPANs relay the signal to ascending as well as descending interneurons. Above the site of stimulus, ascending interneurons activate excitatory motor neurons that release acetylcholine and substance P, producing contraction of the smooth muscle. At the same time below the site of stimulus, descending cholinergic interneurons activate inhibitory motor neurons that release nitric oxide (NO), vasoactive intestinal peptide (VIP) and adenosine triphosphate (ATP), causing smooth muscle relaxation ahead of the stimulus. As result of the ascending contraction and descending relaxation, the food bolus is propelled in anterograde direction. These local reflexes involved in peristalsis can be influenced by 5-HT and its receptors since the neurons and smooth muscle cells involved here are endowed with 5-HT receptors, such as the 5-HT_4 receptor.

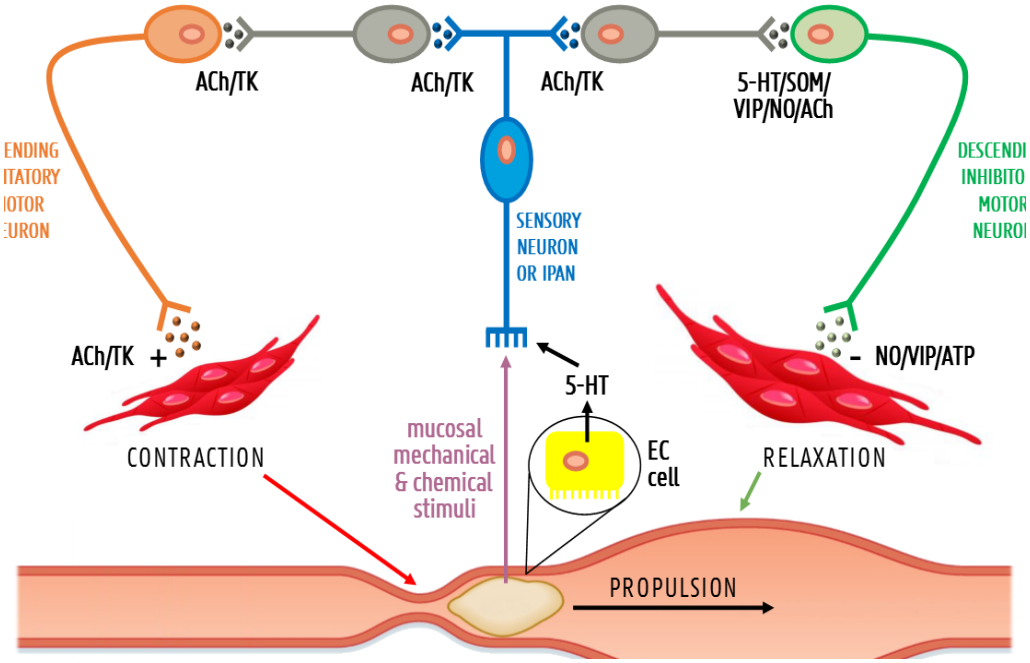


Figure I.5 - Neuronal regulation of peristalsis: intrinsic reflex circuits. Mucosal chemical (e.g. pH, osmolality, nutrients) and/or mechanical stimulation (wall distension) stimulate sensory neurons directly or via 5-HT released from enterochromaffin (EC) cells. The sensory neurons trigger ascending as well as descending interneurons. Ascending interneurons release acetylcholine (ACh) and substance P; descending interneurons release 5-HT, somatostatin (SOM), vasoactive intestinal peptide (VIP), nitric oxide (NO), ACh and other mediators. The ascending interneurons activate ascending excitatory motor neurons that release Ach and tachykinins (TK) at the muscular junction, which leads to smooth muscle contraction; whereas descending interneurons activate descending inhibitory motor neurons that release NO, VIP or adenosine triphosphate (ATP) (depends on the level of the GI tract and animal species) at the muscular junction, which induces smooth muscle relaxation. The ascending contraction owing to the ascending excitatory reflex and the descending relaxation owing to the descending inhibitory reflex happen simultaneously and result in propulsion of the food bolus. Based upon De Ponti, 2004; Koeppen and Stanton, 2008.

I.2 5-HT$_4$ receptors

I.2.1 5-HT and its receptors

5-HT is a biogenic monoamine; it was isolated and characterized 70 years ago by Rapport et al. (Rapport *et al.*, 1948). Even though 5-HT is best known for its role in the brain, about 95% of 5-HT in the human body is located in the periphery mostly stored in GI EC cells and platelets (Gershon and Tack, 2007; Mohammad-Zadeh *et al.*, 2008); of the human body 5-HT, approximately 5-8% is present in platelets and about 90-95% is located in the GI tract in EC cells (Szeitz and Bandiera, 2018). Already in 1937, Vittorio Erspamer extracted a substance from EC cells in the GI tract that was causing smooth muscle contraction. The molecule was then called enteramine and in 1952 it was found to be the same as 5-HT (Erspamer and Asero, 1952). 5-HT is a hormone, neurotransmitter and mitogen (mitosis stimulating or inducing substance), which is present in many tissues including brain, lung, kidney, platelets and the GI tract, where it is involved in normal physiology with amongst others effects on sleep and appetite, and mediation of behavioral effects, GI motility, vascular tone and platelet aggregation, as well as in the pathogenesis of certain diseases (Mohammad-Zadeh *et al.*, 2008; Ruddell *et al.*, 2008; Berger *et al.*, 2009) (Figure I.6). Diseases of the brain where 5-HT has been implicated include psychological disorders such as depression and schizophrenia, migraine, Alzheimer's disease; peripheral diseases associated with 5-HT are cardiovascular diseases such as systemic and pulmonary hypertension and GI disorders such as irritable bowel syndrome (Mohammad-Zadeh *et al.*, 2008; Ruddell *et al.*, 2008; Berger *et al.*, 2009).

5-HT is **synthesized** from the essential amino acid tryptophan (Figure I.7) and accounts for 5% of the total metabolism of tryptophan (the other 95% is enzymatically conversed in the liver by tryptophan pyrrolase to kynurenine). It is a two-step process with involvement of the enzymes tryptophan hydroxylase (TPH), more specific TPH1 expressed in the periphery and TPH2 in the brain and enteric neurons, and L-aromatic amino acid decarboxylase (Mohammad-Zadeh *et al.*, 2008; Gershon, 2013; Kendig and Grider, 2015; Coates *et al.*, 2017). L-aromatic amino acid decarboxylase has affinity for many L-amino acids and is present in most tissues (Mohammad-Zadeh *et al.*, 2008). This contrasts to the specific TPH which is the rate-limiting enzyme with only limited affinity for other amino acids than L-tryptophan and a tissue distribution limited to the brain, EC cells and myenteric neurons, and to a much lesser extent, platelets (Nicholson *et al.*, 1991). 5-HT is thus synthesized in the CNS (< 1% of 5-HT in human body is synthesized in serotonergic neurons in the brain), but also peripherally in the GI tract, mainly in EC cells (accounts for the synthesis of 90% of 5-HT in human body) and to a lesser extent in enteric neurons, and to a small extent in platelets, that have very little ability to produce 5-HT but are a major storage site for it (Mohammad-Zadeh *et al.*, 2008; Ruddell *et al.*, 2008; Szeitz and Bandiera, 2018). In mice, but not in man (in normal physiological condition), mast cells in the mucosal lamina propria are also capable of synthesizing and releasing 5-HT (Mawe and Hoffman, 2013).

5-HT is **metabolized** by the intracellular enzyme monoamine oxidase (MAO) to 5-hydroxyindoleacetaldehyde which is further degraded by an aldehyde dehydrogenase leading to the major stable metabolite 5-hydroxyindoleacetic acid (5-HIAA) which is excreted primarily in the urine (Mohammad-Zadeh *et al.*,

2008; Mawe and Hoffman, 2013). MAO-A is responsible for the major portion of the 5-HT metabolism while MAO-B, being the predominant MAO isoform in platelets, is only responsible for a small portion of 5-HT metabolism (Beavo, 1995). Major sites of MAO activity are the brain, GI tract, platelets, lungs and liver (Mohammad-Zadeh *et al.*, 2008). Although metabolism occurs very rapidly, **storage**, mainly in EC cells, platelets and neurons, protects 5-HT against metabolism by MAO (Mohammad-Zadeh *et al.*, 2008). Other peripheral cells that contain 5-HT are macrophages and mast cells.

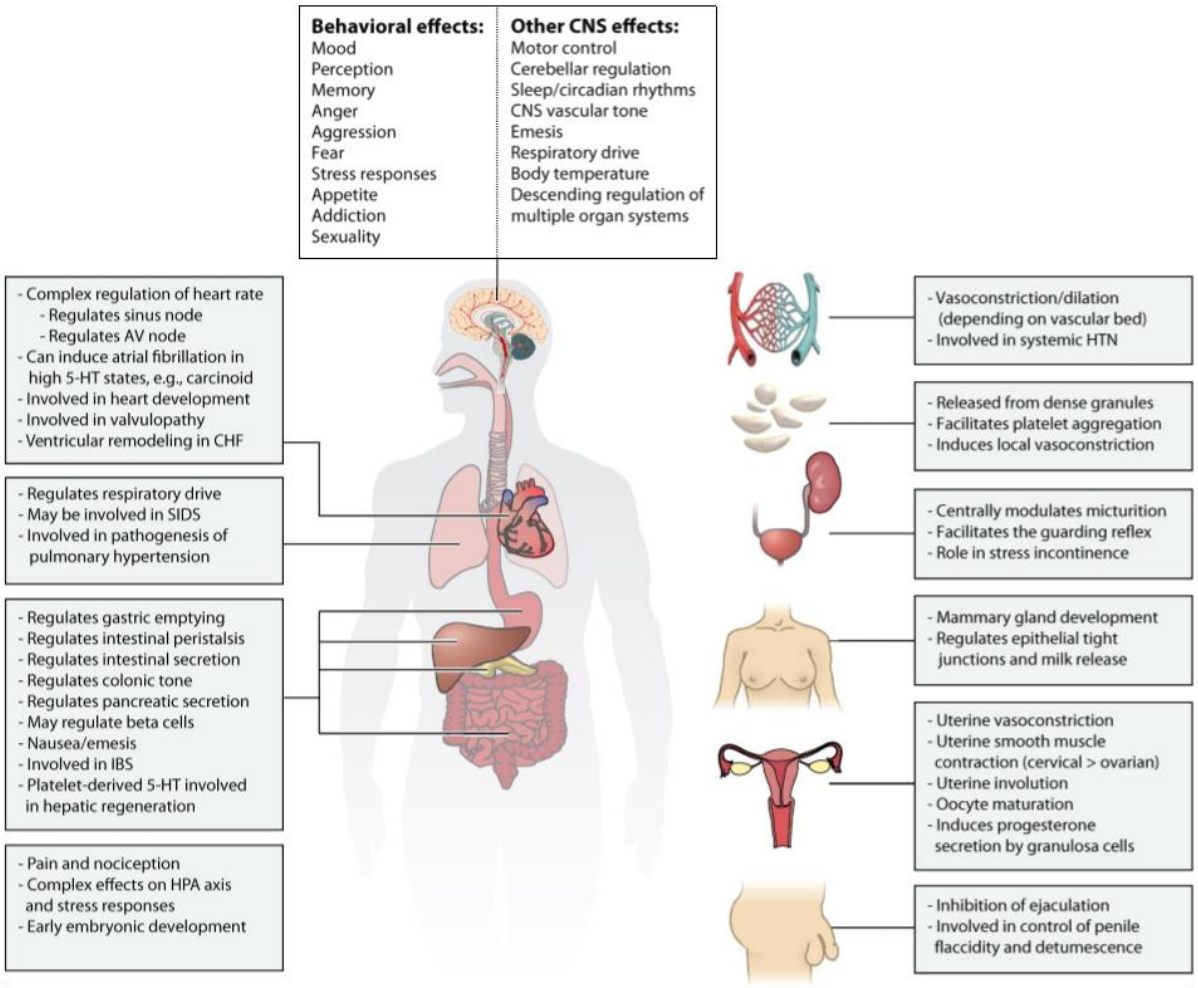


Figure I.6 - Myriad central and peripheral effects of 5-HT. Abbreviations: AV, atrioventricular; CHF, congestive heart failure; CNS, central nervous system; HPA, hypothalamic-pituitary-adrenal; HTN, hypertension; IBS, irritable bowel syndrome; SIDS, sudden infant death syndrome. Adapted from Berger et al., 2009.

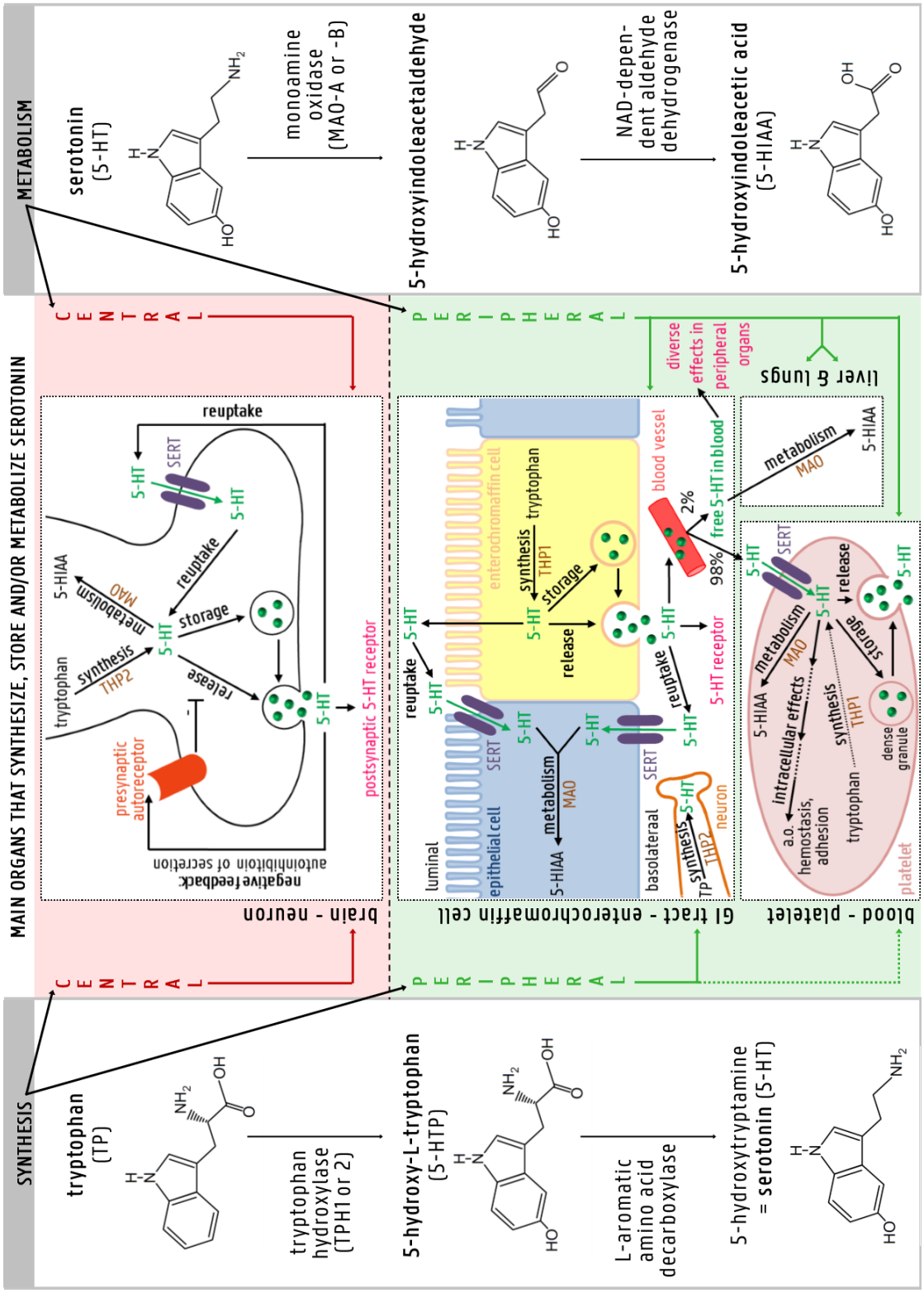


Figure I.7 - Main localizations of 5-HT synthesis, storage and metabolism. Synthesis in the brain, GI tract (mainly enterochromaffin cells and to a lesser extent in enteric neurons) and, to a much lesser extent, platelets: tryptophan (TP) is hydroxylated by tryptophan hydroxylase (TPH) type 1 (peripheral) or 2 (brain and enteric neurons) to 5-hydroxytryptophan (5-HTP), which is decarboxylated by L-aromatic amino acid decarboxylase to form 5-hydroxytryptamine (5-HT). Metabolism in the brain, GI enterochromaffin cells, platelets, liver and lungs: monoamine oxidase (MAO) A and B, and NAD-dependent aldehyde dehydrogenase metabolize 5-HT to the major metabolite 5-hydroxyindoleacetic acid (5-HIAA) which is excreted primarily in the urine. 5-HT is mainly stored centrally in the brain and peripherally in the GI tract and platelets. Based upon Mohammad-Zadeh *et al.*, 2008; Ruddell *et al.*, 2008; Herr *et al.*, 2017.

5-HT can be released from neurons into the synapse and from EC cells predominantly at the basolateral side, but also apically (Mawe and Hoffman, 2013), after which 5-HT can be taken up via the highly selective Na^+/Cl^- dependent 5-HT transporter (SERT; identified in CNS, GI tract, pulmonary and peripheral vasculature, and platelets). Not taken up 5-HT can bind to its receptors, the 5-HT receptors, which are present on amongst others many GI cell types (discussed in I.2.2) and mediate diverse effects, or it can enter the blood circulation via the dense capillary bed of the lamina propria for 5-HT released by EC cells (Mohammad-Zadeh *et al.*, 2008). In the blood, 98% of 5-HT is taken up into platelets expressing SERT and 2% remains free reaching other organs via the blood stream (Mohammad-Zadeh *et al.*, 2008; Mawe and Hoffman, 2013). Virtually all 5-HT in the blood is derived from EC cells, since the blood-brain barrier is impermeable to 5-HT and the capacity of platelets to synthesize 5-HT is limited (Gershon and Tack, 2007; Mohammad-Zadeh *et al.*, 2008).

5-HT receptors are grouped into seven families based on their structural and transductional properties (Table I.1): 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7, several of them being further subdivided (Berger *et al.*, 2009). Except for the 5-HT_3 receptor which is a ligand-gated Na^+/K^+ ion channel (Mohammad-Zadeh *et al.*, 2008), all 5-HT receptor types are G protein-coupled receptors (GPCR). They are integral membrane proteins with 7 hydrophobic transmembrane domains connected by 3 intracellular loops and 3 extracellular loops (Raymond *et al.*, 2001). The intracellular domain can interact with G proteins that are able to initiate a signal transduction cascade depending on the G protein type coupled (Figure I.8). The 5-HT receptor subtypes are widely distributed throughout the body and are involved in very diverse functions (Table I.1); their distribution and function in the GI tract is further discussed below.

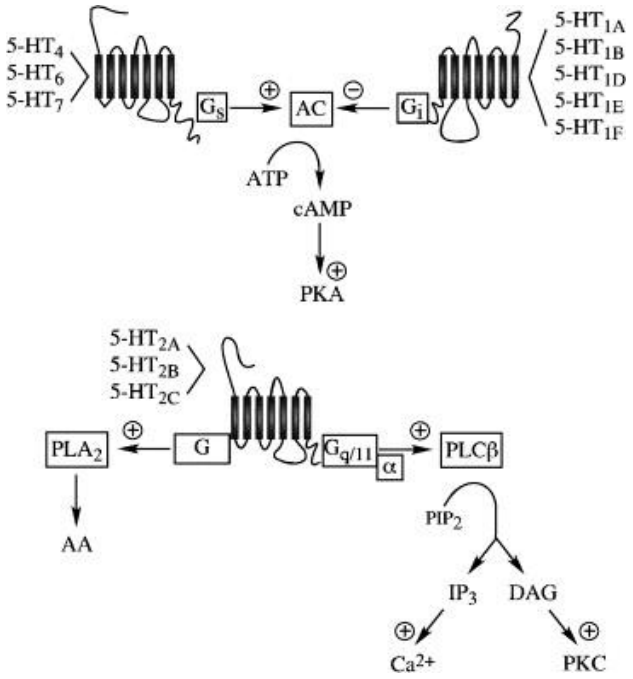


Figure I.8 - Prototypical signaling enzyme linkages of the G protein-coupled 5-HT receptors. 5-HT_5 receptor is not shown as the common signaling linkage is not yet known. Abbreviations: AA, arachidonic acid; AC, adenylyl cyclase; cAMP, cyclic AMP; DAG, diacylglycerol; IP_3, inositol trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC-β, phospholipase C isotype β. From Raymond *et al.*, 2001.

Table I.1 – Overview of 5-HT receptor families and subtypes.

5-HT receptor family	**subtype** (Alexander *et al.*, 2017a)	**primary transduction mechanism** (Barnes *et al.*)	**common signaling linkage *** (Raymond *et al.*, 2001)	**location/main function** (Mohammad-Zadeh *et al.*, 2008; Ruddell *et al.*, 2008; Sanger, 2008; Stasi *et al.*, 2014)
5-HT$_1$ GPCR	5-HT$_{1A}$	$G_{i/o}$	inhibits AC activates K^+ channels stimulates ERK inhibits Ca^{2+} conductance	CNS: neuronal inhibition/hyperpolarization, behavioral effects (sleep, feeding, thermoregulation, and anxiety) GI tract: ENS, enterocytes, release of mediators
	5-HT$_{1B}$	$G_{i/o}$	inhibits AC stimulates ERK	CNS: inhibition of neurotransmitter release, autoreceptor, behavioral effects vascular: pulmonary vasoconstriction GI tract: ENS, muscle, extrinsic nerves, increase of transmitter release, muscle relaxation
	5-HT$_{1D}$	$G_{i/o}$	inhibits AC	CNS: inhibition of neurotransmitter release intracranial vessels: vascular smooth muscle contraction GI tract: ENS, muscle, extrinsic nerves, increase of transmitter release, muscle relaxation
	5-ht$_{1e}$ [1]	$G_{i/o}$	inhibits AC	CNS: unknown
	5-HT$_{1F}$	$G_{i/o}$	inhibits AC	CNS: limbic functions GI tract: suggestion of presence in stomach
5-HT$_2$ GPCR	5-HT$_{2A}$	$G_{q/11}$	activates PLC & PKC stimulates ERK activates PLA_2	CNS: neuronal excitation, behavioral effects and learning smooth muscle: contraction, vasoconstriction/dilatation platelets: aggregation GI tract: ENS, enterocytes, muscle, muscle contraction
	5-HT$_{2B}$	$G_{q/11}$	activates PLC activates ERK activates PLA_2	GI tract: ENS, muscle, GI motility
	5-HT$_{2C}$	$G_{q/11}$	activates PLC activates PKC activates PLA_2	choroid plexus, CSF secretion CNS: regulation of emotional states
5-HT$_3$ ligand-gated cation channel	ion channel; subunits: 5-HT3A, 5-HT3B [2], 5-HT3C [2], 5-HT3D [2], 5-HT3E [2]		results in plasma membrane depolarization	peripheral and central neurons: depolarization ENS: modulation of the release of other neurotransmitters such as dopamine, GABA, substance P and acetylcholine GI tract: sensory and enteric nerves, enterocytes, EC, ICC, extrinsic nerves, emesis, GI motility, pain transmission
5-HT$_4$ GPCR	-	G_s	activates AC & PKA	CNS: neuronal excitation, memory, cognitive function, affective symptoms GI tract: ENS, enterocytes, EC, ICC, muscle, neurotransmitter release, GI motility heart: tachycardia

5-HT receptor family	**subtype** (Alexander *et al.*, 2017a)	**primary transduction mechanism** (Barnes *et al.*)	**common signaling linkage *** (Raymond *et al.*, 2001)	**location/main function** (Mohammad-Zadeh *et al.*, 2008; Ruddell *et al.*, 2008; Sanger, 2008; Stasi *et al.*, 2014)
5-HT$_5$ GPCR	5-HT$_{5A}$	G_i/G_o	unknown	CNS: regulation of affective states, learning, sensory perception, neuroendocrine functions and memory
	5-ht$_{5b}$[1]	N.I.	unknown	not yet characterized
5-HT$_6$ GPCR	-	G_s	activates AC & PKA	CNS: regulation of affective states
5-HT$_7$ GPCR	-	G_s	activates AC & PKA	CNS: Regulation of affective states GI tract: ENS, muscle, muscle relaxation blood vessels: function unknown

Abbreviations: AC, adenylyl cyclase; CNS, central nervous system; ERK, extracellular signal-regulated kinase; GI, gastrointestinal; GPCR, G protein-coupled receptor; ICC, interstitial cell of Cajal; N.I., not identified; PKA, protein kinase A; PKC, protein kinase C; PLA, phospholipase A; PLC, phospholipase C.

* not common signal linkages implicated in receptor function have been identified but are not included.

[1] is yet to achieve receptor status since a robust response mediated via the protein is yet to be reported in the literature; lower-case appellation is used to denote this.

[2] do not appear to form functional homomeric receptors although assemble into heteromeric 5-HT$_3$ receptors along with the 5-HT3A subunit.

This table is not intended to be complete.

Several 5-HT receptors have attracted attention as pharmacological target. When exaggerated stimulation of 5-HT receptors by endogenous 5-HT contributes to a pathophysiological mechanism, antagonism of the receptor involved can be clinically meaningful. Chemotherapy and radiotherapy induced nausea and emesis are related to stimulation of 5-HT$_3$ receptors on vagal afferents in the GI tract by 5-HT released from the EC cells and 5-HT$_3$ receptor antagonists are now standard therapy for this condition (Gershon and Tack, 2007; Sanger, 2008). These 5-HT$_3$ receptor antagonists are also studied to reduce pain and diarrhea in some forms of irritable bowel syndrome (Gershon and Tack, 2007; Sanger, 2008; Walstab *et al.*, 2010). The non-selective 5-HT receptor antagonist methysergide was previously used for prevention of migraine attacks in view of a possible role of 5-HT in triggering migraine attacks (Dahlof and Van Den Brink, 2012). In other conditions, 5-HT receptor agonists are used. Triptans are 5-HT$_{1B}$/5-HT$_{1D}$ agonists used to treat an ongoing migraine attack; they induce constriction of cerebral blood vessels reducing mechanical stimulation of surrounding trigeminal afferents; they also reduce the release of pro-inflammatory peptides as calcitonin gene-related peptide from these afferents (Durham, 2006; Dahlof and Van Den Brink, 2012). 5-HT$_4$ receptor agonists are used as gastroprokinetics and this will be discussed in detail below. Many psychotropic drugs such chlorpromazine, clozapine, pimozide, have affinity for one or more 5-HT receptor subtypes such as 5-HT$_{2A}$, 5-HT$_6$ and 5-HT$_7$ but the importance of this interaction for their clinical effects are not clear (Glennon, 2003).

I.2.2 5-HT and its receptors in the GI tract

In the GI tract, 5-HT is an important neurotransmitter and mucosal signaling molecule targeting enterocytes, smooth muscle cells, and enteric and extrinsic neurons (De Ponti, 2004). It plays a role in GI motility (segmentation and propulsion by peristalsis and MMCs; Mawe and Hoffman, 2013; Kendig and Grider, 2015), sensation, ENS development, growth/maintenance of the intestinal mucosa and immunomodulation (Gershon and Tack, 2007; Gershon, 2013; Mawe and Hoffman, 2013). Evidence suggests that 5-HT is also involved in the control of intestinal secretion and absorption (Gershon and Tack, 2007). GI 5-HT is secreted by EC cells and neurons and both are involved in GI motility. 5-HT secretion by neurons appears to be essential for normal GI motility, while the absolute requirement of 5-HT secreted by EC cells is still under debate, but it certainly modifies intestinal motility and is capable of inducing peristalsis (Gershon, 2013; Kendig and Grider, 2015).

Given the diverse functions of 5-HT in the GI tract, it is not surprising that 5 of the 7 receptor families are expressed in the GI tract and can affect gut functions: 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4 and 5-HT_7. They are present on several classes of myenteric neurons (sensory neurons, inter- and (secreto)motor neurons, and vagal afferents), on smooth muscle cells and on epithelial cells (Table I.2 and Figure I.9). Neuronal 5-HT receptors may enhance (5-HT_3 and 5-HT_4) or inhibit (5-HT_{1A}) transmitter release; smooth muscle 5-HT receptors may contract (5-HT_{2A}) or relax (5-HT_4 and 5-HT_7) smooth muscle (De Ponti, 2004).

Table I.2 – Main 5-HT receptor subtypes in the gut.

receptor subtype	**distribution in the gut** (De Ponti, 2004; Tonini and Pace, 2006)	**functional response on stimulation** (De Ponti, 2004; Tonini and Pace, 2006)
5-HT_{1A}	enteric neurons	transmitter release ↓
5-$HT_{1B/D}$	enteric neurons (?) circular smooth muscle (?)	transmitter release ↑ relaxation
5-HT_{2A}	smooth muscle	contraction
5-HT_{2B}	longitudinal smooth muscle	contraction
5-HT_3	enteric neurons extrinsic vagal afferents	fast EPSP transmitter release ↑ secretion ↑
5-HT_4	enteric neurons smooth muscle enterocytes	transmitter release ↑ relaxation secretion ↑
5-HT_7	smooth muscle enteric neurons (?)	relaxation slow EPSP transmitter release ↑

Abbreviations: (?), indicates there is debate in the literature; ↓, decrease; ↑, increase; EPSP, excitatory postsynaptic potential.
More details about localization and functional role can be found in Beattie and Smith, 2008.

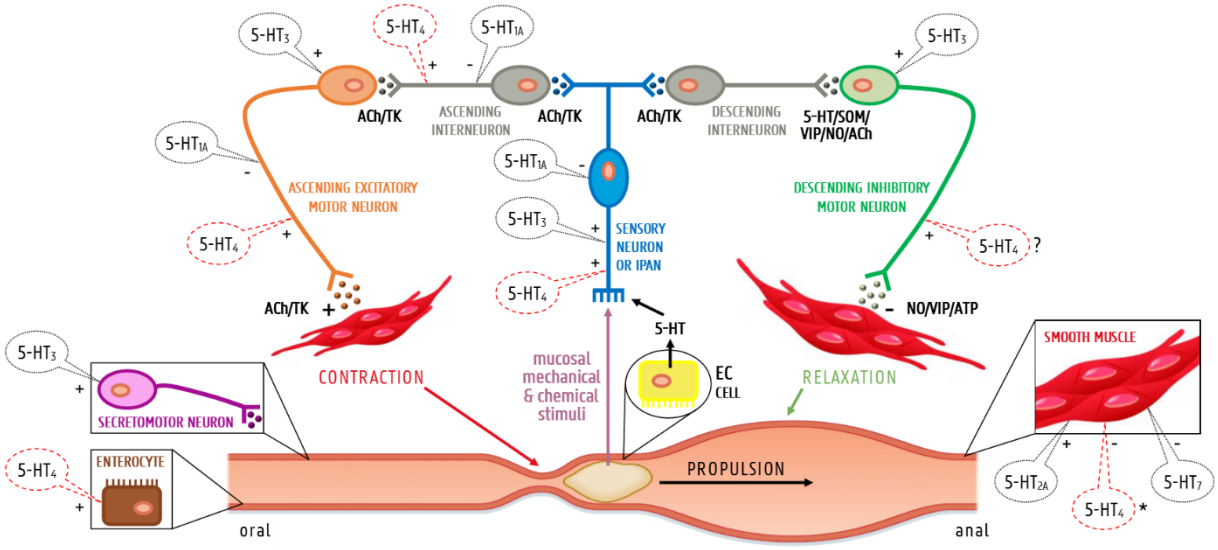


Figure I.9 - Presence of 5-HT receptor subtypes in the GI tract (data obtained in different species). For receptors located on neurons, the scheme only intends to illustrate what receptors influence a given neuron without indication whether this is at the level of the cell body, axon or nerve terminal. Abbreviations: ACh, acetylcholine; ATP, adenosine triphosphate; NO, nitric oxide; SOM, somatostatin; TK, tachykinins; VIP, vasoactive intestinal peptide; +, stimulation; -, inhibition; *, not systemically confirmed; ?, limited data. Based upon De Ponti, 2004; Koeppen and Stanton, 2008.

Since it was discovered that the gastroprokinetic effect of substituted benzamides as metoclopramide and cisapride is related to activation of 5-HT_4 receptors (Sanger, 1987; Dumuis *et al.*, 1989; Buchheit and Buhl, 1991; Craig and Clarke, 1991), these GI 5-HT_4 receptors have been further investigated for therapeutic application and these are now discussed in detail.

I.2.3 5-HT_4 receptors: structure and signal transduction

The 5-HT_4 receptor is positively coupled to the G_s protein/adenylyl cyclase system and thus cAMP formation (Langlois and Fischmeister, 2003). The 5-HT_4 receptor thus belongs to the GPCR family and its **structure** is similar to that proposed for nearly all GPCRs: a membrane protein made up of seven hydrophobic transmembrane helices connected by three intracellular and three extracellular loops (Figure I.10). The amino terminus is oriented toward the extracellular space and the carboxyl terminus (C-terminus) toward the cytoplasm. The 5-HT_4 receptor possesses common sites for posttranslational modifications including extracellular (e.g. glycosylation domains) as well as intracellular domains for interacting with regulatory proteins like G proteins (Roth, 2006).

The 5-HT_4 receptor is transcribed from a complex gene located on chromosome 5 (bands q31-q33; 700 kb, 38 exons) (Hannon and Hoyer, 2008). The coding region includes common and alternative splice exons leading to multiple splicing combinations (Bockaert *et al.*, 2006). To date, eleven functional splice variants, also called isoforms (Figure I.10), generated by alternative splicing of the human transcript, have been described (Bockaert *et al.*, 2006; Coupar *et al.*, 2007). Ten of them are identical up to Leu^{358} and thereafter the DNA sequence encoding the intracellular C-terminus is unique for each splice variant, differing in length and composition. For the c variant a long and short form exists containing respectively 53 (long) and 22 (short) amino acids from Leu^{358} on. The eleventh splice variant, 5-$HT_{4(h)}$, is an insertion of 14 amino acids in the second extracellular loop (Hannon and Hoyer, 2008). So far it has

only been found in combination with the b variant, named 5-HT$_{4(hb)}$ (De Maeyer *et al.*, 2008a). The splice variant expression was found to be different between species (De Maeyer *et al.*, 2008a) and within the same species the tissue distribution can differ (Coupar *et al.*, 2007; Hannon and Hoyer, 2008). For example, the 5-HT$_{4(d)}$ isoform has only been found in human small intestine and colon and is up till now not found in other tissues (Coupar *et al.*, 2007); the 5-HT$_{4(a)}$ receptor subtype is the only splice variant present in the bladder (Hannon and Hoyer, 2008). Tissue specific (or predominant) expression could potentially be used as a basis for new drug development. So far, no major differences in affinity of splice variants for agonists or antagonists have been reported. Although the design of selective ligands for a given isoform is currently not realistic as the variations in the receptor sequence are located intracellularly, in the future the concept of agonist-directed trafficking of the stimulus response might open up new avenues in this direction (De Maeyer *et al.*, 2008b; Hannon and Hoyer, 2008).

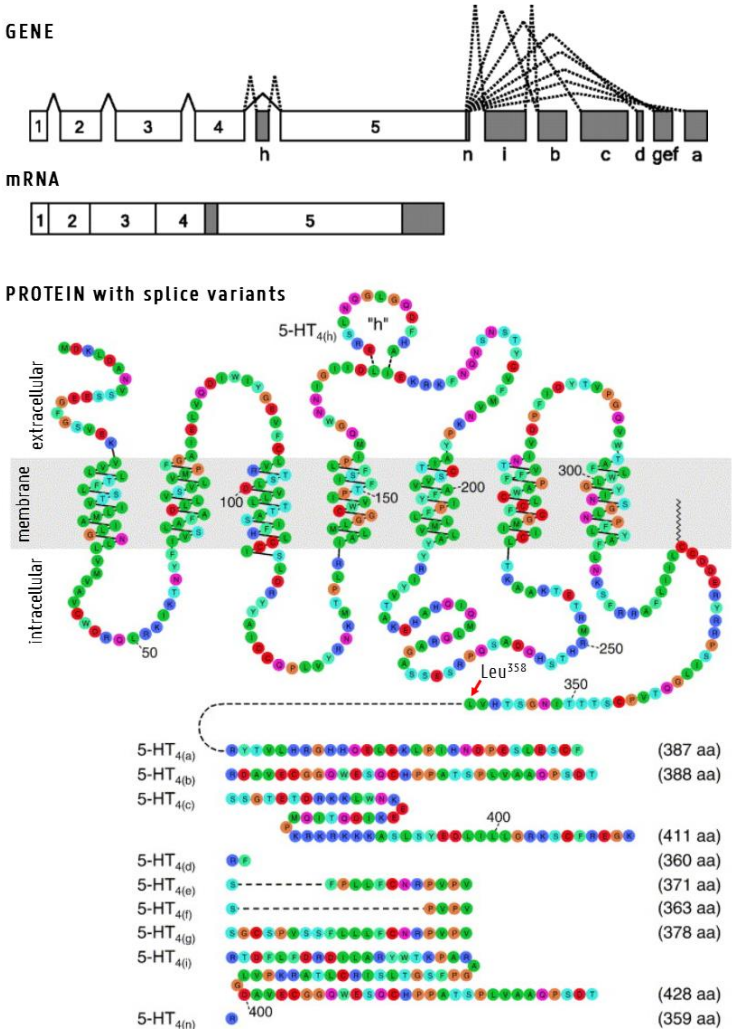


Figure I.10 - Schematic representation of the organization of the coding part of the human HTR4 gene, the mRNA transcript and the translated protein with the splice variants of the 5-HT$_4$ receptor. In the gene structure, the white boxes represent common exons, the grey boxes represent alternatively spliced exons, and the space between the exons represents the location of the introns (not in scale). The protein consists of 7 transmembrane helices, 3 extracellular and 3 intracellular loops, an extracellular NH_2-terminus and a variable intracellular COOH-terminus caused by alternative splicing. Ten of the eleven known human splice variants are given in this figure with their size (number of amino acids) between brackets; for the 'c' splice variant a long (shown) and short (not shown) variant exists. From Kaumann and Levy, 2006; De Maeyer *et al.*, 2008a.

Once 5-HT_4 receptors are formed, they are transported and embedded in the cell surface, where they can respond to their ligands and take on their functions (Coupar *et al.*, 2007). Since 5-HT_4 receptors are GPCR the general G protein-mediated **signaling cascade** is applicable. Upon ligand binding, GPCRs undergo a conformational change that facilitates interaction between the intracellular domain of the receptor and a heterotrimeric guanine nucleotide binding protein (G protein) (Woehler and Ponimaskin, 2009) (Figure I.11). The latter interaction results in activation of the G protein by facilitating the exchange of bound guanosine diphosphate (GDP) with guanosine triphosphate (GTP) and changing the G protein conformation. The activated G protein then dissociates from the receptor into the active GTP-bound G_α subunit and $G_{\beta\gamma}$ dimer. In their activated form G protein subunits activate primary effectors which modulate second messenger or ion concentrations that are subsequently responsible for the cellular response. The G_α subunit remains active until GTP is hydrolyzed resulting in the inactive GDP–bound G_α subunit that eventually re-associates with the $G_{\beta\gamma}$ unit and awaits further activation/inactivetion cycles. GPCRs are coupled to one corresponding type of G protein (Woehler and Ponimaskin, 2009); for 5-HT_4 receptors, all splice variants are preferentially coupled to the G_s protein and promote cAMP formation, by activation of adenylyl cyclase (Figure I.8) (Hannon and Hoyer, 2008). cAMP is an intracellular second messenger that interacts with various targets, such as the phosphorylating enzyme protein kinase A (PKA), but also cyclic nucleotide-gated ion channels, leading to modulation of calcium ion flux and membrane excitability and other cellular processes. The signaling cascade further downstream of PKA depends on the cell type. In myenteric cholinergic neurons, the activation of voltage sensitive Ca^{2+} channels is regulated, leading to enhanced acetylcholine release. This is considered to be responsible for the GI prokinetic actions of 5-HT_4 receptor agonists as described in detail in I.2.5.

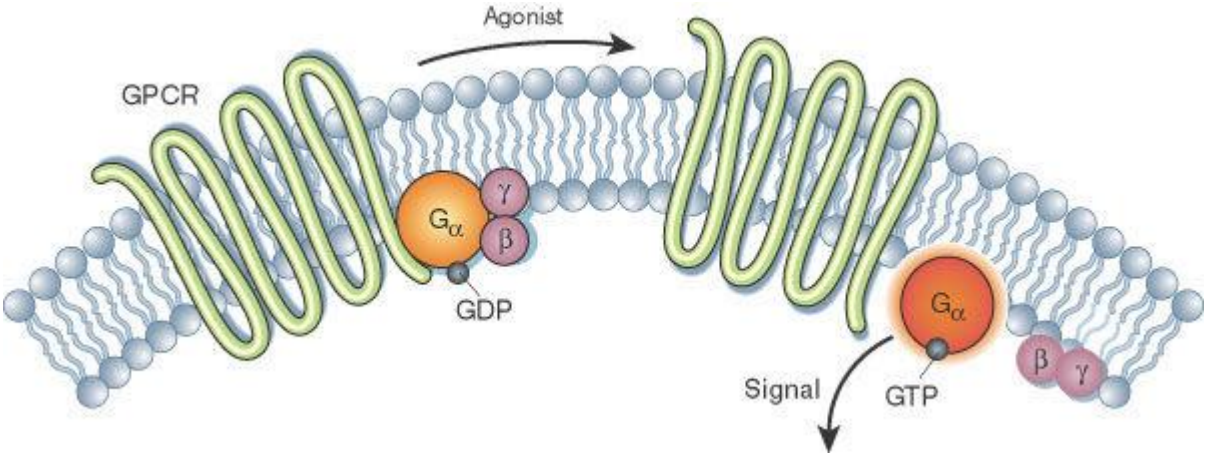


Figure I.11 – Schematic representation of the conformational change of G protein-coupled receptors (GPCRs) upon ligand binding. In unstimulated cells, the state of G_α subunit is defined by its interaction with GDP, $G_{\beta\gamma}$ and a GPCR. Upon receptor stimulation by a ligand (5-HT or agonist), its state is changed owing to its dissociation from the receptor and $G_{\beta\gamma}$, and the exchange of GTP for GDP, leading to G_α activation which can activate other molecules in the cell. From Li *et al.*, 2002.

I.2.4 Distribution and role of 5-HT$_4$ receptors in the body

In 1988, Bockaert and co-workers first described the 5-HT$_4$ receptor in murine colliculi and hippocampal neurons as a receptor positively coupled to adenylyl cyclase (Dumuis *et al.*, 1988). Soon after its discovery the 5-HT$_4$ receptor was also found in the heart (Kaumann *et al.*, 1990) and GI tract (Eglen *et al.*, 1990). Currently, the 5-HT$_4$ receptor has been identified in a wide variety of tissues and species. In man, 5-HT$_4$ receptors are present in the brain, heart, GI tract, urinary bladder and adrenal gland (Hoyer *et al.*, 1994; Langlois and Fischmeister, 2003).

In the CNS, 5-HT$_4$ receptors appear to be located on neurons where they may promote neurotransmitter release and thereby enhance synaptic transmission. They are possibly involved in motor coordination, and visual perception, in addition to learning and memory (Hoyer *et al.*, 1994). In the heart, 5-HT$_4$ receptor activation evokes tachycardia in the right atria and has a positive inotropic effect in left atria of man, pig and monkey (Hoyer *et al.*, 1994). Other species like guinea-pig, dog, rat and mouse, lack functional 5-HT$_4$ receptors in the heart under normal physiological conditions (De Maeyer *et al.*, 2006a; De Maeyer *et al.*, 2006b). In the urinary bladder, 5-HT$_4$ receptors enhance smooth muscle contraction and in the adrenal gland, they mediate steroid secretion (Hoyer *et al.*, 1994).

In the GI tract, 5-HT$_4$ receptors have been described on many cell types, such as neurons, enterocytes and EC cells. Evidence obtained in different species is summarized in Table I.3. The effect of 5-HT$_4$ receptor activation depends on the cell type. E.g activation of 5-HT$_4$ receptors on enterocytes elicits chloride and HCO_3^- secretion (Ning *et al.*, 2004); on IPANs it evokes a neuronal reflex that stimulates chloride secretion (Kellum *et al.*, 1999); on EC cells it inhibits (guinea pig ileum, Gebauer *et al.*, 1993 and porcine small intestine, Schworer and Ramadori, 1998) or stimulates the release of 5-HT (rat ileum, Minami *et al.*, 1995). Recently 5-HT$_4$ receptors were suggested to be present on cholinergic neurons associated with macrophages, thereby facilitating the vagal cholinergic anti-inflammatory pathway upon stimulation (Tsuchida *et al.*, 2011; Gomez-Pinilla *et al.*, 2014). Most interest has gone to 5-HT$_4$ receptors involved in motility. The observation that gastric emptying is delayed and small intestine transit time is slow in mice lacking 5-HT$_4$ receptors (5-HT$_4^{-/-}$ mice) suggests that they might be physiologically involved in maintaining normal motility (Liu *et al.*, 2009). In man, the selective 5-HT$_4$ receptor antagonist SB-207266 tended to delay colonic transit in healthy subjects (Bharucha *et al.*, 2000). Exogenous 5-HT$_4$ receptor agonists *in vivo* have a gastroprokinetic effect from stomach to colon (Emmanuel *et al.*, 1998; Bouras *et al.*, 1999; Poen *et al.*, 1999; Bouras *et al.*, 2001; Emmanuel *et al.*, 2002; Coremans *et al.*, 2003). To what extent 5-HT$_4$ receptors present on circular muscle cells, inducing relaxation, might contribute to this effect is not clear. Smooth muscle relaxation by 5-HT$_4$ receptor activation was reported in human colonic circular smooth muscle (Mclean *et al.*, 1995; Tam *et al.*, 1995; McLean and Coupar, 1996b, a) but these relaxant 5-HT$_4$ receptors in human were not systematically confirmed (Cellek *et al.*, 2006); neither could they be detected in pig colon circular muscle (Priem and Lefebvre, 2011). 5-HT$_4$ receptors have been reported to be present on nitrergic neurons innervating circular muscle in human colon enhancing nitric oxide release (Cellek *et al.*, 2006). When this would lead to more pronounced relaxation in front of the bolus during peristalsis, this might indeed contribute to

Table I.3 - Summary (non-limitative) of 5-HT_4 receptor location on GI cell types in different species.

	guinea pig [1]	rat [2]	mouse [3]	dog [4]	pig [5]	man [6]
EC cells	+				+	+
muscularis mucosae	+	+	+			
enterocytes	+	+	+			+
circular muscle		+	+	+		+
IPAN	+	+	+			+
nitrergic neurons CM						+
ascending interneurons	+					
ICC	+		+			
cholinergic neurons CM	+			+	+	+
cholinergic neurons LM	+	+		+	+	+
cholinergic neurons toward macrophages		+	+			
submucosal neurons			+	+	+	+
mast cells						+
extrinsic afferents		+		+		

Abbreviations: CM, circular smooth muscle; EC, enterochromaffin, ICC, interstitial cell of Cajal; IPAN, intrinsic primary afferent neuron; LM, longitudinal smooth muscle.

Direct and indirect (functional) evidence for 5-HT_4 receptors in the GI tract of:

[1] **guinea pig:** Eglen *et al.*, 1990; Buchheit and Buhl, 1991; Craig and Clarke, 1991; Buchheit *et al.*, 1992; Kilbinger and Wolf, 1992; Tonini *et al.*, 1992; Buchheit and Buhl, 1994; Pan and Galligan, 1994; Leung *et al.*, 1995; McLean and Coupar, 1995; Briejer and Schuurkes, 1996; Foxx-Orenstein *et al.*, 1996; Kojima and Shimo, 1996; Jin *et al.*, 1999; Sakurai-Yamashita *et al.*, 1999a; Sakurai-Yamashita *et al.*, 1999b; Takemura *et al.*, 1999; Kojima *et al.*, 2000; Galligan *et al.*, 2003; Tonini *et al.*, 2003; LePard *et al.*, 2004; Poole *et al.*, 2006; Fang *et al.*, 2008.

[2] **rat:** Baxter *et al.*, 1991; Hegde *et al.*, 1995; Amemiya *et al.*, 1996; Budhoo *et al.*, 1996b; Clayton and Gale, 1996; Tuladhar *et al.*, 1996; Yamano *et al.*, 1997; Grider *et al.*, 1998; Beattie *et al.*, 2004; Ning *et al.*, 2004; Sun and Luo, 2004; Greenwood-Van Meerveld *et al.*, 2006; Safsten *et al.*, 2006; Komada and Yano, 2007; Tsuchida *et al.*, 2011.

[3] **mouse:** Hedge *et al.*, 1994; Banner *et al.*, 1996; Sanger *et al.*, 1998; Pascual *et al.*, 2002; Grider, 2003; Tuo *et al.*, 2004; Liu *et al.*, 2005; Poole *et al.*, 2006; Gershon and Tack, 2007; Wang *et al.*, 2007; Gomez-Pinilla *et al.*, 2014.

[4] **dog:** Schuurkes, 1990; Gullikson *et al.*, 1993; Fukui *et al.*, 1994; Bingham *et al.*, 1995; Prins *et al.*, 1999; Prins *et al.*, 2000; Briejer *et al.*, 2001; Prins *et al.*, 2001a; Prins *et al.*, 2001b; Makimoto *et al.*, 2002.

[5] **pig:** Hansen, 1994, 1995; Schworer and Ramadori, 1998; De Maeyer *et al.*, 2006a; Priem and Lefebvre, 2011; Priem *et al.*, 2012; Priem *et al.*, 2013.

[6] **man:** Budhoo *et al.*, 1996a; Foxx-Orenstein *et al.*, 1996; McLean and Coupar, 1996b; Tomita *et al.*, 1997; Grider *et al.*, 1998; Schworer and Ramadori, 1998; Kellum *et al.*, 1999; Prins *et al.*, 1999; Sakurai-Yamashita *et al.*, 1999a; Sakurai-Yamashita *et al.*, 1999b; Prins *et al.*, 2000; Sakurai-Yamashita *et al.*, 2000; Leclere and Lefebvre, 2002; Gill *et al.*, 2005; Leclere *et al.*, 2005; Cellek *et al.*, 2006; Poole *et al.*, 2006; Streutker *et al.*, 2006.

the prokinetic effect of 5-HT_4 receptor agonists as peristalsis is the result of a combination of ascending contraction and descending relaxation (Figure I.9). These 5-HT_4 receptors on nitrergic neurons were not detected in pig colonic circular muscle (Priem and Lefebvre, 2011). But 5-HT_4 receptors enhancing acetylcholine release from cholinergic neurons innervating circular as well as longitudinal muscle have been systematically found throughout species (Table I.3). This mechanism leading to enhanced contraction is considered to be the predominant mechanism of action for the prokinetic effect of clinically used 5-HT_4 receptor agonists and is discussed in detail in the next section.

I.2.5 5-HT_4 receptors on GI excitatory cholinergic neurons

5-HT_4 receptor agonists are potent prokinetic agents (Talley, 2002; Tonini and Pace, 2006; Gershon and Tack, 2007) and have considerable therapeutic potential in the treatment of GI hypomotility disorders (Table I.4) such as gastroparesis and constipation (Schiller, 2004; Galligan and Vanner, 2005). The clinical efficacy of the 5-HT_4 receptor agonists cisapride, tegaserod, prucalopride, renzapride, mosapride and velusetrag (TD-5108), has been reported in patients with functional dyspepsia, gastroparesis, chronic constipation and irritable bowel syndrome with constipation (IBS-C) (Beattie and Smith, 2008). The highly selective 5-HT_4 receptor agonist prucalopride (Resolor®) is marketed for chronic idiopathic constipation. *In vivo*, prucalopride has gastroprokinetic properties and accelerates gastric emptying and stimulates colonic transit in healthy as well as in constipated people (Emmanuel *et al.*, 1998; Bouras *et al.*, 1999; Poen *et al.*, 1999; Bouras *et al.*, 2001; Emmanuel *et al.*, 2002; Coremans *et al.*, 2003).

Table I.4 - 5-HT_4 receptor agonists approved (according to the European Medicines Agency), withdrawn and in development (according to clinicaltrials.gov) for the treatment of GI motility disorders.

5-HT_4 receptor agonist	stage of development	indication
APPROVED		
prucalopride (Resolor®)	marketed since 2009	chronic idiopathic constipation
	phase 3, recruiting	postoperative ileus
metoclopramide (Primperan®)	marketed since 1960s, but restricted use since 2013	nausea and vomiting, gastro-esophageal reflux disease, diabetic gastroparesis since 2013: restricted to indications involving short-term use
mosapride (Gasmotin®)	marketed since 1998: in several Asian countries, not in Europa nor in U.S.A	functional dyspepsia, gastritis (AdisInsight database: adisinsight.springer.com/drugs/800000624)
	phase 1, completed	gastritis
	phase 3, terminated (poor recruitment, high dropout rate)	constipation-predominant irritable bowel syndrome
	phase 3, recruiting	postoperative ileus upon cesarean section

5-HT_4 receptor agonist	stage of development	indication
	phase 4, unknown	chronic constipation, constipation-predominant irritable bowel syndrome
	phase 4, unknown	postoperative ileus upon colorectal surgery
	phase 4, unknown	diabetic gastroparesis
IN DEVELOPMENT		
TD-8954 (McKinnell et al., 2013)	phase 1, terminated (for business reasons)	gastrointestinal motility disorder
DA-6886 (Lee *et al.*, 2014)	phase 1, recruiting	constipation-predominant irritable bowel syndrome
velusetrag (TD-5108) (Goldberg *et al.*, 2010; Manini *et al.*, 2010; Ahn *et al.*, 2015)	phase 2, completed	constipation, gastroparesis
naronapride (ATI-7505) (Bowersox *et al.*, 2011)	phase 2, terminated	chronic idiopathic constipation
YKP10811 (Shin et al., 2015)	phase 2, completed	constipation-predominant irritable bowel syndrome, functional constipation
PF-00885706 (Komada et al., 2009)	phase 2, terminated (on clinical hold by the sponsor due to operational reasons)	gastro-esophageal reflux disease
renzapride (Mozaffari *et al.*, 2014)	phase 3, terminated (due to insufficient efficacy over placebo in study ATL1251/038/CL.)	constipation-predominant irritable bowel syndrome
WITHDRAWN		
tegaserod (Zelnorm®) (Pasricha, 2007)	approved in 2002, but withdrawn in 2007 [1]	constipation-predominant irritable bowel syndrome
cisapride (Prepulsid®) (Barbey *et al.*, 2002)	approved in 1988, but withdrawn in 2000 [2]	gastro-esophageal reflux disease, gastroparesis and some forms of constipation

[1] because of an increased risk for atherosclerotic obstructive events (Pasricha, 2007)
[2] because of rare but life-threatening cardiac arrhythmias (Barbey *et al.*, 2002)

Used terminology (according to clinicaltrials.gov):
- Recruiting: The study is currently recruiting participants.
- Terminated: The study has stopped early and will not start again. Participants are no longer being examined or treated.
- Completed: The study has ended normally, and participants are no longer being examined or treated (that is, the last participant's last visit has occurred).
- Unknown: A study whose last known status was recruiting; not yet recruiting; or active, not recruiting but that has passed its completion date, and the status has not been last verified within the past 2 years.

The GI prokinetic properties of 5-HT_4 receptor agonists are attributed to the activation of excitatory 5-HT_4 receptors on cholinergic myenteric motor neurons exciting smooth muscle by released acetylcholine (Gershon and Tack, 2007). This location of 5-HT_4 receptors was already shown in several species, including pig and man, and receptor activation results in facilitation of ongoing acetylcholine release leading to increased smooth muscle contractions (Prins *et al.*, 2000; Leclere and Lefebvre, 2002; Leclere *et al.*, 2005; Cellek *et al.*, 2006; Cellek *et al.*, 2008; Priem and Lefebvre, 2011; Priem *et al.*, 2012). Figure I.12 illustrates the facilitating effect of the 5-HT_4 receptor agonist prucalopride on electrically induced cholinergic contractions in porcine stomach and human colon.

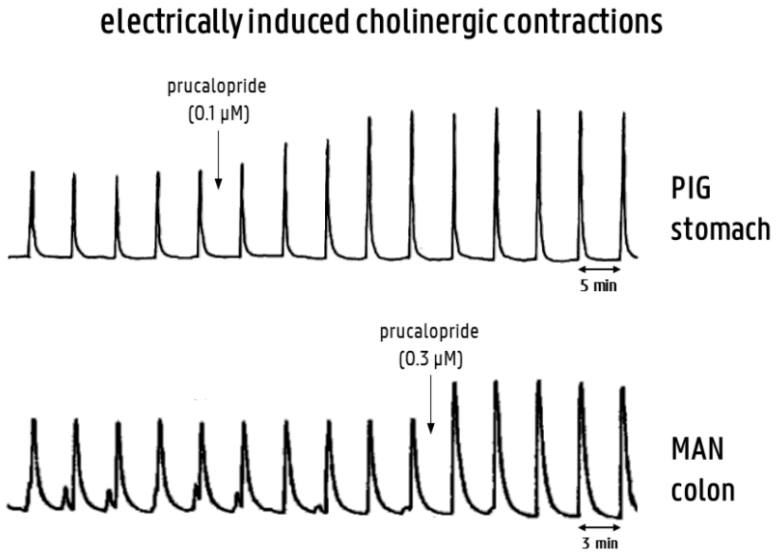


Figure I.12 – Facilitating effect of prucalopride on electrically induced cholinergic contractions in porcine stomach circular muscle (from Priem *et al.*, 2012) and in human colon longitudinal muscle (from Prins *et al.*, 2000)

The cellular mechanism that facilitates acetylcholine release upon 5-HT_4 receptor stimulation is not fully established in myenteric neurons. In CNS neurons, 5-HT_4 receptors activate the adenylyl cyclase/PKA signaling cascade, resulting in an inhibition of some potassium channels causing neuronal excitation which results in acetylcholine release (Galligan *et al.*, 2003). Current knowledge about the 5-HT_4 receptor signal transduction in myenteric neurons (Figure I.13) is based on a study in guinea pig ileum (Galligan *et al.*, 2003). In parallel with CNS neurons, the 5-HT_4 receptor-mediated facilitation of electrically induced acetylcholine release is coupled to an increase of cAMP and depends on PKA activation in myenteric neurons. But 5-HT_4 receptor activation does not change neuronal excitability at the level of the cell body, so the targets of PKA activity in the nerve terminal remains to be determined and could include calcium or other ion channels, synaptic vesicle proteins or other proteins involved in synaptic vesicle exocytosis. Direct actions of cAMP on ion channels or PKA independent signaling pathways were not seen (Galligan *et al.*, 2003). The released acetylcholine acts at muscarinic receptors on the smooth muscle cells leading to contraction. Recently, control of the 5-HT_4 receptor pathway in cholinergic neurons toward smooth muscle by a cAMP-specific phosphodiesterase was described in porcine stomach (Priem *et al.*, 2012) and colon (Priem *et al.*, 2013).

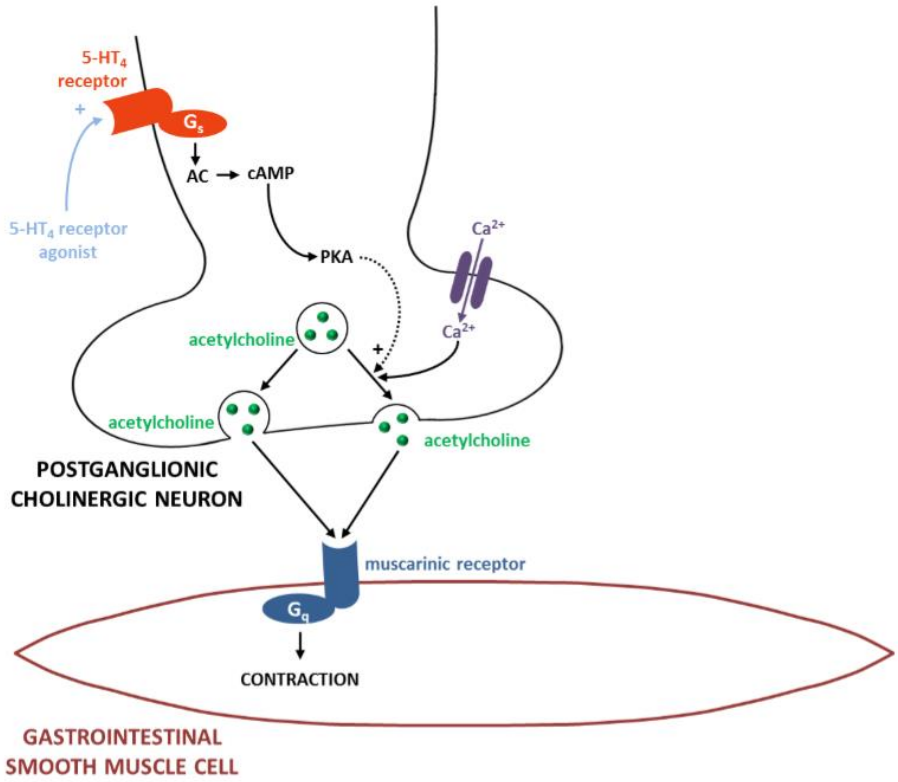


Figure I.13 – Schematic overview of the intraneuronal signal transduction of 5-HT_4 receptors in cholinergic neurons innervating GI smooth muscle resulting in enhancement of acetylcholine release that finally leads to increased smooth muscle contraction. Abbreviations: AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A.

I.3 Phosphodiesterases

I.3.1 Definition and history

Phosphodiesterase (PDE) refers to cyclic nucleotide phosphodiesterase. The PDEs form a diverse family of enzymes that degrade the cyclic nucleotides, cAMP and cGMP, to their respective inactive products 5'-AMP and 5'-GMP by hydrolyzing the 3' cyclic phosphate bond (Figure I.14). PDEs thus play a key role in modulating intracellular levels of the second messengers cAMP and cGMP, and hence are regulators of cell function (Boswell-Smith *et al.*, 2006). The synthesis and metabolism of both cyclic nucleotides are visualized in Figure I.14.

The story of PDEs goes back to 1880. The asthmatic Henry Hyde Salter, known as the author of the best book on asthma in the nineteenth century, advocated a strong cup of coffee to control an asthma attack (Sakula, 1985). Although the mechanism of action at that time was unknown, it has since been discovered in 1958 that caffeine acts as a weak non-selective PDE inhibitor (Sutherland and Rall, 1958). PDE inhibition increases the cAMP level in the bronchi which promotes airway smooth muscle relaxation resulting in bronchodilation (Tilley, 2011).

Earl Sutherland and Ted Rall identified the nucleotide cAMP as a second messenger present in several tissues and suggested that it mediated many of the cellular effects of neurotransmitters and hormones (Rall and Sutherland, 1958; Sutherland and Rall, 1958). The enzymatic capacity of PDEs to inactivate cAMP was also noticed. The discovery of a second intracellular messenger cGMP followed 5 years later (Ashman *et al.*, 1963).

In subsequent years the crucial role of cAMP, cGMP and PDEs in health and disease (Conti and Beavo, 2007; Francis *et al.*, 2011; Keravis and Lugnier, 2012) was confirmed and many families of PDEs were discovered (see I.3.2 Classification). A century after its first mention, PDE inhibitors are still tested for the treatment of asthma and over the years their therapeutic applications were extended with amongst others chronic obstructive pulmonary disease (COPD), erectile dysfunction and congestive heart failure (see I.3.3 PDE inhibitors) (Miller, 2015).

I.3.2 Classification

The different PDE isoenzymes, numbered according to their elucidation order, are distinguished primarily by their substrate specificity and sensitivity to calcium-calmodulin (Boswell-Smith *et al.*, 2006). A first notion of the existence of different PDEs arose in 1970 by Beavo et al. (1970). Initially, three enzymes were identified: Ca^{2+}/calmodulin-PDE, cAMP-PDE and cGMP-PDE. They were further characterized by the use of selective inhibitors (Nicholson *et al.*, 1991) and the arrival of the molecular age led to a strong increase in the number of PDE isoenzymes and isoforms. In 1995, a standardized nomenclature for the PDE family was introduced by Beavo (1995). Meanwhile, the counter stands at 11 identified isoenzyme families, namely PDE1-11 (Alexander *et al.*, 2017b) and these 11 families comprise at least 21 genes generating over 40 isoforms (Miller, 2015). The PDE isoenzymes differ in their substrate specificity, regulatory mechanisms and tissue distribution (DeNinno, 2012); an overview is given in Table I.5.

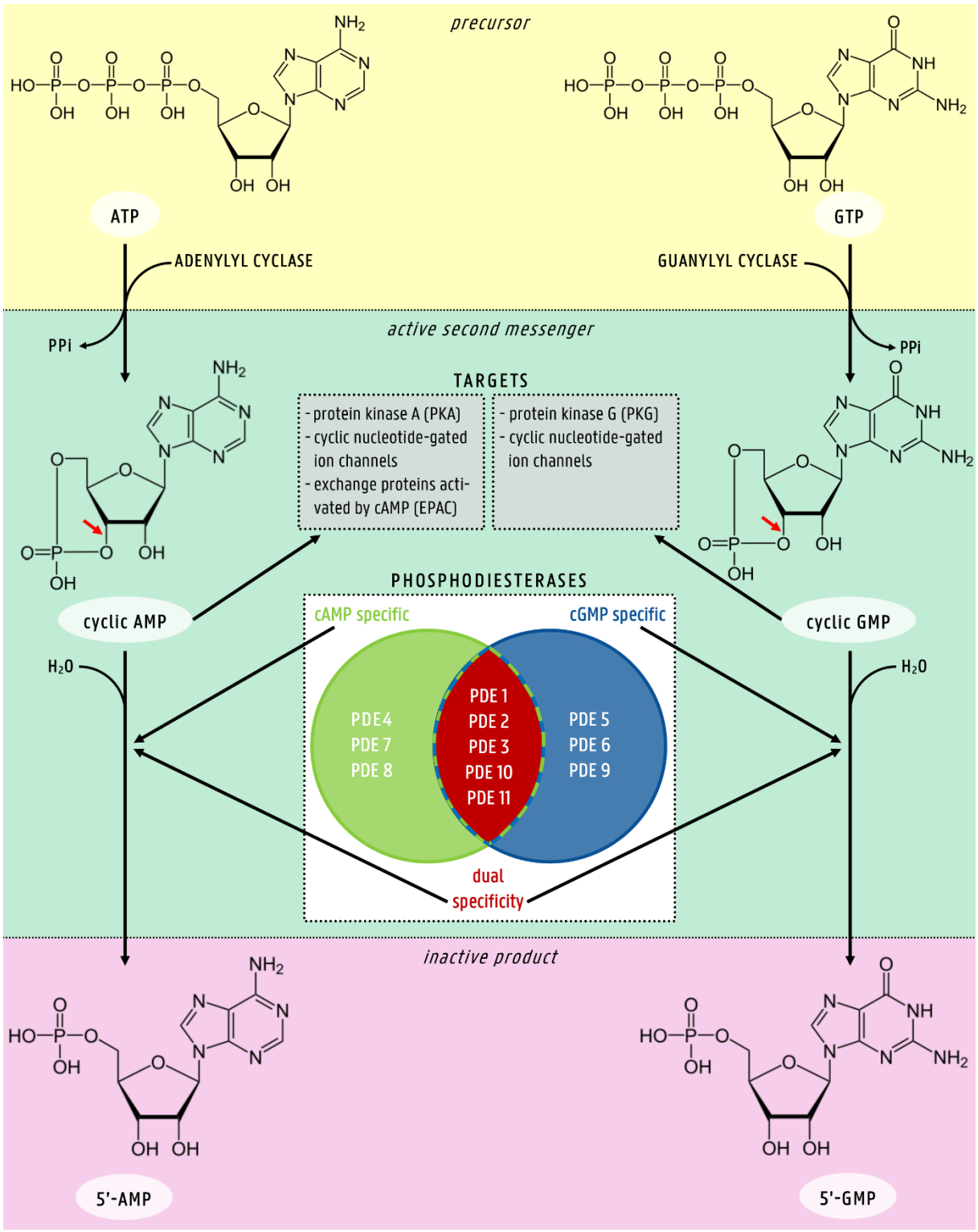


Figure I.14 - Cyclic nucleotide metabolism: synthesis and degradation. The cyclic nucleotides, cAMP and cGMP, are active second messengers. They are synthesized from their precursors, ATP and GTP, by adenylyl and guanylyl cyclase. These second messengers target the cAMP- or cGMP activated protein kinases, namely protein kinase A (PKA) and G (PKG), cyclic nucleotide-gated ion channels (leading to intracellular Ca^{2+} elevation), and exchange proteins activated by cAMP (EPAC). cAMP-, cGMP- and/or dual specific phosphodiesterases (PDE) degrade the active second messengers to their inactive products 5'-AMP and 5'-GMP by hydrolyzing the 3' cyclic phosphate bond (indicated by red arrows). Abbreviations: PPi, pyrophosphate.

Concerning their substrate specificity, three categories exist, as illustrated in the central panel of Figure I.14: (1) PDE4, 7 and 8 are cAMP specific, (2) PDE5, 6 and 9 are cGMP specific, and (3) PDE1, 2, 3, 10 and 11 are dual substrate PDEs and can hydrolyze both cAMP and cGMP although not at similar rates (Lugnier, 2006; DeNinno, 2012). The widespread distribution of PDEs, as illustrated in Table I.5, offers therapeutic possibilities for selective PDE inhibitors. The knowledge about all PDE families has grown, but PDE1 to 5 remain the classic PDE isoenzymes which are well-characterized; they have a widespread tissue distribution and selective inhibitors are available.

Table I.5 - Overview of the 11 PDE families with their substrate(s), regulatory mechanism(s) and tissue distribution.

PDE isoenzyme	**substrate(s)** (Lugnier, 2006)	**regulatory mechanism(s)** (Lugnier, 2006)	**tissue distribution** (Keravis and Lugnier, 2012)
PDE1	cAMP, cGMP	Ca^{2+}-calmodulin stimulated	heart, brain, lung, vascular smooth muscle
PDE2	cAMP, cGMP	cGMP-stimulated	adrenal gland, heart, lung, liver, platelets, endothelial cells
PDE3	cAMP, cGMP	cGMP-inhibited	heart, vascular smooth muscle, lung, liver, platelets, adipocytes, immunocytes
PDE4	cAMP	cGMP-insensitive	brain, Sertoli cells, kidney, liver, heart, vascular smooth muscle, lung, endothelial cells, immunocytes
PDE5	cGMP	PKA/PKG phosphorylated	lung, platelets, vascular smooth muscle, heart, endothelial cells, brain
PDE6	cGMP	transducin-activated	photoreceptors, pineal gland, lung
PDE7	cAMP	rolipram-insensitive	skeletal muscle, heart, kidney, brain, pancreas, T lymphocytes
PDE8	cAMP	rolipram-insensitive IBMX-insensitive	testes, eye, liver, skeletal muscle, heart, kidney, ovary, brain, T lymphocytes, thyroid
PDE9	cGMP	IBMX-insensitive	kidney, liver, lung, brain
PDE10	cAMP, cGMP	unknown	testes, brain, thyroid
PDE11	cAMP, cGMP	unknown	skeletal muscle, prostate, pituitary gland, liver, heart

Abbreviations: cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; PKA, protein kinase A; PKG, protein kinase G.
This table is not intended to be complete.

I.3.3 PDE inhibitors

Inhibition of PDEs leads to increasing intracellular concentrations of endogenous cAMP/cGMP (Bender and Beavo, 2006) and can thus enhance a variety of physiological mechanisms at different cell and organ levels. Xanthine derivates, including caffeine and theophylline, were clinically used as bronchodilatators, diuretics and ionotropic agents before they were identified as non-selective PDE inhibitors (Maurice *et al.*, 2014). Since the 1970s PDE inhibitors have been investigated as potential

treatment for several medical conditions (Miller, 2015) and these compounds include both non-selective PDE inhibitors such as 3-isobutyl-1-methylxanthine (IBMX) as well as selective PDE inhibitors that target a specific PDE family such as vinpocetine, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), cilostamide and rolipram (Table I.6).

Table I.6 - Overview of some examples of inhibitors for the PDE isoenzymes and their possible medical applications.

PDE isoenzyme	Inhibitors *	possible medical applications *
PDE1	vinpocetine, nimodipine, dioclein, IC86340, IC224, IC295, SCH51866	memory loss, dementia
PDE2	EHNA, oxindole, BAY607550, PDP, IC933, ND7001	acute respiratory distress syndrome, sepsis, memory loss
PDE3	cilostamide, anagrelide, cilostazol, milrinone, inamrinone, siguazodan , trequinsin, OPC-33540	congestive heart failure, pulmonary hypertension, thrombosis, glomerulonephritis
PDE4	rolipram, roflumilast, cilomilast, apremilast, CDP840, Ro20-1724, YM976, NCS 613, AWD 12-281, SCH351591, V-11294A	glomerulonephritis, asthma, COPD, bipolar disease
PDE5	sildenafil, tadalafil, avanafil, vardenafil, zaprinast, T0156, SCH51866, DMPPO, E4021, DA-8159	erectile dysfunction, benign prostatic hyperplasia, pulmonary hypertension, chronic renal failure
PDE6	sildenafil, zaprinast, vardenafil, DMPPO, E4021	no clinical applications
PDE7	dipyridamole, BRL50481, SCH51866, ICI242, ASB16165	immunologic disorders, lung disease
PDE8	dipyridamole, PF-04957325	immunologic disorders
PDE9	zaprinast, SCH51866, BAY-73-6691, PF-04447943	possible hypoglycemic effects
PDE10	dipyridamole, papaverine, TP-10, MP-10	schizophrenia and psychiatric disorders
PDE11	tadalafil, BC11-38	possible improvement in testicular function

Abbreviations: COPD, chronic pulmonary obstructive disease; PDE, phosphodiesterase.
* The inhibitors are based on Bender and Beavo, 2006; Lugnier, 2006; Keravis and Lugnier, 2012; Miller, 2015; Alexander *et al.*, 2017b and clinical implications on Miller, 2015.
This table is not intended to be complete.

Although many PDE inhibitors are available and the clinical possibilities are broad, their therapeutic success was impeded by lack of efficacy and intolerable side effects, and till now only a few of them proved safe enough for clinical use (Maurice *et al.*, 2014). The PDE5 inhibitor sildenafil is one of them and is approved for erectile dysfunction since 1998 (DeNinno, 2012). Clinical trials originally assessing the potential of sildenafil in the treatment of coronary artery disease found an off-target tissue response, namely smooth muscle relaxation in the arteries and corpora cavernosa of the penis. Because of this off-target tissue response sildenafil, also known as Viagra (Pfizer), became very successful (1998-2006: 177 million prescriptions in 120 countries (Boswell-Smith *et al.*, 2006)) for the treatment of erectile dysfunction. It rekindled the pharmaceutical interest in PDE inhibitors as promising therapeutic targets for many diseases. Meanwhile, 3 other PDE5 inhibitors, namely avanafil, tadalafil and vardenafil are approved for erectile dysfunction and sildenafil and tadalafil are now also approved for pulmonary hypertension (Noel *et al.*, 2012). More recently (in 2010) the PDE4 inhibitor roflumilast was approved for COPD (Giembycz and Field, 2010). Research involving PDEs is ongoing; genetics, tissue distribution, functions, signal transduction and therapeutic targetability are being further unraveled. New inhibitors are under development. Recently, a link between 5-HT_4 receptors and PDEs was found.

I.4 5-HT$_4$ receptors and PDEs

The 5-HT$_4$ receptor agonist cisapride (Prepulsid®) was used very frequently for treatment of GI motility disorders, but was withdrawn from the market in 2000 because of rare but life-threatening cardiac arrhythmias (Barbey *et al.*, 2002). These are due to disturbed repolarization of cardiomyocytes, linked to the potent blockade of the cardiac human ether-a-go-go-related gene (hERG) potassium channel by plasma concentrations of cisapride that can occur in clinical practice (Mohammad *et al.*, 1997). Tegaserod is another non-selective 5-HT$_4$ receptor agonist, withdrawn from the market in 2007 because of cardiovascular safety concerns (Pasricha, 2007). Tegaserod does not induce arrhythmias but increases the possibility of atherosclerosis-related events such as myocardial infarction. The mechanism is less clear, but its interaction with a wide range of receptors other than the 5-HT$_4$ receptor, such as 5-HT$_1$ and/or 5-HT$_2$ receptors, might be involved (Beattie and Smith, 2008; De Maeyer *et al.*, 2008b). The withdrawal of these compounds dampened the interest in further development of new highly selective 5-HT$_4$ receptor agonists for several years. Although these highly selective 5-HT$_4$ receptor agonists do not block hERG potassium channels and are not pro-ischemic (De Maeyer *et al.*, 2008b; Tack *et al.*, 2012; Keating, 2013), a mild effect in the heart can indeed be observed via cardiac 5-HT$_4$ receptors. The attention was drawn to these cardiac 5-HT$_4$ receptors with further unraveling of their signaling pathway. This led to the discovery of the very tight control of cardiac 5-HT$_4$ receptors by PDEs.

I.4.1 PDE-mediated control of cardiac 5-HT$_4$ receptors

5-HT$_4$ receptors are expressed on cardiomyocytes in porcine and human atrium (Parker *et al.*, 1995), and to a lower extent in ventricles. Next to positive chronotropic and lusitropic responses, 5-HT$_4$ receptor agonism leads to a rather weak and transient inotropic effect in porcine and human cardiac tissue (Krobert *et al.*, 2005; De Maeyer *et al.*, 2006b). The quick fade of the inotropic effect is caused by PDEs that quickly degrade cAMP formed after 5-HT$_4$ receptor stimulation; this can experimentally be prevented by PDE inhibition (De Maeyer *et al.*, 2006b; Galindo-Tovar *et al.*, 2009). Further studies indicated which PDE(s) are involved: in porcine atrium (Galindo-Tovar *et al.*, 2009; Weninger *et al.*, 2012) both PDE3 and PDE4 control the response to 5-HT$_4$ receptor stimulation in a redundant way, while in human atrium (Afzal *et al.*, 2008; Galindo-Tovar *et al.*, 2009) solely PDE3 is involved.
The highly selective 5-HT$_4$ receptor agonist prucalopride (Resolor®) was marketed for chronic idiopathic constipation in 2009. Subsequent surveys reported that prucalopride seems cardiovascularly safe (Manabe *et al.*, 2010; Tack *et al.*, 2012; Keating, 2013). Still, concern for the cardiovascular safety of prokinetic compounds remains actual (Giudicessi *et al.*, 2018).

I.4.2 PDE-mediated control of GI 5-HT$_4$ receptors

In contrast to cardiac responses to 5-HT$_4$ receptor agonists, their facilitating effect on acetylcholine release and cholinergic contraction in the GI tract is pronounced and sustained (Figure I.15). Still moderate PDE-mediated control of the signal transduction of 5-HT$_4$ receptors in myenteric cholinergic neurons innervating smooth muscle was observed. In porcine stomach (Priem *et al.*, 2012) and colon (Priem *et al.*, 2013), the facilitating effect of the 5-HT$_4$ receptor agonist prucalopride on acetylcholine

release and cholinergic smooth muscle contraction was enhanced by the selective PDE4 inhibitor rolipram. This might create an opportunity for combination therapy *in vivo*. Indeed, although the new generation of highly selective 5-HT_4 receptor agonists seem cardiovascularly safe, a combination therapy allowing to obtain optimal GI prokinetic effects with a lower dose and thus plasma concentrations of the 5-HT_4 receptor agonist, would still further reduce the risk of cardiac adverse events. The observation that in the porcine GI tract, the effect of a 5-HT_4 receptor agonist is enhanced in the presence of a selective PDE4 inhibitor was therefore further investigated in this PhD thesis.

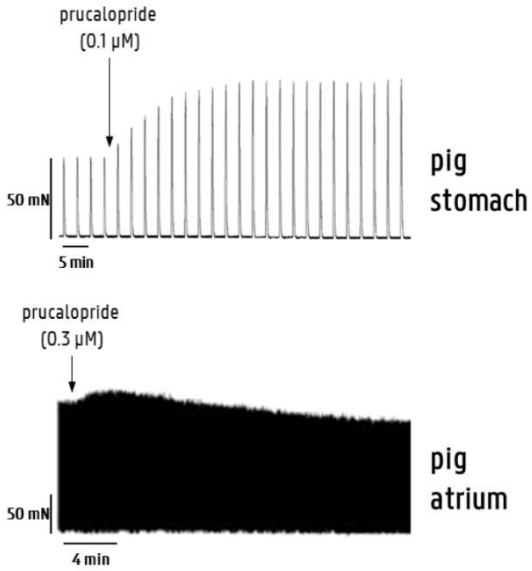


Figure I.15 - Response to prucalopride (single administration) in cardiac versus GI tissue. Above, a pronounced and sustained enhancement of electrically induced cholinergic contractions in porcine stomach longitudinal muscle (from De Maeyer *et al.*, 2006a). Below, a weak and transient inotropic effect in porcine left atrial pectinate muscle (De Maeyer *et al.*, 2006b).

I.5 References

Afzal F., Andressen K.W., Mork H.K., Aronsen J.M., Sjaastad I., Dahl C.P., Skomedal T., Levy F.O., Osnes J.B., and Qvigstad E. (2008). 5-HT4-elicited positive inotropic response is mediated by cAMP and regulated by PDE3 in failing rat and human cardiac ventricles. *Br J Pharmacol* 155**,** 1005-1014. DOI: 10.1038/bjp.2008.339

Alexander S.P.H., Christopoulos A., Davenport A.P., Kelly E., Marrion N.V., Peters J.A., Faccenda E., Harding S.D., Pawson A.J., Sharman J.L.*, et al.* (2017a). THE CONCISE GUIDE TO PHARMACOLOGY 2017/18: G protein-coupled receptors. *Br J Pharmacol* 174**,** S17-S129. DOI: 10.1111/bph.13878

Alexander S.P.H., Fabbro D., Kelly E., Marrion N.V., Peters J.A., Faccenda E., Harding S.D., Pawson A.J., Sharman J.L., Southan C.*, et al.* (2017b). THE CONCISE GUIDE TO PHARMACOLOGY 2017/18: Enzymes. *Br J Pharmacol* 174**,** S272-S359. DOI: 10.1111/bph.13877

Amemiya N., Hatta S., Takemura H., and Ohshika H. (1996). Characterization of the contractile response induced by 5-methoxytryptamine in rat stomach fundus strips. *Eur J Pharmacol* 318**,** 403-409. DOI: 10.1016/S0014-2999(96)00777-7

Ashman D.F., Price T.D., Melicow M.M., and Lipton R. (1963). Isolation of Adenosine 3,5-Monophosphate and Guanosine 3,5-Monophosphate from Rat Urine. *Biochem Biophys Res Commun* 11**,** 330-&. DOI: 10.1016/0006-291x(63)90566-7

Banner S.E., Smith M.I., Bywater D., Gaster L.M., and Sanger G.J. (1996). Increased defaecation caused by 5-HT4 receptor activation in the mouse. *Eur J Pharmacol* 308**,** 181-186. DOI: 10.1016/0014-2999(96)00296-8

Barbey J.T., Lazzara R., and Zipes D.P. (2002). Spontaneous adverse event reports of serious ventricular arrhythmias, QT prolongation, syncope, and sudden death in patients treated with cisapride. *J Cardiovasc Pharmacol Ther* 7**,** 65-76. DOI: 10.1177/107424840200700202

Barnes N.M., Hoyer D., Andrade R., Baxter G., Bockaert J., Branchek T., Cohen M.L., Dumuis A., Eglen R.M., Göthert M.*, et al. 5-Hydroxytryptamine receptors* [Online]. Available: http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=1 [Accessed 02/03/2018].

Barrett E.K., Barman S.M., Boitano S., and Brooks H.L. (2010). *Ganong's Review of Medical Physiology 23rd Edition.* United States: The McGraw-Hill Companies.

Baxter G.S., Craig D.A., and Clarke D.E. (1991). 5-Hydroxytryptamine4 Receptors Mediate Relaxation of the Rat Esophageal Tunica Muscularis Mucosae. *Naunyn Schmiedebergs Arch Pharmacol* 343**,** 439-446. DOI: 10.1007/BF00169544

Beattie D.T., Smith J.A., Marquess D., Vickery R.G., Armstrong S.R., Pulido-Rios T., Mccullough J.L., Sandlund C., Richardson C., Mai N.*, et al.* (2004). The 5-HT4 receptor agonist, tegaserod, is a potent 5-HT2B receptor antagonist in vitro and in vivo. *Br J Pharmacol* 143**,** 549-560. DOI: 10.1038/sj.bjp.0705929

Beattie D.T., and Smith J.A. (2008). Serotonin pharmacology in the gastrointestinal tract: a review. *Naunyn Schmiedebergs Arch Pharmacol* 377**,** 181-203. DOI: 10.1007/s00210-008-0276-9

Beavo J.A., Hardman J.G., and Sutherla.Ew (1970). Hydrolysis of Cyclic Guanosine and Adenosine 3',5'-Monophosphates by Rat and Bovine Tissues. *J Biol Chem* 245**,** 5649-5655.

Beavo J.A. (1995). Cyclic-Nucleotide Phosphodiesterases - Functional Implications of Multiple Isoforms. *Physiol Rev* 75**,** 725-748. DOI: 10.1152/physrev.1995.75.4.725

Bender A.T., and Beavo J.A. (2006). Cyclic nucleotide phosphodiesterases: Molecular regulation to clinical use. *Pharmacol Rev* 58**,** 488-520. DOI: 10.1124/pr.58.3.5

Berger M., Gray J.A., and Roth B.L. (2009). The Expanded Biology of Serotonin. *Annu Rev Med* 60, 355-366. DOI: 10.1146/annurev.med.60.042307.110802

Bharucha A.E., Camilleri M., Haydock S., Ferber I., Burton D., Cooper S., Tompson D., Fitzpatrick K., Higgins R., and Zinsmeister A.R. (2000). Effects of a serotonin 5-HT4 receptor antagonist SE-207266 on gastrointestinal motor and sensory function in humans. *Gut* 47, 667-674. DOI: 10.1136/gut.47.5.667

Bingham S., King B.F., Rushant B., Smith M.I., Gaster L., and Sanger G.J. (1995). Antagonism by SB 204070 of 5-HT-evoked contractions in the dog stomach: an in-vivo model of 5-HT4 receptor function. *J Pharm Pharmacol* 47, 219-222. DOI: 10.1111/j.2042-7158.1995.tb05782.x

Bockaert J., Claeysen S., Becamel C., Dumuis A., and Marin P. (2006). Neuronal 5-HT metabotropic receptors: fine-tuning of their structure, signaling, and roles in synaptic modulation. *Cell Tissue Res* 326, 553-572. DOI: 10.1007/s00441-006-0286-1

Boron W.F., and Boulpaep E.L. (2009). Medical Physiology: A Cellular and Molecular Approach 2nd edition. Canada: Saunders Elsevier.

Boswell-Smith V., Spina D., and Page C.P. (2006). Phosphodiesterase inhibitors. *Br J Pharmacol* 147, S252-S257. DOI: 10.1038/sj.bjp.0706495

Bouras E.P., Camilleri M., Burton D.D., and Mckinzie S. (1999). Selective stimulation of colonic transit by the benzofuran 5HT(4) agonist, prucalopride, in healthy humans. *Gut* 44, 682-686. DOI: 10.1136/gut.44.5.682

Bouras E.P., Camilleri M., Burton D.D., Thomforde G., Mckinzie S., and Zinsmeister A.R. (2001). Prucalopride accelerates gastrointestinal and colonic transit in patients with constipation without a rectal evacuation disorder. *Gastroenterology* 120, 354-360. DOI: 10.1053/gast.2001.21166

Briejer M.R., and Schuurkes J.A. (1996). 5-HT3 and 5-HT4 receptors and cholinergic and tachykininergic neurotransmission in the guinea-pig proximal colon. *Eur J Pharmacol* 308, 173-180. DOI: 10.1016/0014-2999(96)00297-X

Briejer M.R., Prins N.H., and Schuurkes J.a.J. (2001). Effects of the enterokinetic prucalopride (R093877) on colonic motility in fasted dogs. *Neurogastroenterol Motil* 13, 465-472. DOI: 10.1046/j.1365-2982.2001.00280.x

Browning K.N., and Travagli R.A. (2014). Central nervous system control of gastrointestinal motility and secretion and modulation of gastrointestinal functions. *Compr Physiol* 4, 1339-1368. DOI: 10.1002/cphy.c130055

Buchheit K.H., and Buhl T. (1991). Prokinetic benzamides stimulate peristaltic activity in the isolated guinea pig ileum by activation of 5-HT4 receptors. *Eur J Pharmacol* 205, 203-208. DOI: 10.1016/0014-2999(91)90821-7

Buchheit K.H., Gamse R., and Pfannkuche H.J. (1992). SDZ 205-557, a selective, surmountable antagonist for 5-HT4 receptors in the isolated guinea pig ileum. *Naunyn Schmiedebergs Arch Pharmacol* 345, 387-393. DOI: 10.1007/BF00176615

Buchheit K.H., and Buhl T. (1994). Stimulant Effects of 5-Hydroxytryptamine on Guinea-Pig Stomach Preparations in-Vitro. *Eur J Pharmacol* 262, 91-97. DOI: 10.1016/0014-2999(94)90031-0

Budhoo M.R., Harris R.P., and Kellum J.M. (1996a). The role of the 5-HT4 receptor in Cl- secretion in human jejunal mucosa. *Eur J Pharmacol* 314, 109-114. DOI: 10.1016/S0014-2999(96)00474-8

Budhoo M.R., Harris R.P., and Kellum J.M. (1996b). 5-Hydroxytryptamine-induced Cl- transport is mediated by 5-HT3 and 5-HT4 receptors in the rat distal colon. *Eur J Pharmacol* 298, 137-144. DOI: 10.1016/0014-2999(95)00752-0

Cellek S., John A.K., Thangiah R., Dass N.B., Bassil A.K., Jarvie E.M., Lalude O., Vivekanandan S., and Sanger G.J. (2006). 5-HT4 receptor agonists enhance both cholinergic and nitrergic activities in human isolated colon circular muscle. *Neurogastroenterol Motil* 18, 853-861. DOI: 10.1111/j.1365-2982.2006.00810.x

Cellek S., Thangiah R., Jarvie E.M., Vivekanandan S., Lalude O., and Sanger G.J. (2008). Synergy between 5-HT4 receptor activation and acetylcholinesterase inhibition in human colon and rat forestomach. *Neurogastroenterol Motil* 20, 539-545. DOI: 10.1111/j.1365-2982.2007.01062.x

Clayton N.M., and Gale J.D. (1996). 5-HT4 receptors are not involved in the control of small intestinal transit in the fasted conscious rat. *Neurogastroenterol Motil* 8, 1-8. DOI: 10.1111/j.1365-2982.1996.tb00236.x

Coates M.D., Tekin I., Vrana K.E., and Mawe G.M. (2017). Review article: the many potential roles of intestinal serotonin (5-hydroxytryptamine, 5-HT) signalling in inflammatory bowel disease. *Aliment Pharmacol Ther* 46, 569-580. DOI: 10.1111/apt.14226

Conti M., and Beavo J. (2007). Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu Rev Biochem* 76, 481-511. DOI: 10.1146/annurev.biochem.76.060305.150444

Coremans G., Kerstens R., De Pauw M., and Stevens M. (2003). Prucalopride is effective in patients with severe chronic constipation in whom laxatives fail to provide adequate relief - Results of a double-blind, placebo-controlled clinical trial. *Digestion* 67, 82-89. DOI: 10.1159/000070202

Coupar I.M., Desmond P.V., and Irving H.R. (2007). Human 5-HT(4) and 5-HT(7) receptor splice variants: are they important? *Curr Neuropharmacol* 5, 224-231. DOI: 10.2174/157015907782793621

Craig D.A., and Clarke D.E. (1991). Peristalsis evoked by 5-HT and renzapride: evidence for putative 5-HT4 receptor activation. *Br J Pharmacol* 102, 563-564. DOI: 10.1111/j.1476-5381.1991.tb12211.x

Dahlof C., and Van Den Brink A.M. (2012). Dihydroergotamine, Ergotamine, Methysergide and Sumatriptan - Basic Science in Relation to Migraine Treatment. *Headache* 52, 707-714. DOI: 10.1111/j.1526-4610.2012.02124.x

De Maeyer J.H., Prins N.H., Schuurkes J.a.J., and Lefebvre R.A. (2006a). Differential effects of 5-hydroxytryptamine4 receptor agonists at gastric versus cardiac receptors: an operational framework to explain and quantify organ-specific behavior. *J Pharmacol Exp Ther* 317, 955-964. DOI: 10.1124/jpet.106.101329

De Maeyer J.H., Straetemans R., Schuurkes J.a.J., and Lefebvre R.A. (2006b). Porcine left atrial and sinoatrial 5-HT4 receptor-induced responses: fading of the response and influence of development. *Br J Pharmacol* 147, 140-157. DOI: 10.1038/sj.bjp.0706497

De Maeyer J.H., Aerssens J., Verhasselt P., and Lefebvre R.A. (2008a). Alternative splicing and exon duplication generates 10 unique porcine 5-HT(4) receptor splice variants including a functional homofusion variant. *Physiol Genomics* 34, 22-33. DOI: 10.1152/physiolgenomics.00038.2008

De Maeyer J.H., Lefebvre R.A., and Schuurkes J.a.J. (2008b). 5-HT4 receptor agonists: similar but not the same. *Neurogastroenterol Motil* 20, 99-112. DOI: 10.1111/j.1365-2982.2007.01059.x

De Ponti F. (2004). Pharmacology of serotonin: What a clinician should know. *Gut* 53, 1520-1535. DOI: 10.1136/gut.2003.035568

Deninno M.P. (2012). Future directions in phosphodiesterase drug discovery. *Bioorg Med Chem Lett* 22, 6794-6800. DOI: 10.1016/j.bmcl.2012.09.028

Dumuis A., Bouhelal R., Sebben M., Cory R., and Bockaert J. (1988). A nonclassical 5-hydroxytryptamine receptor positively coupled with adenylate cyclase in the central nervous system. *Mol Pharmacol* 34, 880-887.

Dumuis A., Sebben M., and Bockaert J. (1989). The gastrointestinal prokinetic benzamide derivatives are agonists at the non-classical 5-HT receptor (5-HT4) positively coupled to adenylate cyclase in neurons. *Naunyn Schmiedebergs Arch Pharmacol* 340, 403-410. DOI: 10.1007/BF00167041

Durham P.L. (2006). Calcitonin gene-related peptide (CGRP) and migraine. *Headache* 46, S3-S8. DOI: 10.1111/j.1526-4610.2006.00483.x

Eglen R.M., Swank S.R., Walsh L.K., and Whiting R.L. (1990). Characterization of 5-HT3 and 'atypical' 5-HT receptors mediating guinea-pig ileal contractions in vitro. *Br J Pharmacol* 101, 513-520. DOI: 10.1111/j.1476-5381.1990.tb14113.x

Emmanuel A.V., Kamm M.A., Roy A.J., and Antonelli K. (1998). Effect of a novel prokinetic drug, R093877, on gastrointestinal transit in healthy volunteers. *Gut* 42, 511-516. DOI: 10.1136/gut.42.4.511

Emmanuel A.V., Roy A.J., Nicholls T.J., and Kamm M.A. (2002). Prucalopride, a systemic enterokinetic, for the treatment of constipation. *Aliment Pharmacol Ther* 16, 1347-1356. DOI: 10.1046/j.1365-2036.2002.01272.x

Erspamer V., and Asero B. (1952). Identification of enteramine, the specific hormone of the enterochromaffin cell system, as 5-hydroxytryptamine. *Nature* 169, 800-801. DOI: 10.1038/169800b0

Fang X., Liu S., Wang X.Y., Gao N., Hu H.Z., Wang G.D., Cook C.H., Needleman B.J., Mikami D.J., Xia Y.*, et al.* (2008). Neurogastroenterology of tegaserod (HTF 919) in the submucosal division of the guinea-pig and human enteric nervous system. *Neurogastroenterol Motil* 20, 80-93. DOI: 10.1111/j.1365-2982.2007.00983.x

Foxx-Orenstein A.E., Kuemmerle J.F., and Grider J.R. (1996). Distinct 5-HT receptors mediate the peristaltic reflex induced by mucosal stimuli in human and guinea pig intestine. *Gastroenterology* 111, 1281-1290. DOI: 10.1053/gast.1996.v111.pm8898642

Francis S.H., Blount M.A., and Corbin J.D. (2011). Mammalian cyclic nucleotide phosphodiesterases: molecular mechanisms and physiological functions. *Physiol Rev* 91, 651-690. DOI: 10.1152/physrev.00030.2010

Fukui H., Yamamoto M., Sasaki S., and Sato S. (1994). Possible involvement of peripheral 5-HT4 receptors in copper sulfate-induced vomiting in dogs. *Eur J Pharmacol* 257, 47-52. DOI: 10.1016/0014-2999(94)90692-0

Galindo-Tovar A., Vargas M.L., Escudero E., and Kaumann A.J. (2009). Ontogenic changes of the control by phosphodiesterase-3 and -4 of 5-HT responses in porcine heart and relevance to human atrial 5-HT4 receptors. *Br J Pharmacol* 156, 237-249. DOI: 10.1111/j.1476-5381.2008.00023.x

Galligan J.J., Pan H., and Messori E. (2003). Signalling mechanism coupled to 5-hydroxytryptamine4 receptor-mediated facilitation of fast synaptic transmission in the guinea-pig ileum myenteric plexus. *Neurogastroenterol Motil* 15, 523-529. DOI: 10.1046/j.1365-2982.2003.00428.x

Galligan J.J., and Vanner S. (2005). Basic and clinical pharmacology of new motility promoting agents. *Neurogastroenterol Motil* 17, 643-653. DOI: 10.1111/j.1365-2982.2005.00675.x

Gebauer A., Merger M., and Kilbinger H. (1993). Modulation by 5-HT3 and 5-HT4 receptors of the release of 5-hydroxytryptamine from the guinea-pig small intestine. *Naunyn Schmiedebergs Arch Pharmacol* 347, 137-140.

Gershon M.D., and Tack J. (2007). The serotonin signaling system: From basic understanding to drug development-for functional GI disorders. *Gastroenterology* 132, 397-414. DOI: 10.1053/j.gastro.2006.11.002

Gershon M.D. (2013). 5-Hydroxytryptamine (serotonin) in the gastrointestinal tract. *Curr Opin Endocrinol Diabetes Obes* 20, 14-21. DOI: 10.1097/MED.0b013e32835bc703

Giembycz M.A., and Field S.K. (2010). Roflumilast: first phosphodiesterase 4 inhibitor approved for treatment of COPD. *Drug Des Devel Ther* 4, 147-158. DOI: 10.2147/DDDT.S7667

Gill R.K., Saksena S., Tyagi S., Alrefai W.A., Malakooti J., Sarwar Z., Turner J.R., Ramaswamy K., and Dudeja P.K. (2005). Serotonin inhibits Na+/H+ exchange activity via 5-HT4 receptors and activation of PKC alpha in human intestinal epithelial cells. *Gastroenterology* 128, 962-974. DOI: 10.1053/j.gastro.2005.02.01

Giudicessi J.R., Ackerman M.J., and Camilleri M. (2018). Cardiovascular safety of prokinetic agents: A focus on drug-induced arrhythmias. *Neurogastroenterol Motil*. DOI: 10.1111/nmo.13302

Glennon R.A. (2003). Higher-end serotonin receptors: 5-HT5, 5-HT6, and 5-HT7. *J Med Chem* 46, 2795-2812. DOI: 10.1021/jm030030n

Gomez-Pinilla P.J., Di Giovangiulio M., Nemethova A., Stakenborg N., Farro G., Bosmans G., Matteoli G., and Boeckxstaens G.E. (2014). Prucalopride Activates the Intestinal Cholinergic Anti-Inflammatory Pathway and Prevents Postoperative Ileus (Abstract). *Gastroenterology* 146, S-89 DOI: 10.1016/S0016-5085(14)60324-3

Greenwood-Van Meerveld B., Venkova K., Hicks G., Dennis E., and Crowell M.D. (2006). Activation of peripheral 5-HT4 receptors attenuates colonic sensitivity to intraluminal distension. *Neurogastroenterol Motil* 18, 76-86. DOI: 10.1111/j.1365-2982.2005.00723.x

Grider J.R., Foxx-Orenstein A.E., and Jin J.G. (1998). 5-Hydroxytryptamine4 receptor agonists initiate the peristaltic reflex in human, rat, and guinea pig intestine. *Gastroenterology* 115, 370-380. DOI: 10.1016/S0016-5085(98)70203-3

Grider J.R. (2003). Neurotransmitters mediating the intestinal peristaltic reflex in the mouse. *J Pharmacol Exp Ther* 307, 460-467. DOI: 10.1124/jpet.103.053512

Gullikson G.W., Virina M.A., Loeffler R.F., Yang D.C., Goldstin B., Wang S.X., Moummi C., Flynn D.L., and Zabrowski D.L. (1993). SC-49518 enhances gastric emptying of solid and liquid meals and stimulates gastrointestinal motility in dogs by a 5-hydroxytryptamine4 receptor mechanism. *J Pharmacol Exp Ther* 264, 240-248.

Hannon J., and Hoyer D. (2008). Molecular biology of 5-HT receptors. *Behav Brain Res* 195, 198-213. DOI: 10.1016/j.bbr.2008.03.020

Hansen M.B. (1994). ICS 205-930 reduces 5-methoxytryptamine-induced short-circuit current in stripped pig jejunum. *Can J Physiol Pharmacol* 72, 227-232. DOI: 10.1139/y94-035

Hansen M.B. (1995). Tropisetron and octreotide reduce serotonin-induced fluid hypersecretion in pig jejunum. *Pharmacol Toxicol* 76, 136-140. DOI: 10.1111/j.1600-0773.1995.tb00119.x

Hedge S.S., Moy T.M., Perry M.R., Loeb M., and Eglen R.M. (1994). Evidence for the involvement of 5-hydroxytryptamine 4 receptors in 5-hydroxytryptophan-induced diarrhea in mice. *J Pharmacol Exp Ther* 271, 741-747.

Hegde S.S., Wong A.G., Perry M.R., Ku P., Moy T.M., Loeb M., and Eglen R.M. (1995). 5-Ht4 Receptor-Mediated Stimulation of Gastric-Emptying in Rats. *Naunyn Schmiedebergs Arch Pharmacol* 351, 589-595. DOI: 10.1007/BF00170157

Herr N., Bode C., and Duerschmied D. (2017). The Effects of Serotonin in Immune Cells. *Front Cardiovasc Med* 4, 48. DOI: 10.3389/fcvm.2017.00048

Hightower N.C., Sircus W., Dworken H.J., and Keeton W.T. *Human Digestive System* [Online]. Available: https://www.britannica.com/science/human-digestive-system/images-videos [Accessed 25/01/2018].

Hoyer D., Clarke D.E., Fozard J.R., Hartig P.R., Martin G.R., Mylecharane E.J., Saxena P.R., and Humphrey P.P. (1994). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol Rev* 46, 157-203.

Jin J.G., Foxx-Orenstein A.E., and Grider J.R. (1999). Propulsion in guinea pig colon induced by 5-hydroxytryptamine (HT) via 5-HT4 and 5-HT3 receptors. *J Pharmacol Exp Ther* 288, 93-97.

Kaumann A.J., Sanders L., Brown A.M., Murray K.J., and Brown M.J. (1990). A 5-hydroxytryptamine receptor in human atrium. *Br J Pharmacol* 100, 879-885. DOI: 10.1111/j.1476-5381.1990.tb14108.x

Kaumann A.J., and Levy F.O. (2006). 5-hydroxytryptamine receptors in the human cardiovascular system. *Pharmacol Ther* 111, 674-706. DOI: 10.1016/j.pharmthera.2005.12.004

Keating G.M. (2013). Prucalopride: A Review of Its Use in the Management of Chronic Constipation. *Drugs* 73, 1935-1950. DOI: 10.1007/s40265-013-0140-1

Kellum J.M., Albuquerque F.C., Stoner M.C., and Harris R.P. (1999). Stroking human jejunal mucosa induces 5-HT release and Cl- secretion via afferent neurons and 5-HT4 receptors. *Am J Physiol* 277, G515-520. DOI: 10.1152/ajpgi.1999.277.3.G515

Kendig D.M., and Grider J.R. (2015). Serotonin and colonic motility. *Neurogastroenterol Motil* 27, 899-905. DOI: 10.1111/nmo.12617

Keravis T., and Lugnier C. (2012). Cyclic nucleotide phosphodiesterase (PDE) isozymes as targets of the intracellular signalling network: benefits of PDE inhibitors in various diseases and perspectives for future therapeutic developments. *Br J Pharmacol* 165, 1288-1305. DOI: 10.1111/j.1476-5381.2011.01729.x

Kilbinger H., and Wolf D. (1992). Effects of 5-HT4 receptor stimulation on basal and electrically evoked release of acetylcholine from guinea-pig myenteric plexus. *Naunyn Schmiedebergs Arch Pharmacol* 345, 270-275. DOI: 10.1007/BF00168686

Koeppen B.M., and Stanton B.A. (2008). *Berne and Levy Physiology 6th edition.* Canada: Mosby Elsevier.

Kojima S., and Shimo Y. (1996). Investigation into the 5-hydroxytryptamine-induced atropine-resistant neurogenic contraction of guinea-pig proximal colon. *Br J Pharmacol* 117, 1613-1618. DOI: 10.1111/j.1476-5381.1996.tb15330.x

Kojima S., Ikeda M., and Kamikawa Y. (2000). Investigation into the 5-hydroxytryptophan-evoked luminal 5-hydroxytryptamine release from the guinea pig colon. *Jpn J Pharmacol* 84, 174-178. DOI: 10.1254/jjp.84.174

Komada T., and Yano S. (2007). Pharmacological characterization of 5-hydroxytryptamine-receptor subtypes in circular muscle from the rat stomach. *Biol Pharm Bull* 30, 508-513. DOI: 10.1248/bpb.30.508

Krobert K.A., Brattelid T., Levy F.O., and Kaumann A.J. (2005). Prucalopride is a partial agonist through human and porcine atrial 5-HT4 receptors: comparison with recombinant human 5-HT4 splice variants. *Naunyn Schmiedebergs Arch Pharmacol* 371, 473-479. DOI: 10.1007/s00210-005-1068-0

Langlois M., and Fischmeister R. (2003). 5-HT4 receptor ligands: Applications and new prospects. *J Med Chem* 46, 319-344. DOI: 10.1021/jm020099f

Leclere P.G., and Lefebvre R.A. (2002). Presynaptic modulation of cholinergic neurotransmission in the human proximal stomach. *Br J Pharmacol* 135, 135-142. DOI: 10.1038/sj.bjp.0704471

Leclere P.G., Prins N.H., Schuurkes J.a.J., and Lefebvre R.A. (2005). 5-HT4 receptors located on cholinergic nerves in human colon circular muscle. *Neurogastroenterol Motil* 17, 366-375. DOI: 10.1111/j.1365-2982.2005.00621.x

Lepard K.J., Ren J., and Galligan J.J. (2004). Presynaptic modulation of cholinergic and non-cholinergic fast synaptic transmission in the myenteric plexus of guinea pig ileum. *Neurogastroenterol Motil* 16, 355-364. DOI: 10.1111/j.1365-2982.2004.00505.x

Leung E., Blissard D., Jett M.F., and Eglen R.M. (1995). Investigation of the 5-hydroxytryptamine receptor mediating the "transient" short-circuit current response in guinea-pig ileal mucosa. *Naunyn Schmiedebergs Arch Pharmacol* 351, 596-602. DOI: 10.1007/BF00170158

Li J., Ning Y.H., Hedley W., Saunders B., Chen Y.S., Tindill N., Hannay T., and Subramaniam S. (2002). The Molecule Pages database. *Nature* 420, 716-717. DOI: 10.1038/nature01307

Liu M., Geddis M.S., Wen Y., Setlik W., and Gershon M.D. (2005). Expression and function of 5-HT4 receptors in the mouse enteric nervous system. *Am J Physiol Gastrointest Liver Physiol* 289, G1148-1163. DOI: 10.1152/ajpgi.00245.2005

Liu M.T., Kuan Y.H., Wang J.W., Hen R., and Gershon M.D. (2009). 5-HT4 Receptor-Mediated Neuroprotection and Neurogenesis in the Enteric Nervous System of Adult Mice. *J Neurosci* 29, 9683-9699. DOI: 10.1523/Jneurosci.1145-09.2009

Lugnier C. (2006). Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. *Pharmacol Ther* 109, 366-398. DOI: 10.1016/j.pharmthera.2005.07.003

Makimoto N., Sakurai-Yamashita Y., Furuichi A., Kawakami S., Enjoji A., Kanematsu T., and Taniyama K. (2002). In vivo assessment of acceleration of motor activity associated with acetylcholine release via 5-hydroxytryptamine(4) receptor in dog intestine. *Jpn J Pharmacol* 90, 28-35. DOI: 10.1254/jjp.90.28

Manabe N., Wong B.S., and Camilleri M. (2010). New-generation 5-HT4 receptor agonists: potential for treatment of gastrointestinal motility disorders. *Expert Opin Investig Drugs* 19, 765-775. DOI: 10.1517/13543784.2010.482927

Maurice D.H., Ke H.M., Ahmad F., Wang Y.S., Chung J., and Manganiello V.C. (2014). Advances in targeting cyclic nucleotide phosphodiesterases. *Nat Rev Drug Discov* 13, 290-314. DOI: 10.1038/nrd4228

Mawe G.M., and Hoffman J.M. (2013). Serotonin signalling in the gut-functions, dysfunctions and therapeutic targets. *Nat Rev Gastroenterol Hepatol* 10, 473-486. DOI: 10.1038/nrgastro.2013.105

Mclean P.G., and Coupar I.M. (1995). 5-HT4 receptor antagonist affinities of SB207710, SB205008, and SB203186 in the human colon, rat oesophagus, and guinea-pig ileum peristaltic reflex. *Naunyn Schmiedebergs Arch Pharmacol* 352, 132-140. DOI: 10.1007/BF00176766

Mclean P.G., Coupar I.M., and Molenaar P. (1995). A Comparative-Study of Functional 5-Ht4 Receptors in Human Colon, Rat Esophagus and Rat Ileum. *Br J Pharmacol* 115, 47-56. DOI: 10.1111/j.1476-5381.1995.tb16318.x

Mclean P.G., and Coupar I.M. (1996a). Further investigation into the signal transduction mechanism of the 5-HT4-like receptor in the circular smooth muscle of human colon. *Br J Pharmacol* 118, 1058-1064. DOI: 10.1111/j.1476-5381.1996.tb15506.x

Mclean P.G., and Coupar I.M. (1996b). Stimulation of cyclic AMP formation in the circular smooth muscle of human colon by activation of 5-HT4-like receptors. *Br J Pharmacol* 117, 238-239. DOI: 10.1111/j.1476-5381.1996.tb15182.x

Miller M.S. (2015). Phosphodiesterase inhibition in the treatment of autoimmune and inflammatory diseases: current status and potential. *J Receptor Ligand Channel Res* 8, 19-30. DOI: 10.2147/Jrlcr.S50401

Minami M., Tamakai H., Ogawa T., Endo T., Hamaue N., Hirafuji M., Yoshioka M., and Blower P.R. (1995). Chemical modulation of 5-HT3 and 5-HT4 receptors affects the release of 5-hydroxytryptamine from the ferret and rat intestine. *Res Commun Mol Pathol Pharmacol* 89, 131-142.

Mohammad-Zadeh L.F., Moses L., and Gwaltney-Brant S.M. (2008). Serotonin: a review. *J Vet Pharmacol Ther* 31, 187-199. DOI: 10.1111/j.1365-2885.2008.00944.x

Mohammad S., Zhou Z.F., Gong Q.M., and January C.T. (1997). Blockage of the HERG human cardiac K+ channel by the gastrointestinal prokinetic agent cisapride. *Am J Physiol Heart Circ Physiol* 273**,** H2534-H2538. DOI: 10.1152/ajpheart.1997.273.5.H2534

Nicholson C.D., Challiss R.a.J., and Shahid M. (1991). Differential Modulation of Tissue Function and Therapeutic Potential of Selective Inhibitors of Cyclic-Nucleotide Phosphodiesterase Isoenzymes. *Trends Pharmacol Sci* 12**,** 19-27. DOI: 10.1016/0165-6147(91)90484-A

Ning Y., Zhu J.X., and Chan H.C. (2004). Regulation of ion transport by 5-hydroxytryptamine in rat colon. *Clin Exp Pharmacol Physiol* 31**,** 424-428. DOI: 10.1111/j.1440-1681.2004.04015.x

Noel S., Dhooghe B., and Leal T. (2012). PDE5 inhibitors as potential tools in the treatment of cystic fibrosis. *Front Pharmacol* 3. DOI: 10.3389/fphar.2012.00167

Openstax - Rice University (2013). *Anatomy and Physiology.* OpenStax CNX; Rice University. Available from: http://cnx.org/contents/14fb4ad7-39a1-4eee-ab6e-3ef2482e3e22@8.24.

Pan H., and Galligan J.J. (1994). 5-HT1A and 5-HT4 receptors mediate inhibition and facilitation of fast synaptic transmission in enteric neurons. *Am J Physiol* 266**,** G230-238. DOI: 10.1152/ajpgi.1994.266.2.G230

Parker S.G., Taylor E.M., Hamburger S.A., Vimal M., and Kaumann A.J. (1995). Blockade of human and porcine myocardial 5-HT4 receptors by SB 203186. *Naunyn Schmiedebergs Arch Pharmacol* 353**,** 28-35. DOI: 10.1007/BF00168912

Pascual D., Alsasua A., Goicoechea C., and Martin M.I. (2002). The involvement of 5-HT3 and 5-HT4 receptors in two models of gastrointestinal transit in mice. *Neurosci Lett* 326**,** 163-166. DOI: 10.1016/S0304-3940(02)00251-3

Pasricha P.J. (2007). Desperately seeking serotonin... A commentary on the withdrawal of tegaserod and the state of drug development for functional and motility disorders. *Gastroenterology* 132**,** 2287-2290. DOI: 10.1053/j.gastro.2007.04.057

Poen A.C., Felt-Bersma R.J., Van Dongen P.A., and Meuwissen S.G. (1999). Effect of prucalopride, a new enterokinetic agent, on gastrointestinal transit and anorectal function in healthy volunteers. *Aliment Pharmacol Ther* 13**,** 1493-1497. DOI: 10.1046/j.1365-2036.1999.00629.x

Poole D.P., Xu B., Koh S.L., Hunne B., Coupar I.M., Irving H.R., Shinjo K., and Furness J.B. (2006). Identification of neurons that express 5-hydroxytryptamine4 receptors in intestine. *Cell Tissue Res* 325**,** 413-422. DOI: 10.1007/s00441-006-0181-9

Priem E.K.V., and Lefebvre R.A. (2011). Investigation of neurogenic excitatory and inhibitory motor responses and their control by 5-HT4 receptors in circular smooth muscle of pig descending colon. *Eur J Pharmacol* 667**,** 365-374. DOI: 10.1016/j.ejphar.2011.06.021

Priem E.K.V., Van Colen I., De Maeyer J.H., and Lefebvre R.A. (2012). The facilitating effect of prucalopride on cholinergic neurotransmission in pig gastric circular muscle is regulated by phosphodiesterase 4. *Neuropharmacology* 62**,** 2126-2135. DOI: 10.1016/j.neuropharm.2011.12.020

Priem E.K.V., De Maeyer J.H., and Lefebvre R.A. (2013). Influence of phosphodiesterases on basal and 5-HT4 receptor facilitated cholinergic contractility in pig descending colon. *Eur J Pharmacol* 705**,** 156-163. DOI: 10.1016/j.ejphar.2013.02.011

Prins N.H., Van Haselen J.F., Lefebvre R.A., Briejer M.R., Akkermans L.M., and Schuurkes J.A. (1999). Pharmacological characterization of 5-HT4 receptors mediating relaxation of canine isolated rectum circular smooth muscle. *Br J Pharmacol* 127**,** 1431-1437. DOI: 10.1038/sj.bjp.0702665

Prins N.H., Akkermans L.M.A., Lefebvre R.A., and Schuurkes J.a.J. (2000). 5-HT4 receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. *Br J Pharmacol* 131, 927-932. DOI: 10.1038/sj.bjp.0703615

Prins N.H., Akkermans L.M.A., Lefebvre R.A., and Schuurkes J.a.J. (2001a). Characterization of the receptors involved in the 5-HT-induced excitation of canine antral longitudinal muscle. *Br J Pharmacol* 134, 1351-1359. DOI: 10.1038/sj.bjp.0704376

Prins N.H., Van Der Grijn A., Lefebvre R.A., Akkermans L.M.A., and Schuurkes J.a.J. (2001b). 5-HT4 receptors mediating enhancement of contractility in canine stomach; an in vitro and in vivo study. *Br J Pharmacol* 132, 1941-1947. DOI: 10.1038/sj.bjp.0703985

Rall T.W., and Sutherland E.W. (1958). Formation of a Cyclic Adenine Ribonucleotide by Tissue Particles. *J Biol Chem* 232, 1065-1076.

Rapport M.M., Green A.A., and Page I.H. (1948). Serum vasoconstrictor, serotonin; isolation and characterization. *J Biol Chem* 176, 1243-1251.

Raymond J.R., Mukhin Y.V., Gelasco A., Turner J., Collinsworth G., Gettys T.W., Grewal J.S., and Garnovskaya M.N. (2001). Multiplicity of mechanisms of serotonin receptor signal transduction. *Pharmacol Ther* 92, 179-212. DOI: 10.1016/S0163-7258(01)00169-3

Roth B.L. (2006). The Serotonin Receptors: From molecular pharmacology to human therapeutics. United States: Humana Press.

Ruddell R.G., Mann D.A., and Ramm G.A. (2008). The function of serotonin within the liver. *J Hepatol* 48, 666-675. DOI: 10.1016/j.jhep.2008.01.006

Safsten B., Sjoblom M., and Flemstrom G. (2006). Serotonin increases protective duodenal bicarbonate secretion via enteric ganglia and a 5-HT4-dependent pathway. *Scand J Gastroenterol* 41, 1279-1289. DOI: 10.1080/00365520600641480

Sakula A. (1985). Henry Hyde Salter (1823-71): a biographical sketch. *Thorax* 40, 887-888. DOI: 10.1136/thx.40.12.887

Sakurai-Yamashita Y., Takada K., Takemura K., Yamashita K., Enjoji A., Kanematsu T., and Taniyama K. (1999a). Ability of mosapride to bind to 5-HT4 receptor in the human stomach. *Jpn J Pharmacol* 79, 493-496. DOI: 10.1254/jjp.79.493

Sakurai-Yamashita Y., Yamashita K., Kanematsu T., and Taniyama K. (1999b). Localization of the 5-HT(4) receptor in the human and the guinea pig colon. *Eur J Pharmacol* 383, 281-285. DOI: 10.1016/S0014-2999(99)00642-1

Sakurai-Yamashita Y., Yamashita K., Yoshimura M., and Taniyama K. (2000). Differential localization of 5-hydroxytryptamine3 and 5-hydroxytryptamine4 receptors in the human rectum. *Life Sci* 66, 31-34. DOI: 10.1016/S0024-3205(99)00558-5

Sanger G.J. (1987). Activation of a myenteric 5-hydroxytryptamine-like receptor by metoclopramide. *J Pharm Pharmacol* 39, 449-453. DOI: 10.1111/j.2042-7158.1987.tb03418.x

Sanger G.J., Banner S.E., Smith M.I., and Wardle K.A. (1998). SB-207266: 5-HT4 receptor antagonism in human isolated gut and prevention of 5-HT-evoked sensitization of peristalsis and increased defaecation in animal models. *Neurogastroenterol Motil* 10, 271-279. DOI: 10.1046/j.1365-2982.1998.00106.x

Sanger G.J. (2008). 5-hydroxytryptamine and the gastrointestinal tract: where next? *Trends Pharmacol Sci* 29, 465-471. DOI: 10.1016/j.tips.2008.06.008

Schiller L.R. (2004). New and emerging treatment options for chronic constipation. *Rev Gastroenterol Disord* 4 Suppl 2, S43-51.

Schuurkes J. (1990). Effect of cisapride on gastric motility. *Z Gastroenterol* 28 Suppl 1, 27-30; discussion 44.

Schworer H., and Ramadori G. (1998). Autoreceptors can modulate 5-hydroxytryptamine release from porcine and human small intestine in vitro. *Naunyn Schmiedebergs Arch Pharmacol* 357, 548-552. DOI: 10.1007/PL00005206

Seeley R., Stephens T., and Tate P. (2004). *Anatomy and Physiology, 6th Edition.* The McGraw−Hill Companies. Available from: http://bio.bsu.by/t/temp/holik/24%20Digestive%20System.pdf.

Spiller R., Grundy D., Azpiroz F., Aziz Q., Barbara G., Bharucha A.E., Bielefeldt K., Blackshaw L.A., Brooks S., Bueno L., *et al.* (2004). *Pathophysiology of the Enteric Nervous System: a Basis for Understanding Functional Diseases 1st Edition.* Slovenia: Blackwell Publishing.

Stasi C., Bellini M., Bassotti G., Blandizzi C., and Milani S. (2014). Serotonin receptors and their role in the pathophysiology and therapy of irritable bowel syndrome. *Tech Coloproctol* 18, 613-621. DOI: 10.1007/s10151-013-1106-8

Streutker C., Colley E.C., Hillsley K., Hicks G.A., Kelly S.M., and Stead R.H. (2006). 5-HT4 receptor-immunoreactivity (5-HT4-IR) is expressed in non-neuronal cells of the human gastrointestinal (GI) tract (abstract). *Neurogastroenterol Motil* 18, 672.

Sun Y.N., and Luo J.Y. (2004). Effects of tegaserod on Fos, substance P and calcitonin gene-related peptide expression induced by colon inflammation in lumbarsacral spinal cord. *World J Gastroenterol* 10, 1830-1833. DOI: 10.3748/wjg.v10.i12.1830

Sutherland E.W., and Rall T.W. (1958). Fractionation and Characterization of a Cyclic Adenine Ribonucleotide Formed by Tissue Particles. *J Biol Chem* 232, 1077-1091.

Szeitz A., and Bandiera S.M. (2018). Analysis and measurement of serotonin. *Biomed Chromatogr* 32. DOI: 10.1002/bmc.4135

Tack J., Camilleri M., Chang L., Chey W.D., Galligan J.J., Lacy B.E., Muller-Lissner S., Quigley E.M., Schuurkes J., De Maeyer J.H., *et al.* (2012). Systematic review: cardiovascular safety profile of 5-HT(4) agonists developed for gastrointestinal disorders. *Aliment Pharmacol Ther* 35, 745-767. DOI: 10.1111/j.1365-2036.2012.05011.x

Takemura K., Takada K., Mameya S., Kaibara M., and Taniyama K. (1999). Regional and functional differences of 5-hydroxytryptamine-receptor subtypes in guinea pig stomach. *Jpn J Pharmacol* 79, 41-49. DOI: 10.1254/jjp.79.41

Talley N.J. (2002). New and emerging treatments for irritable bowel syndrome and functional dyspepsia. *Expert Opin Emerg Drugs* 7, 91-98. DOI: 10.1517/14728214.7.1.91

Tam F.S., Hillier K., Bunce K.T., and Grossman C. (1995). Differences in response to 5-HT4 receptor agonists and antagonists of the 5-HT4-like receptor in human colon circular smooth muscle. *Br J Pharmacol* 115, 172-176. DOI: 10.1111/j.1476-5381.1995.tb16335.x

Tilley S.L. (2011). Methylxanthines in asthma. *Handb Exp Pharmacol*, 439-456. DOI: 10.1007/978-3-642-13443-2_17

Tomita R., Tanjoh K., and Munakata K. (1997). The role of motilin and cisapride in the enteric nervous system of the lower esophageal sphincter in humans. *Surg Today* 27, 985-992. DOI: 10.1007/Bf02385776

Tonini M., Candura S.M., Onori L., Coccini T., Manzo L., and Rizzi C.A. (1992). 5-Hydroxytryptamine4 receptor agonists facilitate cholinergic transmission in the circular muscle of guinea-pig ileum: antagonism by tropisetron and DAU-6285. *Life Sci* 50, Pl173-Pl178. DOI: 10.1016/0024-3205(92)90453-V

Tonini M., De Giorgio R., Spelta V., Bassotti G., Di Nucci A., Anselmi L., Balestra B., and De Ponti F. (2003). 5-HT4 receptors contribute to the motor stimulating effect of levosulpiride in the guinea-pig gastrointestinal tract. *Dig Liver Dis* 35, 244-250. DOI: 10.1016/S1590-8658(03)00061-6

Tonini M., and Pace F. (2006). Drugs acting on serotonin receptors for the treatment of functional GI disorders. *Dig Dis* 24, 59-69. DOI: 10.1159/000090309

Tsuchida Y., Hatao F., Fujisawa M., Murata T., Kaminishi M., Seto Y., Hori M., and Ozaki H. (2011). Neuronal stimulation with 5-hydroxytryptamine 4 receptor induces anti-inflammatory actions via alpha7nACh receptors on muscularis macrophages associated with postoperative ileus. *Gut* 60, 638-647. DOI: 10.1136/gut.2010.227546

Tuladhar B.R., Costall B., and Naylor R.J. (1996). Pharmacological characterization of the 5-hydroxytryptamine receptor mediating relaxation in the rat isolated ileum. *Br J Pharmacol* 119, 303-310. DOI: 10.1111/j.1476-5381.1996.tb15986.x

Tuo B.G., Sellers Z., Paulus P., Barrett K.E., and Isenberg J.I. (2004). 5-HT induces duodenal mucosal bicarbonate secretion via cAMP- and Ca2+-dependent signaling pathways and 5-HT4 receptors in mice. *Am J Physiol Gastrointest Liver Physiol* 286, G444-G451. DOI: 10.1152/ajpgi.00105.2003

Villarreal M.R. (2016). *The gastrointestinal tract: Digestive system diagram* [Online]. Available: https://en.wikibooks.org/wiki/Human_Physiology/The_gastrointestinal_system#/media/File:Digestive_system_diagram_en.svg [Accessed 25/01/2018].

Walstab J., Rappold G., and Niesler B. (2010). 5-HT3 receptors: Role in disease and target of drugs. *Pharmacol Ther* 128, 146-169. DOI: 10.1016/j.pharmthera.2010.07.001

Wang L., Martinez V., Kimura H., and Tache Y. (2007). 5-Hydroxytryptophan activates colonic myenteric neurons and propulsive motor function through 5-HT4 receptors in conscious mice. *Am J Physiol Gastrointest Liver Physiol* 292, G419-428. DOI: 10.1152/ajpgi.00289.2006

Weninger S., De Maeyer J.H., and Lefebvre R.A. (2012). Study of the regulation of the inotropic response to 5-HT4 receptor activation via phosphodiesterases and its cross-talk with C-type natriuretic peptide in porcine left atrium. Naunyn Schmiedebergs Arch Pharmacol 385, 565-577. DOI: 10.1007/s00210-012-0746-y

Woehler A., and Ponimaskin E.G. (2009). G protein--mediated signaling: same receptor, multiple effectors. *Curr Mol Pharmacol* 2, 237-248. DOI: 10.2174/1874467210902030237

Yamano M., Kamato T., and Miyata K. (1997). Participation of a cholinergic mechanism in 5-hydroxytryptamine (5-HT)3 and 5-HT4 receptor-mediated stimulation of gastric emptying in rats. *Arzneimittelforschung* 47, 1242-1246.

CHAPTER II

Aims

CHAPTER II - Aims

Two of the first prokinetic 5-HT_4 receptor agonists, cisapride and tegaserod, used for treatment of GI hypomotility disorders, were withdrawn because of cardiovascular side effects (Barbey *et al.*, 2002; Pasricha, 2007). Although the cardiovascular side effects of cisapride and tegaserod were non-5-HT_4 receptor related, this has influenced the perception on the use of 5-HT_4 receptor agonists for GI disorders (De Maeyer *et al.*, 2008) and dampened for several years the interest to clinically further develop highly selective 5-HT_4 receptor agonists, that had been synthetized and experimentally investigated (Giudicessi *et al.*, 2018). 5-HT_4 receptors are also present in the cardiac atria but stimulation of these receptors only induces mild and transient effects due to the tight control of the induced cAMP response by PDEs (De Maeyer et al., 2006; Galindo-Tovar et al., 2009; Weninger et al., 2012). The nonclinical cardiovascular studies of the highly selective 5-HT_4 receptor agonist prucalopride showed that no relevant cardiovascular and cardiac electrophysiological effects were observed at prucalopride concentrations of at least 50 times the therapeutic C_{max} in man, except for small transient increases in heart rate and blood pressure in anesthetized pigs at concentrations 10 times higher than this human therapeutic C_{max} (Conlon *et al.*, 2018). Prucalopride was marketed for chronic idiopathic constipation in 2009 and was found to be cardiovascularly safe till now (Manabe *et al.*, 2010; Tack *et al.*, 2012; Keating, 2013).

Still, combination therapy that would allow to induce the same desired GI prokinetic effects with a lower dose of the 5-HT_4 receptor agonist, will still further decrease the plasma concentration to which cardiac 5-HT_4 receptors are exposed. Combination of the 5-HT_4 receptor agonist prucalopride with the acetylcholinesterase inhibitor neostigmine had a synergistic effect on *in vitro* cholinergic activity in human colon (Cellek *et al.*, 2008) and this was confirmed with the acetylcholinesterase inhibitor donepezil (Broad *et al.*, 2013). Combination of tegaserod with neostigmine also had a synergistic effect on *in vivo* colonic transit time in rat (Campbell-Dittmeyer *et al.*, 2009).

Our group has previously found that the *in vitro* facilitating effect of prucalopride on acetylcholine release and cholinergic contraction is enhanced by the selective PDE4 inhibitor rolipram in circular muscle of porcine stomach and colon (Priem *et al.*, 2012; Priem *et al.*, 2013) suggesting PDE4-mediated control of 5-HT_4 receptors in the cholinergic neurons innervating smooth muscle. Combination of a 5-HT_4 receptor agonist with a selective PDE4 inhibitor might thus also be considered to enhance the prokinetic effect of a 5-HT_4 receptor agonist, administered in a lower dose. The evidence for synergy between a 5-HT_4 receptor agonist and a PDE4 inhibitor in the porcine GI tract is limited to only one combination of 5-HT_4 receptor agonist and PDE4 inhibitor, namely prucalopride and rolipram.

The first aim of this thesis (**CHAPTER III**) was therefore to investigate whether the regulatory role of PDE4 for GI 5-HT_4 receptors in porcine stomach could be confirmed with velusetrag (5-HT_4 receptor agonist) and roflumilast (PDE4 inhibitor). Velusetrag is another new generation highly selective 5-HT_4 receptor agonist that is in phase 2 (completed) of clinical development for chronic constipation

(Goldberg *et al.*, 2010; Manini *et al.*, 2010); roflumilast has been marketed for treatment of COPD (Rabe, 2011; Garnock-Jones, 2015).

As the results of CHAPTER III confirmed the PDE4-mediated control of 5-HT_4 receptors on cholinergic neurons innervating circular muscle in the porcine GI tract, the second aim (**CHAPTER IV**) was to investigate whether PDE4 also has a regulatory role for GI 5-HT_4 receptors in human large intestine. This study corroborated the possible clinical usefulness of combining a 5-HT_4 receptor agonist with a selective PDE4 inhibitor in man.

Then it was investigated whether the mouse might be an adequate model to take the step to *in vivo* testing of the combination, as methods to measure GI transit *in vivo* in pigs are scarce and the financial costs exclude large series. It was therefore first investigated whether 5-HT_4 receptors enhancing neuron-mediated cholinergic contraction of circular muscle are also present in the murine GI tract (**CHAPTER V**). As this was indeed detected in murine stomach, jejunum and colon, the last aim (**CHAPTER VI**) was to investigate whether PDEs regulate murine GI 5-HT_4 receptors; additionally, the involvement of PDEs in controlling contraction at the smooth muscle level was investigated.

An overview of the main pharmacological property of the compounds used throughout CHAPTER III - VI is given in Table II.1.

Table II.1 - Overview of the main pharmacological property of the most important compounds used throughout CHAPTER III - VI.

COMPOUND (concentration*)	MAIN PHARMACOLOGICAL PROPERTY
5-HT receptor-related	
GR 113808 (0.3 µM)	selective 5-HT_4 receptor antagonist
granisetron (0.3 µM)	5-HT_3 receptor antagonist
methysergide (1 µM)	5-HT_1, 5-HT_2, 5-HT_5, 5-HT_6 and 5-HT_7 receptor antagonist
prucalopride	selective 5-HT_4 receptor agonist
velusetrag	selective 5-HT_4 receptor agonist
PDE-related	
cilostamide	selective PDE3 inhibitor
IBMX	non-selective PDE inhibitor
EHNA	selective PDE2 inhibitor
rolipram	selective PDE4 inhibitor
roflumilast	selective PDE4 inhibitor
vinpocetine	selective PDE1 inhibitor
other	
atropine (1 µM)	muscarinic receptor antagonist
carbachol	muscarinic receptor agonist
guanethidine (4 µM)	noradrenergic neuron blocker
hemicholinium-3 (10 µM)	choline transporter blocker
hexamethonium (0.5 mM)	nicotinic acetylcholine receptor antagonist
L-NAME (300 µM) (N_ω-nitro-L-arginine methyl ester hydrochloride)	nitric oxide synthase inhibitor
mecamylamine (30 µM)	nicotinic acetylcholine receptor antagonist
MRS 2500 (1 µM)	$P2Y_1$ receptor antagonist
physostigmine (10 µM)	cholinesterase inhibitor
tetrodotoxin (TTX) (3 µM)	voltage-gated Na^+ channel blocker

* when multiple concentrations of the compound were used in the protocols of Chapter III to VI, they are for clarity not included in this table. For the exact concentrations we refer to the 'Materials and methods' of each chapter.

II.1 References

Barbey J.T., Lazzara R., and Zipes D.P. (2002). Spontaneous adverse event reports of serious ventricular arrhythmias, QT prolongation, syncope, and sudden death in patients treated with cisapride. *J Cardiovasc Pharmacol Ther* 7, 65-76. DOI: 10.1177/107424840200700202

Broad J., Kung V.W., Boundouki G., Aziz Q., De Maeyer J.H., Knowles C.H., and Sanger G.J. (2013). Cholinergic interactions between donepezil and prucalopride in human colon: potential to treat severe intestinal dysmotility. *Br J Pharmacol* 170, 1253-1261. DOI: 10.1111/bph.12397

Campbell-Dittmeyer K., Hicks G.A., Earnest D.L., and Greenwood-Van Meerveld B. (2009). Increased colonic transit in rats produced by a combination of a cholinesterase inhibitor with a 5-HT4 receptor agonist. *Neurogastroenterol Motil* 21, 1197-e1108. DOI: 10.1111/j.1365-2982.2008.01238.x

Cellek S., Thangiah R., Jarvie E.M., Vivekanandan S., Lalude O., and Sanger G.J. (2008). Synergy between 5-HT4 receptor activation and acetylcholinesterase inhibition in human colon and rat forestomach. *Neurogastroenterol Motil* 20, 539-545. DOI: 10.1111/j.1365-2982.2007.01062.x

Conlon K., De Maeyer J.H., Bruce C., Schuurkes J.a.J., Christie L., Mcredmond J., Derakhchan K., and Wade P.R. (2018). Nonclinical Cardiovascular Studies of Prucalopride, a Highly Selective 5-Hydroxytryptamine 4 Receptor Agonist. *J Pharmacol Exp Ther* 364, 156-169. DOI: 10.1124/jpet.117.244079

De Maeyer J.H., Lefebvre R.A., and Schuurkes J.a.J. (2008). 5-HT4 receptor agonists: similar but not the same. *Neurogastroenterol Motil* 20, 99-112. DOI: 10.1111/j.1365-2982.2007.01059.x

Garnock-Jones K.P. (2015). Roflumilast: A Review in COPD. *Drugs* 75, 1645-1656. DOI: 10.1007/s40265-015-0463-1

Giudicessi J.R., Ackerman M.J., and Camilleri M. (2018). Cardiovascular safety of prokinetic agents: A focus on drug-induced arrhythmias. *Neurogastroenterol Motil*. DOI: 10.1111/nmo.13302

Goldberg M., Li Y.P., Johanson J.F., Mangel A.W., Kitt M., Beattie D.T., Kersey K., and Daniels O. (2010). Clinical trial: the efficacy and tolerability of velusetrag, a selective 5-HT4 agonist with high intrinsic activity, in chronic idiopathic constipation - a 4-week, randomized, double-blind, placebo-controlled, dose-response study. *Aliment Pharmacol Ther* 32, 1102-1112. DOI: 10.1111/j.1365-2036.2010.04456.x

Keating G.M. (2013). Prucalopride: A Review of Its Use in the Management of Chronic Constipation. *Drugs* 73, 1935-1950. DOI: 10.1007/s40265-013-0140-1

Manabe N., Wong B.S., and Camilleri M. (2010). New-generation 5-HT4 receptor agonists: potential for treatment of gastrointestinal motility disorders. *Expert Opin Investig Drugs* 19, 765-775. DOI: 10.1517/13543784.2010.482927

Manini M.L., Camilleri M., Goldberg M., Sweetser S., Mckinzie S., Burton D., Wong S., Kitt M.M., Li Y.P., and Zinsmeister A.R. (2010). Effects of Velusetrag (TD-5108) on gastrointestinal transit and bowel function in health and pharmacokinetics in health and constipation. *Neurogastroenterol Motil* 22, 42-49, e47-48. DOI: 10.1111/j.1365-2982.2009.01378.x

Pasricha P.J. (2007). Desperately seeking serotonin... A commentary on the withdrawal of tegaserod and the state of drug development for functional and motility disorders. *Gastroenterology* 132, 2287-2290. DOI: 10.1053/j.gastro.2007.04.057

Priem E.K.V., Van Colen I., De Maeyer J.H., and Lefebvre R.A. (2012). The facilitating effect of prucalopride on cholinergic neurotransmission in pig gastric circular muscle is regulated by phosphodiesterase 4. *Neuropharmacology* 62, 2126-2135. DOI: 10.1016/j.neuropharm.2011.12.020

Priem E.K.V., De Maeyer J.H., and Lefebvre R.A. (2013). Influence of phosphodiesterases on basal and 5-HT4 receptor facilitated cholinergic contractility in pig descending colon. *Eur J Pharmacol* 705**,** 156-163. DOI: 10.1016/j.ejphar.2013.02.011

Rabe K.F. (2011). Update on roflumilast, a phosphodiesterase 4 inhibitor for the treatment of chronic obstructive pulmonary disease. *Br J Pharmacol* 163**,** 53-67. DOI: 10.1111/j.1476-5381.2011.01218.x

Tack J., Camilleri M., Chang L., Chey W.D., Galligan J.J., Lacy B.E., Muller-Lissner S., Quigley E.M., Schuurkes J., De Maeyer J.H.*, et al.* (2012). Systematic review: cardiovascular safety profile of 5-HT(4) agonists developed for gastrointestinal disorders. *Aliment Pharmacol Ther* 35**,** 745-767. DOI: 10.1111/j.1365-2036.2012.05011.x

CHAPTER III

Confirmation of control of 5-HT_4 receptors by PDE in porcine stomach

Synergistic effect between 5-HT_4 receptor agonist and phosphodiesterase 4 inhibitor in releasing acetylcholine in pig gastric circular muscle *in vitro*

Romain A. Lefebvre[1], Inge Van Colen[1], Vicky Pauwelyn[1] and Joris H. De Maeyer[2]

[1] Department of Pharmacology - Heymans Institute, Ghent University, Ghent, Belgium
[2] Shire-Movetis NV, Turnhout, Belgium

European Journal of Pharmacology 2016; 781: 76-82. DOI: 10.1016/j.ejphar.2016.04.003
https://doi.org/10.1016/j.ejphar.2016.04.003

CHAPTER III -
Confirmation of control of 5-HT_4 receptors by PDE in porcine stomach

Synergistic effect between 5-HT_4 receptor agonist and phosphodiesterase 4 inhibitor in releasing acetylcholine in pig gastric circular muscle *in vitro*

III.1 Abstract

III.1.1 Background

5-HT_4 receptor agonists have a gastroprokinetic effect by facilitating acetylcholine release from cholinergic nerves innervating gastrointestinal smooth muscle. The role of phosphodiesterase (PDE) 4 in the signal transduction pathway of the 5-HT_4 receptors located on the cholinergic neurons toward the circular muscle layer in pig stomach was investigated by analysis of acetylcholine release.

III.1.2 Methods

Circular muscle strips were prepared from pig proximal stomach and tritium outflow, induced by electrical field stimulation, was studied as a marker for acetylcholine release after incubation with [^{3}H]-choline.

III.1.3 Results

The PDE4 inhibitor roflumilast concentration-dependently (0.1 - 1 µM) enhanced the facilitating effect of a submaximally effective concentration of the 5-HT_4 receptor agonist prucalopride (0.01 µM) on electrically induced acetylcholine release. Roflumilast (0.3 µM) enhanced acetylcholine release per se but in the combined presence of roflumilast and prucalopride, acetylcholine release was enhanced more than the sum of the effect of the two compounds alone. The 5-HT_4 receptor agonist velusetrag concentration-dependently (0.01 - 0.1 µM) enhanced acetylcholine release; the effect of the minimally effective concentration (0.01 µM) was significantly enhanced by 1 µM of the PDE4 inhibitor rolipram, again to a level higher than the sum of the effect of the two compounds alone.

III.1.4 Conclusions

The synergistic effect between 5-HT_4 receptor agonists and PDE4 inhibitors demonstrates that the intracellular pathway of the 5-HT_4 receptors located on cholinergic neurons toward pig gastric circular muscle is controlled by PDE4. Combining a 5-HT_4 receptor agonist with a PDE4 inhibitor might thus enhance its gastroprokinetic effect.

III.1.5 Keywords

5-HT_4 receptor, phosphodiesterase 4, stomach, acetylcholine release, prucalopride, velusetrag

III.2 Introduction

5-hydroxytryptamine (5-HT) 4 receptors are present in the human gastrointestinal (GI) tract, brain, heart, adrenal cortex and bladder. In the GI tract, the facilitation of cholinergic transmission via 5-HT_4 receptors was first shown in guinea pig ileum (Craig and Clarke, 1990). This mechanism through 5-HT_4 receptors on enteric cholinergic nerves, also shown to be present in other species such as dog (Prins *et al.*, 2000; Prins *et al.*, 2001), pig (Priem and Lefebvre, 2011; Priem *et al.*, 2012) and man (Prins *et al.*, 2000; Leclere *et al.*, 2005) is now believed to underlie the GI prokinetic effects of 5-HT_4 receptor agonists; the latter are therefore actually developed for gastrointestinal hypomotility disorders such as gastroparesis and constipation (Galligan and Vanner, 2005).

The substituted benzamide cisapride was frequently prescribed for gastro-esophageal reflux and gastroparesis (Wiseman and Faulds, 1994). Its marketing was suspended because of possible induction of severe ventricular arrhythmias (Barbey *et al.*, 2002). This attracted the attention to the cardiovascular safety profile of 5-HT_4 receptor agonists (Tack *et al.*, 2012). The arrhythmogenic potential of cisapride is due to its non-selectivity with inhibition of the cardiac human ether-a-go-go-related gene (hERG) potassium channel in concentrations less than 10 fold higher than needed to stimulate 5-HT_4 receptors (De Maeyer *et al.*, 2008). The benzofurancarboxamide prucalopride is a highly selective 5-HT_4 receptor agonist that was marketed for constipation and no cardiovascular safety issues have arisen during the clinical trials in patients with chronic constipation (Keating, 2013). While 5-HT_4 receptors are present in human and porcine atria (Parker *et al.*, 1995), prucalopride behaves as a partial agonist in atrial tissue having only a weak and transient inotropic effect (Krobert *et al.*, 2005; De Maeyer *et al.*, 2006). This is because cardiac 5-HT_4 receptors, being G_s-protein-coupled receptors linked to adenylyl cyclase and generation of cAMP, are under tight control of phosphodiesterases (PDEs) degrading the second messenger cAMP and preventing a pronounced effect of 5-HT_4 receptor agonists. This control is mediated by PDE3 in human (Afzal *et al.*, 2008) and PDE3 plus PDE4 in pig heart (Galindo-Tovar *et al.*, 2009; Weninger *et al.*, 2012).

In contrast, the facilitating effect of prucalopride on enteric cholinergic neurotransmission in isolated gastrointestinal tissues is pronounced and sustained. Still, we recently showed that amongst PDE1, 2, 3 and 4 inhibitors, the PDE4 inhibitor rolipram is able to enhance the effect of prucalopride on cholinergic transmission in pig stomach and descending colon (Priem *et al.*, 2012; Priem *et al.*, 2013), suggesting that the transduction pathway of the neuronal 5-HT_4 receptors in pig stomach and colon is also regulated by PDE4.

In the present study we used the same porcine model system to evaluate the interaction potential between the clinically used 5-HT_4 receptor agonist prucalopride and PDE4 inhibitor roflumilast on gastric acetylcholine release. Roflumilast is a selective PDE4 inhibitor marketed for severe chronic obstructive pulmonary disease (COPD; Rabe, 2011; Michalski *et al.*, 2012). In addition, we further validated the interaction pharmacology between velusetrag, a highly selective 5-HT_4 receptor agonist belonging to another chemical class than prucalopride (Long *et al.*, 2012a; Long *et al.*, 2012b) which is under development for constipation and gastroparesis, and the PDE4 inhibitor rolipram. If confirmed *in vivo*

in humans, our results suggest a potential for drug interaction when combining a 5-HT_4 receptor agonist and a PDE4 inhibitor resulting in increased GI motility.

III.3 Materials and methods

III.3.1 Animals and tissue preparation

All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.

About 2 months old male piglets (Line 36; n = 25; weight 19.0 ± 0.7 kg) from Rattlerow Seghers (Lokeren, Belgium) were used. On the morning of the experiments, the piglets were anesthetized by intramuscular injection of 5 ml Zoletil 100 (containing 50 mg/ml tiletamine and 50 mg/ml zolazepam; Virbac Belgium S.A., Belgium). After exsanguination for 90 s, the entire stomach was isolated, cut open along the lesser curvature and kept in physiological salt solution (composition in mM: 112 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 11.5 glucose and 25 $NaHCO_3$) at room temperature. The mucosa was removed and 4 full thickness circular muscle strips of approximately 1.5 cm in length and 0.3 cm in width were prepared from the ventral side of the proximal stomach. All strips were used on the day of preparation.

Each strip was mounted vertically between two platinum wire electrodes under a load of 2 g in a 2 ml organ bath containing physiological salt solution supplemented with choline (0.0015 mM) and ascorbic acid (0.057 mM) at 37 °C and gassed with carbogen (95% O_2, 5% CO_2). Electrical field stimulation (EFS) was performed by means of a stimulator (Grass S88, United States) with a constant voltage unit.

III.3.2 Drugs

L-ascorbic acid, atropine sulphate, choline chloride, dimethyl sulfoxide (DMSO), hemicholinium-3-bromide, physostigmine salicylate (Sigma-Aldrich, Belgium), [methyl ^{3}H]-choline chloride (Perkin Elmer, Belgium), prucalopride succinate, velusetrag (Shire-Movetis, Belgium), rolipram and roflumilast (Tocris, U.K.). Stock solutions were prepared in deionized water, except for rolipram, roflumilast and velusetrag, which were dissolved in DMSO, yielding a DMSO concentration of 0.01 (rolipram) or 0.1% in the organ bath (Figure III.1).

III.3.3 Acetylcholine release

The same method as described before was used (Leclere and Lefebvre, 2001). The tissues were equilibrated for 1 h, superfusing them with physiological salt solution at a rate of 2 ml/min (Gilson Minipuls, France). During the last 20 min, EFS (40 V, 1 ms, 0.5 Hz) was applied continuously. After superfusion was stopped, the tissues were incubated for 30 min with [^{3}H]-choline (5 µCi/ml) under continuous EFS (40 V, 1 ms, 2 Hz) in order to label their cholinergic transmitter stores. Incubation with [^{3}H]-choline and EFS was stopped and the strips were superfused (2 ml/min) for 90 min to remove loosely bound radioactivity. From now on the physiological salt solution contained hemicholinium-3 (10 µM), physostigmine (10 µM) and atropine (1 µM) to prevent re-uptake of choline, hydrolysis of acetylcholine and auto-inhibition of acetylcholine release, respectively.

After the washout period, the organ baths were filled with 1 ml of physiological salt solution. The content was collected and replaced every 3 min for a total of 37 samples. The strips were stimulated twice (15 V, 1 ms, 4 Hz) for 2 min at the 13th (sample 5; S_1) and 73rd (sample 25; S_2) min after the end of the washout

period. Drugs were added before S_2 and remained present until the end of the experiment: PDE4 inhibitors (roflumilast or rolipram) from min 37 (sample 13; 36 min before S_2) and 5-HT_4 receptor agonists (prucalopride or velusetrag) from min 58 (sample 20; 15 min before S_2) on. At the end of the experiment, the tissues were blotted and weighed.

For each sample, 0.5 ml was mixed with 2 ml scintillator containing solution Ultima Gold (Perkin Elmer, United States). Radioactivity was measured as disintegrations per min (dpm) by liquid scintillation counting (Packard Tri-Carb 2100 TR, Packard Instrument Company, United States) with external standardization to correct for counting efficiency. The total tritium outflow (a mixture of [^{3}H]-acetylcholine, [^{3}H]-phosphorylcholine, and [^{3}H]-choline) can be considered as a marker for acetylcholine release, because separation of these radioactive compounds by HPLC showed that EFS predominantly increases [^{3}H]-acetylcholine release and that EFS-induced changes in total tritium outflow parallel those in [^{3}H]-acetylcholine (Leclere and Lefebvre, 2001).

III.3.4 Experimental protocols (Figure III.1)

The sequence of drug administration in the four experimental series is given in Figure III.1.

- *Concentration finding of roflumilast*: four parallel strips (same animal), one with administration of 0.01 µM prucalopride before S_2 and three where prucalopride was added after previous administration of 0.1, 0.3 or 1 µM roflumilast, respectively. The concentration of prucalopride had been shown before to be minimally effective on acetylcholine release in pig stomach, allowing enhancement of its effect (Priem *et al.*, 2012). The concentrations of roflumilast were selected on the basis of results in a functional assay on ciliary beating frequency in rat central airways, where effects were observed in the 0.01 to 10 µM range (Wohlsen *et al.*, 2010).
- *Influence of 0.3 µM roflumilast on the effect of 0.01 µM prucalopride*: four parallel strips receiving prucalopride, roflumilast, roflumilast and prucalopride or only the solvent of roflumilast (0.1% DMSO).
- *Concentration finding of velusetrag*: four parallel strips with 0.01, 0.03 or 0.1 µM velusetrag and one control, receiving the solvent of velusetrag (0.1% DMSO). The concentrations were selected on the basis of the concentration-response curve for the contractile effect of velusetrag in the guinea pig colonic longitudinal muscle/myenteric plexus preparation (Beattie *et al.*, 2011).
- *Influence of 1 µM rolipram on the effect of 0.01 µM velusetrag*: four parallel strips with velusetrag alone, rolipram alone, velusetrag in the presence of rolipram and a control not receiving active compounds. The solvents of rolipram (0.01% DMSO) and velusetrag (0.1% DMSO) were taken in account. The concentration of rolipram significantly enhances the facilitating effect of 0.01 µM prucalopride on cholinergic transmission in pig stomach (Priem *et al.*, 2012).

III.3.5 Data analysis and statistics

EFS induced an increase in tritium outflow in samples 5 up to 7 for S_1 and 25 up to 27 for S_2. Basal tritium outflow was calculated by fitting a regression line through the 4 samples just before stimulation and

the 4 samples from where outflow had returned to basal values after stimulation. The EFS-induced increase in tritium outflow was determined by reducing the values of the samples with increased outflow with basal tritium outflow. The S_2/S_1 ratio was calculated for all experiments in which the graph of the S_2-induced release peak had the same pattern as that of S_1. If the S_1-peak showed an aberrant pattern (e.g. double-peak pattern) from the S_2-peak or vice versa, the S_2/S_1 ratio was not calculated and the tissue was not taken in account (2/20 tissues in series 1; none on 24 tissues in series 2; 3/28 tissues in series 3; 1/28 tissues in series 4).

Results are expressed as means ± S.E.M., *n* referring to tissues obtained from different animals. Data obtained in parallel tissue groups were compared by one-way analysis of variance (ANOVA) followed by a post hoc *t*-test corrected for multiple comparisons (Bonferroni procedure). *P*-values less than 0.05 were considered statistically significant.

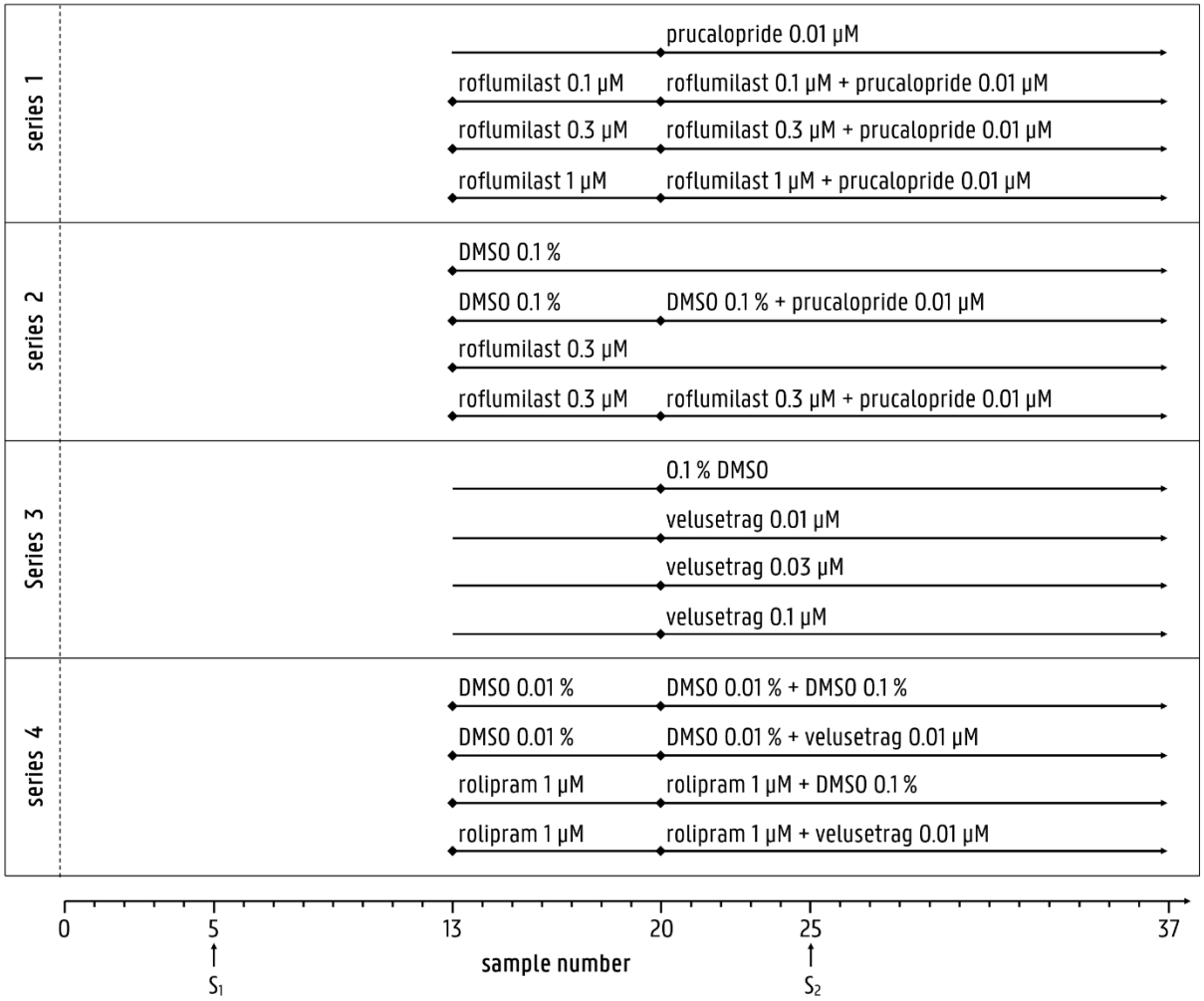


Figure III.1 - Protocols of the four sets of experiments. Samples were obtained at 3 min intervals for a total of 37 samples. The strips were stimulated twice for 2 min at sample 5 (S_1) and sample 25 (S_2). PDE inhibitors roflumilast or rolipram were added from sample 13 (36 min before S_2); 5-HT$_4$ receptor agonists prucalopride or velusetrag were added from sample 20 (15 min before S_2).

III.4 Results

III.4.1 Concentration finding of roflumilast (Figure III.2)

The influence of different concentrations of roflumilast (0.1, 0.3 and 1 µM) was tested versus 0.01 µM prucalopride. The S_2/S_1 ratio was 0.84 ± 0.04 (n = 4) in tissues where only prucalopride was administered before S_2. When roflumilast was administered before prucalopride, a clear-cut significant increase in EFS-induced tritium outflow was obtained with all concentrations tested, with a maximum increase for 0.3 µM roflumilast (S_2/S_1: 1.26 ± 0.05; n = 5). A concentration of 0.3 µM roflumilast was used for the experiments in series 2.

III.4.2 Influence of roflumilast on the effect of prucalopride (Figure III.3)

Prucalopride (0.01 µM) and roflumilast (0.3 µM) both evoked a moderate, but significant effect on the EFS-induced tritium outflow compared to control tissues. The S_2/S_1 ratio in the presence of prucalopride or roflumilast was 0.85 ± 0.05 (n = 6) and 0.85 ± 0.02 (n = 6), respectively, versus 0.62 ± 0.02 (n = 6) for the control strips. When roflumilast (0.3 µM) was administered before prucalopride (0.01 µM), a clear-cut significant increase in EFS-induced tritium outflow versus that in the presence of prucalopride or roflumilast alone was obtained (S_2/S_1 ratio of 1.22 ± 0.09, n = 6).

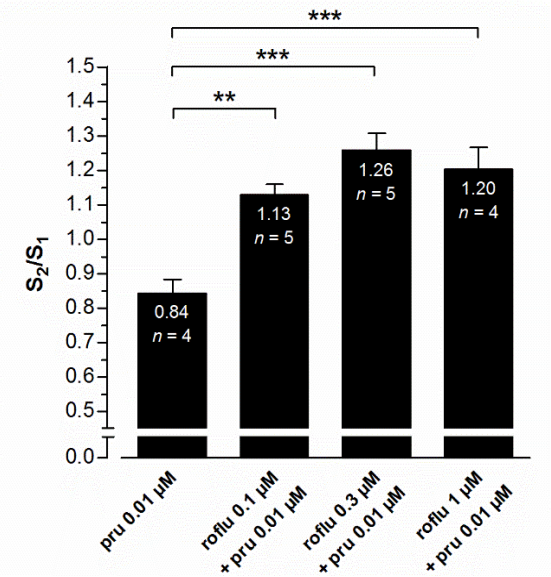


Figure III.2 - Influence of prucalopride (pru, 0.01 µM) alone and in the presence of different concentrations of roflumilast (roflu, 0.1, 0.3 and 1 µM) on the S_2/S_1 ratio of electrically evoked total radioactivity release. Tissues were stimulated twice (S_1 and S_2; 15 V, 1 ms, 4 Hz, 2 min). Roflumilast was added 36 min and prucalopride 15 min before S_2. Means ± S.E.M. of the S_2/S_1 ratio of n = 4 – 5 tissues are shown. ** P < 0.01; *** P < 0.001: significantly different from 0.01 µM prucalopride. ANOVA followed by a Bonferroni multiple comparisons t-test.

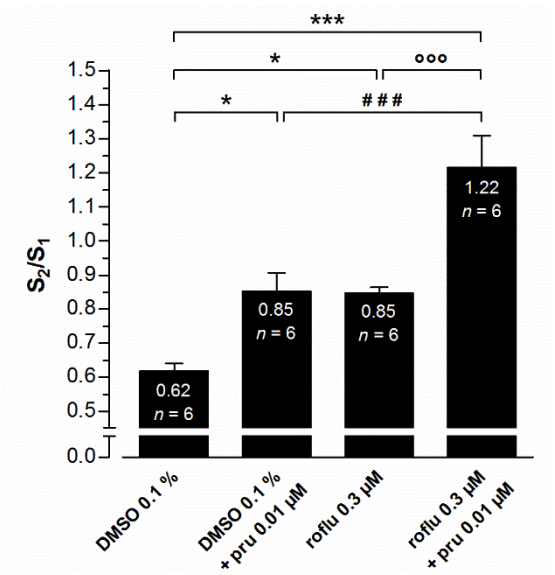


Figure III.3 - Influence of 0.01 µM prucalopride (pru), 0.3 µM roflumilast (roflu) and prucalopride in the presence of roflumilast on the S_2/S_1 ratio of EFS-induced total radioactivity release. Tissues were stimulated twice (S_1 and S_2; 15 V, 1 ms, 4 Hz, 2 min). Roflumilast was added 36 min and prucalopride was added 15 min before S_2. Means ± S.E.M. of the S_2/S_1 ratio of n = 6 tissues are shown. * P < 0.05; *** P < 0.001: significantly different from control (0.1% DMSO). ### P < 0.001: significantly different from 0.01 µM prucalopride. ooo P < 0.001: significantly different from 0.3 µM roflumilast. ANOVA followed by a Bonferroni multiple comparisons t-test.

III.4.3 Concentration finding of velusetrag (Figure III.4)

To further validate the interaction between 5-HT$_4$ and PDE4 on gastric acetylcholine release, we also evaluated the effect of a second 5-HT$_4$ receptor agonist belonging to a different chemical class than prucalopride. To select an optimal concentration, we first tested the influence of different concentrations (0.01, 0.03 and 0.1 µM) of velusetrag on EFS-induced tritium outflow. In these experiments, velusetrag showed a concentration-dependent effect on the electrically evoked efflux of total radioactivity. While 0.01 µM velusetrag only tended to increase the electrically evoked tritium outflow (S_2/S_1 of 0.64 ± 0.05, n = 7, versus 0.57 ± 0.03 in controls, n = 7), both 0.03 and 0.1 µM velusetrag (S_2/S_1: 0.80 ± 0.04, n = 5; 0.92 ± 0.05, n = 6) significantly enhanced EFS-induced tritium outflow versus control. The concentration of 0.01 µM velusetrag was selected for the interaction study with rolipram in series 4.

III.4.4 Influence of rolipram on the effect of velusetrag (Figure III.5)

Velusetrag (0.01 µM; S_2/S_1 ratio 0.70 ± 0.03, n = 6) showed a minimal effect on EFS-induced tritium outflow versus control tissues (S_2/S_1 ratio 0.60 ± 0.02, n = 7). Rolipram (1 µM) significantly increased EFS-induced tritium outflow (S_2/S_1: 0.82 ± 0.03, n = 7). In the presence of rolipram and velusetrag, the S_2/S_1 ratio of total radioactivity outflow (1.17 ± 0.06, n = 7) was significantly enhanced compared to that in the presence of velusetrag or rolipram alone.

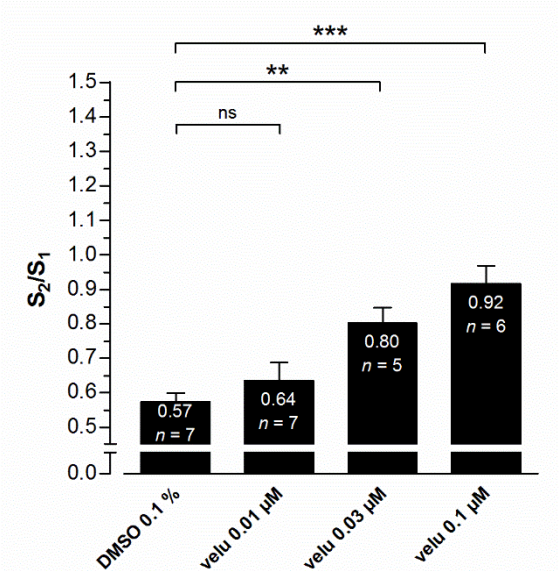


Figure III.4 - Influence of different concentrations of velusetrag (velu 0.01, 0.03 and 0.1 µM) on the S_2/S_1 ratio of electrically evoked total radioactivity release. Tissues were stimulated twice (S_1 and S_2; 15 V, 1 ms, 4 Hz, 2 min). Velusetrag was added 15 min before S_2. Means ± S.E.M. of the S_2/S_1 ratio of n = 5 to 7 tissues are shown. ns not significant, ** P< 0.01; *** P< 0.001: significantly different from control (DMSO 0.1%). ANOVA followed by a Bonferroni multiple comparisons t-test.

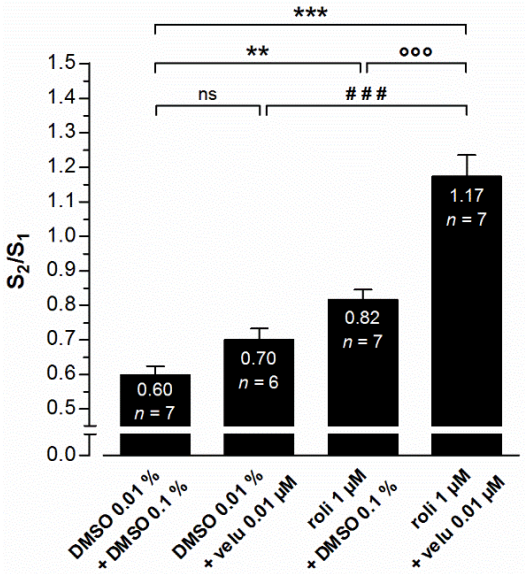


Figure III.5 - Influence of 0.01 µM velusetrag (velu), 1 µM rolipram (roli) and velusetrag in the presence of rolipram on the S_2/S_1 ratio of EFS-induced outflow of total radioactivity. Tissues were stimulated twice (S_1 and S_2; 15 V, 1 ms, 4 Hz, 2 min). Rolipram was added 36 min and velusetrag was added 15 min before S_2. Means ± S.E.M. of the S_2/S_1 ratio of n = 6 - 7 tissues are shown. ns not significant, ** P< 0.01; *** P< 0.001: significantly different from control (0.01% DMSO - 0.1% DMSO). ### P < 0.001: significantly different from 0.01 µM velusetrag, ooo P < 0.001: significantly different from 1 µM rolipram. ANOVA followed by a Bonferroni multiple comparisons t-test.

III.5 Discussion

5-HT_4 receptors are G_s-protein coupled receptors linked to adenylyl cyclase, generating cAMP. cAMP increases acetylcholine release from cerebral and from peripheral myenteric cholinergic neurons (Wiley and Owyang, 1987; Katsura *et al.*, 1991). The facilitating effect of the 5-HT_4 receptor agonist renzapride on acetylcholine release from guinea pig myenteric neurons is related to activation of the adenylyl cyclase-protein kinase A pathway (Ren *et al.*, 2008). The cellular level of cyclic nucleotides is regulated by PDEs. Yau et al. (1987) showed that the stimulating effect of the adenylyl cyclase activator forskolin on acetylcholine release in the guinea pig ileum was enhanced in the presence of the non-selective PDE inhibitor 3-isobutyl-1-methylxanthine, suggesting that the cAMP-mediated influence on acetylcholine release from myenteric neurons is regulated by PDEs. We recently reported that the facilitating effect of the 5-HT_4 receptor agonist prucalopride on electrically induced acetylcholine release and cholinergic contraction in pig stomach is concentration-dependently antagonized by the 5-HT_4 receptor antagonist GR 113808 confirming interaction with 5-HT_4 receptors. The effect of prucalopride on acetylcholine release is selectively enhanced by the PDE4 inhibitor rolipram while inhibitors of PDE1, 2 and 3 have no influence, suggesting that the intraneuronal transduction pathway of myenteric 5-HT_4 receptors in pig stomach is regulated by PDE4 (Priem *et al.*, 2012). In the present study we extended these data by evaluating the interaction potential between the clinically used 5-HT_4 receptor agonist prucalopride and PDE4 inhibitor roflumilast. In addition, we further validated the interaction pharmacology by studying the influence of rolipram versus velusetrag, a highly selective 5-HT_4 receptor agonist belonging to another chemical class than prucalopride.

The S_2/S_1 ratio in the control tissues receiving DMSO solvent before S_2 (0.57-0.62) was similar as the ratio previously obtained in control tissues not receiving any solvent (Priem *et al.*, 2012), excluding an effect per se of DMSO on electrically induced acetylcholine release. In the present study, we used a concentration of prucalopride (0.01 µM) that had shown only a minimal, non-significant facilitation of electrically induced acetylcholine release in our previous study. In the actual study though, 0.01 µM prucalopride significantly increased acetylcholine release. The degree of facilitation was the same in series 1 and 2 (S_2/S_1 ratio of 0.84 and 0.85), excluding an influence of previous administration of DMSO on the effect of prucalopride as this was only done in series 2. Although the effect of 0.01 µM prucalopride was larger than anticipated, it still allowed to study further facilitation as maximal concentrations of prucalopride induced S_2/S_1 ratios higher than 1 (Priem *et al.*, 2012). The effect of 0.01 µM prucalopride was indeed greatly enhanced in the presence of the selective PDE4 inhibitor roflumilast. In view of a predominant expression of PDE4 in inflammatory cells such as eosinophils, dendritic cells and mast cells, and in structural cells such as airway epithelial cells, roflumilast was developed and marketed for airway diseases in particular COPD, where it suppresses exacerbations (Wan *et al.*, 2015). Roflumilast inhibits the 4 PDE4 isozymes (A-D) with an IC_{50} of about 1 nM (Tralau-Stewart *et al.*, 2011). In functional assays such as inhibition of ovalbumin-induced contractions in isolated trachea from ovalbumin-sensitized guinea pigs (Bundschuh *et al.*, 2001) and increase of ciliary beating frequency in airways of rat lung slices (Wohlsen *et al.*, 2010), EC_{50} values of 20 to 74 nM were reported. To select a

maximally effective concentration for the interaction experiments, we therefore first tested three concentrations of roflumilast above these EC_{50} values (0.1, 0.3 and 1 µM). In this concentration range roflumilast indeed enhanced the effect of 0.01 µM prucalopride on electrically induced acetylcholine release, reaching a maximum at 0.3 µM. We therefore selected this concentration for an interaction study with the necessary control tissues. In this series, a significant effect of roflumilast on evoked acetylcholine release was revealed. In our previous study in pig stomach (Priem *et al.*, 2012), the non-selective PDE inhibitor 3-isobutyl-1-methylxanthine and 1 µM of the PDE4 inhibitor rolipram tended to enhance acetylcholine release but this did not reach significance. Because in the actual study, also 1 µM rolipram induced a significant increase of evoked acetylcholine release, our data suggest that the basal content of cAMP in pig stomach cholinergic neurons is constitutively regulated by PDE4. Our data show that also roflumilast is able to enhance the facilitating effect of prucalopride on acetylcholine release as the increase in the S_2/S_1 ratio in their combined presence (from 0.62 to 1.22) was higher than the sum of that induced by roflumilast or prucalopride alone (for each from 0.62 to 0.85), suggesting a synergistic interaction.

Velusetrag has more than 500-fold selectivity for 5-HT_4 receptors over other 5-HT receptors, non-5-HT receptors and ion channels (Smith *et al.*, 2008; Beattie *et al.*, 2011). The pK_i of velusetrag at transfected human 5-$HT_{4(c)}$ receptors is 7.7 (Smith *et al.*, 2008) versus 7.6 (Beattie *et al.*, 2011) for prucalopride. In the guinea pig colonic longitudinal muscle/myenteric plexus preparation, an established functional assay for 5-HT_4 receptors (Wardle and Sanger, 1993), velusetrag showed a pEC_{50} of 8.6 and its maximal effect was reached at 0.1 µM (Beattie *et al.*, 2011). We therefore tested 0.01, 0.03 and 0.1 µM velusetrag which concentration-dependently increased electrically evoked acetylcholine release in pig stomach. The S_2/S_1 ratio obtained with the highest concentration (0.92) is lower than that with maximally effective concentrations of prucalopride (Priem *et al.*, 2012). We cannot exclude that a higher concentration than 0.1 µM velusetrag might induce a still higher S_2/S_1 ratio, but the observation might also correlate with its lower maximal agonist effect in the guinea pig assay (55% of maximum by 5-HT) compared to prucalopride (81%; Beattie *et al.*, 2011). Because 0.01 µM prucalopride had a clear-cut effect per se, decreasing the facilitation window for an interacting compound, we now selected the lowest concentration of velusetrag (0.01 µM) for the interaction study with rolipram. This series confirmed the mild effect of 0.01 µM velusetrag per se that was, however, largely enhanced in the presence of 1 µM rolipram to a S_2/S_1 ratio of 1.17. This increase from 0.60 in control tissues is much higher than the sum of the increase induced by velusetrag (to 0.70) and rolipram (to 0.82), corroborating a synergistic interaction.

Our results confirm that the intraneuronal transduction pathway of the facilitatory 5-HT_4 receptors on myenteric cholinergic neurons in pig stomach is regulated by PDE4; this can be extrapolated to the pig distal colon as we previously showed that rolipram also enhances the influence of prucalopride on cholinergic neurotransmission in this tissue (Priem *et al.*, 2013). If confirmed in humans, our results suggest a potential for drug interaction when combining a 5-HT_4 receptor agonist and a PDE4 inhibitor. This might open the possibility of synergistic combination therapy with a 5-HT_4 receptor agonist and a PDE4 inhibitor for conditions with gastrointestinal hypomotility such as gastroparesis and constipation.

Prucalopride (Resolor™) is marketed for constipation. Also velusetrag increased the weekly frequency of spontaneous bowel movements with feeling of complete evacuation in patients with chronic constipation (Goldberg *et al.*, 2010) and is under investigation for treatment of idiopathic and diabetic gastroparesis in a large phase 2b study (www.clinicaltrials.gov; NCT02267525) to confirm positive results obtained in a small phase 2 study (Ahn *et al.*, 2015). Although not converging at the level of the signal transduction pathway, also combination of a 5-HT$_4$ receptor agonist with a cholinesterase inhibitor had a synergistic effect on rat colonic transit *in vivo* (Campbell-Dittmeyer *et al.*, 2009) and on electrically evoked cholinergic contractions in isolated human colon (Broad *et al.*, 2013), and was therefore proposed as a possible therapeutic approach for conditions with severe gastrointestinal hypomotility. Combination therapy with a 5-HT$_4$ receptor agonist and a PDE4 inhibitor in humans will not potentiate the cardiac effects of the 5-HT$_4$ receptor agonist as the human atrial 5-HT$_4$ receptor is under the sole control of PDE3 (Galindo-Tovar *et al.*, 2009). In human colon, 5-HT$_4$ receptors on circular smooth muscle cells, inducing relaxation through the adenylyl cyclase/protein kinase A pathway, have been described (McLean and Coupar, 1996). If these receptors contribute to the clinical efficacy of 5-HT$_4$ receptor agonists, their inhibitory effect must be balanced by the excitatory action via the 5-HT$_4$ receptors on the cholinergic neurons, as acceleration of colonic transit is the effect observed (Manini *et al.*, 2010; Emmanuel *et al.*, 2014). If the PDE involved in controlling cAMP in human colonic circular muscle was PDE4, combination of a PDE4 inhibitor with a 5-HT$_4$ receptor agonist might enhance its relaxant effect, functionally antagonizing the gain in excitatory effect via the 5-HT$_4$ receptors on the cholinergic neurons aimed at in patients with constipation. As far as we know, the predominant PDE isoform in human colonic circular muscle is not known. In porcine colonic circular muscle, it is PDE3 (Priem *et al.*, 2013); if confirmed in humans, the colonic prokinetic influence of a 5-HT$_4$ receptor agonist plus a PDE4 inhibitor will thus not be diminished by concomitant enhancement of muscular relaxation.

III.6 Conclusion

In conclusion, the intraneuronal pathway of 5-HT$_4$ receptors on cholinergic neurons in porcine stomach is under control of PDE4, leading to synergy of a 5-HT$_4$ receptor agonist and a PDE4 inhibitor on acetylcholine release. This synergy could be explored as a possible combination therapy for gastrointestinal hypomotility; on the other hand the potential for a negative drug interaction should be taken in account when administering a full dose of a 5-HT$_4$ receptor agonist in a patient under therapy with a PDE4 inhibitor.

III.7 Acknowledgements

The study was financially supported by Shire-Movetis NV (A11/TT/1382).

III.8 Disclosure

J.H. De Maeyer was employed by Shire-Movetis NV. R.A. Lefebvre was scientifically involved in a Research and Development Project of Shire-Movetis NV on extra-gastrointestinal effects of 5-HT$_4$ receptor agonists, financially supported by IWT (Agency for Innovation by Science and Technology; IWT100551).

III.9 References

Afzal F., Andressen K.W., Mork H.K., Aronsen J.M., Sjaastad I., Dahl C.P., Skomedal T., Levy F.O., Osnes J.B., and Qvigstad E. (2008). 5-HT4-elicited positive inotropic response is mediated by cAMP and regulated by PDE3 in failing rat and human cardiac ventricles. *Br J Pharmacol* 155, 1005-1014. DOI: 10.1038/bjp.2008.339

Ahn A., Barnes C., Shaywitz D., Grimaldi M., and Canafax D.M. (2015). Velusetrag improves gastric emptying time in subjects with diabetic or idiopathic gastroparesis. *Gastroenterology* 148, S-507. DOI: 10.1016/S0016-5085(15)31697-8

Barbey J.T., Lazzara R., and Zipes D.P. (2002). Spontaneous adverse event reports of serious ventricular arrhythmias, QT prolongation, syncope, and sudden death in patients treated with cisapride. *J Cardiovasc Pharmacol Ther* 7, 65-76. DOI: 10.1177/107424840200700202

Beattie D.T., Armstrong S.R., Vickery R.G., Tsuruda P.R., Campbell C.B., Richardson C., Mccullough J.L., Daniels O., Kersey K., Li Y.P., *et al.* (2011). The Pharmacology of TD-8954, a Potent and Selective 5-HT(4) Receptor Agonist with Gastrointestinal Prokinetic Properties. *Front Pharmacol* 2, 25. DOI: 10.3389/fphar.2011.00025

Broad J., Kung V.W., Boundouki G., Aziz Q., De Maeyer J.H., Knowles C.H., and Sanger G.J. (2013). Cholinergic interactions between donepezil and prucalopride in human colon: potential to treat severe intestinal dysmotility. *Br J Pharmacol* 170, 1253-1261. DOI: 10.1111/bph.12397

Bundschuh D.S., Eltze M., Barsig J., Wollin L., Hatzelmann A., and Beume R. (2001). In vivo efficacy in airway disease models of roflumilast, a novel orally active PDE4 inhibitor. *J Pharmacol Exp Ther* 297, 280-290.

Campbell-Dittmeyer K., Hicks G.A., Earnest D.L., and Greenwood-Van Meerveld B. (2009). Increased colonic transit in rats produced by a combination of a cholinesterase inhibitor with a 5-HT4 receptor agonist. *Neurogastroenterol Motil* 21, 1197-e1108. DOI: 10.1111/j.1365-2982.2008.01238.x

Craig D.A., and Clarke D.E. (1990). Pharmacological characterization of a neuronal receptor for 5-hydroxytryptamine in guinea-pig ileum with properties similar to the 5-hydroxytryptamine-4 receptor. *J Pharmacol Exp Ther* 252, 1378-1386.

De Maeyer J.H., Straetemans R., Schuurkes J.a.J., and Lefebvre R.A. (2006). Porcine left atrial and sinoatrial 5-HT4 receptor-induced responses: fading of the response and influence of development. *Br J Pharmacol* 147, 140-157. DOI: 10.1038/sj.bjp.0706497

De Maeyer J.H., Lefebvre R.A., and Schuurkes J.a.J. (2008). 5-HT4 receptor agonists: similar but not the same. *Neurogastroenterol Motil* 20, 99-112. DOI: 10.1111/j.1365-2982.2007.01059.x

Emmanuel A., Cools M., Vandeplassche L., and Kerstens R. (2014). Prucalopride improves bowel function and colonic transit time in patients with chronic constipation: an integrated analysis. *Am J Gastroenterol* 109, 887-894. DOI: 10.1038/ajg.2014.74

Galindo-Tovar A., Vargas M.L., Escudero E., and Kaumann A.J. (2009). Ontogenic changes of the control by phosphodiesterase-3 and -4 of 5-HT responses in porcine heart and relevance to human atrial 5-HT4 receptors. *Br J Pharmacol* 156, 237-249. DOI: 10.1111/j.1476-5381.2008.00023.x

Galligan J.J., and Vanner S. (2005). Basic and clinical pharmacology of new motility promoting agents. *Neurogastroenterol Motil* 17, 643-653. DOI: 10.1111/j.1365-2982.2005.00675.x

Goldberg M., Li Y.P., Johanson J.F., Mangel A.W., Kitt M., Beattie D.T., Kersey K., and Daniels O. (2010). Clinical trial: the efficacy and tolerability of velusetrag, a selective 5-HT4 agonist with high intrinsic activity, in chronic idiopathic constipation - a 4-week, randomized, double-blind, placebo-controlled, dose-response study. *Aliment Pharmacol Ther* 32, 1102-1112. 10.1111/j.1365-2036.2010.04456.x

Katsura M., Hashimoto T., and Kuriyama K. (1991). Effect of 1,3-di-n-butyl-7-(2-oxopropyl)-xanthine (denbufylline) on metabolism and function of cerebral cholinergic neurons. *Jpn J Pharmacol* 55, 233-240. DOI: 10.1254/jjp.55.233

Keating G.M. (2013). Prucalopride: A Review of Its Use in the Management of Chronic Constipation. *Drugs* 73, 1935-1950. DOI: 10.1007/s40265-013-0140-1

Krobert K.A., Brattelid T., Levy F.O., and Kaumann A.J. (2005). Prucalopride is a partial agonist through human and porcine atrial 5-HT4 receptors: comparison with recombinant human 5-HT4 splice variants. *Naunyn Schmiedebergs Arch Pharmacol* 371, 473-479. DOI: 10.1007/s00210-005-1068-0

Leclere P.G., and Lefebvre R.A. (2001). Influence of nitric oxide donors and of the alpha(2)-agonist UK-14,304 on acetylcholine release in the pig gastric fundus. *Neuropharmacology* 40, 270-278. DOI: 10.1016/S0028-3908(00)00123-4

Leclere P.G., Prins N.H., Schuurkes J.a.J., and Lefebvre R.A. (2005). 5-HT4 receptors located on cholinergic nerves in human colon circular muscle. *Neurogastroenterol Motil* 17, 366-375. DOI: 10.1111/j.1365-2982.2005.00621.x

Long D.D., Armstrong S.R., Beattie D.T., Choi S.K., Fatheree P.R., Gendron R.A., Goldblum A.A., Humphrey P.P., Marquess D.G., Shaw J.P.*, et al.* (2012a). Discovery, oral pharmacokinetics and in vivo efficacy of a highly selective 5-HT4 receptor agonist: clinical compound TD-2749. *Bioorg Med Chem Lett* 22, 4849-4853. DOI: 10.1016/j.bmcl.2012.05.034

Long D.D., Armstrong S.R., Beattie D.T., Choi S.K., Fatheree P.R., Gendron R.a.L., Genov D., Goldblum A.A., Humphrey P.P., Jiang L.*, et al.* (2012b). Discovery, oral pharmacokinetics and in vivo efficacy of velusetrag, a highly selective 5-HT4 receptor agonist that has achieved proof-of-concept in patients with chronic idiopathic constipation. *Bioorg Med Chem Lett* 22, 6048-6052. DOI: 10.1016/j.bmcl.2012.08.051

Manini M.L., Camilleri M., Goldberg M., Sweetser S., Mckinzie S., Burton D., Wong S., Kitt M.M., Li Y.P., and Zinsmeister A.R. (2010). Effects of Velusetrag (TD-5108) on gastrointestinal transit and bowel function in health and pharmacokinetics in health and constipation. *Neurogastroenterol Motil* 22, 42-49, e47-48. DOI: 10.1111/j.1365-2982.2009.01378.x

Mclean P.G., and Coupar I.M. (1996). Further investigation into the signal transduction mechanism of the 5-HT4-like receptor in the circular smooth muscle of human colon. *Br J Pharmacol* 118, 1058-1064. DOI: 10.1111/j.1476-5381.1996.tb15506.x

Michalski J.M., Golden G., Ikari J., and Rennard S.I. (2012). PDE4: a novel target in the treatment of chronic obstructive pulmonary disease. *Clin Pharmacol Ther* 91, 134-142. DOI: 10.1038/clpt.2011.266

Parker S.G., Taylor E.M., Hamburger S.A., Vimal M., and Kaumann A.J. (1995). Blockade of human and porcine myocardial 5-HT4 receptors by SB 203186. *Naunyn Schmiedebergs Arch Pharmacol* 353, 28-35. DOI: 10.1007/BF00168912

Priem E.K.V., and Lefebvre R.A. (2011). Investigation of neurogenic excitatory and inhibitory motor responses and their control by 5-HT4 receptors in circular smooth muscle of pig descending colon. *Eur J Pharmacol* 667, 365-374. DOI: 10.1016/j.ejphar.2011.06.021

Priem E.K.V., Van Colen I., De Maeyer J.H., and Lefebvre R.A. (2012). The facilitating effect of prucalopride on cholinergic neurotransmission in pig gastric circular muscle is regulated by phosphodiesterase 4. *Neuropharmacology* 62, 2126-2135. DOI: 10.1016/j.neuropharm.2011.12.020

Priem E.K.V., De Maeyer J.H., and Lefebvre R.A. (2013). Influence of phosphodiesterases on basal and 5-HT4 receptor facilitated cholinergic contractility in pig descending colon. *Eur J Pharmacol* 705, 156-163. DOI: 10.1016/j.ejphar.2013.02.011

Prins N.H., Akkermans L.M.A., Lefebvre R.A., and Schuurkes J.a.J. (2000). 5-HT4 receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. *Br J Pharmacol* 131, 927-932. DOI: 10.1038/sj.bjp.0703615

Prins N.H., Van Der Grijn A., Lefebvre R.A., Akkermans L.M.A., and Schuurkes J.a.J. (2001). 5-HT4 receptors mediating enhancement of contractility in canine stomach; an in vitro and in vivo study. *Br J Pharmacol* 132, 1941-1947. DOI: 10.1038/sj.bjp.0703985

Rabe K.F. (2011). Update on roflumilast, a phosphodiesterase 4 inhibitor for the treatment of chronic obstructive pulmonary disease. *Br J Pharmacol* 163, 53-67. 10.1111/j.1476-5381.2011.01218.x

Ren J., Zhou X., and Galligan J.J. (2008). 5-HT4 receptor activation facilitates recovery from synaptic rundown and increases transmitter release from single varicosities of myenteric neurons. *Am J Physiol Gastrointest Liver Physiol* 294, G1376-1383. DOI: 10.1152/ajpgi.00078.2008

Smith J.A., Beattie D.T., Marquess D., Shaw J.P., Vickery R.G., and Humphrey P.P. (2008). The in vitro pharmacological profile of TD-5108, a selective 5-HT(4) receptor agonist with high intrinsic activity. *Naunyn Schmiedebergs Arch Pharmacol* 378, 125-137. DOI: 10.1007/s00210-008-0282-y

Tack J., Camilleri M., Chang L., Chey W.D., Galligan J.J., Lacy B.E., Muller-Lissner S., Quigley E.M., Schuurkes J., De Maeyer J.H., *et al.* (2012). Systematic review: cardiovascular safety profile of 5-HT(4) agonists developed for gastrointestinal disorders. *Aliment Pharmacol Ther* 35, 745-767. DOI: 10.1111/j.1365-2036.2012.05011.x

Tralau-Stewart C.J., Williamson R.A., Nials A.T., Gascoigne M., Dawson J., Hart G.J., Angell A.D., Solanke Y.E., Lucas F.S., Wiseman J., *et al.* (2011). GSK256066, an exceptionally high-affinity and selective inhibitor of phosphodiesterase 4 suitable for administration by inhalation: in vitro, kinetic, and in vivo characterization. *J Pharmacol Exp Ther* 337, 145-154. DOI: 10.1124/jpet.110.173690

Wan Y., Sun S.X., Corman S., Huang X., Gao X., and Shorr A.F. (2015). A longitudinal, retrospective cohort study on the impact of roflumilast on exacerbations and economic burden among chronic obstructive pulmonary disease patients in the real world. *Int J Chron Obstruct Pulmon Dis* 10, 2127-2136. DOI: 10.2147/COPD.S80106

Wardle K.A., and Sanger G.J. (1993). The guinea-pig distal colon--a sensitive preparation for the investigation of 5-HT4 receptor-mediated contractions. *British Journal of Pharmacology*

110, 1593-1599. DOI: 10.1111/j.1476-5381.1993.tb14006.x

Weninger S., De Maeyer J.H., and Lefebvre R.A. (2012). Study of the regulation of the inotropic response to 5-HT4 receptor activation via phosphodiesterases and its cross-talk with C-type natriuretic peptide in porcine left atrium. *Naunyn Schmiedebergs Arch Pharmacol* 385, 565-577. 10.1007/s00210-012-0746-y

Wiley J., and Owyang C. (1987). Somatostatin inhibits cAMP-mediated cholinergic transmission in the myenteric plexus. *Am J Physiol Gastrointest Liver Physiol* 253, G607-612. DOI: 10.1152/ajpgi.1987.253.5.G607

Wiseman L.R., and Faulds D. (1994). Cisapride. An updated review of its pharmacology and therapeutic efficacy as a prokinetic agent in gastrointestinal motility disorders. *Drugs* 47, 116-152. DOI: 10.2165/00003495-199447010-00008

Wohlsen A., Hirrle A., Tenor H., Marx D., and Beume R. (2010). Effect of cyclic AMP-elevating agents on airway ciliary beat frequency in central and lateral airways in rat precision-cut lung slices. *Eur J Pharmacol* 635, 177-183. DOI: 10.1016/j.ejphar.2010.03.005

Yau W.M., Dorsett J.A., and Youther M.L. (1987). Stimulation of acetylcholine-release from myenteric neurons of guinea-pig small-intestine by forskolin and cyclic AMP. *J Pharmacol Exp Ther* 243, 507-510.

CHAPTER IV

PDE-mediated control of 5-HT_4 receptors in human large intestine

Synergy between 5-HT_4 receptor stimulation and phosphodiesterase 4 inhibition in facilitating acetylcholine release in human large intestinal circular muscle

Vicky Pauwelyn[1], Wim Ceelen[2] and Romain A. Lefebvre[1]

[1] Department of Pharmacology - Heymans Institute, Ghent University, Ghent, Belgium
[2] Department of Gastrointestinal Surgery, Ghent University Hospital, Ghent, Belgium

Neurogastroenterology & Motility 2018; 30(2): e13162. DOI: 10.1111/nmo.13162
https://doi.org/10.1111/nmo.13162
Supplementary table was integrated.

CHAPTER IV -
PDE-mediated control of 5-HT_4 receptors in human large intestine

Synergy between 5-HT_4 receptor stimulation and phosphodiesterase 4 inhibition in facilitating acetylcholine release in human large intestinal circular muscle

IV.1 Abstract

IV.1.1 Background

Gastroprokinetic properties of 5-HT_4 receptor agonists, such as prucalopride, are attributed to activation of 5-HT_4 receptors on cholinergic nerves innervating smooth muscle in the gastrointestinal smooth muscle layer, increasing acetylcholine release and muscle contraction. In porcine stomach and colon, phosphodiesterase (PDE) 4 has been shown to control the signaling pathway of these 5-HT_4 receptors. The aim of this study was to investigate the PDE-mediated control of these 5-HT_4 receptors in human large intestine.

IV.1.2 Methods

Circular smooth muscle strips were prepared from human large intestine; after incubation with [^{3}H]-choline, electrically induced tritium outflow was determined as a measure for acetylcholine release. The influence of PDE inhibition on the facilitating effect of prucalopride on electrically induced acetylcholine release was studied.

IV.1.3 Key results

The non-selective PDE inhibitor IBMX enhanced the facilitating effect of prucalopride on electrically induced acetylcholine release. The selective inhibitors vinpocetine (PDE1), EHNA (PDE2) and cilostamide (PDE3) did not influence, while rolipram and roflumilast (PDE4) enhanced the prucalopride-induced facilitation to the same extent as IBMX.

IV.1.4 Conclusions & inferences

In human large intestinal circular muscle, the intracellular pathway of 5-HT_4 receptors facilitating cholinergic neurotransmission to large intestinal circular smooth muscle is controlled by PDE4. If the synergy between 5-HT_4 receptor agonism and PDE4 inhibition is confirmed in a functional assay with electrically induced cholinergic contractions of human large intestinal circular smooth muscle strips, combination of a selective 5-HT_4 receptor agonist with a selective PDE4 inhibitor might enhance the *in vivo* prokinetic effect of the 5-HT_4 receptor agonist in the large intestine.

IV.1.5 Keywords

5-HT$_4$ receptor, phosphodiesterase, acetylcholine release, human large intestine, prucalopride

IV.2 Key points

- In porcine stomach and large intestine, phosphodiesterase (PDE) 4 has been shown to control the signaling pathway of 5-HT$_4$ receptors on cholinergic nerves innervating the smooth muscle layer; this was now investigated in the human large intestine.
- In human large intestinal circular smooth muscle strips, the facilitating effect of the 5-HT$_4$ receptor agonist prucalopride on electrically induced acetylcholine release was enhanced by non-selective PDE inhibition with IBMX. The selective PDE4 inhibitors rolipram and roflumilast mimicked the effect of IBMX, while inhibitors of PDE1 (vinpocetine), PDE2 (EHNA) and PDE3 (cilostamide) did not.
- The signaling pathway of 5-HT$_4$ receptors facilitating cholinergic neurotransmission toward human large intestinal circular smooth muscle is thus controlled by PDE4. If the synergy between 5-HT$_4$ receptor agonism and PDE4 inhibition is confirmed in a functional assay with electrically induced cholinergic contractions, combination of a 5-HT$_4$ receptor agonist with a selective PDE4 inhibitor might also *in vivo* enhance the prokinetic effect of the 5-HT$_4$ receptor agonist.

IV.3 Graphical abstract

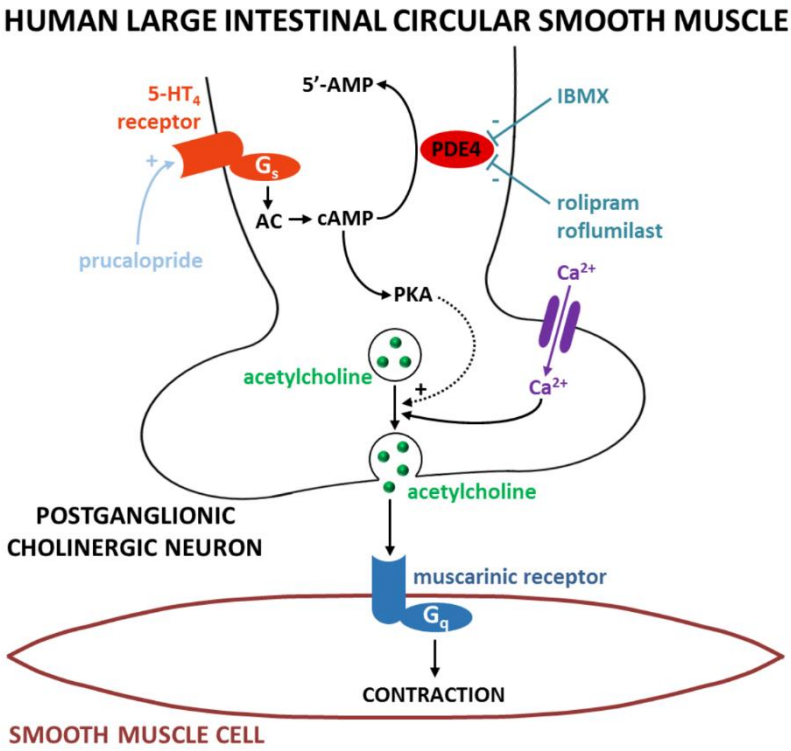


Figure IV.1 – Graphical abstract. Abbreviations: AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; IBMX, 3-isobutyl-1-methylxanthine; PDE4, phosphodiesterase 4; PKA, protein kinase A.

IV.4 Introduction

5-HT$_4$ receptor agonists are used for gastrointestinal (GI) hypomotility disorders such as gastroparesis and constipation (Galligan and Vanner, 2005). Their GI prokinetic effects are attributed to activation of 5-HT$_4$ receptors on cholinergic neurons innervating the smooth muscle layer (Gershon and Tack, 2007). These receptors, facilitating acetylcholine release and GI contraction, were first shown in the GI tract of the guinea-pig (Craig and Clarke, 1990; Tonini *et al.*, 1992) and later of several species as rat (Cellek *et al.*, 2008), dog (Prins *et al.*, 2000; Prins *et al.*, 2001), pig (Priem and Lefebvre, 2011; Priem *et al.*, 2012) and man (Prins *et al.*, 2000; Leclere and Lefebvre, 2002; Leclere *et al.*, 2005; Cellek *et al.*, 2006). 5-HT$_4$ receptors are also present in porcine and human atria (Parker *et al.*, 1995). The highly selective 5-HT$_4$ receptor agonist prucalopride, which is marketed for chronic idiopathic constipation, only induces a weak and transient inotropic effect in porcine and human atrial tissue (Krobert *et al.*, 2005; De Maeyer *et al.*, 2006b). 5-HT$_4$ receptors are G_s protein-coupled receptors, linked to adenylyl cyclase and signaling via generation of the second messenger cAMP. The weak response to 5-HT$_4$ receptor agonism in cardiac tissue is due to the pronounced control of the cAMP signal by phosphodiesterases (PDEs), degrading cAMP and limiting the effect of 5-HT$_4$ receptor agonists (De Maeyer *et al.*, 2006b). This regulation is mediated by PDE3 plus PDE4 in porcine heart (Galindo-Tovar *et al.*, 2009; Weninger *et al.*, 2012) and by PDE3 in human heart (Afzal *et al.*, 2008; Galindo-Tovar *et al.*, 2009).

The facilitation of enteric cholinergic neurotransmission by prucalopride in the porcine and human GI tract is pronounced and sustained (Prins *et al.*, 2000; De Maeyer *et al.*, 2006a; Broad *et al.*, 2014a; Broad *et al.*, 2014b). Still, this effect was shown to be enhanced by the selective PDE4 inhibitor rolipram in porcine stomach and colon (Priem *et al.*, 2012; Priem *et al.*, 2013). We recently confirmed this (Lefebvre *et al.*, 2016) with the selective PDE4 inhibitor roflumilast, marketed for chronic obstructive pulmonary disease (Rabe, 2011), and the highly selective 5-HT$_4$ receptor agonist velusetrag (Smith *et al.*, 2008), that is actually under clinical development. These results demonstrate that the intracellular pathway of the 5-HT$_4$ receptors on the myenteric cholinergic neurons in the porcine GI tract is controlled by PDE4. If confirmed in humans, combination of a 5-HT$_4$ receptor agonist with a selective PDE4 inhibitor might thus enhance the gastroprokinetic effect of the 5-HT$_4$ receptor agonist without potentiation of its cardiac effects, which are controlled by PDE3.

The aim of the present study was therefore to investigate whether the signal transduction pathway of 5-HT$_4$ receptors in myenteric cholinergic neurons innervating human large intestinal circular muscle is controlled by PDEs, and if so whether PDE4 is the isozyme involved. This was done by measuring the influence of PDE inhibitors on the facilitating effect of prucalopride on electrically induced acetylcholine release.

IV.5 Materials and methods

IV.5.1 Patients and tissue preparation

The study was approved by the Ethical Committee of Ghent University Hospital. All patients (n = 59), including 17 women and 42 men with a mean age of 66 ± 2 years, provided written informed consent and underwent resection of the large intestine because of cancer.

A specimen from the macroscopic healthy segment of the resected large intestinal tissue (3 caecum, 5 ascending, 9 transverse, 7 descending, 20 sigmoid colon and 15 rectum) was placed in oxygenated (95% O_2 + 5% CO_2) ice-chilled Krebs-Henseleit solution (composition in mM: NaCl 118.0, KCl 4.69, $MgSO_4$ 1.18, KH_2PO_4 1.18, $CaCl_2$ 2.51, glucose 11.1 and $NaHCO_3$ 25.0) and transported to the laboratory. Adipose tissue, adhering mesentery and mucosa were removed by blunt dissection and the tissue was stored overnight in Krebs-Henseleit solution at 4 °C. Within 24 h after surgery, four full thickness intertaenial smooth muscle strips of approximately 1 to 1.5 cm in length and 0.3 cm in width were cut in the direction of the circular smooth muscle from each human large intestinal specimen to study acetylcholine release.

IV.5.2 Measuring acetylcholine release

Circular smooth muscle strips were mounted vertically between two platinum wire electrodes (40 x 0.5 mm, 4 mm apart) under a load of 2 g in organ baths (2 ml) filled with oxygenated Krebs-Henseleit solution from now on containing choline (1.5 µM), ascorbic acid (57 µM) and guanethidine (4 µM) at 37 °C. During 1 h of equilibration, the tissues were superfused (2 ml/min) with Krebs-Henseleit solution using a peristaltic pump (Minipuls 3; Gilson S.A.S., Villiers le Bel, France). The last 20 min, continuous electrical field stimulation (EFS; 1 ms monophasic square wave pulses, 40 V, 0.5 Hz) was applied by means of a stimulator (Grass S88; Grass Technologies, West Warwick, RI, United States) with a constant voltage unit. After superfusion was stopped, the cholinergic transmitter stores were labeled by incubating the tissues with [^{3}H]-choline (5 µCi/mL) under continuous EFS (1 ms, 40 V, 2 Hz) for 30 min. EFS and incubation were stopped and loosely bound radioactivity was washed-out by superfusing (2 ml/min) the tissues for 90 min with Krebs-Henseleit solution from now on also containing hemicholinium-3 (10 µM), physostigmine (10 µM) and atropine (1 µM) to prevent re-uptake of choline, hydrolysis of acetylcholine and auto-inhibition of acetylcholine release, respectively.

After ending superfusion for wash-out, the organ baths were filled with 1 ml Krebs-Henseleit solution. The organ bath content was collected and replaced every 3 min for a total of 35 samples. EFS (1 ms, 15 V, 4 Hz) was applied twice for 2 min (S_1 and S_2): S_1 started at the 13th (sample 5) and S_2 at the 73rd (sample 25) min after finishing the wash-out period. Drugs were added before S_2 and remained present until the end of the experiment: PDE inhibitors or their solvent from the 37th minute (sample 13; 36 min before S_2) and prucalopride from the 58th minute (sample 20; 15 min before S_2). Tissue strips were blotted and weighed at the end of sample collection (28.75 ± 1.48 mg for the 184 strips included in the results).

0.5 ml of each sample was mixed with 2 ml scintillator containing solution Ultima Gold (Perkin Elmer, Waltham, MA, United States). Total tritium outflow was measured as disintegrations per minute (dpm) by liquid scintillation counting (Packard Tri-Carb 2100 TR; Packard Instrument Company, Downers Grove,

IL, United States) with external standardization to correct for counting efficiency. Total tritium outflow, containing [^{3}H]-acetylcholine, [^{3}H]-phosphorylcholine and [^{3}H]-choline, can be considered as a marker for acetylcholine release as we previously showed by separation of the three radioactive compounds that EFS predominantly increases [^{3}H]-acetylcholine release and that EFS-induced changes in total tritium outflow parallel those in [^{3}H]-acetylcholine (Leclere *et al.*, 2005).

IV.5.3 Experimental protocols and design (Figure IV.2)

The sequence of drug administration in the seven experimental series is given in Figure IV.2. In four separate series, the influence of 10 µM IBMX (non-selective PDE inhibitor) on the effect of 0.03 µM prucalopride and the influence of 10 µM IBMX, 1 µM rolipram or 0.3 µM roflumilast (both selective PDE4 inhibitors) on the effect of 0.01 µM prucalopride was studied. Four parallel strips (same patient) were used for each series: either PDE inhibitor alone, prucalopride alone, or prucalopride in the presence of the PDE inhibitor was added before S_2; the fourth control strip did not receive active compounds.
The influence of 100 µM vinpocetine (PDE1 inhibitor), 30 µM EHNA (PDE2 inhibitor) and 1 µM cilostamide (PDE3 inhibitor) on the effect of 0.01 µM prucalopride was studied in a fifth series with four parallel strips: one with administration of prucalopride alone, and three where prucalopride was added after previous administration of vinpocetine, EHNA or cilostamide.
In two final series the solvent of IBMX, ethanol, and of vinpocetine, cilostamide, rolipram and roflumilast, DMSO, was tested in the highest concentration used upon administration of the PDE inhibitors: 0.05% for ethanol and 1% for DMSO. Their possible influence on the effect of prucalopride was tested by four parallel strips receiving the solvent alone, prucalopride alone, prucalopride in the presence of the solvent and a control not receiving active compounds.
The four experimental conditions per series were rotated over the four organ baths from experiment to experiment.

IV.5.4 Data and statistical analysis

EFS induced a clear-cut increase in tritium outflow, by S_1 in samples 5 up to 7 and by S_2 in samples 25 up to 27. The EFS-induced increase in tritium outflow was determined by subtracting the corresponding basal tritium outflow, which was calculated by fitting a regression line through the values of four samples just before stimulation and the values of the four samples starting from the sixth sample after stimulation. S_2/S_1 ratio was then calculated: the sum of the tritium outflow above baseline of samples 25 to 27 as ratio of the sum of the tritium outflow above baseline of samples 5 to 7.
Strips were excluded from analysis when: the S_1- and/or S_2-induced release peak showed an aberrant pattern e.g. double peak (24 out of 236 strips), the basal outflow was unstable (18 out of 236 strips) or a technical defect occurred mainly a defect of electrodes (10 out of 236 strips) meaning that the results of 184 strips are given. This exclusion from data analysis also explains unequal group sizes within an experimental series.

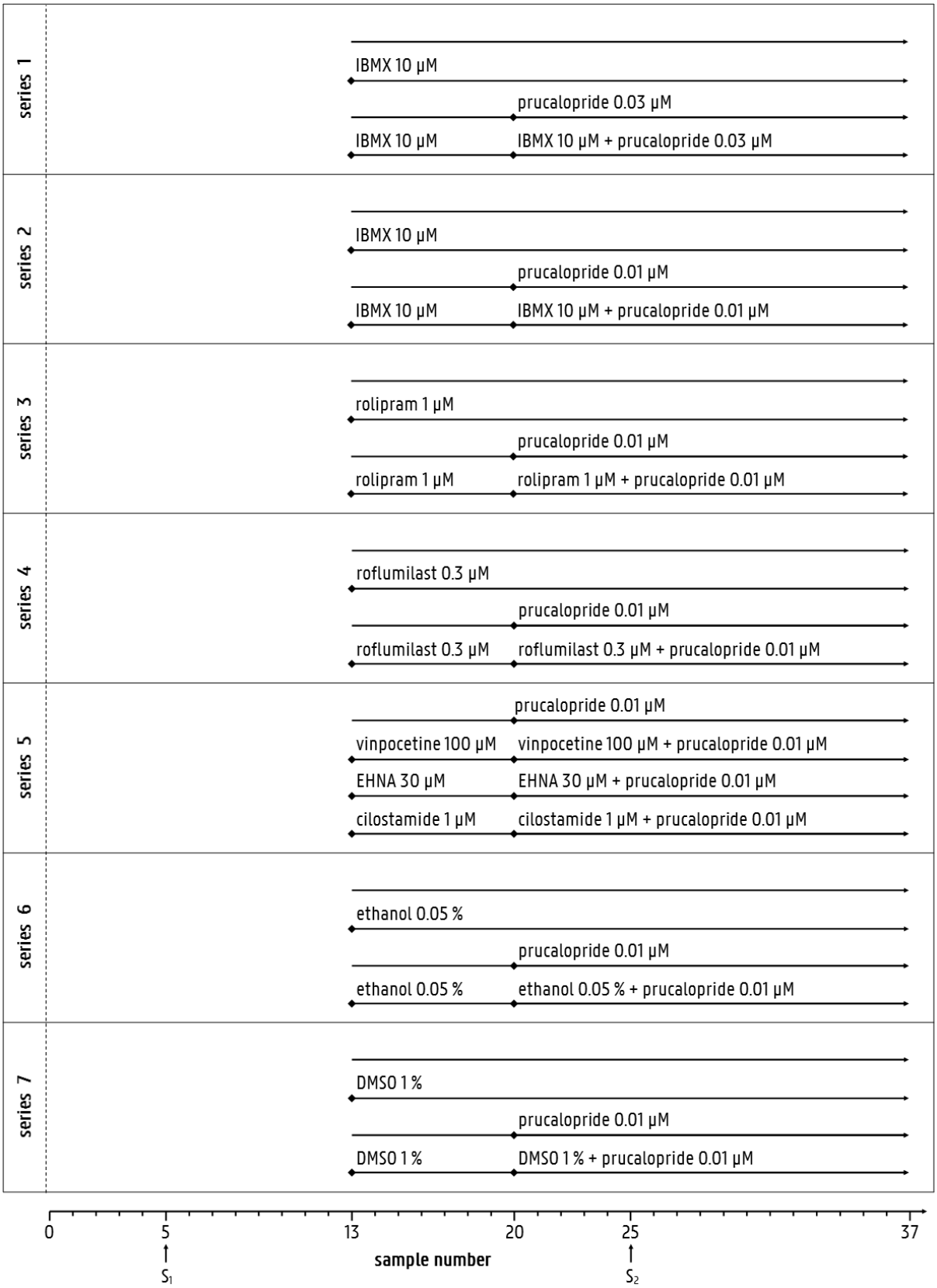


Figure IV.2 - Protocols of the seven sets of experiments. Samples were obtained at 3 min intervals for a total of 37 samples. The strips were stimulated twice for 2 min at sample 5 (S_1) and sample 25 (S_2). In each experimental set, a control strip not receiving active compounds was included. PDE inhibitors IBMX, rolipram, roflumilast, vinpocetine, EHNA or cilostamide were added from sample 13 (36 min before S_2); the 5-HT$_4$ receptor agonist prucalopride was added from sample 20 (15 min before S_2).

Data are expressed as mean ± S.E.M. and *n* refers to the number of tissues obtained from different patients. As data were homogenous (Levene's test), they were compared by one-way ANOVA with unpaired *t*-tests with Bonferroni correction for multiple comparisons; the Bonferroni corrected *t*-tests were only conducted if the ANOVA showed significance. *P*-values less than 0.05 were considered statistically significant. Statistical analysis was performed with GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, United States).

IV.5.5 Drugs

L-ascorbic acid, atropine sulfate salt, choline chloride, guanethidine sulfate, hemicholinium-3 bromide, 3-isobutyl-1-methylxanthine (IBMX), eserine salicylate salt (physostigmine), roflumilast were obtained from Sigma-Aldrich (St. Louis, MO, United States); cilostamide, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) hydrochloride, rolipram, vinpocetine from Tocris Bioscience (Bristol, UK); [methyl-^{3}H]-choline chloride from Perkin Elmer (Boston, MA, United States) and prucalopride succinate from Selleck Chemicals (Houston, TX, United States).

Drugs were dissolved and diluted in distilled water, except for IBMX which was dissolved in ethanol, yielding a concentration of 0.05% in the organ bath, and vinpocetine, cilostamide, rolipram and roflumilast which were dissolved in DMSO, yielding a concentration of respectively 1, 0.01, 0.01 and 0.003% in the organ bath.

IV.6 Results

IV.6.1 Influence of IBMX on the effect of prucalopride (Figure IV.3 and Figure IV.4).

We previously have shown that 0.3 µM prucalopride induces a pronounced increase in EFS-induced acetylcholine release in human colon (Leclere *et al.*, 2005; Lefebvre *et al.*, 2010). A 10-fold lower concentration of prucalopride was therefore used in the first series of the actual study to test the influence of 10 µM IBMX. EFS induced a clear-cut increase in tritium outflow above the basal level in up to three samples after EFS and at the 6th sample after EFS, tritium outflow returned to baseline values (Figure IV.3). The S_2-induced tritium outflow was lower than that induced by S_1 (Figure IV.3 A), yielding a S_2/S_1 ratio of 0.60 ± 0.03 in the control group (n = 9; Figure IV.4 A). Prucalopride as well as IBMX did not influence basal tritium outflow (Figure IV.3 B and C); IBMX had no significant effect on the S_2-induced tritium outflow (n = 8; Figure IV.3 B and Figure IV.4 A). Prucalopride (0.03 µM) however significantly enhanced EFS-induced tritium outflow (Figure IV.3 C) yielding a S_2/S_1 ratio of 0.88 ± 0.04 (n = 10) versus 0.60 ± 0.03 in control tissues (Figure IV.4 A). No apparent regional differences in the effect of prucalopride were observed (Table IV.1). Prucalopride (0.03 µM) in the presence of IBMX (10 µM) led to a S_2/S_1 ratio of 0.94 ± 0.05 (n = 10), which is somewhat higher than with prucalopride alone but this increase did not reach significance (Figure IV.4 A).

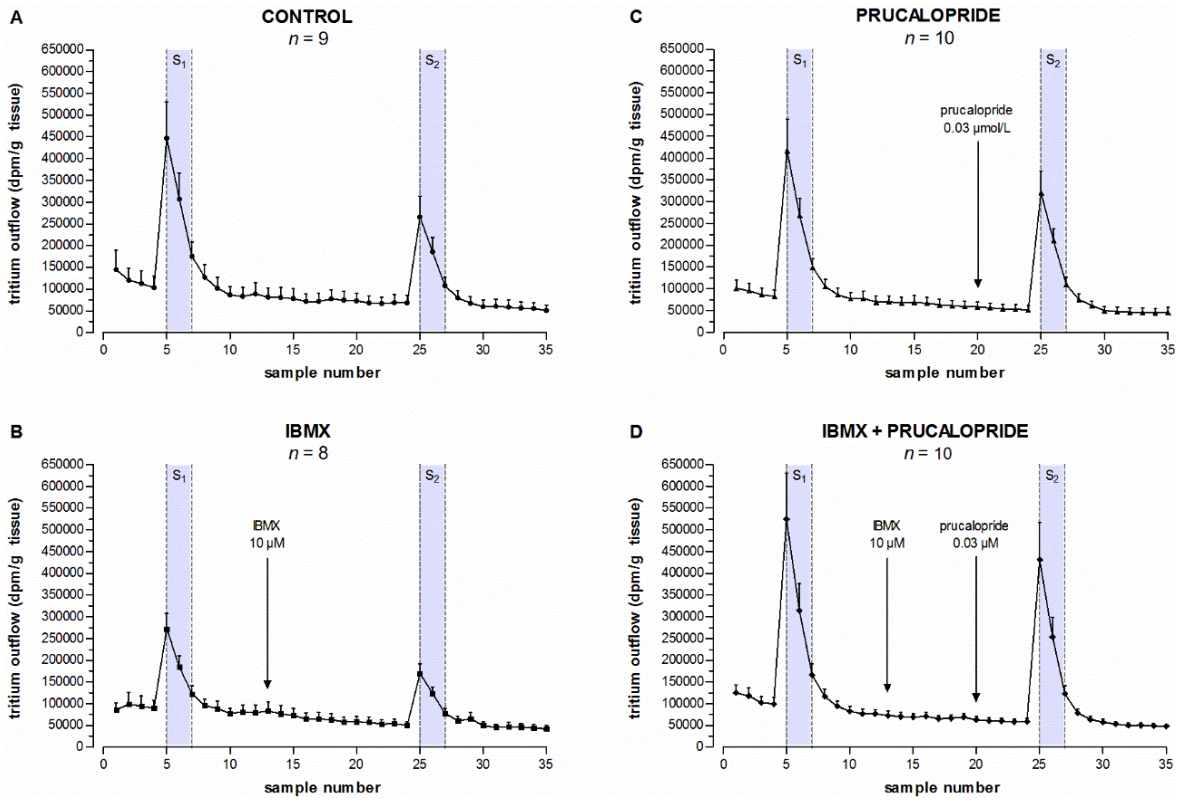


Figure IV.3 - Influence of IBMX and prucalopride on basal and electrically induced tritium outflow. Influence of 10 µM IBMX (B), 0.03 µM prucalopride (C), and prucalopride in the presence of IBMX (D) on basal and electrically induced total tritium outflow; parallel control tissues are shown in A. Samples were collected every 3 min for measurement of total tritium outflow. Tissues were stimulated twice (1 ms, 15 V, 4 Hz, 2 min) at the 5th (S_1) and 25th (S_2) sample inducing a stimulation-induced peak of the tritium outflow as indicated by grey shading; IBMX and prucalopride were added before S_2 as indicated by the arrows. Means ± S.E.M. are shown.

Table IV.1 - Influence of prucalopride in series 1 according to large intestinal region, age and gender.

region	age (years)	gender (M/F)	S_2/S_1 ratio	
			control	prucalopride
caecum	84	F	0.52	0.94
ascending colon	54	M	ND	0.90
transverse colon	59	F	0.57	0.90
sigmoid colon	51	M	0.56	0.83
	63	M	0.51	0.84
	57	M	ND	0.63
	67	F	0.54	ND
	37	M	ND	1.11
	61	M	0.79	ND
	76	M	0.64	ND
rectum	71	F	ND	0.91
	56	M	0.63	0.87
	52	M	0.64	0.83

Influence of 0.03 µM prucalopride on the S_2/S_1 ratio of electrically induced total tritium outflow, compared to control tissues not receiving compound. Tissues were stimulated twice (S_1 and S_2; 1 ms, 15 V, 4 Hz, 2 min); prucalopride was added 15 min before S_2.
Abbreviations: F, female; M, male; ND, not determinable due to technical reasons (see IV.5.4).

IBMX (10 µM) was therefore tested versus a 3-fold lower concentration of prucalopride (0.01 µM). In this series, the S_2/S_1 ratio was 0.74 ± 0.04 (n = 7) in the control tissues, 0.76 ± 0.05 (n = 11) in the strips where IBMX alone was added and 0.86 ± 0.03 (n = 9) in the strips where prucalopride alone was added; this tendency to increase the S_2/S_1 ratio by 0.01 µM prucalopride did not reach significance (Figure IV.4 B). However, prucalopride (0.01 µM) in the presence of IBMX (10 µM) induced a pronounced increase of the S_2-induced tritium outflow with a S_2/S_1 ratio of 1.06 ± 0.07 (n = 10), being significantly different from controls as well as from strips treated with prucalopride alone (Figure IV.4 B).

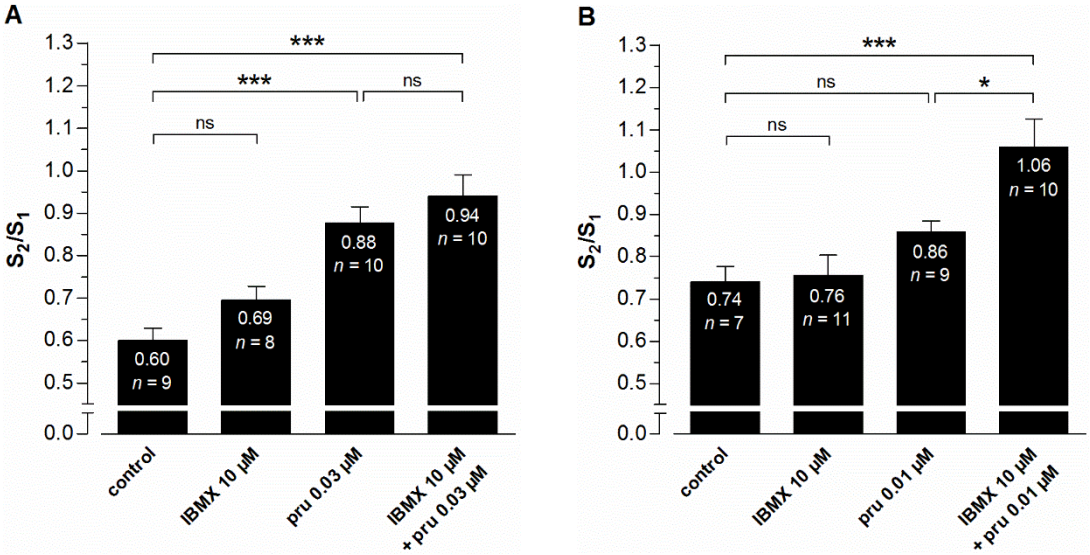


Figure IV.4 - S_2/S_1 ratios illustrating the influence of IBMX on the effect of prucalopride. Influence of 10 µM IBMX, 0.03 (A) and 0.01 (B) µM prucalopride (pru), and 0.03 (A) and 0.01 (B) prucalopride in the presence of IBMX on the S_2/S_1 ratio of electrically induced total tritium outflow. Tissues were stimulated twice (S_1 and S_2; 1 ms, 15 V, 4 Hz, 2 min); IBMX was added 36 min and prucalopride 15 min before S_2. Mean S_2/S_1 ± S.E.M.; one-way ANOVA followed by Bonferroni corrected t-test with ns not significant, * $P < 0.05$ and *** $P < 0.001$.

IV.6.2 Influence of rolipram and roflumilast on the effect of prucalopride (Figure IV.5 and Figure IV.6)

Rolipram (1 µM) did not influence basal or EFS-induced tritium outflow (S_2/S_1 ratio of 0.72 ± 0.02 [n = 7], versus 0.72 ± 0.03 [n = 9] in controls; Figure IV.5). Prucalopride (0.01 µM) alone did not significantly facilitate EFS-induced tritium outflow (S_2/S_1 ratio of 0.78 ± 0.04, n = 8). In the presence of rolipram, prucalopride (0.01 µM) induced a pronounced increase of EFS-induced tritium outflow with a S_2/S_1 ratio of 0.95 ± 0.03 (n = 8), being significantly different from that in control strips and in strips where prucalopride alone was administered.

This was confirmed with another selective PDE4 inhibitor roflumilast that also did not influence basal tritium outflow. Roflumilast (0.3 µM) and prucalopride (0.01 µM) alone had no significant influence on the EFS-induced tritium outflow with S_2/S_1 ratios of respectively 0.74 ± 0.05 (n = 7) and 0.75 ± 0.03 (n = 7) versus 0.72 ± 0.02 (n = 7) in control tissues (Figure IV.6). Prucalopride in the presence of roflumilast significantly enhanced the S_2/S_1 ratio up to 0.98 ± 0.07 (n = 8), when compared with control tissues and tissues receiving prucalopride alone. This enhancement in the effect of prucalopride by roflumilast is of similar magnitude as that found with rolipram.

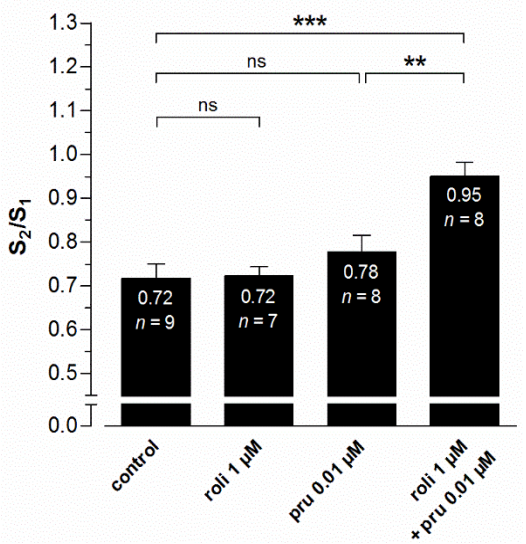


Figure IV.5 - S_2/S_1 ratios illustrating the influence of rolipram on the effect of prucalopride. Influence of 1 µM rolipram (roli), 0.01 µM prucalopride (pru) and prucalopride in the presence of rolipram on the S_2/S_1 ratio of electrically induced total tritium outflow. Tissues were stimulated twice (S_1 and S_2; 1 ms, 15 V, 4 Hz, 2 min); rolipram was added 36 min and prucalopride 15 min before S_2. Mean S_2/S_1 ± S.E.M.; one-way ANOVA followed by Bonferroni corrected t-test with ns not significant, ** P < 0.01 and *** P < 0.001.

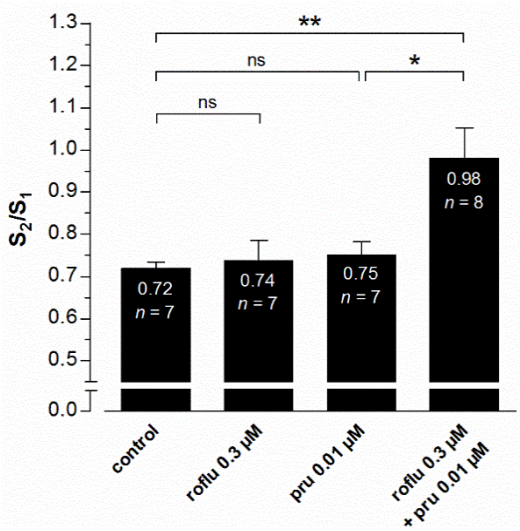


Figure IV.6 - S_2/S_1 ratios illustrating the influence of roflumilast on the effect of prucalopride. Influence of 0.3 µM roflumilast (roflu), 0.01 µM prucalopride (pru) and prucalopride in the presence of roflumilast on the S_2/S_1 ratio of electrically induced total tritium outflow. Tissues were stimulated twice (S_1 and S_2; 1 ms, 15 V, 4 Hz, 2 min); roflumilast was added 36 min and prucalopride 15 min before S_2. Mean S_2/S_1 ± S.E.M.; one-way ANOVA followed by Bonferroni corrected t-test with ns not significant, * P < 0.05 and ** P < 0.01.

IV.6.3 Influence of vinpocetine, EHNA and cilostamide on the effect of prucalopride (Figure IV.7)

In order to be able to study the three PDE inhibitors in one series, there were no control strips without addition of active compounds before S_2 included, but the strips where prucalopride alone was administered were used for comparison. In the presence of prucalopride (0.01 µM) a S_2/S_1 value of

0.74 ± 0.03 (n = 5) was obtained. This was not influenced by previous addition of vinpocetine (100 µM), EHNA (30 µM) and cilostamide (1 µM) where S_2/S_1 ratios of respectively 0.72 ± 0.07 (n = 4), 0.72 ± 0.05 (n = 6) and 0.72 ± 0.03 (n = 6) were obtained (Figure IV.7). A one-way ANOVA did not reach significance. Vinpocetine, EHNA and cilostamide had no effect on the basal tritium outflow.

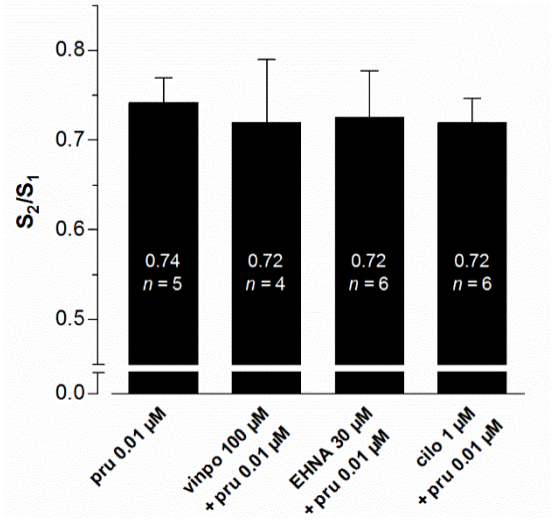


Figure IV.7 - S_2/S_1 ratios illustrating the influence of vinpocetine, EHNA and cilostamide on the effect of prucalopride. Influence of 0.01 µM prucalopride (pru) alone or in the presence of 100 µM vinpocetine (vinpo), 30 µM EHNA or 1 µM cilostamide (cilo) on the S_2/S_1 ratio of electrically induced total tritium outflow. Tissues were stimulated twice (S_1 and S_2; 1 ms, 15 V, 4 Hz, 2 min); vinpocetine, EHNA and rolipram were added 36 min and prucalopride 15 min before S_2. Mean S_2/S_1 ± S.E.M.; one-way ANOVA did not reach significance.

IV.6.4 Influence of ethanol and DMSO on the effect of prucalopride

The solvents were tested in two small series. Ethanol (0.05%) did not influence basal tritium outflow. In the presence of ethanol, the S_2/S_1 ratio of 0.64 ± 0.06 (n = 3) was the same as that in controls (0.64 ± 0.03; n = 3). Prucalopride (0.01 µM) alone led to a S_2/S_1 ratio of 0.68 ± 0.07 (n = 3), which was not influenced by previous addition of ethanol (S_2/S_1 ratio of 0.65 ± 0.08; n = 3).

DMSO (1%) had no effect on basal and EFS-induced tritium outflow with a S_2/S_1 ratio of 0.72 ± 0.05 (n = 4) versus 0.70 ± 0.02 (n = 4) in controls. In the presence of prucalopride (0.01 µM) the S_2/S_1 ratio was 0.81 ± 0.07 (n = 4), which is comparable to 0.83 ± 0.05 (n = 4) measured for prucalopride in the presence of DMSO.

IV.7 Discussion

Prucalopride (Resolor™), marketed for constipation, stimulates whole gut transit and colonic transit in healthy humans (Emmanuel *et al.*, 1998; Bouras *et al.*, 1999) and increases stool frequency and decreases stool consistency in patients with constipation (Emmanuel *et al.*, 2002; Sloots *et al.*, 2002). The underlying mechanism for these prokinetic properties of prucalopride and other 5-HT$_4$ receptor agonists is thought to be interaction with stimulating 5-HT$_4$ receptors localized on cholinergic neurons toward GI smooth muscle, facilitating acetylcholine release and cholinergic contraction in the GI tract. This mechanism was clearly shown in human stomach (Leclere and Lefebvre, 2002) and colon (Leclere *et al.*, 2005) as prucalopride increased electrically induced acetylcholine release, which was antagonized by selective 5-HT$_4$ receptor antagonists confirming interaction with 5-HT$_4$ receptors; the facilitating effect of 0.3 µM prucalopride, which is 10- to 30-fold higher than used in this study, was antagonized by 1 nM SB204070 in human stomach (Leclere and Lefebvre, 2002) and by 0.01 µM GR 113808 in human colon (Leclere *et al.*, 2005). Cardiovascular adverse events were reported with the 5-HT$_4$ receptor agonists cisapride and tegaserod, but these adverse events are not 5-HT$_4$ receptor-related (cisapride by interaction with human ether-a-go-go-related gene potassium channels and tegaserod possibly by interaction with 5-HT$_1$ and/or 5-HT$_2$ receptors (De Maeyer *et al.*, 2008)). These cardiovascular side effects should thus not occur with the new generation of highly selective 5-HT$_4$ receptor agonists such as prucalopride. Prucalopride was indeed found to be cardiovascularly safe in several randomized placebo-controlled double-blind trials in patients with chronic constipation (Keating, 2013); nevertheless combination therapies are explored to lower the dose and risk of adverse events. Combination of a 5-HT$_4$ receptor agonist with the acetylcholinesterase inhibitor neostigmine had a synergistic effect on cholinergic activity *in vitro* in human colon (Cellek *et al.*, 2008) and on colonic transit time *in vivo* in rat (Campbell-Dittmeyer *et al.*, 2009); the synergistic effect on cholinergic activity *in vitro* in human colon was confirmed with the acetylcholinesterase inhibitor donepezil (Broad *et al.*, 2013). Combination of a 5-HT$_4$ receptor agonist with a PDE4 inhibitor might also be considered. 5-HT$_4$ receptors are G_s-protein coupled receptors linked to adenylyl cyclase and the generation of the second messenger cAMP, and PDEs are the sole family of isozymes degrading cAMP. A tight controlling role of the signal transduction of cardiac 5-HT$_4$ receptors was shown: by PDE3 plus PDE4 in the porcine heart (Galindo-Tovar *et al.*, 2009; Weninger *et al.*, 2012) and by PDE3 in the human heart (Afzal *et al.*, 2008). As 5-HT$_4$ receptors enhancing cholinergic neurotransmission in porcine stomach and colon are controlled by PDE4, we proposed that combining a 5-HT$_4$ receptor agonist with a PDE4 inhibitor might enhance its gastroprokinetic effect (Priem *et al.*, 2012; Priem *et al.*, 2013; Lefebvre *et al.*, 2016). The actual study therefore investigated whether 5-HT$_4$ receptors enhancing cholinergic neurotransmission in human large intestine are also controlled by PDEs, by studying the effect of PDE inhibition on the facilitating effect of prucalopride on acetylcholine release.

In order to be able to observe a possible facilitating influence of PDE inhibition on the effect of prucalopride, the influence of 10 µM of the non-selective PDE inhibitor IBMX, which was shown to be effective in enhancing the effect of prucalopride on acetylcholine release in porcine gastric circular

muscle (Priem *et al.*, 2012), was first tested versus 0.03 µM prucalopride, a tenfold lower concentration than the one shown before to clearly enhance electrically induced acetylcholine release in human colon (Leclere *et al.*, 2005). However this concentration of prucalopride still induced a pronounced facilitation of the electrically induced acetylcholine release with a S_2/S_1 ratio of 0.88 (versus 0.60 in the control group) which is in the range of the effect previously reported with 0.3 µM prucalopride in human colon circular muscle (Leclere *et al.*, 2005; Lefebvre *et al.*, 2010); the effect of 0.03 µM prucalopride was not significantly enhanced by IBMX. When further decreasing the prucalopride concentration to 0.01 µM, a non-significant increase in S_2/S_1 ratio to 0.86 (versus 0.74 in the control group) was observed. But in the presence of IBMX, that had no influence on the electrically induced acetylcholine release, 0.01 µM prucalopride significantly enhanced the S_2/S_1 ratio to 1.06. This degree of potentiation with IBMX is in agreement with the level obtained in porcine stomach circular muscle (Priem *et al.*, 2012). The effect of IBMX is not due to an effect of its solvent ethanol, given the non-effect of ethanol on electrically induced acetylcholine release and its potentiation by prucalopride. The intraneuronal pathway of 5-HT_4 receptors on cholinergic neurons, facilitating acetylcholine release, in human large intestinal circular muscle is thus also regulated by PDEs.

To identify the responsible PDE subtype, selective PDE inhibitors were investigated. As cAMP is the cyclic nucleotide involved in the 5-HT_4 receptor pathway, only the cAMP degrading classic PDE subtypes were investigated: PDE1, 2 and 3 degrade both cAMP and cGMP and PDE4 is cAMP specific (Beavo and Reifsnyder, 1990; Maurice *et al.*, 2003) and are selectively inhibited by respectively vinpocetine, EHNA, cilostamide and rolipram (Alexander *et al.*, 2017). 100 µM vinpocetine, 30 µM EHNA and 1 µM cilostamide did not influence the effect of prucalopride on electrically induced acetylcholine release. These concentrations were definitely high enough to inhibit their respective PDE subtype given the reported K_i-values of 14 µM, 1 µM and 0.02 µM (Maurice *et al.*, 2003) and IC_{50} values of 8-50 µM (Hagiwara *et al.*, 1984; Saeki and Saito, 1993; Loughney *et al.*, 1996), 1-5 µM (Podzuweit *et al.*, 1995; Michie *et al.*, 1996; O'Grady *et al.*, 2002) and 0.005-0.13 µM (Hidaka *et al.*, 1979; Elks and Manganiello, 1984; Beavo and Reifsnyder, 1990; Sudo *et al.*, 2000; O'Grady *et al.*, 2002) for respectively vinpocetine, EHNA and cilostamide. Rolipram in a concentration of 1 µM, which is able to inhibit all PDE4 isozymes (Wang *et al.*, 1997), did not influence the electrically induced acetylcholine release, but significantly enhanced the effect of prucalopride; this is not related to its solvent DMSO that was without effect. The increase in S_2/S_1 ratio from 0.78 with prucalopride alone to 0.95 with prucalopride in the presence of rolipram is of the same magnitude as obtained in the series with IBMX (from 0.86 to 1.06). Roflumilast is another selective PDE4 inhibitor which is already used to suppress exacerbations of chronic obstructive pulmonary disease (Rabe, 2011; Michalski *et al.*, 2012; Wan *et al.*, 2015). Roflumilast, inhibiting the four PDE4 isozymes with an IC_{50} of about 1 nM (Tralau-Stewart *et al.*, 2011), reached a maximal effect in a concentration of 0.3 µM when tested for its enhancement of prucalopride-induced facilitation of acetylcholine release in porcine stomach (Lefebvre *et al.*, 2016). In human large intestinal circular smooth muscle, this concentration of roflumilast did not influence acetylcholine release, but potentiated the facilitating effect of prucalopride to the same extent as rolipram with an increase in S_2/S_1 ratio from 0.75 with prucalopride alone to 0.98 with prucalopride in the presence of roflumilast.

The results with rolipram and roflumilast illustrate that the intraneuronal pathway of acetylcholine release facilitating 5-HT_4 receptors, localized on cholinergic neurons in human large intestinal circular muscle, is regulated exclusively by PDE4, just as in the porcine stomach and colon. From their functional study in human colon circular muscle, Cellek et al. (Cellek *et al.*, 2008) suggested possible poor coupling of the 5-HT_4 receptor to acetylcholine release; this might be related to the regulation of the cAMP response to receptor activation by PDE4. In the porcine stomach, rolipram or roflumilast alone enhanced electrically induced acetylcholine release (Lefebvre *et al.*, 2016) suggesting that PDE4 constitutively regulates the basal content of cAMP in porcine stomach cholinergic neurons. This is not the case in human large intestine, as rolipram or roflumilast alone did not influence acetylcholine release.
The 5-HT_4 receptor pathway in cholinergic neurons in human large intestinal circular muscle is thus also regulated by PDE4. If the synergy between 5-HT_4 receptor agonism and PDE4 inhibition as shown here on acetylcholine release can also be obtained on EFS-induced cholinergic contraction, *in vivo* combination therapy of prucalopride or another selective 5-HT_4 receptor agonist with a selective PDE4 inhibitor can be considered for constipation. This combination therapy might allow to use a low dose of the 5-HT_4 receptor agonist, while the prokinetic effect is maintained. The recommended dose of prucalopride is 2 mg once daily. In healthy volunteers, taking 2 mg prucalopride per os once daily for 7 days, pharmacokinetic steady state was attained within 3 days and the C_{max} plasma concentration on day 7 was 8.09 ng/ml, at a median time of 2 h post dose; C_{min} and $C_{average}$ were 2.21 and 4.32 ng/ml respectively (Chen *et al.*, 2012) . Similar values were obtained in elderly patients with constipation. Upon intake of 0.5, 1 or 2 mg prucalopride once daily for 4 weeks, steady state was reached between days 4 and 7 of treatment and peak plasma concentrations were attained 2-3 h postdose (Camilleri *et al.*, 2009); the near C_{max} plasma concentrations determined at 3 h postdose on day 7 were 2, 4 and 7 ng/ml, corresponding to 0.005, 0.011 and 0.019 µM. The prucalopride concentration of 0.01 µM that shows synergy with PDE4 inhibition in the actual study thus correlates to the C_{max} obtained upon repeated dosing with 1 mg. When combining a 5-HT_4 receptor agonist with a PDE4 inhibitor, the action of the 5-HT_4 receptor agonist at the level of the cardiac 5-HT_4 receptors will not be influenced, because in the human atrium the 5-HT_4 receptor pathway is under sole control of PDE3 (Galindo-Tovar *et al.*, 2009). However, the role of PDEs in controlling the cyclic nucleotide concentrations at the level of the smooth muscle cells in human large intestinal circular muscle must be taken in account. PDE inhibition might increase the basal level of cyclic nucleotides inducing relaxation. If the PDE involved is PDE4, the PDE4 inhibitor in the combination therapy might induce relaxation, counteracting the prokinetic effect in the large intestine. In porcine colon circular muscle the predominant PDE is PDE3 (Priem *et al.*, 2013). In human rectal circular muscle, PDE1, 2, 3 and 4 inhibitors all reduced histamine-induced tone suggesting a role for several PDEs (Jones *et al.*, 2002). In human colon circular muscle, both IBMX and rolipram concentration-dependently reduced basal tone, rolipram having a smaller maximal effect; other PDE inhibitors were not tested (McLean and Coupar, 1996). An elaborated *in vitro* functional study investigating the effect of PDE inhibitors on cholinergic contractions by electrically released acetylcholine and by an exogenous muscarinic agonist in human large intestinal circular muscle can clarify the muscular role of PDEs. But even when the PDE inhibitor alone induces relaxation, it is possible

to enhance the 5-HT$_4$ receptor-mediated facilitation of cholinergic contraction in a selected concentration range as shown before with IBMX in porcine stomach (Priem *et al.*, 2012). In human colon 5-HT$_4$ receptors have also been described on circular smooth muscle cells and rolipram, in a concentration not influencing basal tone, facilitated the relaxant effect of 5-HT via these receptors (McLean and Coupar, 1996). The possible contribution of these relaxant receptors to the clinical effect of 5-HT$_4$ receptor agonists such as prucalopride at colonic level, that *in vivo* is clear-cut prokinetic (Emmanuel *et al.*, 1998; Bouras *et al.*, 1999), is not well understood. If relevant, the prokinetic effect of a 5-HT$_4$ receptor agonist might depend on a fine balance between its excitatory and inhibitory effects, and facilitation of both effects by rolipram might be beneficial. It should be stated that the presence of relaxant 5-HT$_4$ receptors in human colon circular muscle could not be systematically confirmed (Cellek *et al.*, 2006); they were also not observed in porcine colon (Priem and Lefebvre, 2011).

IV.8 Conclusion and future perspectives

In conclusion, the intraneuronal pathway of 5-HT$_4$ receptors facilitating cholinergic neurotransmission in human large intestinal circular muscle is, just as in the porcine GI tract, controlled by PDE4; PDE4 inhibition leads to an enhancement of the facilitating effect of prucalopride on acetylcholine release. If this synergy is confirmed in a functional assay with electrically induced cholinergic contractions, the *in vivo* prokinetic effect of 5-HT$_4$ receptor agonists in the large intestine might be promoted by combination with a selective PDE4 inhibitor; this can be further explored as possible combination therapy for gastrointestinal hypomotility disorders such as constipation as an alternative option to combination with acetylcholinesterase inhibitors. For both combination strategies safety will have to be evaluated. Muscarinic effects as diarrhea, nausea and vomiting and nicotinic ones as muscle cramps can occur with acetylcholinesterase inhibitors. The most frequently reported adverse events in clinical studies with the PDE4 inhibitor roflumilast for chronic obstructive pulmonary disease also included diarrhea and nausea along with weight loss and headache (Yuan *et al.*, 2016).

IV.9 Acknowledgements

The authors thank Mrs. Inge Van Colen for technical support and Mrs. Saskia De Groote, Mrs. Inge Vandenbroucke and Mrs. Eva Pape from the Department of Gastrointestinal surgery of Ghent University Hospital for help in obtaining the human specimens.

IV.10 References

Afzal F., Andressen K.W., Mork H.K., Aronsen J.M., Sjaastad I., Dahl C.P., Skomedal T., Levy F.O., Osnes J.B., and Qvigstad E. (2008). 5-HT4-elicited positive inotropic response is mediated by cAMP and regulated by PDE3 in failing rat and human cardiac ventricles. *Br J Pharmacol* 155, 1005-1014. DOI: 10.1038/bjp.2008.339

Alexander S.P.H., Fabbro D., Kelly E., Marrion N.V., Peters J.A., Faccenda E., Harding S.D., Pawson A.J., Sharman J.L., Southan C.*, et al.* (2017). THE CONCISE GUIDE TO PHARMACOLOGY 2017/18: Enzymes. *Br J Pharmacol* 174, S272-S359. DOI: 10.1111/bph.13877

Beavo J.A., and Reifsnyder D.H. (1990). Primary Sequence of Cyclic-Nucleotide Phosphodiesterase Isozymes and the Design of Selective Inhibitors. *Trends Pharmacol Sci* 11, 150-155. DOI: 10.1016/0165-6147(90)90066-H

Bouras E.P., Camilleri M., Burton D.D., and Mckinzie S. (1999). Selective stimulation of colonic transit by the benzofuran 5HT(4) agonist, prucalopride, in healthy humans. *Gut* 44, 682-686. DOI: 10.1136/gut.44.5.682

Broad J., Kung V.W., Boundouki G., Aziz Q., De Maeyer J.H., Knowles C.H., and Sanger G.J. (2013). Cholinergic interactions between donepezil and prucalopride in human colon: potential to treat severe intestinal dysmotility. *Br J Pharmacol* 170, 1253-1261. DOI: 10.1111/bph.12397

Broad J., Goralczyk A., Mannur K., Dukes G.E., and Sanger G.J. (2014a). Drugs acting at 5-HT4, D-2, motilin, and ghrelin receptors differ markedly in how they affect neuromuscular functions in human isolated stomach. *Neurogastroenterol Motil* 26, 851-861. DOI: 10.1111/nmo.12338

Broad J., Hughes F., Chin-Aleong J., Sifrim D., and Sanger G.J. (2014b). Regionally dependent neuromuscular functions of motilin and 5-HT4 receptors in human isolated esophageal body and gastric fundus. *Neurogastroenterol Motil* 26, 1311-1322. DOI: 10.1111/nmo.12394

Camilleri M., Beyens G., Kerstens R., Robinson P., and Vandeplassche L. (2009). Safety assessment of prucalopride in elderly patients with constipation: a double-blind, placebo-controlled study. *Neurogastroenterol Motil* 21, 1256-e1117. DOI: 10.1111/j.1365-2982.2009.01398.x

Campbell-Dittmeyer K., Hicks G.A., Earnest D.L., and Greenwood-Van Meerveld B. (2009). Increased colonic transit in rats produced by a combination of a cholinesterase inhibitor with a 5-HT4 receptor agonist. *Neurogastroenterol Motil* 21, 1197-e1108. DOI: 10.1111/j.1365-2982.2008.01238.x

Cellek S., John A.K., Thangiah R., Dass N.B., Bassil A.K., Jarvie E.M., Lalude O., Vivekanandan S., and Sanger G.J. (2006). 5-HT4 receptor agonists enhance both cholinergic and nitrergic activities in human isolated colon circular muscle. *Neurogastroenterol Motil* 18, 853-861. DOI: 10.1111/j.1365-2982.2006.00810.x

Cellek S., Thangiah R., Jarvie E.M., Vivekanandan S., Lalude O., and Sanger G.J. (2008). Synergy between 5-HT4 receptor activation and acetylcholinesterase inhibition in human colon and rat forestomach. *Neurogastroenterol Motil* 20, 539-545. DOI: 10.1111/j.1365-2982.2007.01062.x

Chen X., Jiang J., Liu T., Liu H., Zhong W., and Hu P. (2012). Pharmacokinetics of single and repeated oral doses prucalopride in healthy Chinese volunteers. *Int J Clin Pharmacol Ther* 50, 797-804. DOI: 10.5414/CP201769

Craig D.A., and Clarke D.E. (1990). Pharmacological characterization of a neuronal receptor for 5-hydroxytryptamine in guinea-pig ileum with properties similar to the 5-hydroxytryptamine-4 receptor. *J Pharmacol Exp Ther* 252, 1378-1386.

De Maeyer J.H., Prins N.H., Schuurkes J.A., and Lefebvre R.A. (2006a). Differential effects of 5-hydroxytryptamine4 receptor agonists at gastric versus cardiac receptors: an operational framework to explain and quantify organ-specific behavior. *J. Pharmacol. Exp. Ther.* 317, 955-964. 10.1124/jpet.106.101329

De Maeyer J.H., Straetemans R., Schuurkes J.a.J., and Lefebvre R.A. (2006b). Porcine left atrial and sinoatrial 5-HT4 receptor-induced responses: fading of the response and influence of development. *Br J Pharmacol* 147, 140-157. DOI: 10.1038/sj.bjp.0706497

De Maeyer J.H., Lefebvre R.A., and Schuurkes J.a.J. (2008). 5-HT4 receptor agonists: similar but not the same. *Neurogastroenterol Motil* 20, 99-112. DOI: 10.1111/j.1365-2982.2007.01059.x

Elks M.L., and Manganiello V.C. (1984). Selective Effects of Phosphodiesterase Inhibitors on Different Phosphodiesterases, Adenosine-3',5'-Monophosphate Metabolism, and Lipolysis in 3t3-L1 Adipocytes. *Endocrinology* 115, 1262-1268. DOI: 10.1210/endo-115-4-1262

Emmanuel A.V., Kamm M.A., Roy A.J., and Antonelli K. (1998). Effect of a novel prokinetic drug, R093877, on gastrointestinal transit in healthy volunteers. *Gut* 42, 511-516. DOI: 10.1136/gut.42.4.511

Emmanuel A.V., Roy A.J., Nicholls T.J., and Kamm M.A. (2002). Prucalopride, a systemic enterokinetic, for the treatment of constipation. *Aliment Pharmacol Ther* 16, 1347-1356. DOI: 10.1046/j.1365-2036.2002.01272.x

Galindo-Tovar A., Vargas M.L., Escudero E., and Kaumann A.J. (2009). Ontogenic changes of the control by phosphodiesterase-3 and -4 of 5-HT responses in porcine heart and relevance to human atrial 5-HT4 receptors. *Br J Pharmacol* 156, 237-249. DOI: 10.1111/j.1476-5381.2008.00023.x

Galligan J.J., and Vanner S. (2005). Basic and clinical pharmacology of new motility promoting agents. *Neurogastroenterol Motil* 17, 643-653. DOI: 10.1111/j.1365-2982.2005.00675.x

Gershon M.D., and Tack J. (2007). The serotonin signaling system: From basic understanding to drug development-for functional GI disorders. *Gastroenterology* 132, 397-414. DOI: 10.1053/j.gastro.2006.11.002

Hagiwara M., Endo T., and Hidaka H. (1984). Effects of vinpocetine on cyclic nucleotide metabolism in vascular smooth muscle. *Biochem Pharmacol* 33, 453-457. DOI: 10.1016/0006-2952(84)90240-5

Hidaka H., Hayashi H., Kohri H., Kimura Y., Hosokawa T., Igawa T., and Saitoh Y. (1979). Selective inhibitor of platelet cyclic adenosine monophosphate phosphodiesterase, cilostamide, inhibits platelet aggregation. *J Pharmacol Exp Ther* 211, 26-30.

Jones O.M., Brading A.F., and J. M.M.N. (2002). Phosphodiesterase inhibitors cause relaxation of the internal anal sphincter in vitro. *Dis Colon Rectum* 45, 530-536. DOI: 10.1007/s10350-004-6235-8

Keating G.M. (2013). Prucalopride: A Review of Its Use in the Management of Chronic Constipation. *Drugs* 73, 1935-1950. DOI: 10.1007/s40265-013-0140-1

Krobert K.A., Brattelid T., Levy F.O., and Kaumann A.J. (2005). Prucalopride is a partial agonist through human and porcine atrial 5-HT4 receptors: comparison with recombinant human 5-HT4 splice variants. *Naunyn Schmiedebergs Arch Pharmacol* 371, 473-479. DOI: 10.1007/s00210-005-1068-0

Leclere P.G., and Lefebvre R.A. (2002). Presynaptic modulation of cholinergic neurotransmission in the human proximal stomach. *Br J Pharmacol* 135, 135-142. DOI: 10.1038/sj.bjp.0704471

Leclere P.G., Prins N.H., Schuurkes J.a.J., and Lefebvre R.A. (2005). 5-HT4 receptors located on cholinergic nerves in human colon circular muscle. *Neurogastroenterol Motil* 17, 366-375. DOI: 10.1111/j.1365-2982.2005.00621.x

Lefebvre R.A., Ferrero S., Van Colen I., Dhaese I., Camerini G., Fulcheri E., and Remorgida V. (2010). Influence of 5-HT4 receptor activation on acetylcholine release in human large intestine with endometriosis. *Neurogastroenterol Motil* 22, 557-563, e121-552. DOI: 10.1111/j.1365-2982.2009.01438.x

Lefebvre R.A., Van Colen I., Pauwelyn V., and De Maeyer J.H. (2016). Synergistic effect between 5-HT4 receptor agonist and phosphodiesterase 4-inhibitor in releasing acetylcholine in pig gastric circular muscle in vitro. *Eur J Pharmacol* 781, 76-82. DOI: 10.1016/j.ejphar.2016.04.003

Loughney K., Martins T.J., Harris E.A., Sadhu K., Hicks J.B., Sonnenburg W.K., Beavo J.A., and Ferguson K. (1996). Isolation and characterization of cDNAs corresponding to two human calcium, calmodulin-regulated, 3',5'-cyclic nucleotide phosphodiesterases. *J Biol Chem* 271, 796-806. DOI: 10.1074/jbc.271.2.796

Maurice D.H., Palmer D., Tilley D.G., Dunkerley H.A., Netherton S.J., Raymond D.R., Elbatarny H.S., and Jimmo S.L. (2003). Cyclic nucleotide phosphodiesterase activity, expression, and targeting in cells of the cardiovascular system. *Mol Pharmacol* 64, 533-546. DOI: 10.1124/mol.64.3.533

Mclean P.G., and Coupar I.M. (1996). Further investigation into the signal transduction mechanism of the 5-HT4-like receptor in the circular smooth muscle of human colon. *Br J Pharmacol* 118, 1058-1064. DOI: 10.1111/j.1476-5381.1996.tb15506.x

Michalski J.M., Golden G., Ikari J., and Rennard S.I. (2012). PDE4: A Novel Target in the Treatment of Chronic Obstructive Pulmonary Disease. *Clin. Pharmacol. Ther.* 91, 134-142. 10.1038/clpt.2011.266

Michie A.M., Lobban M., Muller T., Harnett M.M., and Houslay M.D. (1996). Rapid regulation of PDE-2 and PDE-4 cyclic AMP phosphodiesterase activity following ligation of the T cell antigen receptor on thymocytes: analysis using the selective inhibitors erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) and rolipram. *Cell Signal* 8, 97-110. DOI: 10.1016/0898-6568(95)02032-2

O'grady S.M., Jiang X., Maniak P.J., Birmachu W., Scribner L.R., Bulbulian B., and Gullikson G.W. (2002). Cyclic AMP-dependent Cl secretion is regulated by multiple phosphodiesterase subtypes in human colonic epithelial cells. *J Membr Biol* 185, 137-144. DOI: 10.1007/s00232-001-0120-3

Parker S.G., Taylor E.M., Hamburger S.A., Vimal M., and Kaumann A.J. (1995). Blockade of human and porcine myocardial 5-HT4 receptors by SB 203186. *Naunyn Schmiedebergs Arch Pharmacol* 353, 28-35. DOI: 10.1007/BF00168912

Podzuweit T., Nennstiel P., and Muller A. (1995). Isozyme selective inhibition of cGMP-stimulated cyclic nucleotide phosphodiesterases by erythro-9-(2-hydroxy-3-nonyl) adenine. *Cell Signal* 7, 733-738. DOI: 10.1016/0898-6568(95)00042-N

Priem E.K.V., and Lefebvre R.A. (2011). Investigation of neurogenic excitatory and inhibitory motor responses and their control by 5-HT4 receptors in circular smooth muscle of pig descending colon. *Eur J Pharmacol* 667, 365-374. DOI: 10.1016/j.ejphar.2011.06.021

Priem E.K.V., Van Colen I., De Maeyer J.H., and Lefebvre R.A. (2012). The facilitating effect of prucalopride on cholinergic neurotransmission in pig gastric circular muscle is regulated by phosphodiesterase 4. *Neuropharmacology* 62, 2126-2135. DOI: 10.1016/j.neuropharm.2011.12.020

Priem E.K.V., De Maeyer J.H., and Lefebvre R.A. (2013). Influence of phosphodiesterases on basal and 5-HT4 receptor facilitated cholinergic contractility in pig descending colon. *Eur J Pharmacol* 705, 156-163. DOI: 10.1016/j.ejphar.2013.02.011

Prins N.H., Akkermans L.M.A., Lefebvre R.A., and Schuurkes J.a.J. (2000). 5-HT4 receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. *Br J Pharmacol* 131, 927-932. DOI: 10.1038/sj.bjp.0703615

Prins N.H., Van Der Grijn A., Lefebvre R.A., Akkermans L.M.A., and Schuurkes J.a.J. (2001). 5-HT4 receptors mediating enhancement of contractility in canine stomach; an in vitro and in vivo study. *Br J Pharmacol* 132, 1941-1947. DOI: 10.1038/sj.bjp.0703985

Rabe K.F. (2011). Update on roflumilast, a phosphodiesterase 4 inhibitor for the treatment of chronic obstructive pulmonary disease. *Br J Pharmacol* 163, 53-67. DOI: 10.1111/j.1476-5381.2011.01218.x

Saeki T., and Saito I. (1993). Isolation of cyclic nucleotide phosphodiesterase isozymes from pig aorta. *Biochem Pharmacol* 46, 833-839. DOI: 10.1016/0006-2952(93)90492-F

Sloots C.E.J., Poen A.C., Kerstens R., Stevens M., De Pauw M., Van Oene J.C., Meuwissen S.G.M., and Felt-Bersma R.J.F. (2002). Effects of prucalopride on colonic transit, anorectal function and bowel habits in patients with chronic constipation. *Aliment Pharmacol Ther* 16, 759-767. DOI: 10.1046/j.1365-2036.2002.01210.x

Smith J.A., Beattie D.T., Marquess D., Shaw J.P., Vickery R.G., and Humphrey P.P. (2008). The in vitro pharmacological profile of TD-5108, a selective 5-HT(4) receptor agonist with high intrinsic activity. *Naunyn Schmiedebergs Arch Pharmacol* 378, 125-137. DOI: 10.1007/s00210-008-0282-y

Sudo T., Tachibana K., Toga K., Tochizawa S., Inoue Y., Kimura Y., and Hidaka H. (2000). Potent effects of novel anti-platelet aggregatory cilostamide analogues on recombinant cyclic nucleotide phosphodiesterase isozyme activity. *Biochem Pharmacol* 59, 347-356. DOI: 10.1016/S0006-2952(99)00346-9

Tonini M., Candura S.M., Onori L., Coccini T., Manzo L., and Rizzi C.A. (1992). 5-Hydroxytryptamine4 receptor agonists facilitate cholinergic transmission in the circular muscle of guinea-pig ileum: antagonism by tropisetron and DAU-6285. *Life Sci* 50, Pl173-Pl178. DOI: 10.1016/0024-3205(92)90453-V

Tralau-Stewart C.J., Williamson R.A., Nials A.T., Gascoigne M., Dawson J., Hart G.J., Angell A.D., Solanke Y.E., Lucas F.S., Wiseman J*., et al.* (2011). GSK256066, an exceptionally high-affinity and selective inhibitor of phosphodiesterase 4 suitable for administration by inhalation: in vitro, kinetic, and in vivo characterization. *J. Pharmacol. Exp. Ther.* 337, 145-154. 10.1124/jpet.110.173690

Wan Y., Sun S.X., Corman S., Huang X., Gao X., and Shorr A.F. (2015). A longitudinal, retrospective cohort study on the impact of roflumilast on exacerbations and economic burden among chronic obstructive pulmonary disease patients in the real world. *Int J Chron Obstruct Pulmon Dis* 10, 2127-2136. DOI: 10.2147/COPD.S80106

Wang P., Myers J.G., Wu P., Cheewatrakoolpong B., Egan R.W., and Billah M.M. (1997). Expression, purification, and characterization of human cAMP-specific phosphodiesterase (PDE4) subtypes A, B, C, and D. *Biochem Biophys Res Commun* 234, 320-324. DOI: 10.1006/bbrc.1997.6636

Weninger S., De Maeyer J.H., and Lefebvre R.A. (2012). Study of the regulation of the inotropic response to 5-HT4 receptor activation via phosphodiesterases and its cross-talk with C-type natriuretic peptide in porcine left atrium. *Naunyn Schmiedebergs Arch Pharmacol* 385, 565-577. DOI: 10.1007/s00210-012-0746-y

Yuan L., Dai X., Yang M., Cai Q., and Shao N. (2016). Potential treatment benefits and safety of roflumilast in COPD: a systematic review and meta-analysis. *Int J Chron Obstruct Pulmon Dis* 11, 1477-1483. DOI: 10.2147/COPD.S106370

CHAPTER V

5-HT_4 receptors throughout the murine gastrointestinal tract

5-HT_4 receptors facilitate cholinergic neurotransmission throughout the murine gastrointestinal tract

Vicky Pauwelyn and Romain A. Lefebvre

Department of Pharmacology - Heymans Institute, Ghent University, Ghent, Belgium

Neurogastroenterology and Motility 2017; 29(8): e13064. DOI: 10.1111/nmo.13064
https://doi.org/10.1111/nmo.13064
Supplementary material was integrated.

CHAPTER V -
5-HT_4 receptors throughout the murine gastrointestinal tract

5-HT_4 receptors facilitate cholinergic neurotransmission throughout the murine gastrointestinal tract

V.1 Abstract

V.1.1 Background

In the gastrointestinal tract of several species, facilitating 5-HT_4 receptors were proposed on myenteric cholinergic neurons innervating smooth muscle by *in vitro* study of the effect of the selective 5-HT_4 receptor agonist prucalopride on submaximal cholinergic contractions. This was not yet established in the murine gastrointestinal tract.

V.1.2 Methods

In circular smooth muscle strips from murine fundus, jejunum and colon, contractions were induced by electrical field stimulation in the presence of guanethidine, L-NAME and for colon also MRS 2500. Submaximal contractions were induced to study the influence of prucalopride.

V.1.3 Key results

Electrical field stimulation at reduced voltage induced reproducible submaximal neurogenic and cholinergic contractions as the contractions were abolished by TTX and atropine. Hexamethonium had no systematic inhibitory effect but mecamylamine reduced the responses, suggesting that part of the cholinergic response is due to activation of preganglionic neurons.

Prucalopride concentration-dependently increased the submaximal cholinergic contractions in the three tissue types, reaching maximum from 0.03 µM onwards. The facilitation in the different series with 0.03 µM prucalopride ranged from 41 to 104, 30 to 76 and 24 to 74% in fundus, jejunum and colon respectively. The effect of 0.03 µM prucalopride was concentration-dependently inhibited by GR 113808.

V.1.4 Conclusions & inferences

In the murine gastrointestinal tract, activation of 5-HT_4 receptors with prucalopride enhances cholinergic contractions, illustrating facilitation of myenteric cholinergic neurotransmission. The degree of enhancement with prucalopride is of similar magnitude as previously reported in other species, but the effective concentrations are lower than those needed in the gastrointestinal tract of other species.

V.1.5 Keywords

5-HT_4 receptor, cholinergic neurotransmission, gastrointestinal tract, mouse, prucalopride

V.1 Key points

- *In vitro* studies showed that 5-HT_4 receptor stimulation enhances myenteric cholinergic neurotransmission in gastrointestinal muscle of several species, but not yet in mice.
- In smooth muscle strips of murine fundus, jejunum and colon, the selective 5-HT_4 receptor agonist prucalopride enhanced electrically induced submaximal cholinergic contractions; this effect was abolished by selective 5-HT_4 receptor antagonism and confirmed with 5-HT, illustrating that 5-HT_4 receptor stimulation also enhances murine myenteric cholinergic neurotransmission.
- This murine *in vitro* model is useful to further investigate the pharmacology and signal transduction of 5-HT_4 receptors increasing the function of myenteric cholinergic neurons.

V.2 Introduction

Although serotonin or 5-hydroxytryptamine (5-HT) is best known for its role in the brain, approximately 95% of the serotonin in the human body is found in the gastrointestinal (GI) tract, mainly synthetized in enterochromaffin cells and to a small extent in serotonergic neurons of the myenteric plexus (Gershon and Tack, 2007). In the enteric nervous system multiple 5-HT receptor subtypes are expressed, contributing to the regulation of GI motility and secretion (Galligan and Parkman, 2007). 5-HT interacts with 7 subtypes of receptors, one being a ligand-gated ion channel (5-HT$_3$), the 6 others being G protein-coupled receptors. The 5-HT$_4$ receptor is present in the human brain, heart, adrenal cortex, bladder and GI tract. GI 5-HT$_4$ receptors have been implicated in intestinal secretion (Kellum *et al.*, 1999), sensitivity (Greenwood-Van Meerveld *et al.*, 2006), neurogenesis (Liu *et al.*, 2009) and –protection (Bianco *et al.*, 2016), and motility. In the human GI tract, three locations of 5-HT$_4$ receptors influencing motility have been described: (1) on smooth muscle cells, inducing relaxation (McLean and Coupar, 1996; Prins *et al.*, 2000b); (2) on inhibitory nitrergic neurons, inducing nitric oxide release counteracting contraction (Cellek *et al.*, 2006); (3) by far most extensively established, on myenteric excitatory cholinergic neurons innervating the smooth muscle layer, where activation enhances acetylcholine release and smooth muscle contraction (Prins *et al.*, 2000a; Leclere *et al.*, 2005; Cellek *et al.*, 2006). The latter effect was clearly shown *in vitro* for human stomach and colon, as the highly selective 5-HT$_4$ receptor agonist prucalopride enhanced electrically induced submaximal cholinergic contractions (Prins *et al.*, 2000a; Leclere and Lefebvre, 2002; Leclere *et al.*, 2005; Cellek *et al.*, 2006). This paradigm also allowed to illustrate 5-HT$_4$ receptor-mediated facilitation of cholinergic neurotransmission in the rat (Cellek *et al.*, 2008), guinea-pig (Tonini *et al.*, 1992), canine (Prins *et al.*, 2000a; Prins *et al.*, 2001) and porcine (Priem and Lefebvre, 2011; Priem *et al.*, 2012) GI tract. The facilitating effect of prucalopride was observed when either circularly or longitudinally oriented muscle strips were used; when both were studied within the same tissue, facilitation of cholinergic neurotransmission is seen in the circular as well as the longitudinal muscle strips (e.g. in canine stomach (Prins *et al.*, 2001)). The *in vivo* GI prokinetic effects of 5-HT$_4$ receptor agonists are attributed to activation of these 5-HT$_4$ receptors on enteric cholinergic neurons (Gershon and Tack, 2007) and they are therefore developed for treatment of functional gastrointestinal hypomotility disorders such as gastroparesis and constipation (Galligan and Vanner, 2005).

In the murine GI tract, 5-HT$_4$ receptors were shown at RNA and protein level in the submucosal and myenteric plexus (Liu *et al.*, 2005). The receptors also seem to have prokinetic effects. Indeed, gastric emptying and small intestinal transit are delayed in 5-HT$_4$ receptor knockout mice compared to wild-type mice (Liu *et al.*, 2009). Conversely, 5-HT$_4$ receptor agonists have a GI prokinetic effect in mice as RS67506 shortens whole gut transit time (Nagakura *et al.*, 1997) and DA-6886 shortens the colonic transit in normal as well as in loperamide-induced constipated mice (Lee *et al.*, 2014). Using Fos expression as a marker for neuronal activation, 5-hydroxytryptophan, the precursor of 5-HT, was shown to activate myenteric cholinergic neurons; it also induced defecation, both effects being abolished by a selective 5-HT$_4$ receptor antagonist (Wang *et al.*, 2007). Still, the facilitation of myenteric cholinergic

neurotransmission by 5-HT_4 receptor activation, using the paradigm described above for other species, has not yet been established in the murine GI tract.

This study therefore applies this *in vitro* method to investigate whether 5-HT_4 receptors enhance the function of cholinergic neurons inducing circular muscle contraction in three regions of the murine GI tract, namely the gastric fundus, jejunum and colon. After establishing the optimal parameters to induce submaximal neurogenic postganglionic cholinergic smooth muscle contractions by electrical field stimulation (EFS) for each tissue type, the effect of prucalopride on these EFS-induced contractions was tested.

V.3 Materials and methods

V.3.1 Animals and tissue preparation

All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University. Male C57BL/6J mice (minimal 7 weeks, body weight 24.2 ± 0.2 g, mean ± S.E.M. of n = 158) were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and maintained on a normal light-dark cycle with food pellets and water *ad libitum*.

After sacrificing the mice by cervical dislocation, the GI tract was isolated and kept in aerated (95% O_2/5% CO_2) Krebs solution (composition in mM: NaCl 118.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1). The stomach was emptied from its contents and two full thickness fundus strips were prepared by cutting in the direction of the circular smooth muscle layer. For jejunum a segment of the small bowel of approximately 5 cm long, starting 10 cm distally to the pylorus, and for distal colon an approximately 4 cm long segment above the pelvic brim was taken. Jejunum and colon segments were opened along the mesenteric border and pinned with the mucosa side up in Krebs solution. By sharp dissection the mucosa was removed under a microscope and 2 (colon) or 4 (jejunum) smooth muscle strips were cut along the circular axis. To illustrate the weight of the strips, the wet weight (mean ± S.E.M., for each tissue: n = 18 strips out of 9 animals) was 4.84 ± 0.28 mg for fundus, 0.55 ± 0.07 mg for jejunum and 0.51 ± 0.04 mg for colon in the series with construction of voltage-response curves; see V.3.2 Protocols.

After a cotton (fundus) or silk thread (jejunum and colon) was attached to both ends of every strip, they were mounted between two platinum plate electrodes (6-8 mm apart) in a 7 or 15 ml organ bath filled with oxygenated Krebs solution kept at body temperature. Muscle contractions were induced by EFS with a 4-channel custom-made stimulator. Changes in muscle tone were registered by isometric tension recording using Grass FT03 (Grass Instrument co., Quincy, MA, United States) or MLT 050/D force transducers (ADInstruments, Oxford, United Kingdom) and recorded on a Powerlab/8sp data recording system (ADInstruments) with Chart v5.5.6 software (ADInstruments).

V.3.2 Protocols

Strips were studied at optimal load as determined in preliminary experiments via the contractile response to the muscarinic receptor agonist carbachol: 1 g for fundus; 0.25 g for jejunum; 0.5 g for colon. Optimal load was determined in preliminary experiments by repetitively studying the response to a submaximal concentration of the muscarinic receptor agonist carbachol (0.1 µM in fundus and jejunum; 1 µM in colon) at increasing load till the maximal response was obtained. The contact time of carbachol was 3 min which was sufficient to obtain a plateau contraction; the interval between carbachol administrations was 20 min with repetitive rinsing. The optimal load was 1 g for fundus; 0.25 g for jejunum; 0.5 g for colon.

V.3.2.1 Characterization of EFS-induced responses

Experiments were performed in Krebs solution containing 4 µM of the noradrenergic neuron blocker guanethidine and 300 µM of the nitric oxide synthase inhibitor N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) to avoid relaxant influences due to noradrenaline and nitric oxide respectively. For colon tissue, 1 µM MRS 2500 ($P2Y_1$ receptor antagonist) was also added to avoid influences of the relaxant neurotransmitter ATP (Gallego *et al.*, 2012). After 60 min of equilibration with flushing every 15 min, voltage-response curves or frequency-response curves were constructed.

At an interval of 5 min, 10 s trains of EFS (pulse width of 0.5 ms; frequency of 4 Hz; increasing voltage 5-50 V) were applied. The voltage-response curve was repeated in the presence of 3 µM tetrodotoxin (TTX; voltage-gated Na^+ channel blocker; incubated for 30 min) or in its absence (parallel control). Frequency-response curves were obtained by using the same protocol but now fixing the voltage at 30 V and increasing the frequency from 0.5 to 16 Hz. Instead of TTX, the influence of 1 µM atropine (muscarinic receptor antagonist) was now tested.

V.3.2.2 Influence of prucalopride on EFS-induced submaximal cholinergic contractions

All experiments started as follows. Strips were equilibrated for 30 min with rinsing every 10 min whereafter viability and contractile activity of the strips were tested by repetitively studying the response to carbachol (0.1 µM in fundus and jejunum; 1 µM in colon) at 20 min interval with intermediate rinsing until a stable response was obtained. This took at most four administrations. The medium was then switched to Krebs solution containing 4 µM guanethidine, 300 µM L-NAME and for colon also 1 µM MRS 2500. Tissues were allowed to equilibrate 60 min with flushing every 15 min.

To obtain EFS-induced submaximal cholinergic contractions, the stimulation frequency used was intermediate as determined from the frequency-response curves; the voltage used was first supramaximal (30 V), as determined from the voltage-response curves, and then progressively decreased. In fundus and colon, 10 s trains of EFS with a pulse width of 0.5 ms, a frequency of 4 (fundus) or 8 (colon) Hz and supramaximal voltage (V_{max}) of 30 V were applied 10 times at an interval of 3 min. This yielded reproducible contractions. Preliminary experiments in the jejunum with parameters of EFS as for the colon learned that EFS-induced contractions were not stable. Increasing the interval between the trains of EFS to 6 min yielded reproducible contractions so that in jejunum 10 s trains (pulse width of 0.5 ms; frequency of 8 Hz; V_{max} of 30 V) were applied five times in the parallel time period of 10 trains in fundus and colon. EFS was then continued with gradual reduction of the voltage until the amplitude of the contraction was approximately 50% of that obtained at V_{max} ($V_{50\%}$).

Once $V_{50\%}$ was selected and a stable response to EFS at $V_{50\%}$ was obtained, EFS at $V_{50\%}$ was repeated at 5 min interval in the fundus and colon, and 10 min interval in the jejunum. After five contractions were obtained, three parallel strips received 3 µM TTX, 1 µM atropine or 0.5 mM hexamethonium (nicotinic acetylcholine receptor antagonist) and 10 (fundus and colon) or 5 (jejunum) further trains of EFS were applied in the presence of these substances; a 4th parallel control did not receive active compounds.

The same protocol was used to study the influence of three concentrations of prucalopride (first 0.03, 0.1 and 0.3 µM; in a second series 0.003, 0.01 and 0.03 µM); for the colon, the first concentration range

was tested with EFS at 4 instead of 8 Hz. The influence of prucalopride was also studied by adding it cumulatively within the same strip (0.3 nM to 1 µM with half log unit concentration increments).

V.3.2.3 Influence of compounds on the effect of prucalopride

The responses to carbachol and $V_{50\%}$ were first determined as described above. Then, after obtaining five contractions by EFS at $V_{50\%}$, the compound under study was added in one strip, while the parallel control strip did not receive the compound. A further 20 (fundus and colon) or 10 (jejunum) trains of EFS were then applied, with addition of 0.03 µM prucalopride in both strips after 10 (fundus and colon) or 5 (jejunum) trains. The compounds studied versus prucalopride were MRS 2500 (in fundus and jejunum; 1 µM), the selective 5-HT$_4$ receptor antagonist GR 113808 (0.3 µM) and the nicotinic acetylcholine receptor antagonists hexamethonium (0.5 mM) and mecamylamine (30 µM). Using the same protocol, GR 113808 was also studied versus 0.3 µM 5-HT in the presence of 1 µM methysergide and 0.3 µM granisetron to exclude interaction of 5-HT with other 5-HT receptors than 5-HT$_4$ receptors; additionally, GR 113808 was studied versus prucalopride by adding it cumulatively (0.001-0.3 µM with half log unit concentration increments). As GR 113808 cannot be dissolved in distilled water, parallel control tissues received a corresponding amount of its solvent DMSO.

V.3.3 Drugs

Atropine sulfate salt monohydrate, carbamoylcholin chloride, guanethidine sulfate, hexamethonium bromide, N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), mecamylamine hydrochloride and serotonin creatinine sulfate monohydrate (5-HT) were obtained from Sigma-Aldrich (Diegem, Belgium). GR 113808 [(1-[2-[(methylsulfonyl)amino]ethyl)-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate], granisetron hydrochloride, methysergide maleate, MRS 2500 tetraammonium salt [(1R*,2S*)-4-[2-iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt] and tetrodotoxin citrate (TTX) were obtained from Tocris Bioscience (Bristol, United Kingdom) and prucalopride succinate from Selleck Chemicals (Houston, TX, United States). All compounds were dissolved and diluted in distilled water, except for GR 113808 which was dissolved in dimethyl sulfoxide (DMSO), yielding a DMSO concentration of 0.03% in the organ bath.

V.3.4 Data and statistical analysis

The contraction force induced by carbachol or EFS was expressed as gram.second per milligram wet weight of the tissue (g.s/mg wet weight). It was calculated by determining the area under the curve (AUC) during the contraction (3 min for carbachol- and 10 s for EFS-induced contractions), reduced with the AUC during a corresponding time period just before adding carbachol or applying EFS. EFS-induced contractions at $V_{50\%}$ in the presence of substances were expressed as percentage of the mean of the five contractions just before addition (100% reference). For the experiments testing the influence of MRS 2500, hexamethonium or mecamylamine on the effect of prucalopride, contractions after adding prucalopride were also expressed as percentage of the mean of the five contractions in the presence of MRS 2500, hexamethonium or mecamylamine just before adding prucalopride. Strips with unstable

reference responses, defined as when the AUC of minimally one of the five contractions before addition of the substance was outside the range of 75-125%, were excluded from data analysis. To illustrate the exclusion rate, in the series studying TTX, atropine and hexamethonium on electrically induced submaximal contractions seven out of 39, five out of 39 and five out of 40 strips were excluded in the fundus, jejunum and colon respectively.

pEC_{50} values for prucalopride and pIC_{50} values for GR 113808 were calculated by linear interpolation. An estimate of the pK_b of GR 113808 was obtained via the functional version of the Cheng-Prusoff equation for analysis of an antagonist inhibition curve (Craig, 1993). Data are expressed as means ± S.E.M. *n* refers to tissues obtained from different animals. Statistical analysis was performed by use of GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, United States). The EFS-induced contractions induced by the last train of EFS in parallel groups were assessed with an unpaired *t*-test (two groups) or one-way ANOVA followed by a Bonferroni corrected *t*-test (more than two groups). A *P*-value less than 0.05 was considered statistically significant.

V.4 Results

V.4.1 Characterization of EFS-induced contractions (Figure V.1-3)

In the presence of guanethidine, L-NAME and for the colon also MRS 2500, 10 s trains of EFS induced tonic on-contractions that decreased immediately after termination of EFS to reach baseline again within approximately 10 s in the fundus (Figure V.1 A and D) and colon (Figure V.1 C and F). In jejunum EFS induced an increase of the phasic activity, gradually decreasing after termination of EFS (Figure V.1 B and E).

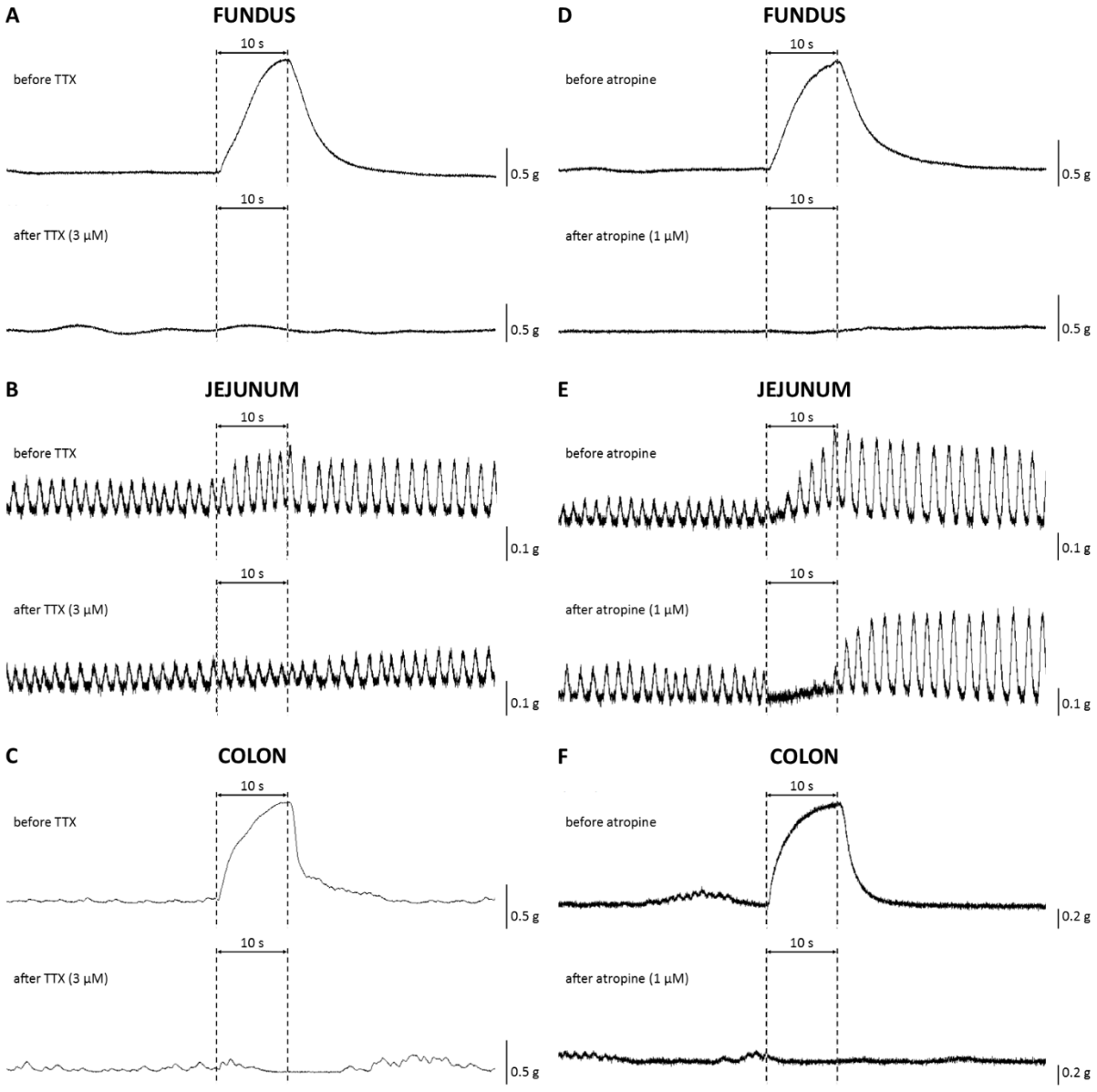


Figure V.1 - Contractile responses to electrical field stimulation before and after adding TTX or atropine. Representative traces showing the influence of 3 µM TTX (A-C) or 1 µM atropine (D-F) on contractions induced by electrical field stimulation (10 s trains with 0.5 ms pulse duration) at 30 V and 4 Hz (A-D) or 8 Hz (E & F). Experiments were performed in the continuous presence of 4 µM guanethidine, 300 µM L-NAME and for colon also 1 µM MRS 2500.

The voltage-response curves with EFS at 4 Hz indicated that maximal contractile response was obtained at 20 V in the three tissues; the responses at increasing voltage were reproducible (Figure V.2 A-C) and abolished by 3 µM TTX except for the responses at 40 and 50 V in the jejunum (Figure V.2 D-F). The frequency-response curves with EFS at 30 V showed frequency-dependent responses, still increasing at the highest frequency applied (16 Hz); the responses at increasing frequency were reproducible (Figure V.3 A-C) and abolished by 1 µM atropine (Figure V.3 D-F). From these experiments, 30 V was determined as supramaximal, and a frequency of 4 (fundus) or 8 Hz (jejunum and colon) was selected as submaximal frequency for further experiments.

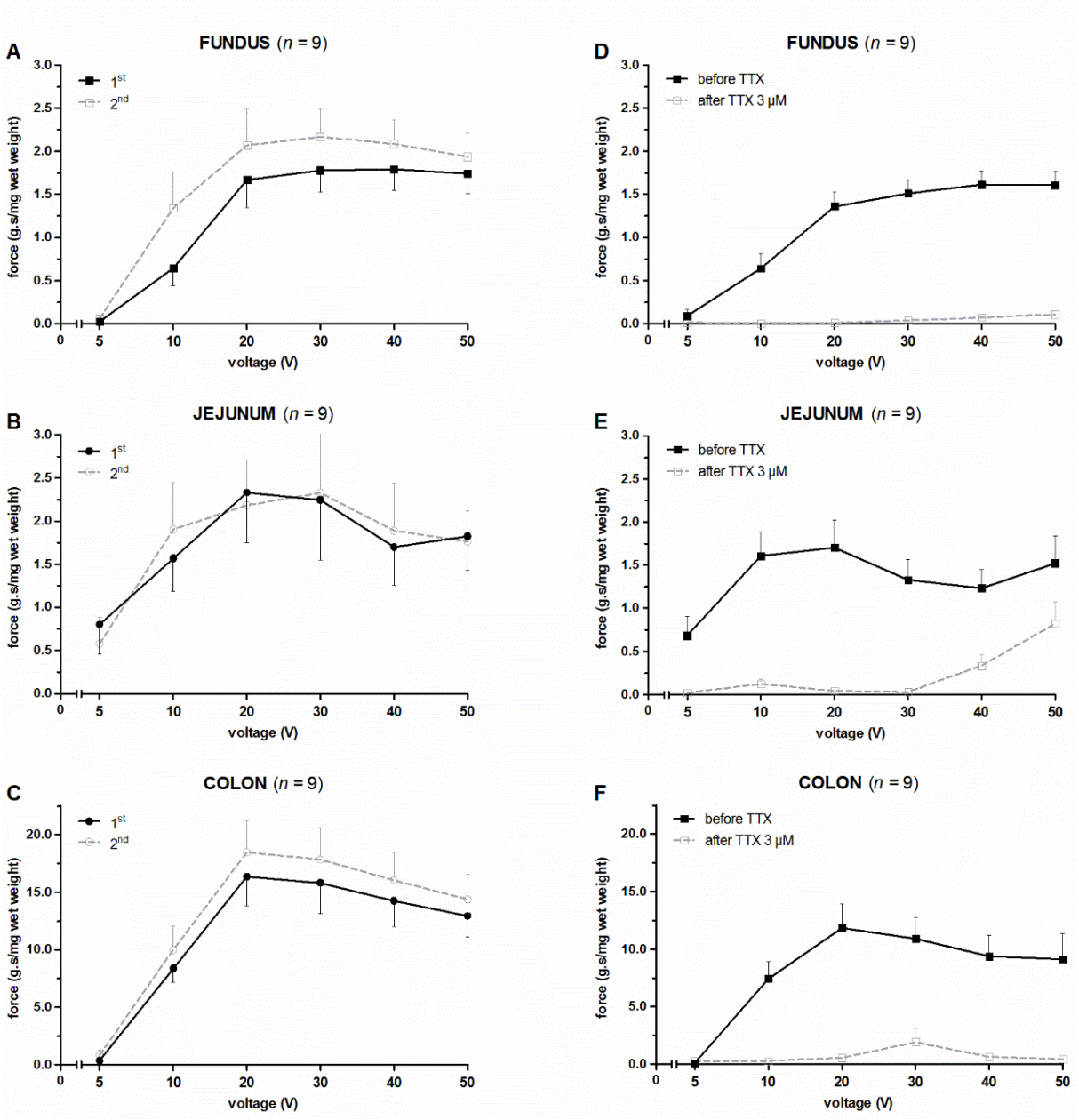


Figure V.2 - Influence of TTX on voltage-response curves. Mean (± S.E.M.) voltage-response curves of electrical field stimulation (10 s trains at 4 Hz, 0.5 ms, 5-50 V, interval of 5 min) in murine fundus, jejunum and colon. Curves were obtained twice in control strips (A-C), or before and in the presence of 3 µM TTX (D-F). Experiments were performed in the continuous presence of 4 µM guanethidine, 300 µM L-NAME and for colon also 1 µM MRS 2500.

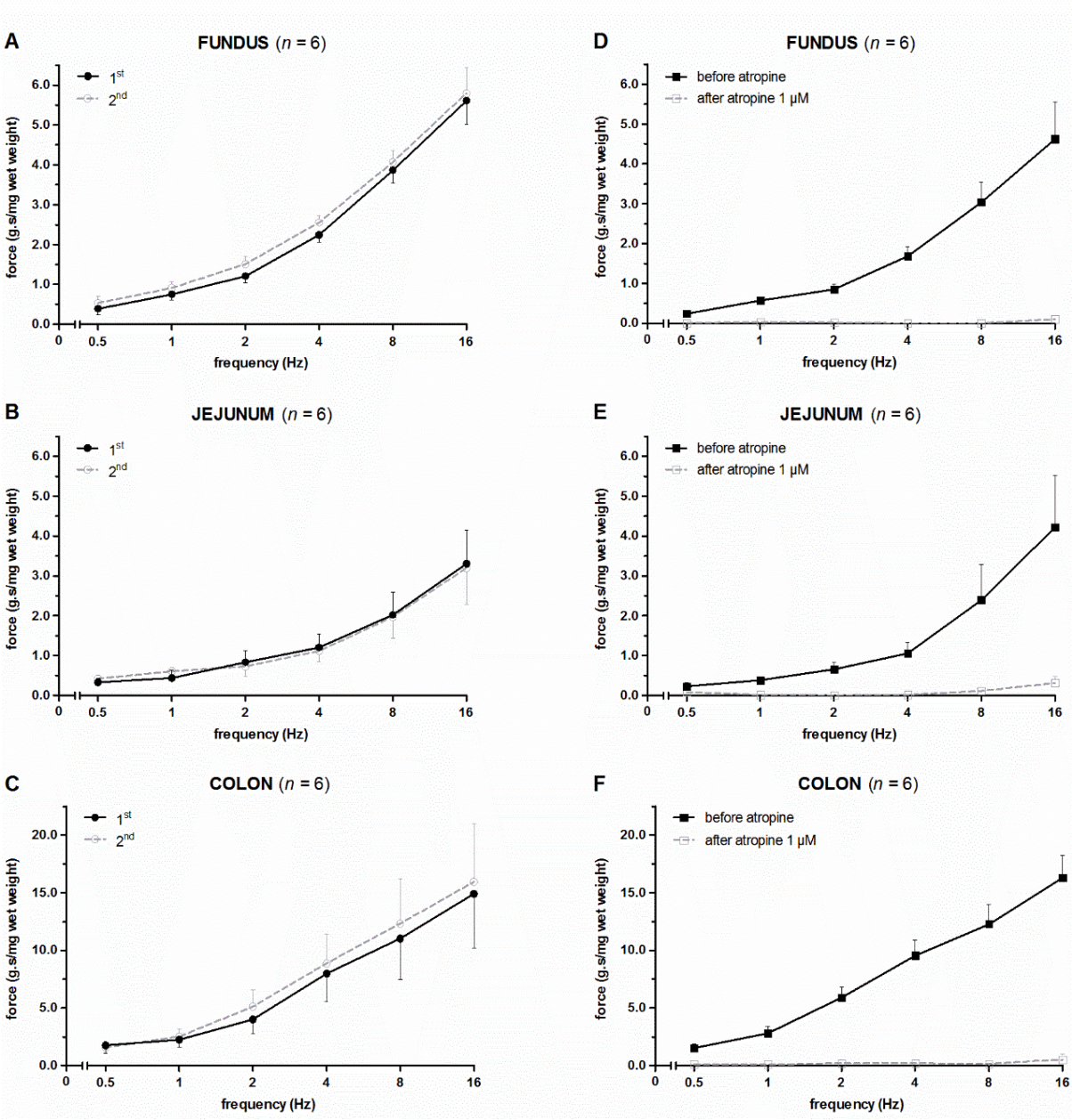


Figure V.3 - Influence of atropine on frequency-response curves. Mean (± S.E.M.) frequency-response curves of electrical field stimulation (10 s trains at 0.5-16 Hz, 0.5 ms, 30 V, interval of 5 min) in murine fundus, jejunum and colon. Curves were obtained twice in control strips (A-C), or before and in the presence of 1 µM atropine (D-F). Experiments were performed in the continuous presence of 4 µM guanethidine, 300 µM L-NAME and for colon also 1 µM MRS 2500.

V.4.2 Characterization of EFS-induced contractions at $V_{50\%}$ (Figure V.4 and Figure V.5)

EFS with 10 s trains (0.5 ms pulse width; supramaximal voltage of 30 V; 4 [fundus] or 8 Hz [jejunum and colon]) at 3 (fundus and colon) or 6 min (jejunum) interval induced reproducible contractions. Upon reduction of the stimulation voltage to $V_{50\%}$, reproducible submaximal contractions were obtained. The neurogenic and cholinergic character of the submaximal EFS-induced contractions at 5 (fundus and colon) or 10 min (jejunum) interval was confirmed in the three tissues as TTX (3 µM) and atropine (1 µM) abolished the contractions. Hexamethonium (0.5 mM) had no influence on the contractions in jejunum and colon while it significantly enhanced the contractions from 95 ± 7% in controls to 141 ± 17% in the fundus (Figure V.4). Results with hexamethonium were quite variable though, as a clear inhibitory effect was obtained in several jejunal strips and an enhancing one in several colonic tissues (Figure V.5).

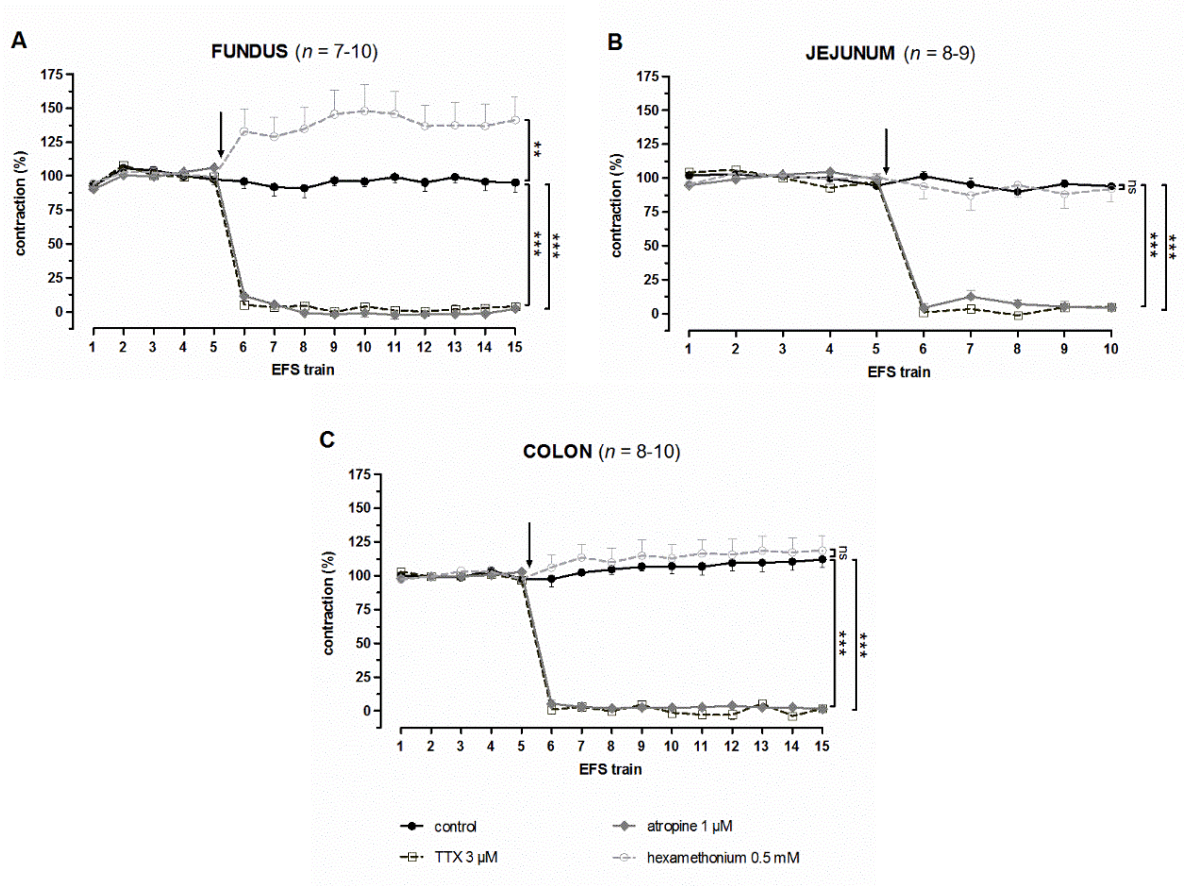


Figure V.4 - Characterization of electrically induced submaximal contractions with TTX, atropine and hexamethonium. Influence of 3 µM TTX, 1 µM atropine and 0.5 mM hexamethonium on submaximal electrically induced contractions at $V_{50\%}$ (10 s trains at 4 Hz [fundus] or 8 Hz [jejunum and colon], 0.5 ms, interval of 5 [fundus and colon] or 10 min [jejunum]) in murine fundus (A), jejunum (B) and colon (C). Contractions are expressed as percentage of the mean of the five contractions before addition of TTX, atropine or hexamethonium. Experiments were performed in the continuous presence of 4 µM guanethidine, 300 µM L-NAME and for colon also 1 µM MRS 2500. Means ± S.E.M.; ns not significant, ** $P < 0.01$, *** $P < 0.001$ versus control (one-way ANOVA with Bonferroni corrected t-test).

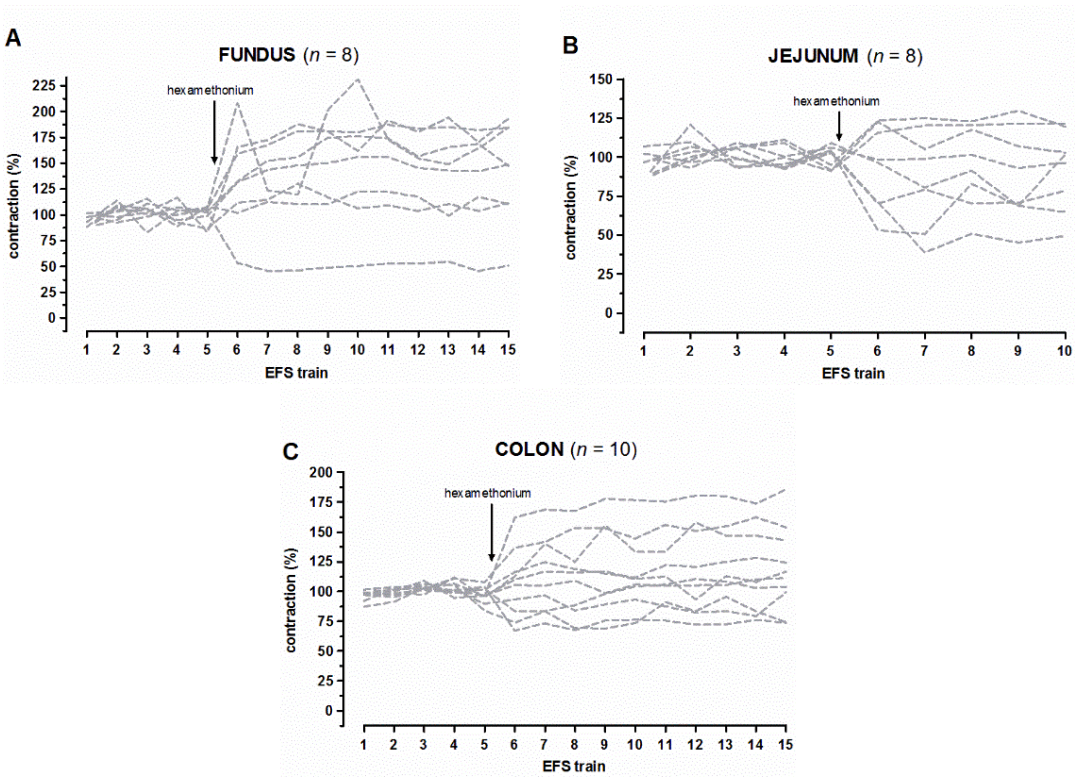


Figure V.5 - Variable influence of hexamethonium on electrically induced submaximal cholinergic contractions. Individual results showing increasing as well as decreasing influences of 0.5 mM hexamethonium on submaximal electrically induced contractions at $V_{50\%}$ (10 s trains at 4 Hz [fundus] or 8 Hz [jejunum and colon], 0.5 ms, interval of 5 [fundus and colon] or 10 min [jejunum]) in murine fundus (A), jejunum (B) and colon (C). Contractions are expressed as percentage of the mean of the five contractions before addition of hexamethonium. Experiments were performed in the continuous presence of 4 µM guanethidine, 300 µM L-NAME and for colon also 1 µM MRS 2500.

V.4.3 Influence of prucalopride (Figure V.6-8)

In a first preliminary series, prucalopride (0.03 µM, 0.1 µM and 0.3 µM administered in separate strips) enhanced the EFS-induced submaximal cholinergic contractions, but a clear-cut concentration-dependency was not present in this concentration range (Figure V.6).

Ten-fold lower concentrations of prucalopride (0.003, 0.01 and 0.03 µM administered in separate strips) concentration-dependently increased the EFS-induced submaximal cholinergic contractions in the fundus (Figure V.7 A). Although less clear than in the fundus, a trend for a concentration-dependent increase by prucalopride was also present in jejunum and colon (Figure V.7 B and C). For the last EFS-induced contraction in the presence of respectively 0.003, 0.01 and 0.03 µM prucalopride mean values of 133 ± 7%, 181 ± 24% ($P < 0.05$) and 204 ± 11% ($P < 0.001$) in fundus, 122 ± 4% ($P < 0.05$), 124 ± 9% ($P < 0.01$) and 138 ± 5% ($P < 0.001$) in jejunum and 133 ± 6%, 136 ± 5% and 152 ± 10% ($P < 0.01$) in colon were measured (P-values obtained from one-way ANOVA with Bonferroni corrected t-test for the three concentrations of prucalopride versus control). In fundus and jejunum, the facilitating effect of 0.03 µM prucalopride was not influenced by 1 µM MRS 2500 (Figure V.8). In the jejunum, MRS 2500 caused a significant reduction of the EFS-induced contractions (Figure V.8 B).

The concentration-dependency of the facilitating effect of prucalopride was corroborated in experiments where prucalopride was added cumulatively within the same strips (Figure V.7 D-F),

showing that the maximal effect was indeed reached from 0.03 µM onwards. pEC$_{50}$ values were 8.37 ± 0.04, 7.83 ± 0.18 and 8.11 ± 0.10 in fundus, jejunum and colon respectively.

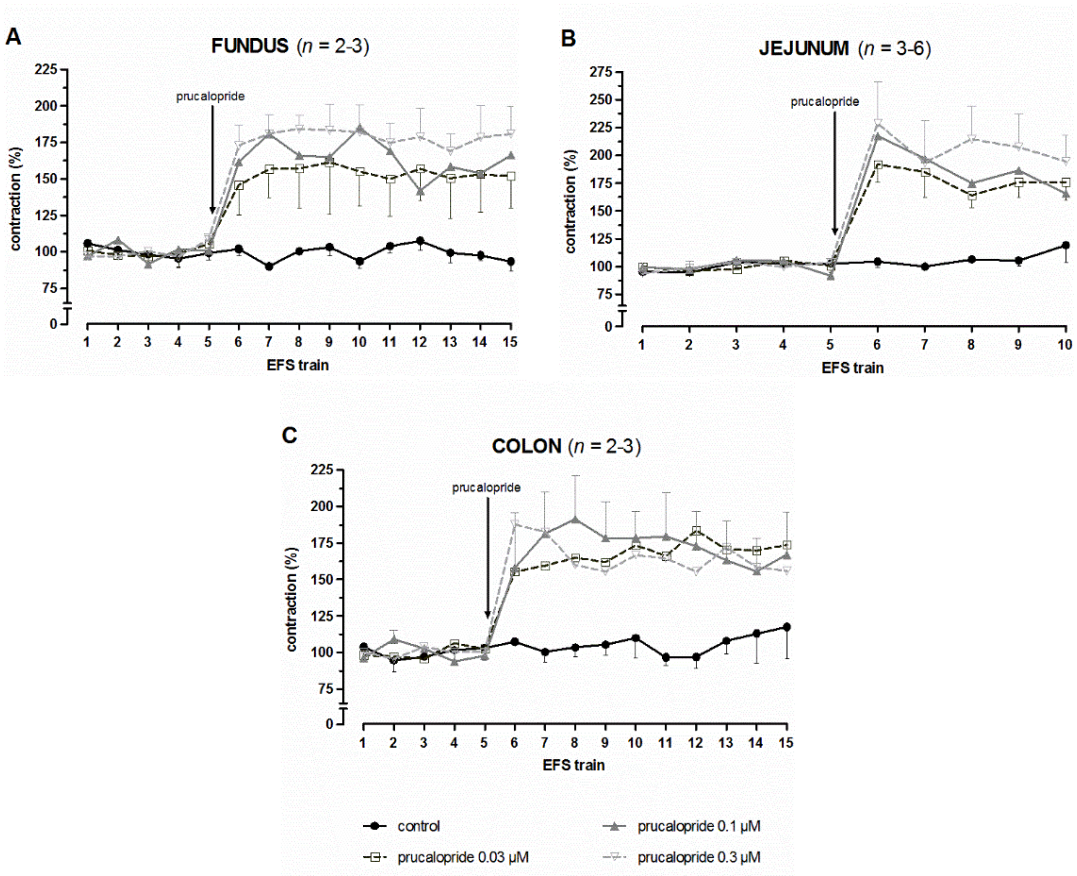


Figure V.6 - Facilitating effect of prucalopride on electrically induced submaximal cholinergic contractions. Influence of 0.03, 0.1 and 0.3 µM prucalopride on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 4 Hz [fundus and colon] or 8 Hz [jejunum], 0.5 ms, interval of 5 [fundus and colon] or 10 min [jejunum]) in murine fundus (A), jejunum (B) and colon (C). Contractions are expressed as percentage of the mean of the five contractions before adding prucalopride. Experiments were performed in the continuous presence of 4 µM guanethidine, 300 µM L-NAME and for colon also 1 µM MRS 2500. Means ± S.E.M.

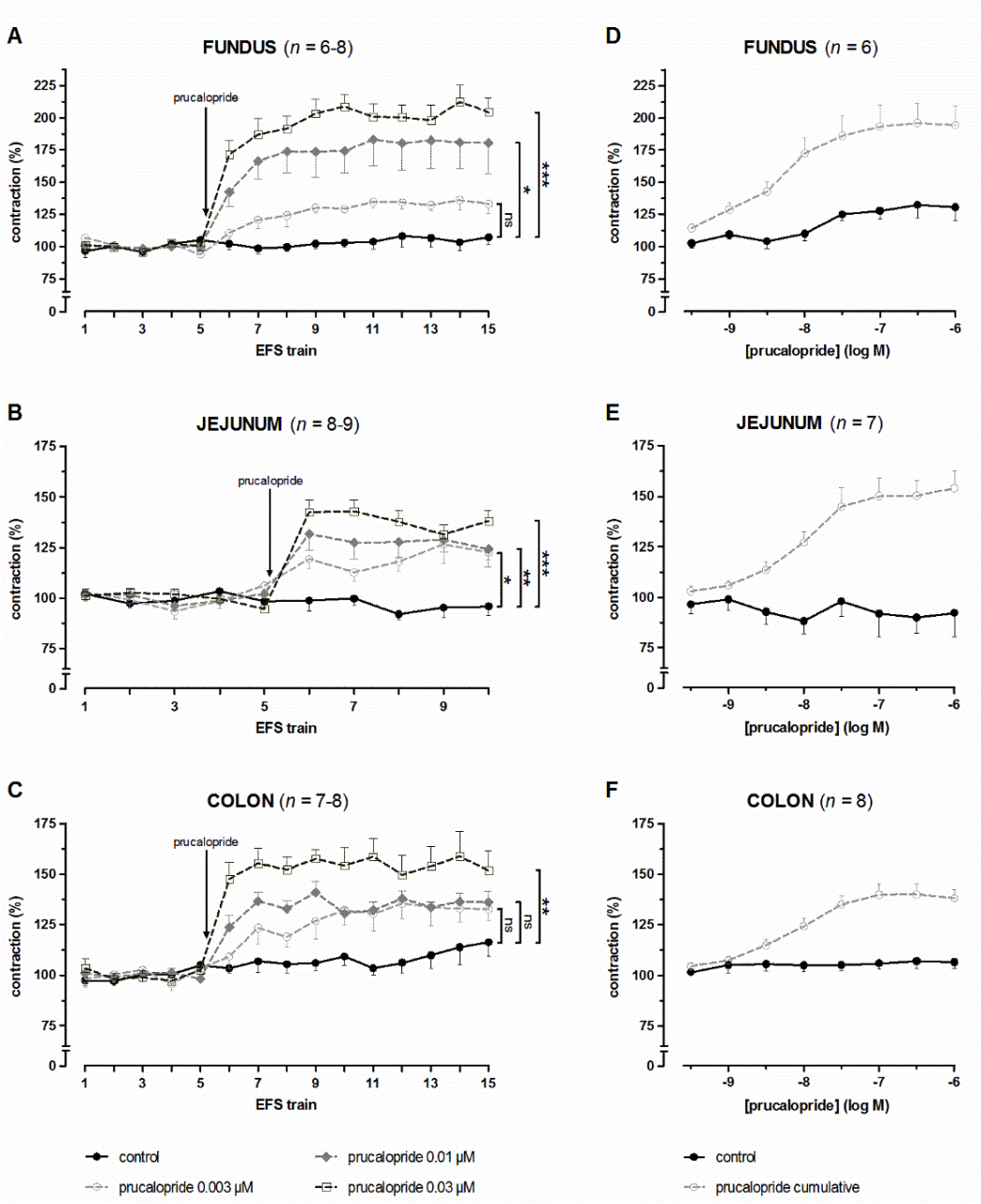


Figure V.7 - Concentration-dependent facilitation by prucalopride of electrically induced submaximal cholinergic contractions. Influence of 0.003, 0.01 and 0.03 µM prucalopride, administered to separate strips (A-C) and of 0.3 nM to 1 µM prucalopride, added cumulatively (D-F) on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 4 Hz [fundus] or 8 Hz [jejunum and colon], 0.5 ms, interval of 5 [fundus and colon] or 10 min [jejunum]) in murine fundus (A & D), jejunum (B & E) and colon (C & F). Contractions are expressed as percentage of the mean of the five contractions before adding prucalopride. Experiments were performed in the continuous presence of 4 µM guanethidine, 300 µM L-NAME and for colon also 1 µM MRS 2500. Means ± S.E.M.; ns not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control (one-way ANOVA with Bonferroni corrected *t*-test).

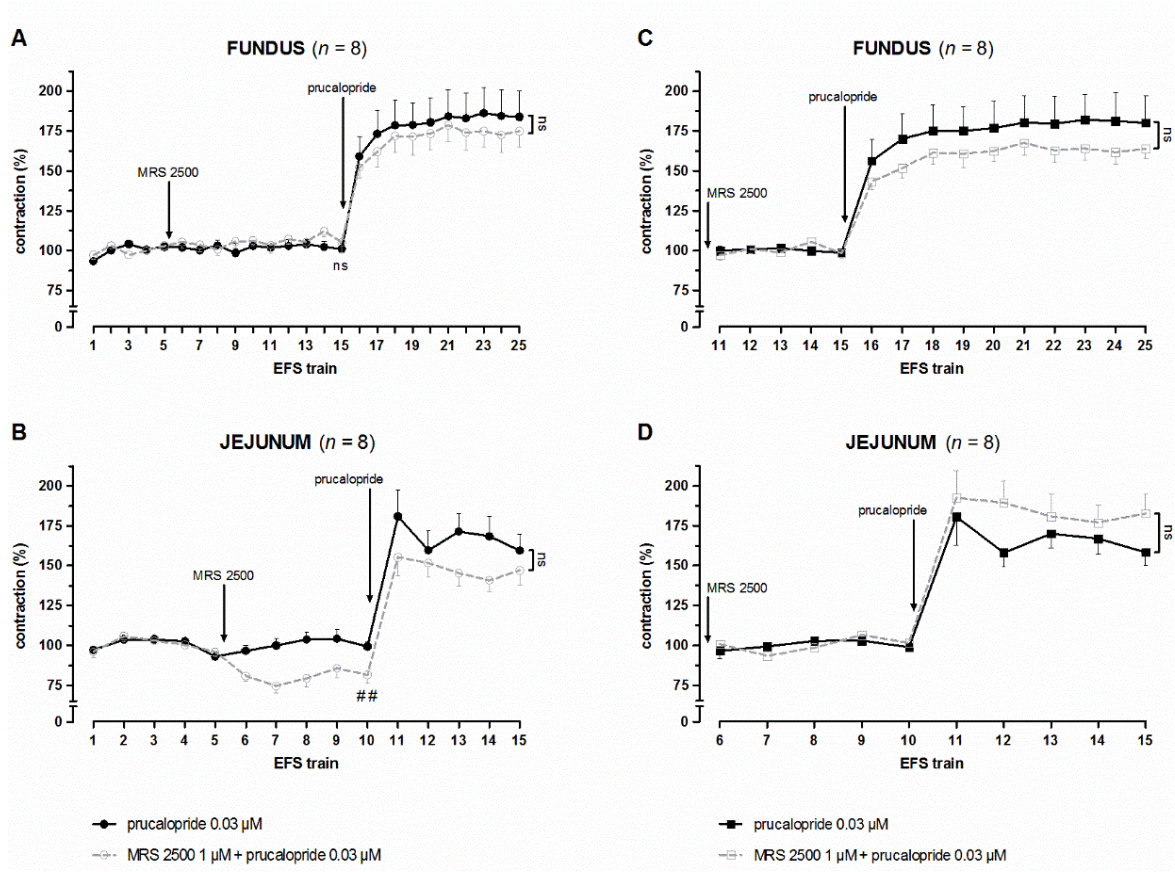


Figure V.8 - Effect of MRS 2500 on the facilitating effect of prucalopride. Influence of 1 µM MRS 2500 on the facilitating effect of 0.03 µM prucalopride on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 4 Hz [fundus] or 8 Hz [jejunum], 0.5 ms, interval of 5 [fundus] or 10 min [jejunum]) in murine fundus (A & C) and jejunum (B & D). Contractions are expressed as percentage of the mean of the five contractions before adding MRS 2500 (A & B) or of the five contractions in the presence of MRS 2500 just before adding prucalopride (C & D). Experiments were performed in the continuous presence of 4 µM guanethidine and 300 µM L-NAME. Means ± S.E.M.; ns not significant, ## $P < 0.01$ versus control not treated with MRS 2500 (unpaired *t*-test).

V.4.4 Influence of GR 113808 on the effect of prucalopride and 5-HT (Figure V.9 and Figure V.10)

GR 113808, added cumulatively (0.001-0.3 µM) once the facilitating effect of prucalopride on EFS-induced submaximal cholinergic contractions was stable, concentration-dependently reduced and finally abolished the effect of prucalopride, while the effect of prucalopride was maintained in the parallel control strip receiving corresponding amounts of the solvent of GR 113808, DMSO. A representative experiment in fundus strips is shown in Figure V.9. pIC_{50} values were 8.15 ± 0.10, 8.33 ± 0.18 and 8.44 ± 0.21 in fundus, jejunum and colon respectively (n = 6 in fundus and jejunum and 5 in colon); the pK_b estimates determined from these values were 9.06 ± 0.10, 8.81 ± 0.18 and 9.14 ± 0.21. When adding prucalopride (0.03 µM) in the presence of DMSO (0.03%), a mean increase of the EFS-induced submaximal cholinergic contractions was observed up to 141 ± 10% in fundus, 130 ± 10% in jejunum and 147 ± 30% in colon (Figure V.10 A-C). In the presence of 0.3 µM GR 113808, prucalopride (0.03 µM) did not enhance the contractions. In bathing medium containing 1 µM methysergide and 0.3 µM granisetron, also 5-HT (0.3 µM) enhanced the EFS-induced cholinergic contractions to 188 ± 14% in fundus, 136 ± 9% in jejunum and 134 ± 4% in colon; GR 113808 (0.3 µM) abolished the effect of 5-HT

in fundus and jejunum, and greatly reduced it in the colon to 107 ± 6% (Figure V.10 D-F). Neither GR 113808 (0.3 µM) nor its solvent DMSO (0.03%) as such influenced the EFS-induced submaximal cholinergic contractions (Figure V.10).

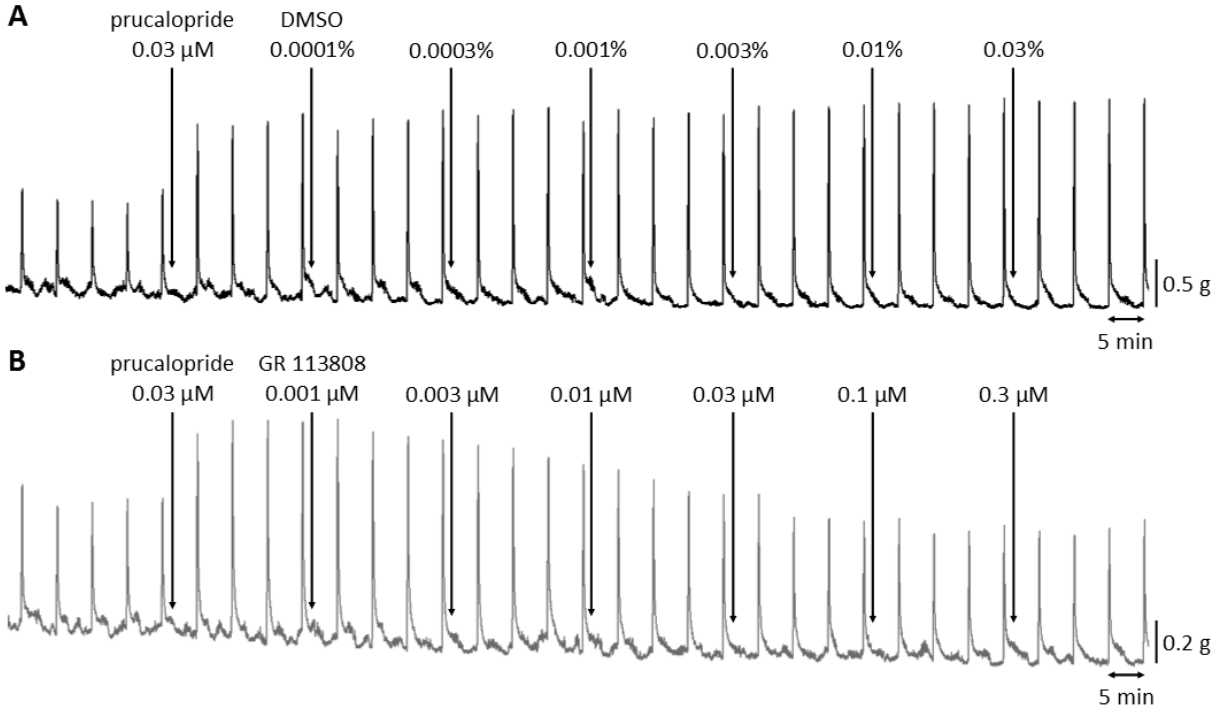


Figure V.9 - Antagonism of prucalopride-induced facilitation by cumulatively added GR 113808. Representative traces showing the response to repetitive electrical field stimulation (10 s trains at 4 Hz, 0.5 ms, $V_{50\%}$) in the continuous presence of 4 µM guanethidine and 300 µM L-NAME in two parallel circular smooth muscle strips of the murine fundus. Both received 0.03 µM prucalopride; when the facilitating effect of prucalopride on the contractions was stable, either GR 113808 (B) or its solvent DMSO (A) was added cumulatively.

V.4.5 Influence of hexamethonium and mecamylamine on the effect of prucalopride (Figure V.11 and Figure V.12)

In the series where hexamethonium (0.5 mM) was tested versus prucalopride, it increased the EFS-induced submaximal cholinergic contractions as such in fundus but also in colon and decreased them in jejunum (Figure V.11 A-C). In view of this effect of hexamethonium as such, the responses in the presence of prucalopride were also expressed as percentage of the mean of the five responses in the presence of hexamethonium just before adding prucalopride; with this analysis the facilitating effect of prucalopride (0.03 µM) on EFS-induced submaximal cholinergic contractions was not significantly influenced by hexamethonium although a decreasing trend was present in fundus and jejunum (Figure V.11 D-F).

In view of the varying effect of hexamethonium on EFS-induced contractions, another nicotinic receptor blocker was studied. Mecamylamine (30 µM) consistently reduced EFS-induced submaximal cholinergic contractions (Figure V.12 A-C). In the presence of mecamylamine, prucalopride still enhanced the EFS-induced contractions. When expressing the responses as percentage of the mean of the five responses in the presence of mecamylamine just before adding prucalopride, the effect of prucalopride was not influenced in jejunum and colon, and significantly reduced in fundus (Figure V.12 D-F).

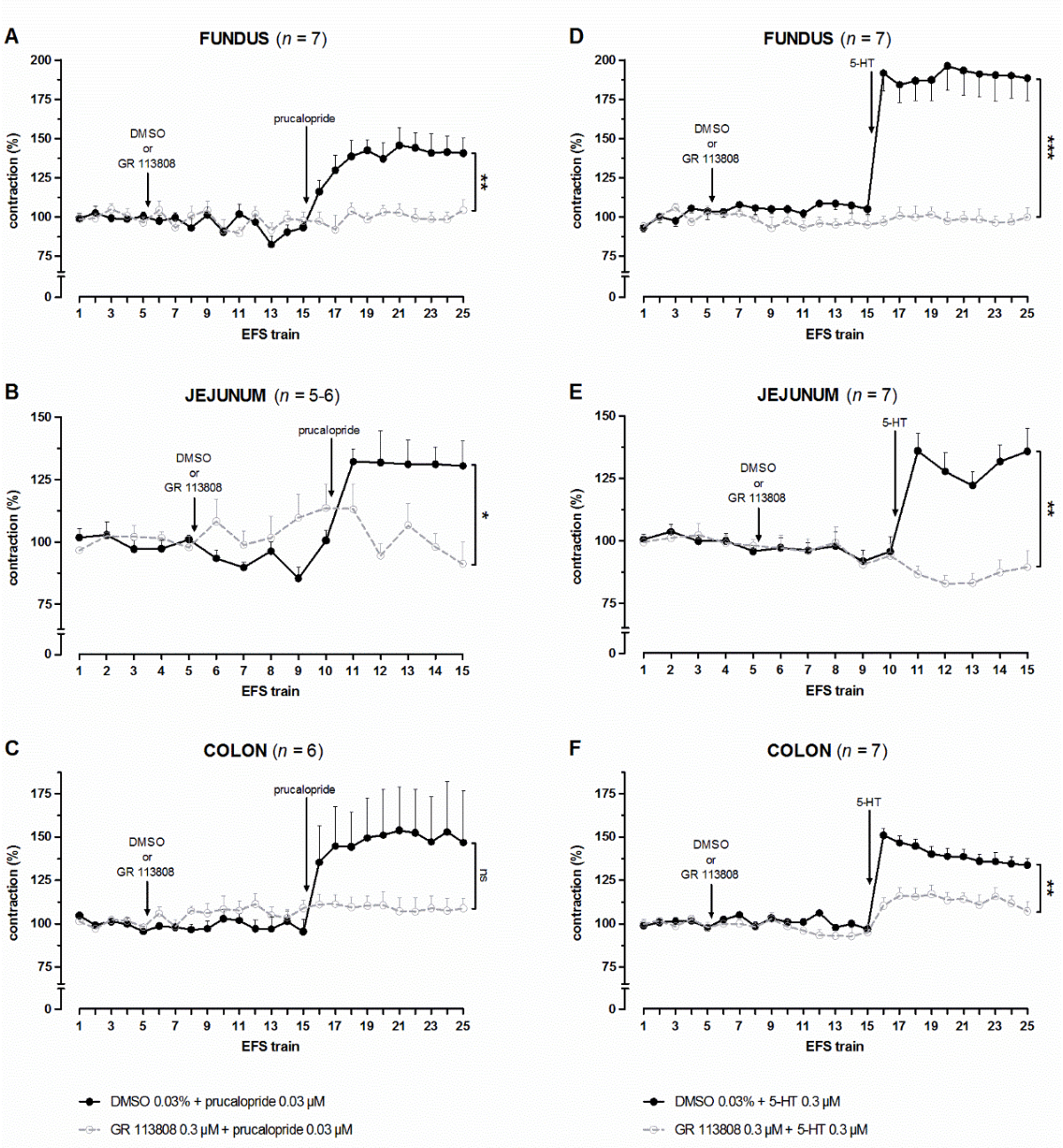


Figure V.10 - Antagonism of prucalopride- and 5-HT-induced facilitation by GR 113808. Influence of 0.3 µM GR 113808 on the facilitating effect of 0.03 µM prucalopride (A-C) and 0.3 µM 5-HT (D-F) on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 4 Hz [fundus] or 8 Hz [jejunum & colon], 0.5 ms, interval of 5 [fundus and colon] or 10 min [jejunum]) in murine fundus (A & D), jejunum (B & E) and colon (C & F). Contractions are expressed as percentage of the mean of the five contractions before adding GR 113808. Experiments were performed in the continuous presence of 4 µM guanethidine, 300 µM L-NAME and for colon also 1 µM MRS 2500; to study the effect of 5-HT, the Krebs solution also contained 1 µM methysergide and 0.3 µM granisetron. Means ± S.E.M.; ns not significant, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus prucalopride or 5-HT in the presence of 0.03% DMSO (unpaired *t*-test).

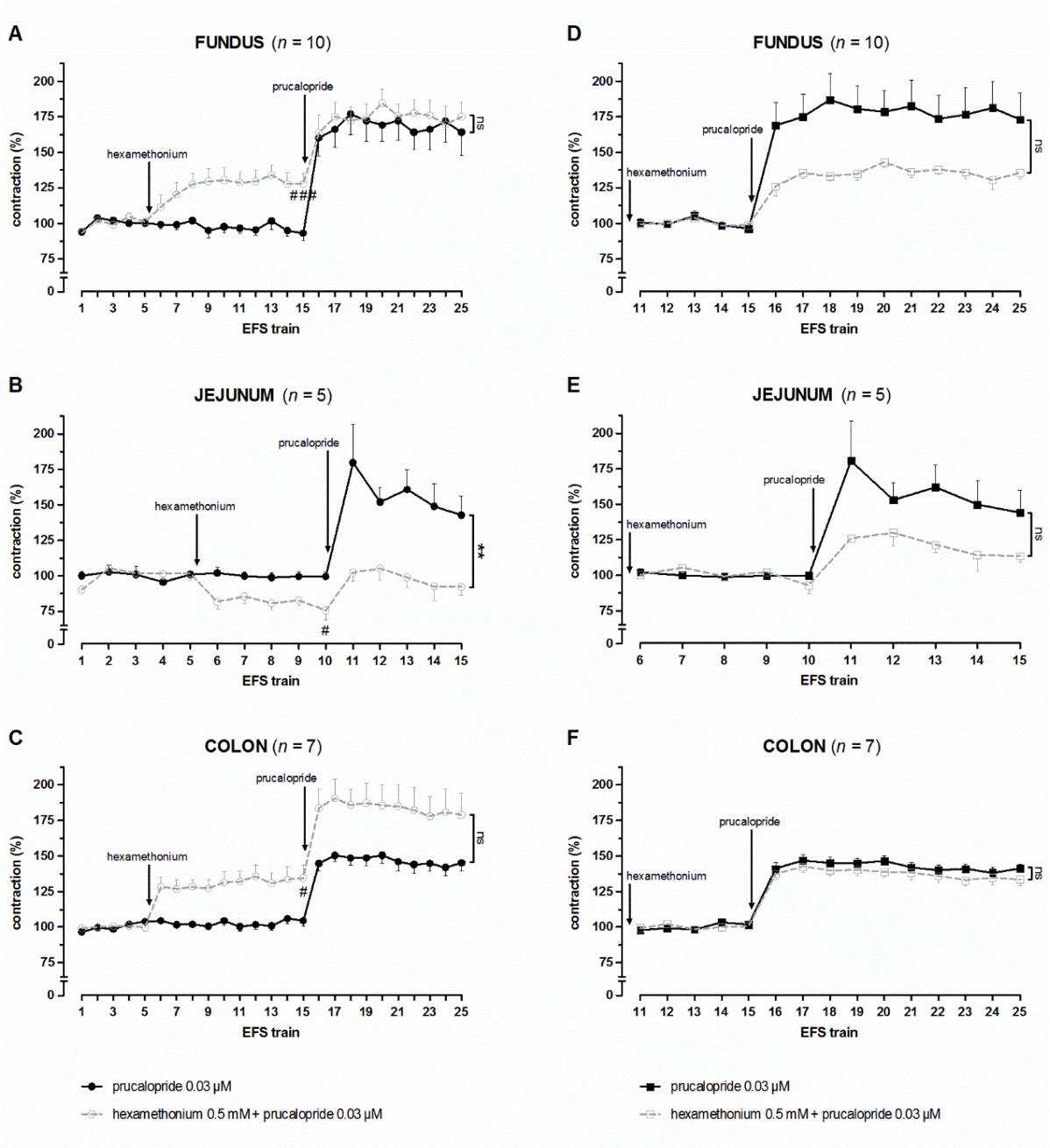


Figure V.11 - Effect of hexamethonium on the facilitating effect of prucalopride. Influence of 0.5 mM hexamethonium on the facilitating effect of 0.03 µM prucalopride on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 4 Hz [fundus] or 8 Hz [jejunum & colon], 0.5 ms, interval of 5 [fundus and colon] or 10 min [jejunum]) in murine fundus (A & D), jejunum (B & E) and colon (C & F). Contractions are expressed as percentage of the mean of the five contractions before adding hexamethonium (A-C) or of the five contractions in the presence of hexamethonium just before adding prucalopride (D-F). Experiments were performed in the continuous presence of 4 µM guanethidine, 300 µM L-NAME and for colon also 1 µM MRS 2500. Means ± S.E.M.; ns not significant, ** $P < 0.01$ versus prucalopride in the absence of hexamethonium (unpaired *t*-test), $^{\#}$ $P < 0.05$ and $^{\#\#\#}$ $P < 0.001$ versus control not treated with hexamethonium (unpaired *t*-test).

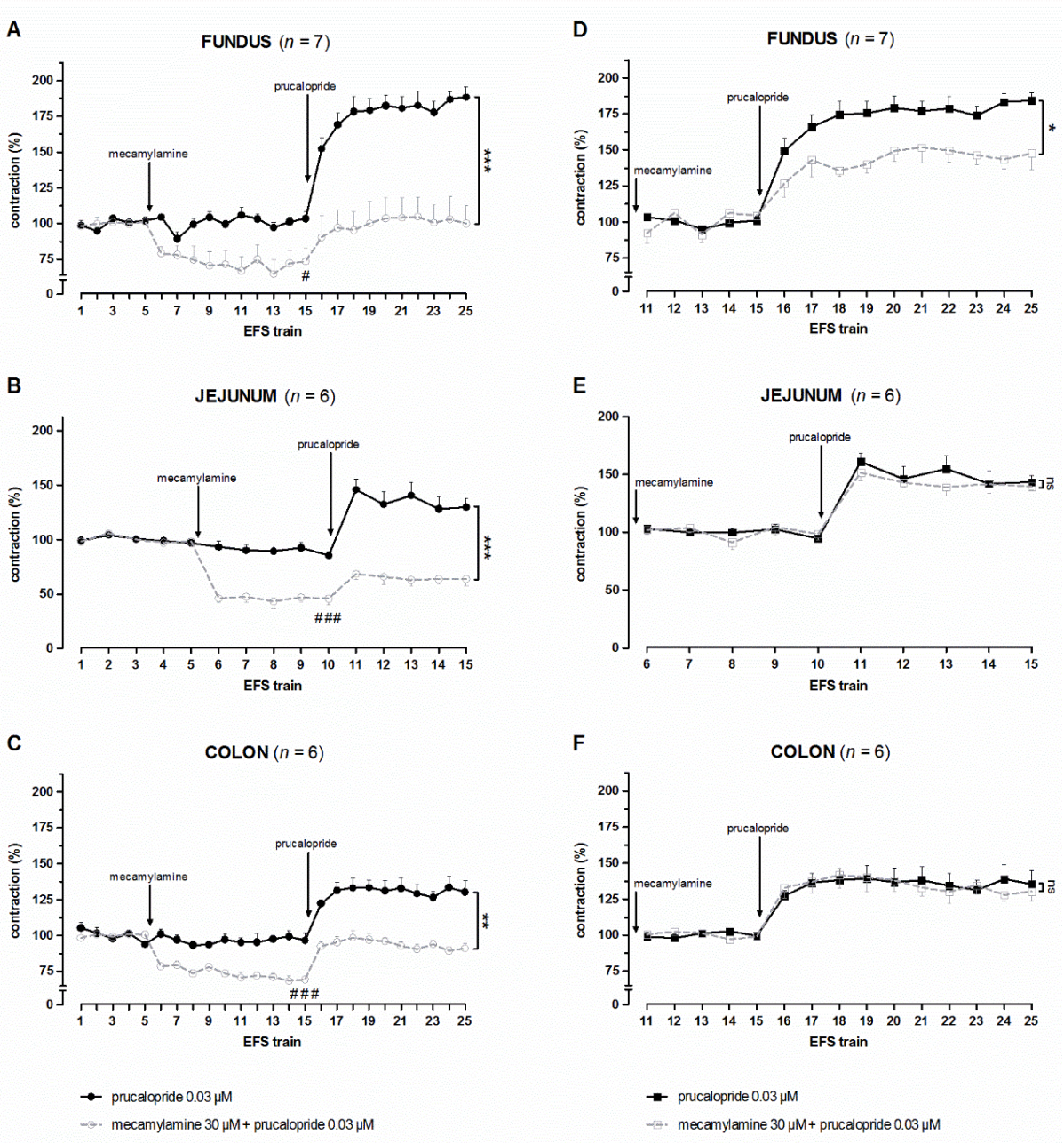


Figure V.12 - Effect of mecamylamine on the facilitating effect of prucalopride. Influence of 30 µM mecamylamine on the facilitating effect of 0.03 µM prucalopride on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 4 Hz [fundus] or 8 Hz [jejunum & colon], 0.5 ms, interval of 5 [fundus and colon] or 10 min [jejunum]) in murine fundus (A & D), jejunum (B & E) and colon (C & F). Contractions are expressed as percentage of the mean of the five contractions before adding mecamylamine (A-C) or of the five contractions in the presence of mecamylamine just before adding prucalopride (D-F). Experiments were performed in the continuous presence of 4 µM guanethidine, 300 µM L-NAME and for colon also 1 µM MRS 2500. Means ± S.E.M.; ns not significant, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus prucalopride in the absence of mecamylamine (unpaired *t*-test), $^{\#}$ $P < 0.05$ and $^{\#\#\#}$ $P < 0.001$ versus control not treated with mecamylamine (unpaired *t*-test).

V.5 Discussion

This study investigated the influence of prucalopride on EFS-induced submaximal cholinergic contractions in murine circular smooth muscle of the fundus, jejunum and colon in order to elucidate whether 5-HT_4 receptors also facilitate myenteric cholinergic neurotransmission in the murine gastrointestinal tract. To induce pure contractile responses by 10 s trains of EFS, guanethidine, L-NAME and for colon also MRS 2500 had to be added. Although also in murine stomach (Gil *et al.*, 2013) (antrum) and jejunum (De Man *et al.*, 2003) evidence for purinergic inhibitory neurotransmission has been provided, we previously showed that non-adrenergic non-cholinergic relaxations induced by EFS in murine fundus and jejunum with similar stimulation parameters as in the actual study were fully nitrergic (Cosyns *et al.*, 2013). In the series where the influence of MRS 2500 on the effect of prucalopride was studied, it was also clear that this $P2Y_1$ receptor antagonist did not influence EFS-induced cholinergic contractions in the fundus and even decreased them in the jejunum. Although there is no clear-cut explanation for the latter effect, the results with MRS 2500 in fundus and jejunum confirm that there is no inhibitory purine counteracting the contractile neurotransmitter during EFS as the response should then increase in the presence of MRS 2500. In the conditions used, the neurogenic nature of the contractions induced at increasing voltage with the frequency fixed at 4 Hz was demonstrated by their abolishment with TTX; only in the jejunum some contractile response persisted with EFS at 40 and 50 V but these voltages were not used in the following experiments. The involved neurotransmitter is acetylcholine, as the contractions induced at increasing frequency with the voltage fixed at 30 V were abolished by the non-selective muscarinic receptor antagonist atropine. The neurogenic cholinergic nature was confirmed when testing TTX and atropine on submaximal contractions at $V_{50\%}$ and 4 or 8 Hz. To investigate to what extent activation of preganglionic neurons might contribute to the EFS-induced contractions, the influence of the nicotinic receptor blocker hexamethonium was tested on the submaximal cholinergic contractions; it had a tissue-dependent influence as it increased the EFS-induced submaximal contractions in the fundus and in some colonic tissues while reducing them clearly in some jejunal tissues. In the series where hexamethonium was tested versus prucalopride, clear-cut increase of the EFS-induced contractions in the fundus and colon and decrease in the jejunum was observed. In view of these variable results, the influence of another nicotinic receptor antagonist mecamylamine was tested; it antagonized neuronal nicotinic receptors in the myenteric plexus with a 30-fold greater potency than hexamethonium (Torocsik *et al.*, 1991). Mecamylamine reduced the EFS-induced submaximal cholinergic contractions in the three tissues, illustrating a contribution of preganglionic neurons via nicotinic receptors. The increase of the submaximal cholinergic contractions by hexamethonium in the fundus and colon is puzzling. This cannot be due to removal of a preganglionically activated inhibitory input, as the adrenergic, nitrergic and when relevant purinergic inhibitory neurotransmission was ruled out. A similar enhancing effect of hexamethonium on electrically induced cholinergic contractions was observed in the guinea-pig ileum longitudinal muscle-myenteric plexus preparation (Donnerer *et al.*, 2014). Based on electrophysiological data obtained in cultured enteric neurons (Zhou and Galligan, 1998) the possibility of a functional cross-

inhibition between nicotinic receptors and P2X receptors on myenteric neurons was suggested; blockade of the nicotinic receptors would then strengthen ganglionic transmission onto the myenteric cholinergic neurons via P2X receptors. But if this mechanism would be present, one would also expect it to occur with mecamylamine which was not the case. Interestingly, in rabbit gastric fundus EFS-evoked contractile responses were significantly enhanced by mecamylamine but not by hexamethonium (Vural *et al.*, 2009).

Prucalopride was first tested in the concentration range of 0.03 to 0.3 µM, which showed concentration-dependent enhancement of submaximal cholinergic contractions in porcine stomach (isolated administration (Priem *et al.*, 2012)) and canine stomach (cumulative administration (Prins *et al.*, 2001)). In the three murine tissues, these three concentrations enhanced the submaximal cholinergic contractions but without concentration-dependency suggesting that the maximal response might already be obtained from 0.03 µM onwards. The range was therefore reduced ten-fold (0.003 to 0.03 µM), yielding clear-cut concentration-dependent enhancement in the mouse gastric fundus, and a trend for it in jejunum and colon. The concentration-dependency of the facilitation by prucalopride was clearly illustrated for the three tissues when it was added cumulatively, also showing that the maximal effect of prucalopride was nearly reached from 0.03 µM onwards. The pEC_{50} values (from 8.37 in the fundus to 7.83 in the jejunum, roughly corresponding with an EC_{50} of 0.004 to 0.015 µM), were similar to those reported for canine (pEC_{50} 7.9) (Prins *et al.*, 2001) and porcine stomach (pEC_{50} 8.25)(De Maeyer *et al.*, 2006) but clearly lower than those reported for rat forestomach (EC_{50} 1.1 µM) (Cellek *et al.*, 2008), human colon (EC_{50} 2.3 µM) (Cellek *et al.*, 2008) and human gastric fundus (pEC_{50} 5.6) (Broad *et al.*, 2014). The facilitating effect of 0.03 µM prucalopride was concentration-dependently depressed by GR 113808 and abolished by 0.3 µM GR 113808. The selective competitive 5-HT_4 receptor antagonist GR 113808 has a high affinity with originally described pA_2 values between 9.2 and 9.7 (Gale *et al.*, 1994). Few affinity estimates of GR 113808 have been reported for its interaction with 5-HT_4 receptors enhancing cholinergic neurotransmission. When tested versus prucalopride in canine stomach, a pA_2 estimate of 9.4 was obtained; testing versus 5-HT yielded a pK_b estimate of 9.1 (Prins *et al.*, 2001). When tested versus the facilitating effect of 5-HT on EFS-induced cholinergic contractions in isolated human detrusor muscle, a pA_2 estimate of 8.9 was obtained (Tonini *et al.*, 1994). The pK_b estimates of GR 113808 obtained in the murine GI tissues (8.81 to 9.14) correspond with these values. Full blockade of the effect of prucalopride by a concentration of GR 113808 approximately 300-fold higher than its affinity can then indeed be expected and further underlines interaction with 5-HT_4 receptors. Enhancement of cholinergic neurotransmission in the murine GI tissues was confirmed with the endogenous agonist 5-HT in the presence of antagonists to inhibit the other 5-HT receptors than 5-HT_4. Only one high concentration of 5-HT (0.3 µM) was studied to proof the principle which might explain that its effect was not completely abolished in colon strips.

In view of the variable effects of hexamethonium as such on EFS-induced contractions but the consistent inhibitory effect of mecamylamine, illustrating a contribution of preganglionic neurons, we concentrate on the results with mecamylamine versus prucalopride to discuss whether interaction with 5-HT_4 receptors on preganglionic neurons contributes to the facilitating effect of prucalopride on cholinergic

neurotransmission. When expressing results as percentage of the responses before adding mecamylamine, the response to prucalopride was significantly reduced in the three tissues. However when taking in account the effect of mecamylamine as such on the contractions by expressing the results as percentage of the responses before adding prucalopride, significant reduction of prucalopride's effect was only maintained in the fundus. This suggests that at least in the fundus, part of the enhancing effect of prucalopride is related to activation of 5-HT$_4$ receptors on preganglionic myenteric neurons activating subsequently cholinergic motor neurons via nicotinic receptors.

The facilitating effect of 0.03 µM prucalopride on submaximal cholinergic contractions varied from series to series; in the fundus, jejunum and colon respectively increases from 41 to 104%, 30 to 76% and 24 to 74% were measured. Variation in the enhancing effect of a given concentration of prucalopride has also been observed before (Priem *et al.*, 2013). In the fundus, the lowest value for the effect of prucalopride was obtained in the series where the control strips received prucalopride in the presence of the solvent of GR 113808, DMSO; but this was not the case for jejunum and colon. DMSO added cumulatively after reaching a stable effect with 0.03 µM prucalopride showed no effect so that it is unlikely that DMSO suppresses the effect of prucalopride at 5-HT$_4$ receptors. When the concentration-dependency of the facilitating effect of prucalopride on cholinergic contractions was studied in rat forestomach (Cellek *et al.*, 2008), canine stomach (Prins *et al.*, 2001), porcine stomach (De Maeyer *et al.*, 2006; Priem *et al.*, 2012), human stomach (Broad *et al.*, 2014) and human colon (Cellek *et al.*, 2006), a similar degree of maximal facilitation was observed as in the murine GI tract but higher concentrations than 0.03 µM prucalopride were required to obtain this maximal effect, even in tissues with a similar pEC$_{50}$ (Prins *et al.*, 2001; De Maeyer *et al.*, 2006). This suggests a higher number and/or a more effective coupling of the 5-HT$_4$ receptors enhancing myenteric cholinergic neurotransmission in the murine gastrointestinal tract, at least fundus and colon, as no comparative data for prucalopride are available for the small intestine in the literature.

V.6 Conclusion and future perspectives

In conclusion, in murine fundus, jejunum and colon, 5-HT$_4$ receptors enhance myenteric cholinergic neurotransmission, inducing circular smooth muscle contraction when activated by prucalopride. The magnitude of the effect of prucalopride in the murine GI tract is similar to that in other species, but the concentration of prucalopride required to induce maximal effects are overall lower than those needed at 5-HT$_4$ receptors in the rat, canine, porcine and human GI tract.

The gastroprokinetic effect of 5-HT$_4$ receptor agonists is attributed to activation of 5-HT$_4$ receptors on myenteric cholinergic neurons toward the smooth muscle layer. To reduce the risk of side effects of gastroprokinetic drugs such as prucalopride, interest exists for lowering the dose by combination therapy. Acetylcholinesterase inhibition (Cellek *et al.*, 2008) and more recently phosphodiesterase inhibition (Priem *et al.*, 2012; Priem *et al.*, 2013) were put forward to combine with 5-HT$_4$ receptor agonists. As the presence of 5-HT$_4$ receptors enhancing myenteric cholinergic neurotransmission toward the circular smooth muscle layer is now confirmed in the murine GI tract, the murine *in vitro* model

described can be used as an easy accessible and less expensive model than canine or porcine tissue for further investigation of these 5-HT$_4$ receptors and their signaling pathway.

V.7 Acknowledgements

The authors thank Mrs. Els Van Deynse for technical support.

V.8 References

Bianco F., Bonora E., Natarajan D., Vargiolu M., Thapar N., Torresan F., Giancola F., Boschetti E., Volta U., Bazzoli F., *et al.* (2016). Prucalopride exerts neuroprotection in human enteric neurons. *Am J Physiol Gastrointest Liver Physiol* 310, G768-G775. DOI: 10.1152/ajpgi.00036.2016

Broad J., Hughes F., Chin-Aleong J., Sifrim D., and Sanger G.J. (2014). Regionally dependent neuromuscular functions of motilin and 5-HT4 receptors in human isolated esophageal body and gastric fundus. *Neurogastroenterol Motil* 26, 1311-1322. DOI: 10.1111/nmo.12394

Cellek S., John A.K., Thangiah R., Dass N.B., Bassil A.K., Jarvie E.M., Lalude O., Vivekanandan S., and Sanger G.J. (2006). 5-HT4 receptor agonists enhance both cholinergic and nitrergic activities in human isolated colon circular muscle. *Neurogastroenterol Motil* 18, 853-861. DOI: 10.1111/j.1365-2982.2006.00810.x

Cellek S., Thangiah R., Jarvie E.M., Vivekanandan S., Lalude O., and Sanger G.J. (2008). Synergy between 5-HT4 receptor activation and acetylcholinesterase inhibition in human colon and rat forestomach. *Neurogastroenterol Motil* 20, 539-545. DOI: 10.1111/j.1365-2982.2007.01062.x

Cosyns S.M.R., Dhaese I., Thoonen R., Buys E.S., Vral A., Brouckaert P., and Lefebvre R.A. (2013). Heme deficiency of soluble guanylate cyclase induces gastroparesis. *Neurogastroenterol Motil* 25, e339-e352. DOI: 10.1111/nmo.12120

Craig D.A. (1993). The Cheng-Prusoff relationship: something lost in the translation. *Trends Pharmacol Sci* 14, 89-91. DOI: 10.1016/0165-6147(93)90070-Z

De Maeyer J.H., Prins N.H., Schuurkes J.a.J., and Lefebvre R.A. (2006). Differential effects of 5-hydroxytryptamine4 receptor agonists at gastric versus cardiac receptors: an operational framework to explain and quantify organ-specific behavior. *J Pharmacol Exp Ther* 317, 955-964. DOI: 10.1124/jpet.106.101329

De Man J.G., De Winter B.Y., Seerden T.C., De Schepper H.U., Herman A.G., and Pelckmans P.A. (2003). 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. DOI: 10.1038/sj.bjp.0705536

Donnerer J., Liebmann I., and Holzer-Petsche U. (2014). Hexamethonium-induced augmentation of the electrical twitch response in the guinea-pig ileum longitudinal muscle-myenteric plexus strip. *Neurosci Lett* 577, 34-37. DOI: 10.1016/j.neulet.2014.06.011

Gale J.D., Grossman C.J., Whitehead J.W.F., Oxford A.W., Bunce K.T., and Humphrey P.P.A. (1994). GR113808: a novel, selective antagonist with high-affinity at the 5-HT4 receptor. *Br J Pharmacol* 111, 332-338. DOI: 10.1111/j.1476-5381.1994.tb14064.x

Gallego D., Gil V., Martinez-Cutillas M., Mane N., Martin M.T., and Jimenez M. (2012). Purinergic neuromuscular transmission is absent in the colon of P2Y1 knocked out mice. *J Physiol* 590, 1943-1956. DOI: 10.1113/jphysiol.2011.224345

Galligan J.J., and Vanner S. (2005). Basic and clinical pharmacology of new motility promoting agents. *Neurogastroenterol Motil* 17, 643-653. DOI: 10.1111/j.1365-2982.2005.00675.x

Galligan J.J., and Parkman H. (2007). Recent advances in understanding the role of serotonin in gastrointestinal motility and functional bowel disorders. *Neurogastroenterol Motil* 19, 1-4. DOI: 10.1111/j.1365-2982.2006.00888.x

Gershon M.D., and Tack J. (2007). The serotonin signaling system: From basic understanding to drug development-for functional GI disorders. *Gastroenterology* 132, 397-414. DOI: 10.1053/j.gastro.2006.11.002

Gil V., Martinez-Cutillas M., Mane N., Martin M.T., Jimenez M., and Gallego D. (2013). P2Y(1) knockout mice lack purinergic neuromuscular transmission in the antrum and cecum. *Neurogastroenterol Motil* 25, e170-182. DOI: 10.1111/nmo.12060

Greenwood-Van Meerveld B., Venkova K., Hicks G., Dennis E., and Crowell M.D. (2006). Activation of peripheral 5-HT4 receptors attenuates colonic sensitivity to intraluminal distension. *Neurogastroenterol Motil* 18, 76-86. DOI: 10.1111/j.1365-2982.2005.00723.x

Kellum J.M., Albuquerque F.C., Stoner M.C., and Harris R.P. (1999). Stroking human jejunal mucosa induces 5-HT release and Cl- secretion via afferent neurons and 5-HT4 receptors. *Am J Physiol Gastrointest Liver Physiol* 277, G515-G520.

Leclere P.G., and Lefebvre R.A. (2002). Presynaptic modulation of cholinergic neurotransmission in the human proximal stomach. *Br J Pharmacol* 135, 135-142. DOI: 10.1038/sj.bjp.0704471

Leclere P.G., Prins N.H., Schuurkes J.a.J., and Lefebvre R.A. (2005). 5-HT4 receptors located on cholinergic nerves in human colon circular muscle. *Neurogastroenterol Motil* 17, 366-375. DOI: 10.1111/j.1365-2982.2005.00621.x

Lee M.J., Cho K.H., Park H.M., Sung H.J., Choi S., and Im W. (2014). Pharmacological profile of DA-6886, a novel 5-HT4 receptor agonist to accelerate colonic motor activity in mice. *Eur J Pharmacol* 735, 115-122. DOI: 10.1016/j.ejphar.2014.03.061

Liu M., Geddis M.S., Wen Y., Setlik W., and Gershon M.D. (2005). Expression and function of 5-HT4 receptors in the mouse enteric nervous system. *Am J Physiol Gastrointest Liver Physiol* 289, G1148-G1163. 10.1152/ajpgi.00245.2005

Liu M.T., Kuan Y.H., Wang J.W., Hen R., and Gershon M.D. (2009). 5-HT4 Receptor-Mediated Neuroprotection and Neurogenesis in the Enteric Nervous System of Adult Mice. *J Neurosci* 29, 9683-9699. DOI: 10.1523/Jneurosci.1145-09.2009

Mclean P.G., and Coupar I.M. (1996). Further investigation into the signal transduction mechanism of the 5-HT4-like receptor in the circular smooth muscle of human colon. *Br J Pharmacol* 118, 1058-1064. DOI: 10.1111/j.1476-5381.1996.tb15506.x

Nagakura Y., Ito H., Kiso T., Naitoh Y., and Miyata K. (1997). The selective 5-hydroxytryptamine (5-HT)(4)-receptor agonist RS67506 enhances lower intestinal propulsion in mice. *Jpn J Pharmacol* 74, 209-212. DOI: 10.1254/jjp.74.209

Priem E.K.V., and Lefebvre R.A. (2011). Investigation of neurogenic excitatory and inhibitory motor responses and their control by 5-HT4 receptors in circular smooth muscle of pig descending colon. *Eur J Pharmacol* 667, 365-374. DOI: 10.1016/j.ejphar.2011.06.021

Priem E.K.V., Van Colen I., De Maeyer J.H., and Lefebvre R.A. (2012). The facilitating effect of prucalopride on cholinergic neurotransmission in pig gastric circular muscle is regulated by phosphodiesterase 4. *Neuropharmacology* 62, 2126-2135. DOI: 10.1016/j.neuropharm.2011.12.020

Priem E.K.V., De Maeyer J.H., and Lefebvre R.A. (2013). Influence of phosphodiesterases on basal and 5-HT4 receptor facilitated cholinergic contractility in pig descending colon. *Eur J Pharmacol* 705, 156-163. DOI: 10.1016/j.ejphar.2013.02.011

Prins N.H., Akkermans L.M.A., Lefebvre R.A., and Schuurkes J.a.J. (2000a). 5-HT4 receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. *Br J Pharmacol* 131, 927-932. DOI: 10.1038/sj.bjp.0703615

Prins N.H., Shankley N.P., Welsh N.J., Briejer M.R., Lefebvre R.A., Akkermans L.M.A., and Schuurkes J.a.J. (2000b). An improved in vitro bioassay for the study of 5-HT4 receptors in the human isolated large intestinal circular muscle. *Br J Pharmacol* 129**,** 1601-1608. DOI: 10.1038/sj.bjp.0703254

Prins N.H., Van Der Grijn A., Lefebvre R.A., Akkermans L.M.A., and Schuurkes J.a.J. (2001). 5-HT4 receptors mediating enhancement of contractility in canine stomach; an in vitro and in vivo study. *Br J Pharmacol* 132**,** 1941-1947. DOI: 10.1038/sj.bjp.0703985

Tonini M., Candura S.M., Onori L., Coccini T., Manzo L., and Rizzi C.A. (1992). 5-Hydroxytryptamine4 receptor agonists facilitate cholinergic transmission in the circular muscle of guinea-pig ileum: antagonism by tropisetron and DAU-6285. *Life Sci* 50**,** Pl173-Pl178. DOI: 10.1016/0024-3205(92)90453-V

Tonini M., Messori E., Franceschetti G.P., Rizzi C.A., Castoldi A.F., Coccini T., and Candura S.M. (1994). Characterization of the 5-HT receptor potentiating neuromuscular cholinergic transmission in strips of human isolated detrusor muscle. *Br J Pharmacol* 113**,** 1-2. DOI: 10.1111/j.1476-5381.1994.tb16163.x

Torocsik A., Oberfrank F., Sershen H., Lajtha A., Nemesy K., and Vizi E.S. (1991). Characterization of somatodendritic neuronal nicotinic receptors located on the myenteric plexus. *Eur J Pharmacol* 202**,** 297-302. DOI: 10.1016/0014-2999(91)90270-Z

Vural I.M., Ozturk Fincan G.S., Bozkurt N.B., Ercan Z.S., and Sarioglu Y. (2009). Role of nicotinic acetylcholine receptor subtypes on nicotine-induced neurogenic contractile response alternation in the rabbit gastric fundus. *Eur J Pharmacol* 602**,** 395-398. DOI: 10.1016/j.ejphar.2008.11.032

Wang L., Martinez V., Kimura H., and Tache Y. (2007). 5-Hydroxytryptophan activates colonic myenteric neurons and propulsive motor function through 5-HT4 receptors in conscious mice. *Am J Physiol Gastrointest Liver Physiol* 292**,** G419-G428. 10.1152/ajpgi.00289.2006

Zhou X.P., and Galligan J.J. (1998). Non-additive interaction between nicotinic cholinergic and P2X purine receptors in guinea-pig enteric neurons in culture. *J Physiol* 513**,** 685-697. DOI: 10.1111/j.1469-7793.1998.685ba.x

CHAPTER VI

PDEs throughout the murine gastrointestinal tract

---

cAMP catalyzing phosphodiesterases control cholinergic muscular activity but their inhibition does not enhance 5-HT_4 receptor-mediated facilitation of cholinergic contractions in the murine gastrointestinal tract

Vicky Pauwelyn and Romain A. Lefebvre

Department of Pharmacology - Heymans Institute, Ghent University, Ghent, Belgium

Frontiers in Pharmacology 2018; 9: 171. DOI: 10.3389/fphar.2018.00171

https://doi.org/10.3389/fphar.2018.00171

Supplementary figures were integrated.

CHAPTER VI - PDEs throughout the murine gastrointestinal tract

cAMP catalyzing phosphodiesterases control cholinergic muscular activity but their inhibition does not enhance 5-HT_4 receptor-mediated facilitation of cholinergic contractions in the murine gastrointestinal tract

VI.1 Abstract

VI.1.1 Background

As the signal transduction of 5-HT_4 receptors on cholinergic neurons innervating smooth muscle is controlled by phosphodiesterase (PDE) 4 in porcine stomach and colon and human large intestine, the *in vivo* gastroprokinetic effects of a 5-HT_4 receptor agonist might be enhanced by combination with a selective PDE4 inhibitor. The presence of 5-HT_4 receptors on cholinergic neurons toward murine gastrointestinal circular muscle was recently shown. If the control of this receptor pathway by PDE4 is also present in mice, this might be a good model for *in vivo* testing of the combination therapy. Therefore this study investigates the role of cAMP catalyzing PDEs in smooth muscle cell activity and in the intraneuronal signal transduction of the 5-HT_4 receptors in the gastrointestinal tract of C57Bl/6J mice.

VI.1.2 Methods

In circular smooth muscle strips from murine fundus, jejunum and colon, submaximal cholinergic contractions were induced by either electrical field stimulation (EFS) or by carbachol (muscarinic receptor agonist). The influence of the PDE inhibitors IBMX (non-selective), vinpocetine (PDE1), EHNA (PDE2), cilostamide (PDE3) and rolipram (PDE4) was tested on these contractions and on the facilitating effect of a submaximal concentration of prucalopride (5-HT_4 receptor agonist) on EFS-induced contractions.

VI.1.3 Results

In the three gastrointestinal regions, IBMX and cilostamide concentration-dependently decreased carbachol- as well as EFS-induced contractions. Some inhibitory effect was also observed with rolipram. In the fundus a non-significant trend for an enhancement of the facilitating effect of prucalopride on EFS-induced contractions was observed with IBMX, but none of the selective PDE inhibitors enhanced the facilitating effect of prucalopride in fundus, jejunum or colon.

VI.1.4 Conclusions

In analogy with the porcine gastrointestinal tract, in murine fundus, jejunum and colon circular smooth muscle PDE3 is the main regulator of the cAMP turnover, with some contribution of PDE4. In contrast to the porcine gastrointestinal tract, the *in vitro* facilitation of electrically induced cholinergic contractions

by 5-HT_4 receptor stimulation could not be enhanced by specific PDE inhibition. The C57Bl/6J murine model is thus not suitable for *in vivo* testing of a 5-HT_4 receptor agonist combined with a selective PDE4 inhibitor.

VI.1.5 Keywords

5-HT_4 receptor, cholinergic neurotransmission, gastrointestinal tract, mouse, phosphodiesterase, prucalopride, smooth muscle

VI.2 Introduction

The gastrointestinal (GI) prokinetic properties of 5-HT_4 receptor agonists, developed for GI hypomotility disorders like gastroparesis and constipation (Galligan and Vanner, 2005), are related to interaction with stimulating 5-HT_4 receptors on cholinergic neurons innervating the GI smooth muscle layer (Gershon and Tack, 2007). The latter 5-HT_4 receptors, facilitating acetylcholine release and cholinergic contraction, were shown in the GI tract of several species: guinea-pig (Craig and Clarke, 1990; Tonini *et al.*, 1992), rat (Cellek *et al.*, 2008), dog (Prins *et al.*, 2000; Prins *et al.*, 2001), pig (Priem and Lefebvre, 2011; Priem *et al.*, 2012) and man (Prins *et al.*, 2000; Leclere and Lefebvre, 2002; Leclere *et al.*, 2005; Cellek *et al.*, 2006).

5-HT_4 receptors are G_s protein-coupled receptors linked to adenylyl cyclase and the generation of cAMP (Raymond *et al.*, 2001). Phosphodiesterases (PDEs) are the sole family of enzymes degrading the cyclic nucleotides cAMP and cGMP. The intraneuronal transduction pathway of stimulating 5-HT_4 receptors on cholinergic neurons is regulated by cAMP specific PDE4 in circular muscle of porcine stomach (Priem *et al.*, 2012; Lefebvre *et al.*, 2016) and colon (Priem *et al.*, 2013). Selective PDE4 inhibition by rolipram enhanced the facilitation by 5-HT_4 receptor stimulation of electrically induced acetylcholine release and cholinergic contraction in circular muscle strips. We recently showed that PDE4 inhibition with rolipram or roflumilast also enhances the facilitating effect of the 5-HT_4 receptor agonist prucalopride on acetylcholine release in circular muscle strips of human large intestine (Pauwelyn *et al.*, 2018). This suggests the possible usefulness of combining a 5-HT_4 receptor agonist with a PDE4 inhibitor in man, allowing enhanced GI prokinetic effects of the 5-HT_4 receptor agonist in low dose. When considering this combination, two possible interferences have to be considered. First, 5-HT_4 receptors are also present in porcine and human atria (Parker *et al.*, 1995); their stimulation only induces weak chrono- and inotropic effects but they are strictly controlled by PDEs and under PDE inhibition, effects become pronounced (Brattelid *et al.*, 2004; De Maeyer *et al.*, 2006). However, porcine cardiac 5-HT_4 receptors are under redundant control of PDE3 plus PDE4 (Galindo-Tovar *et al.*, 2009; Weninger *et al.*, 2012) and human cardiac 5-HT_4 receptors are under sole control of PDE3 (Afzal *et al.*, 2008) so that PDE4 inhibition will not induce cardiac side effects of the 5-HT_4 receptor agonist. Second, PDEs are also involved in controlling the cyclic nucleotide turnover in particular cAMP at the level of intestinal smooth muscle; PDE inhibition increases the cAMP content leading to inhibition of cholinergic contraction (Barnette *et al.*, 1993; Kaneda *et al.*, 2004) which would counteract cholinergic facilitation at the neuronal level. But in porcine colon, PDE3 is the main regulator of circular smooth muscle activity and PDE4 inhibition hardly influenced cholinergic contractions (Priem *et al.*, 2013). If this would also be the case in man, the combination of a PDE4 inhibitor with a 5-HT_4 receptor agonist can be considered for GI hypomotility.

Before taking the step to humans, the prokinetic properties of the combination should be tested *in vivo* in an experimental model. The pig is expensive, not easily accessible and more difficult to handle *in vivo*. Recently it was shown that 5-HT_4 receptors also facilitate cholinergic nerves innervating circular muscle in fundus, jejunum and colon of C57Bl/6J mice (Pauwelyn and Lefebvre, 2017). Whether PDE4 is also selectively regulating the intraneuronal signal transduction of these receptors in the murine GI tract

and what PDEs are involved in the regulation of the cAMP content in murine GI circular muscle is not known. The aim of this study was therefore to investigate the role of cAMP catalyzing PDEs in murine GI circular muscle activity, and in the signal transduction of the 5-HT_4 receptors on the cholinergic nerves to the murine GI circular muscle layer. This was done by investigating the influence of selective PDE inhibitors on cholinergic contractions induced with exogenous carbachol or by endogenous acetylcholine released by electrical field stimulation (EFS), and on submaximal facilitation of EFS-induced cholinergic contractions with the 5-HT_4 receptor agonist prucalopride.

VI.3 Materials and methods

VI.3.1 Animals and tissue preparation

All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University. Male C57Bl/6J mice (minimal 7 weeks, body weight 24.2 ± 0.1 g, mean ± S.E.M. of n = 245) were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and maintained on normal light-dark cycle with food pellets and water *ad libitum*.

Mice were sacrificed by cervical dislocation; the GI tract was isolated and kept in aerated (95% O_2 + 5% CO_2) Krebs solution containing (in mM): NaCl 118.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1. After emptying the stomach from its contents, two full thickness fundus strips were prepared by cutting in the direction of the circular smooth muscle layer. For jejunum an approximately 5 cm long segment of the small bowel, starting 10 cm distally to the pylorus, and for distal colon an approximately 4 cm long segment above the pelvic brim was taken. Jejunum and colon segments were opened along the mesenteric border and pinned with the mucosa side up in Krebs solution. By sharp dissection the mucosa was removed under a microscope and 2 (colon) or 4 (jejunum) smooth muscle strips were cut along the circular axis.

A cotton (fundus) or silk thread (jejunum and colon) was attached to both ends of every strip and they were mounted between two platinum plate electrodes (6-8 mm apart) in a 7 or 15 ml organ bath filled with oxygenated Krebs solution kept at body temperature. EFS was applied with a 4-channel custom-made stimulator. Changes in muscle tone were registered by isometric tension recording using Grass FT03 (Grass Instrument co., Quincy, MA, United States) or MLT 050/D force transducers (ADInstruments, Oxford, United Kingdom) and recorded on a Powerlab/8sp data recording system (ADInstruments) with Chart v5.5.6 software (ADInstruments). The wet weight of the strips was determined at the end of the experiments.

VI.3.2 Protocols

Strips were set at optimal load of 1 g for fundus, 0.25 g for jejunum and 0.5 g for colon as previously determined by studying the contractile response to the muscarinic receptor agonist carbachol (Pauwelyn and Lefebvre, 2017) and equilibrated for 30 min in Krebs solution with flushing every 10 min. Contractions were then induced with carbachol (0.1 µM for fundus and jejunum and 1 µM for colon); within 3 min the contractions reached a plateau. Addition of carbachol was repeated respecting an interval of 20 min after washout of carbachol (with additional flushing after 10 min) until stable responses (defined as a change in amplitude < 25% between consecutive contractions) were obtained. Further submaximal contractions were then induced by carbachol, as described in VI.3.2.1, or by EFS, as described in VI.3.2.2.

VI.3.2.1 Influence of PDE inhibitors on carbachol-induced submaximal contractions

After obtaining stable responses to carbachol, a submaximal concentration of carbachol was added: 0.04 (fundus), 0.05 (jejunum) or 0.3 (colon) µM as based on preliminary concentration-response curves.

The responses reached a plateau after 3 min, which corresponded to approximately 50-65% of the contraction amplitude observed with the carbachol concentration used to stabilize the strip. Carbachol was washed-out and after 10 min the organ bath content was flushed a second time. Addition of carbachol was repeated with an interval of 30 min until stable responses to carbachol (defined as a change in amplitude < 25% between consecutive contractions) were obtained. Some strips were excluded from data analysis since the response to carbachol continued to increase with still > 25% from a 4th to a 5th addition of carbachol, the maximal number of carbachol additions being kept at 5 (13 out of 69 fundus, 5 out of 71 jejunal and 4 out of 64 colon strips). A first concentration of PDE inhibitor (0.01 µM) was then added and left in contact with the tissue for 20 min before administering carbachol again. The cycle was then repeated 7 times, increasing the concentration of PDE inhibitor with half-log units to a maximum of 30 µM. The PDE inhibitors tested were IBMX, vinpocetine, EHNA, cilostamide and rolipram; parallel tissues received the corresponding amount of solvent (ethanol, solvent of IBMX; or DMSO, solvent of vinpocetine, cilostamide and rolipram) or no PDE inhibitor or solvent (pure time control). The eight experimental conditions were rotated over the organ baths from experiment to experiment.

VI.3.2.2 Influence of PDE inhibitors on EFS-induced submaximal cholinergic contractions

When stable responses to carbachol were obtained, the Krebs solution was switched to Krebs containing 4 µM guanethidine (noradrenergic neuron blocker) and 300 µM N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME; nitric oxide synthase inhibitor) to avoid relaxant influences due to noradrenaline and nitric oxide respectively. For colon tissue, also 1 µM MRS 2500 ($P2Y_1$ receptor antagonist) was added to avoid influences of the relaxant neurotransmitter ATP. These conditions were previously shown to allow induction of pure cholinergic on-contractions by EFS (Pauwelyn and Lefebvre, 2017). After 60 min of equilibration (flushing every 15 min), submaximal cholinergic contractions by EFS were obtained as follows. First, 10 s trains of EFS with a pulse width of 0.5 ms, frequency of 4 (fundus) or 8 (jejunum and colon) Hz at supramaximal voltage (V_{max}; 30 V) were repeated at 3 (fundus and colon) or 6 (jejunum) min interval; reproducible contractions were obtained within 10 (fundus and colon) or 5 (jejunum) EFS trains. The voltage was then gradually reduced until the amplitude of the contraction was approximately 50% of that obtained at V_{max} ($V_{50\%}$); stable responses to EFS at $V_{50\%}$ were obtained within 10 (fundus and colon) or 5 (jejunum) trains of EFS. EFS at $V_{50\%}$ was then continued at 5 (fundus and colon) or 10 (jejunum) min interval to register five stable reference contractions.

To study the influence of IBMX, it was administered to three parallel strips (1, 3 and 10 µM); 10 (fundus and colon) or 5 (jejunum) trains of EFS were then applied in its presence. The 4th parallel strip did not receive IBMX (pure time control); in a separate series the influence of ethanol (solvent of IBMX) was tested. For jejunum, also 0.1, 0.3 and 1 µM IBMX were tested with the same protocol.

To study the influence of the selective PDE inhibitors vinpocetine, EHNA, cilostamide or rolipram, they were added cumulatively with half-log unit increments (0.01 to 30 µM), applying 5 (fundus and colon) or 3 (jejunum) trains of EFS at each concentration. The corresponding amount of DMSO (solvent of

vinpocetine, cilostamide and rolipram) was also investigated as well as a pure time control. The six experimental conditions were rotated over the organ baths from experiment to experiment.

VI.3.2.3 Influence of PDE inhibitors on the effect of prucalopride on EFS-induced submaximal cholinergic contractions

These experiments started as described in VI.3.2.2 till five stable contractions by EFS at $V_{50\%}$ were obtained. The influence of the PDE inhibitors was then tested on the facilitating effect of a submaximal concentration of prucalopride (0.003 µM; Pauwelyn and Lefebvre, 2017). The concentrations of the PDE inhibitors studied were selected from their effect on carbachol- and EFS-induced contractions (see VI.4); a single concentration of PDE inhibitor was tested per strip. After adding the PDE inhibitor, 10 (fundus and colon) or 5 (jejunum) trains of EFS were applied; prucalopride was then added and a further series of 10 or 5 trains were applied. Parallel strips received either prucalopride alone, or no active compounds (pure time control). The experimental conditions were rotated over the organ baths from experiment to experiment.

VI.3.3 Data and statistical analysis

The contraction force induced by carbachol or EFS was expressed as gram.second per milligram wet weight of the tissue (g.s/mg wet weight). This was calculated by determining the area under the curve (AUC) during the contraction (3 min for carbachol- and 10 s for EFS-induced contractions) reduced with the AUC during a corresponding time period just before adding carbachol or applying EFS.

Carbachol-induced contractions in the presence of substances were expressed as percentage of the last contraction just before addition (100% reference). EFS-induced contractions at $V_{50\%}$ in the presence of substances were expressed as percentage of the mean of the five stable contractions just before addition (100% reference). For experiments, testing the influence of PDE inhibitors on the effect of prucalopride, the contractions after addition of prucalopride were also expressed as percentage of the mean of the five contractions in the presence of the PDE inhibitor just before adding prucalopride.

Strips where the AUC of minimally one of the five reference contractions by EFS used to calculate the mean 100% reference was outside the range of 75-125%, were excluded from data analysis. As an example of the exclusion rate, in the series studying IBMX on EFS-induced submaximal contractions, 4 out of 24 fundus and 7 out of 48 jejunum strips were excluded; none of the 16 colon strips had to be excluded. Experiments testing the influence of PDE inhibitors on the effect of prucalopride were excluded from data analysis when the response in the presence of 0.003 µM prucalopride alone was lower than 115% for fundus, 107% for jejunum and 116% for colon (as these are the minimal effects for this concentration of prucalopride, observed in our previous study in the murine GI tract; Pauwelyn and Lefebvre, 2017).

Data are expressed as means ± S.E.M.; *n* refers to tissues obtained from different animals. Statistical analysis was performed by use of GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, United States). In experiments with cumulative administration of PDE inhibitor, carbachol- and EFS-induced contractions were assessed with a repeated measures ANOVA followed by a Bonferroni

corrected *t*-test for comparison to the reference contraction in the same strips or with an unpaired *t*-test for comparison with the corresponding control strips. For experiments with single administration of PDE inhibitor, EFS-induced contractions induced by the last train of EFS in parallel groups were compared by a one-way ANOVA followed by a Bonferroni corrected *t*-test. A *P*-value less than 0.05 was considered statistically significant.

VI.3.4 Drugs

Carbamoylcholin chloride, guanethidine sulfate, 3-isobutyl-1-methylxanthine (IBMX) and N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) were obtained from Sigma-Aldrich (St. Louis, MO, United States); cilostamide, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) hydrochloride, MRS 2500 tetra-ammonium salt [(1R*,2S*)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphornooxy)bicyclo-[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt], rolipram and vinpocetine from Tocris Bioscience (Bristol, United Kingdom) and prucalopride succinate from Selleck Chemicals (Houston, TX, United States). Drugs were dissolved and diluted in distilled water, except for IBMX which was dissolved in ethanol, yielding a maximal ethanol concentration of 0.15% in the organ bath, and vinpocetine, cilostamide and rolipram which were dissolved in dimethyl sulfoxide (DMSO), yielding a maximal DMSO concentration of 0.3% in the organ bath.

VI.4 Results

VI.4.1 Influence of PDE inhibitors on carbachol-induced submaximal contractions (Figure VI.1-3)

Since the non-selective PDE inhibitor IBMX is dissolved in ethanol, the control strips received the corresponding amount of ethanol (Figure VI.1 A, Figure VI.2 A and Figure VI.3 A). In the presence of an increasing concentration of ethanol, the carbachol-induced contractions steadily increased. This is not due to ethanol because this trend was also present in pure time control strips not receiving solvent or PDE inhibitor (Figure VI.1 C, Figure VI.2 C and Figure VI.3 C). When comparing the effect of IBMX with the corresponding control strips receiving ethanol, IBMX concentration-dependently decreased the carbachol-induced contractions from 1 µM onwards in the strips from the three GI regions (Figure VI.1 B, Figure VI.2 B and Figure VI.3 B).

The selective PDE2 inhibitor EHNA is dissolved in H_2O and can thus be compared with the pure time controls. Only the highest concentration of EHNA induced a significant decrease of carbachol-induced contraction in fundus (Figure VI.1 D), jejunum (Figure VI.2 D) and colon (Figure VI.3 D). Vinpocetine, cilostamide and rolipram, respectively selective PDE1, PDE3 and PDE4 inhibitors, were dissolved in DMSO. Compared with the control strips receiving the corresponding amount of DMSO (Figure VI.1 E, Figure VI.2 E and Figure VI.3 E), a clear-cut concentration-dependent decrease of carbachol-induced contraction was obtained with cilostamide starting at 0.3 µM in the fundus (Figure VI.1 G) and at 0.03 µM in jejunum and colon (Figure VI.2 G and Figure VI.3 G). Also rolipram concentration-dependently decreased the carbachol-induced contractions over a broad concentration span in strips from the three GI regions (Figure VI.1 H, Figure VI.2 H and Figure VI.3 H), but the decrease was less pronounced compared to cilostamide in particular in the colon. Vinpocetine significantly decreased the contractions in jejunum (Figure VI.2 F) and colon (Figure VI.3 F) in the 2 highest concentrations tested (10-30 µM), but not in the fundus although a similar trend is visible (Figure VI.1 F).

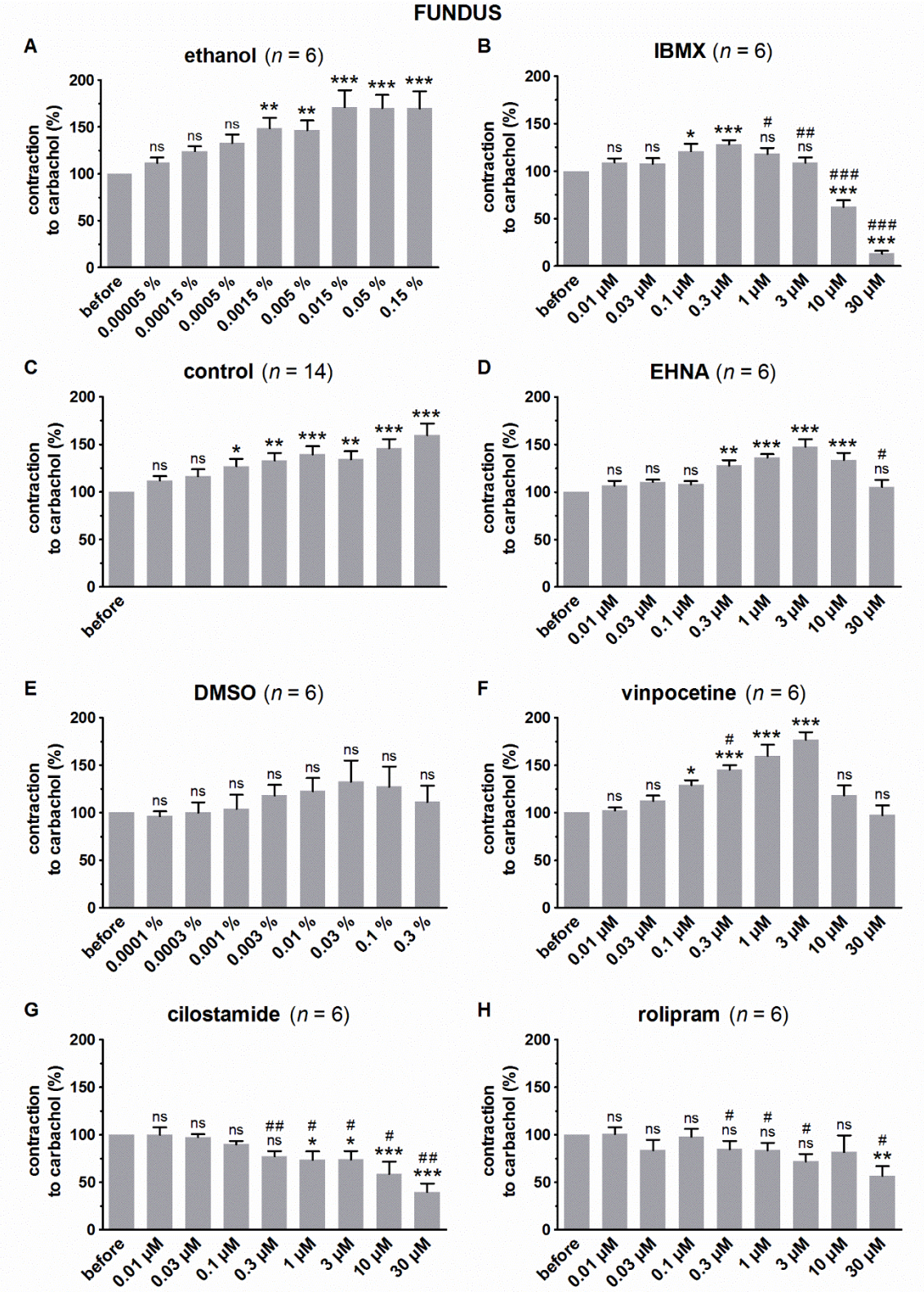


Figure VI.1 - Influence of increasing concentrations (0.01-30 µM) of the PDE inhibitors IBMX (B), EHNA (D), vinpocetine (F), cilostamide (G) and rolipram (H), and the solvents ethanol (A) and DMSO (E) on submaximal carbachol-induced contractions (0.04 µM) in murine fundus circular smooth muscle strips. Parallel time controls not receiving PDE inhibitor or solvent are shown in (C). Contractions in the presence of increasing concentrations of the PDE inhibitor are expressed as percentage of the reference contraction before addition of the PDE inhibitor. Means ± S.E.M.; ns not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus reference before (repeated measures ANOVA with Bonferroni corrected *t*-test) and $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$ versus corresponding control (unpaired *t*-test).

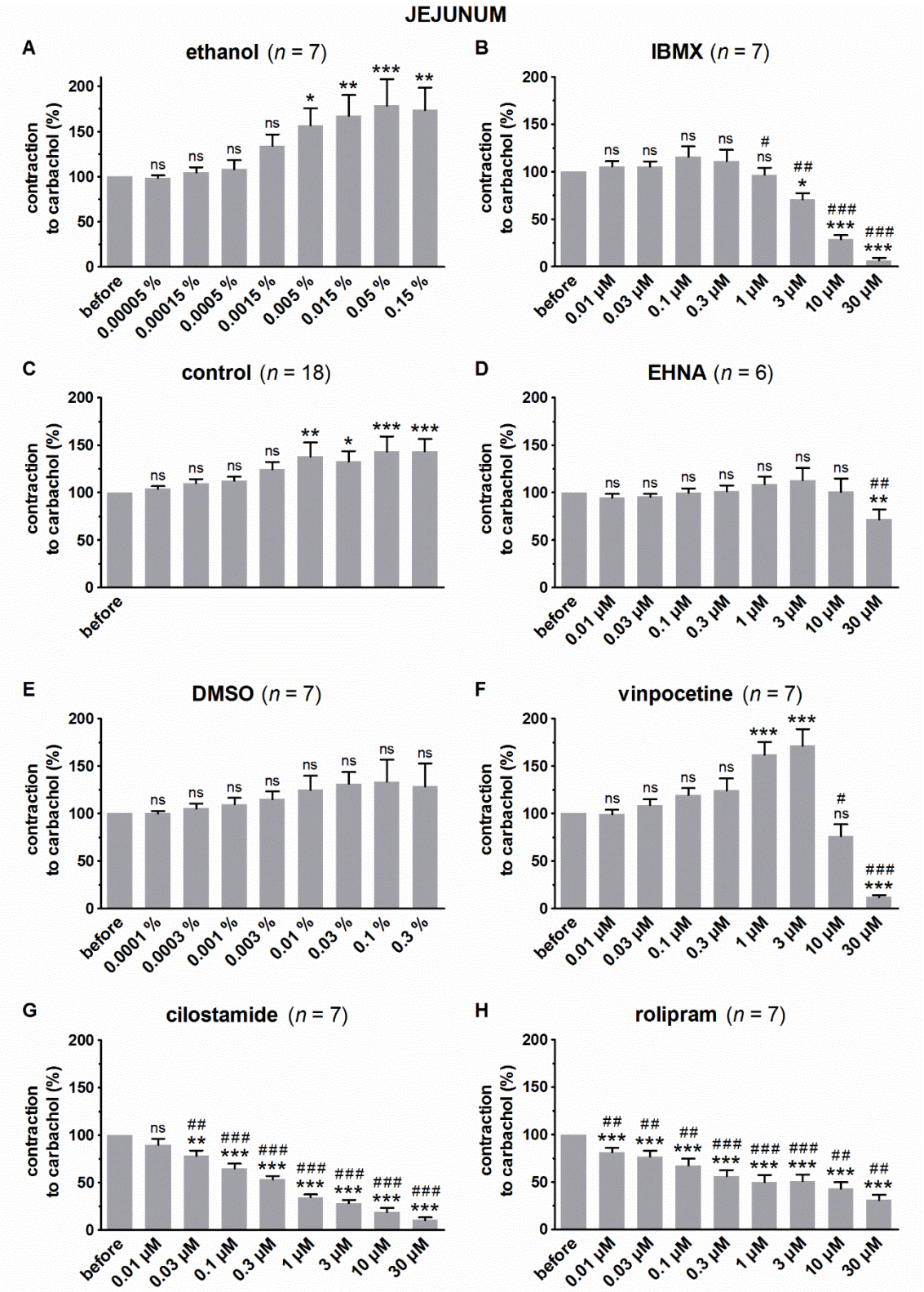


Figure VI.2 - Influence of increasing concentrations (0.01-30 µM) of the PDE inhibitors IBMX (B), EHNA (D), vinpocetine (F), cilostamide (G) and rolipram (H), and the solvents ethanol (A) and DMSO (E) on submaximal carbachol-induced contractions (0.05 µM) in murine jejunum circular smooth muscle strips. Parallel time controls not receiving PDE inhibitor or solvent are shown in (C). Contractions in the presence of increasing concentrations of the PDE inhibitor are expressed as percentage of the reference contraction before addition of the PDE inhibitor. Means ± S.E.M.; ns not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus reference before (repeated measures ANOVA with Bonferroni corrected *t*-test) and # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ versus corresponding control (unpaired *t*-test).

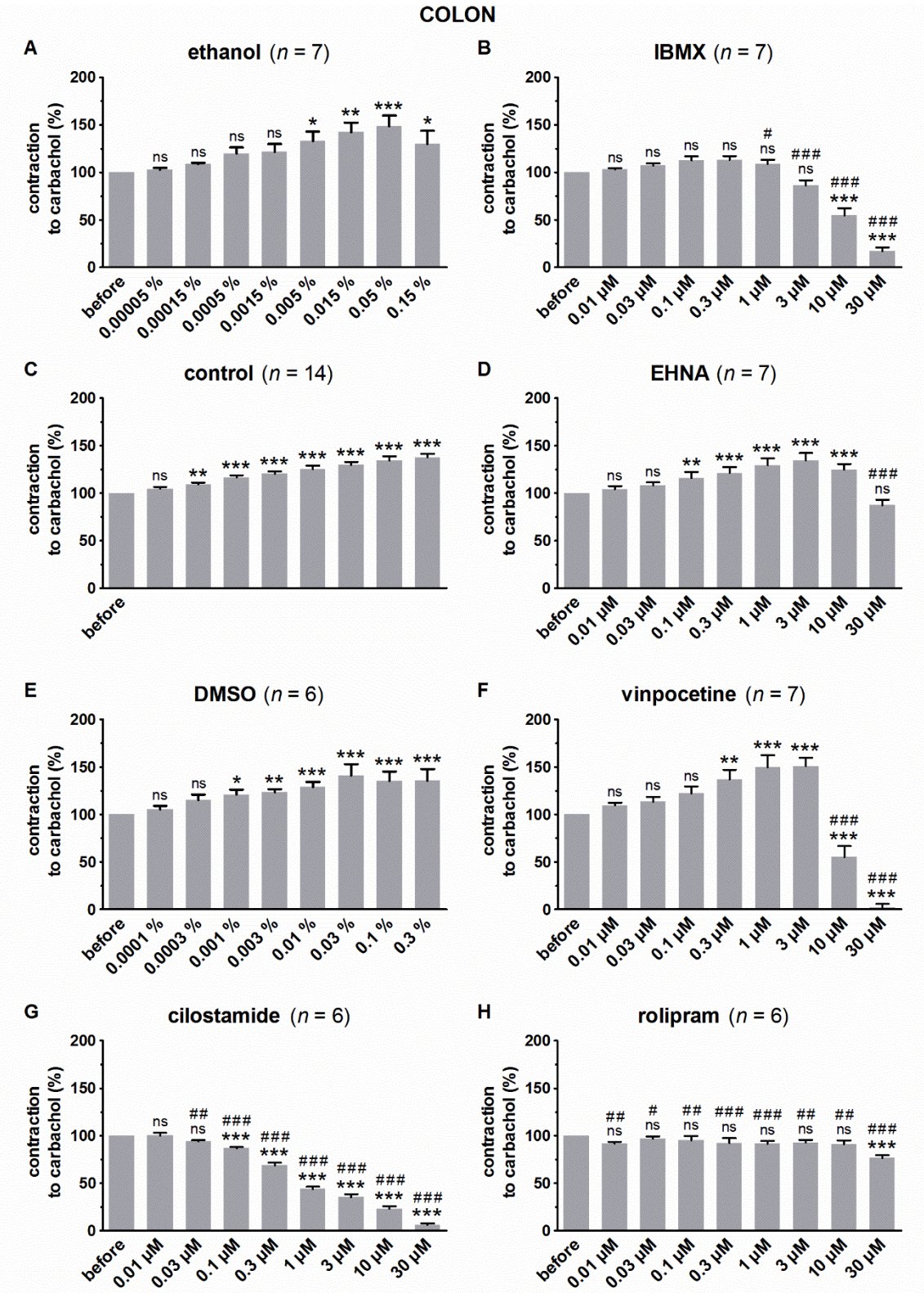


Figure VI.3 - Influence of increasing concentrations (0.01-30 µM) of the PDE inhibitors IBMX (B), EHNA (D), vinpocetine (F), cilostamide (G) and rolipram (H), and the solvents ethanol (A) and DMSO (E) on submaximal carbachol-induced contractions (0.3 µM) in murine colon circular smooth muscle strips. Parallel time controls not receiving PDE inhibitor or solvent are shown in (C). Contractions in the presence of increasing concentrations of the PDE inhibitor are expressed as percentage of the reference contraction before addition of the PDE inhibitor. Means ± S.E.M.; ns not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus reference before (repeated measures ANOVA with Bonferroni corrected *t*-test) and $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$ versus corresponding control (unpaired *t*-test).

VI.4.2 Influence of PDE inhibitors on EFS-induced submaximal contractions (Figure VI.4-7; Table VI.1)

In control strips from the three GI regions, a fairly stable response to EFS was obtained (Figure VI.4). IBMX, 1, 3 and 10 µM, concentration-dependently decreased the EFS-induced submaximal cholinergic contractions (Figure VI.4 A, B, C; Table VI.1 A) in the three regions. As 1 µM IBMX, the lowest concentration tested, had a more pronounced effect on EFS-induced submaximal cholinergic contractions in jejunum than in fundus and colon, 10-fold lower concentrations were also investigated (Figure VI.4 D). These induced concentration-dependent decreases from 103 ± 3% for control strips to 94 ± 9% with 0.1 µM, 83 ± 6% with 0.3 µM and 77 ± 5% ($P < 0.01$) with 1 µM IBMX. In a separate series, the solvent of IBMX (ethanol) did not show any influence (results not shown).

Table VI.1 - Influence of IBMX on EFS-induced submaximal cholinergic contractions (A) and on their facilitation by prucalopride (B) in murine fundus, jejunum and colon. Mean ± S.E.M.

	FUNDUS	JEJUNUM	COLON
A – IBMX on EFS-induced contractions (%)			
control	114 ± 4 (n = 6)	90 ± 6 (n = 3)	104 ± 8 (n = 4)
1 µM IBMX	101 ± 6 ^{ns} (n = 5)	63 ± 6 ^{ns} (n = 4)	90 ± 4 ^{ns} (n = 4)
3 µM IBMX	92 ± 12 ^{ns} (n = 6)	64 ± 2 * (n = 5)	61 ± 4 *** (n = 4)
10 µM IBMX	57 ± 24 * (n = 3)	45 ± 6 * (n = 6)	14 ± 4 *** (n = 4)
B – IBMX on effect of prucalopride on EFS-induced contractions (%)			
control	102 ± 8 (n = 8)	95 ± 5 (n = 8)	111 ± 2 (n = 7)
0.003 µM prucalopride	138 ± 6 ^{ns} (n = 9)	128 ± 5 *** (n = 9)	141 ± 11 * (n = 7)
0.1 µM IBMX + 0.003 µM prucalopride	ND	127 ± 4 ^{ns} (n = 7)	ND
0.3 µM IBMX + 0.003 µM prucalopride	ND	120 ± 4 ^{ns} (n = 7)	129 ± 7 ^{ns} (n = 7)
1 µM IBMX + 0.003 µM prucalopride	150 ± 14 ^{ns} (n = 7)	ND	127 ± 7 ^{ns} (n = 6)
3 µM IBMX + 0.003 µM prucalopride	163 ± 14 ^{ns} (n = 9)	ND	ND

A – Contractile response to the 5th (jejunum) or 10th (fundus and colon) train of EFS in the presence of IBMX as percentage of the mean of the five contractions before adding IBMX. ns not significant, * $P < 0.05$, *** $P < 0.001$ versus control (one-way ANOVA with Bonferroni corrected *t*-test for the three concentrations of IBMX versus control).

B – Contractile response to the 5th (jejunum) or 10th (fundus and colon) train of EFS in the presence of prucalopride as percentage of the mean of the five contractions in the presence of IBMX just before adding prucalopride. ND: Not determined. ns not significant, * P < 0.05, *** P < 0.001 (one-way ANOVA with Bonferroni corrected *t*-test for the three comparisons i.e. prucalopride versus control, and both concentrations of IBMX versus prucalopride).

In pure time controls (Figure VI.5 A, Figure VI.6 A and Figure VI.7 A), but also in strips receiving increasing amounts of DMSO (Figure VI.5 C, Figure VI.6 C and Figure VI.7 C), a fairly stable response to EFS was induced for the three GI regions. Therefore, the statistical analysis was restricted to the comparison of the last EFS-induced contraction in the presence of each concentration of a given PDE inhibitor with the

mean of the five contractions before addition of the PDE inhibitor. In the studied concentration range (0.01-30 µM) EHNA (Figure VI.5 B, Figure VI.6 B and Figure VI.7 B) only mildly reduced the EFS-induced contractions at 30 µM in the fundus and was without effect in jejunum and colon, while vinpocetine (Figure VI.5 D, Figure VI.6 D and Figure VI.7 D) had no influence in the fundus but reduced the EFS-induced contractions at the two highest concentrations tested in jejunum and colon. Cilostamide (Figure VI.5 E, Figure VI.6 E and Figure VI.7 E) decreased the contractions over a broad concentration range in the strips from the three GI regions but no clear concentration-dependency was present in the fundus. Rolipram did not significantly influence the contractions in the fundus (Figure VI.5 F), decreased them in the jejunum (Figure VI.6 F) at the two highest concentrations studied and concentration-dependently decreased them from 0.03 µM onwards in the colon (Figure VI.7 F). The reduction of the contractions with rolipram in the colon was less pronounced than obtained with cilostamide.

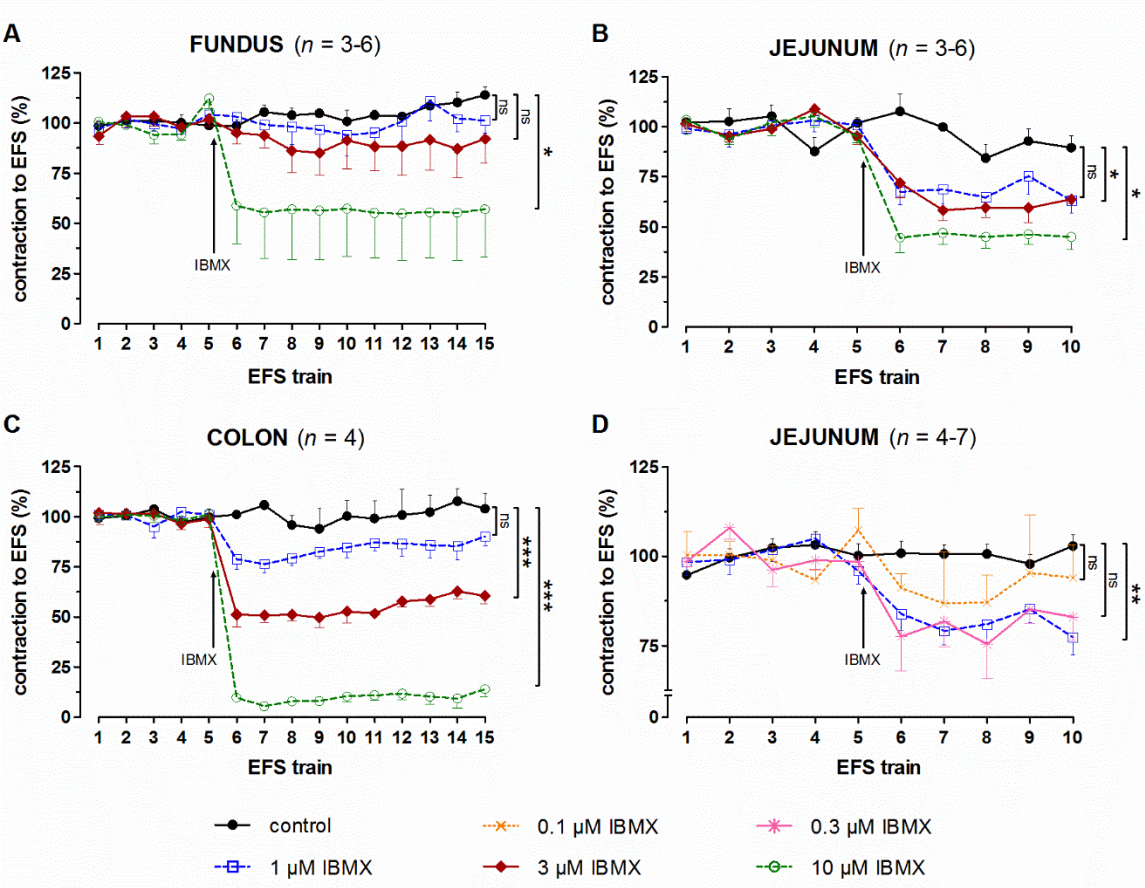


Figure VI.4 - Influence of 1, 3 and 10 µM (A-C) and 0.1, 0.3 and 1 µM (D) IBMX on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 4 [fundus] or 8 [jejunum and colon] Hz, 0.5 ms, interval of 5 [fundus and colon] or 10 [jejunum] min) in murine fundus (A), jejunum (B & D) and colon (C) circular smooth muscle strips. The number of the consecutive stimulation trains before and after adding IBMX is given on the y-axis. Contractions are expressed as percentage of the mean of the five contractions before adding IBMX (trains 1 to 5). Experiments were performed in the continuous presence of 4 µM guanethidine, 300 µM L-NAME and for colon also 1 µM MRS 2500. Means ± S.E.M.; ns not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control (one-way ANOVA with Bonferroni corrected *t*-test for the three concentrations of IBMX versus control).

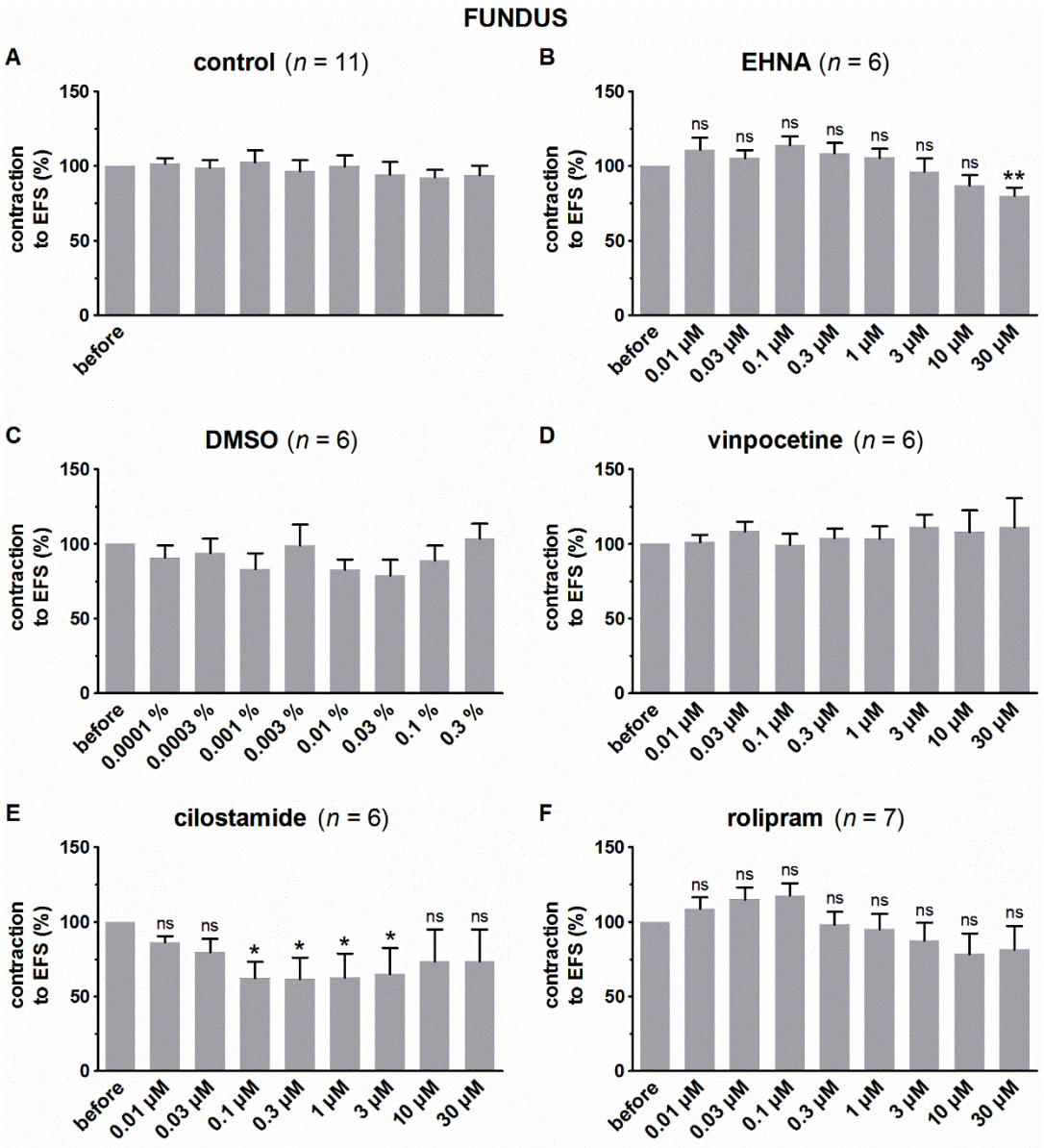


Figure VI.5 - Influence of increasing concentrations (0.01-30 µM) of the PDE inhibitors EHNA (B), vinpocetine (D), cilostamide (E) and rolipram (F), and the solvent DMSO (C) on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 4 Hz, 0.5 ms, interval of 5 min) in murine fundus circular smooth muscle strips. Parallel time controls not receiving PDE inhibitor or solvent are shown in (A). Five contractions were induced in the presence of each concentration of the PDE inhibitor and every 5th contraction is shown, expressed as percentage of the mean of the five contractions before addition of the PDE inhibitor. Experiments were performed in the continuous presence of 4 µM guanethidine and 300 µM L-NAME. Means ± S.E.M.; ns not significant, * $P < 0.05$, ** $P < 0.01$ versus reference before (repeated measures ANOVA with Bonferroni corrected *t*-test). For control, DMSO and vinpocetine, the repeated measures ANOVA was not significant.

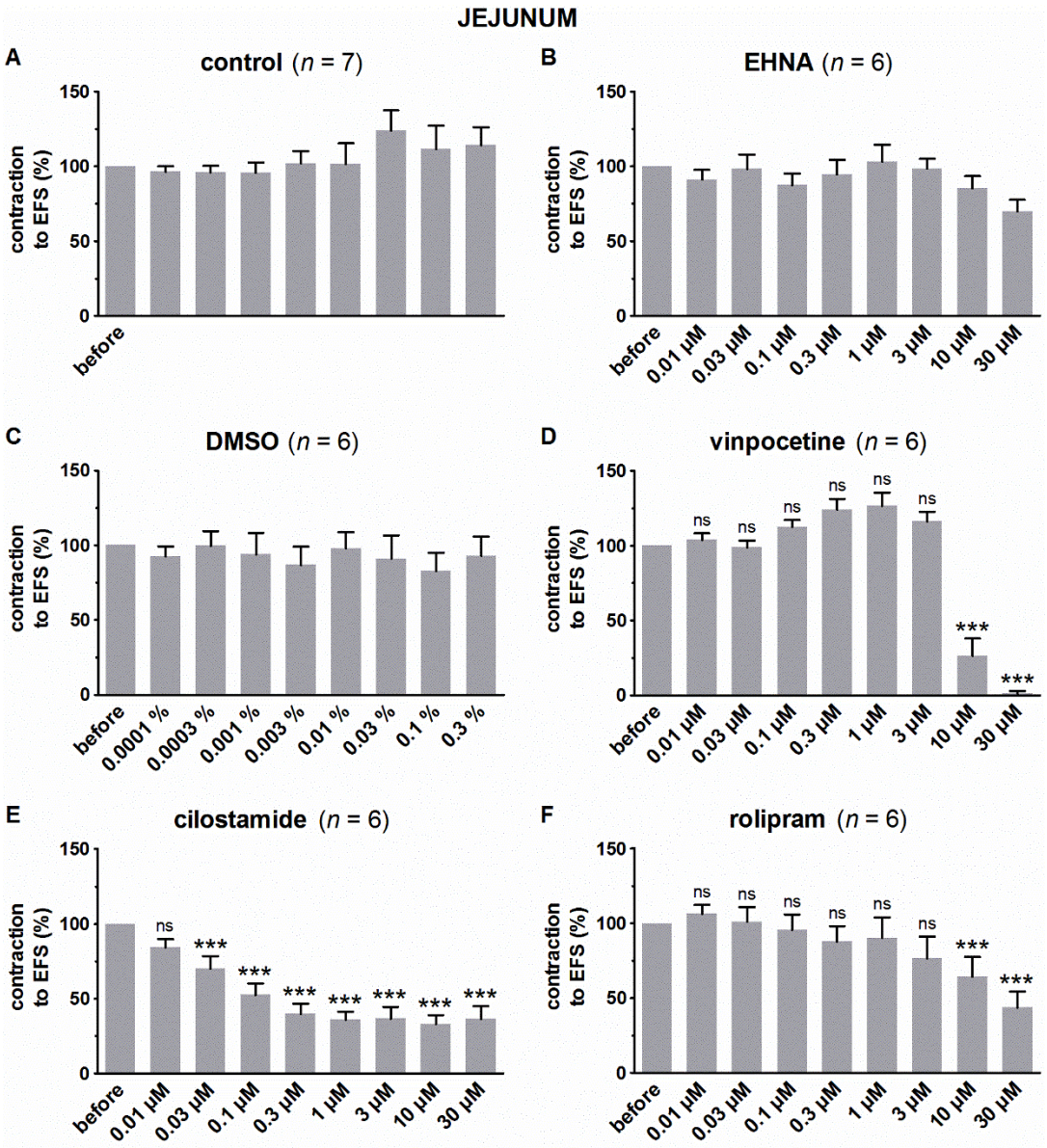


Figure VI.6 - Influence of increasing concentrations (0.01-30 µM) of the PDE inhibitors EHNA (B), vinpocetine (D), cilostamide (E) and rolipram (F), and the solvent DMSO (C) on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 8 Hz, 0.5 ms, interval of 10 min) in murine jejunum circular smooth muscle strips. Parallel time controls not receiving PDE inhibitor or solvent are shown in (A). Three contractions were induced in the presence of each concentration of the PDE inhibitor and every 3rd contraction is shown, expressed as percentage of the mean of the five contractions before addition of the PDE inhibitor. Experiments were performed in the continuous presence of 4 µM guanethidine and 300 µM L-NAME. Means ± S.E.M.; ns not significant, *** $P < 0.001$ versus reference before (repeated measures ANOVA with Bonferroni corrected *t*-test). For control, EHNA and DMSO, the repeated measures ANOVA was not significant.

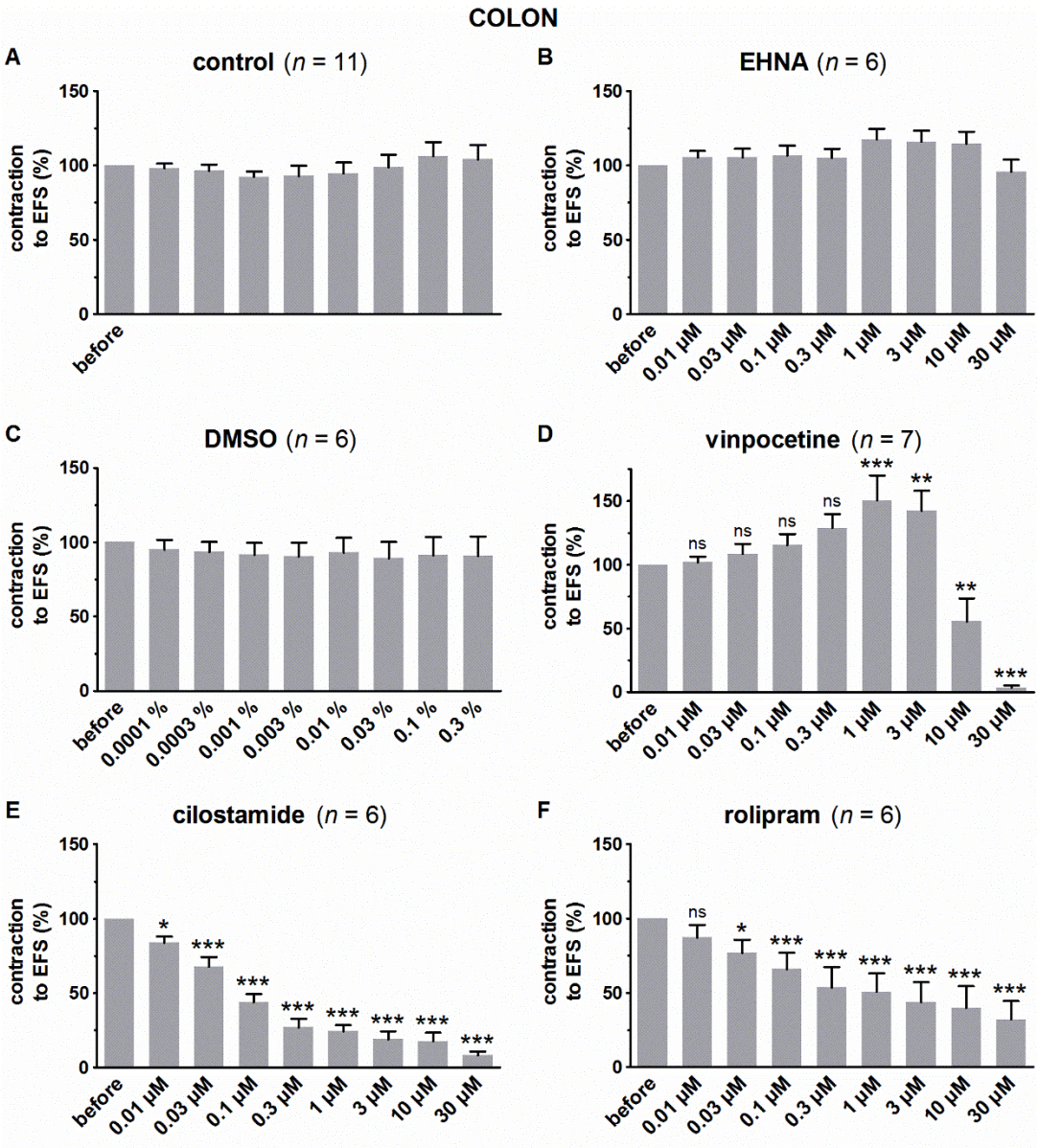


Figure VI.7 - Influence of increasing concentrations (0.01-30 µM) of the PDE inhibitors EHNA (B), vinpocetine (D), cilostamide (E) and rolipram (F), and the solvent DMSO (C) on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 8 Hz, 0.5 ms, interval of 5 min) in murine colon circular smooth muscle strips. Parallel time controls not receiving PDE inhibitor or solvent are shown in (A). Five contractions were induced in the presence of each concentration of the PDE inhibitor and every 5th contraction is shown, expressed as percentage of the mean of the five contractions before addition of the PDE inhibitor. Experiments were performed in the continuous presence of 4 µM guanethidine, 300 µM L-NAME and 1 µM MRS 2500. Means ± S.E.M.; ns not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus reference before (repeated measures ANOVA with Bonferroni corrected *t*-test). For control, EHNA and DMSO, the repeated measures ANOVA was not significant.

VI.4.3 Influence of PDE inhibitors on the effect of prucalopride on EFS-induced submaximal cholinergic contractions (Figure VI.8-14, Table VI.1, Table VI.2)

Taking in account the decreasing effect of IBMX as such on EFS-induced submaximal cholinergic contractions (see VI.4.2; Figure VI.4 and Table VI.1 A), two concentrations of IBMX, inducing less than 20% decrease, were selected to test versus 0.003 µM prucalopride: 1 and 3 µM in fundus, 0.1 and 0.3 µM in jejunum and 0.3 and 1 µM in colon. Also in this series, the decrease of the EFS-induced contractions by these concentrations of IBMX was lower than 20% (Figure VI.8 A, C and E) and only reached significance for 1 µM in the colon ($P < 0.05$ versus controls). Still in view of the effect of IBMX as such, the responses in the presence of prucalopride were also expressed as percentage of the mean of the five contractions just before adding prucalopride (Figure VI.8 B, D and F; Table VI.1 B). This showed that 0.003 µM prucalopride per se facilitated the electrically induced cholinergic contractions, the effect not reaching significance in the fundus, but the presence of IBMX did not significantly enhance the effect of prucalopride although a trend was present in the fundus.

To select the concentrations of the selective PDE inhibitors vinpocetine (PDE1), EHNA (PDE2), cilostamide (PDE3) and rolipram (PDE4) for testing versus prucalopride three elements were taken in account: (1) the effect of the PDE inhibitor as such on the EFS-induced submaximal cholinergic contractions as reported in 3.2; (2) the reported IC_{50} values (Table VI.2), (3) a synergistic effect with prucalopride observed with the inhibitor in similar experiments in porcine GI tissue (Priem *et al.*, 2012; Priem *et al.*, 2013). In principle, for a first test the highest concentration in the range tested (0.01 – 30 µM), not influencing EFS-induced contractions in the three GI regions (see VI.4.2) was studied for a given PDE inhibitor. EHNA at 30 µM significantly reduced EFS-induced submaximal cholinergic contractions in the fundus, so a concentration of 10 µM was selected to test versus prucalopride; this concentration is 10-fold higher than to equal to reported IC_{50} values at PDE2. Vinpocetine was studied versus prucalopride at 3 µM, as this did not decrease EFS-induced contractions in the three tissue types. As this concentration is clearly below reported IC_{50} values at PDE1, also 10 µM vinpocetine was studied although it clearly reduced EFS-induced contractions in jejunum and colon. The lowest concentration of cilostamide tested versus EFS-induced contractions (0.01 µM) still had some decreasing influence in the colon and was first tested. As this concentration is 3- to 13-fold lower than reported IC_{50} values at PDE3, cilostamide was also studied versus prucalopride at 1 µM. For rolipram, 1 and 3 µM were tested versus prucalopride as in porcine GI tissue, these concentrations were able to increase the prucalopride-induced facilitation of EFS-induced contractions (Priem *et al.*, 2012; Priem *et al.*, 2013). 1 µM corresponds to the highest IC_{50} value at PDE4 reported in the literature, although a 20-fold lower value (Bolger *et al.*, 1997) and even an exceptionally 1000-fold lower value (Wang *et al.*, 1997) were also reported (Table VI.2). Both 1 and 3 µM were without effect on EFS-induced submaximal cholinergic contractions in the fundus and jejunum but decreased them in the colon.

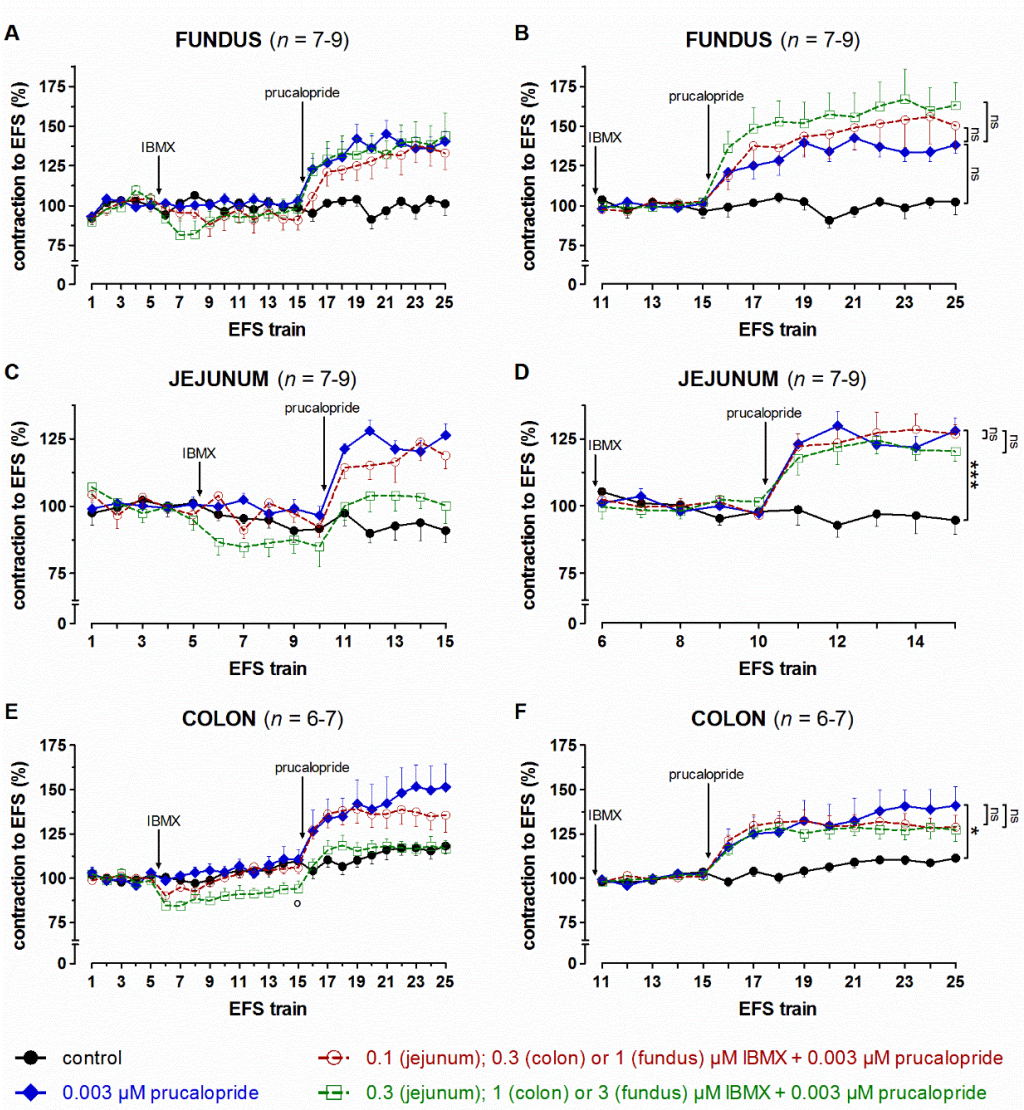


Figure VI.8 - Influence of IBMX in a concentration of 1 and 3 µM (fundus), 0.1 and 0.3 µM (jejunum), and 0.3 and 1 µM (colon) on the facilitating effect of 0.003 µM prucalopride on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 4 [fundus] or 8 [jejunum and colon] Hz, 0.5 ms, interval of 5 [fundus and colon] or 10 [jejunum] min) in murine fundus (A & B), jejunum (C & D) and colon (E & F) circular smooth muscle strips. The number of the consecutive stimulation trains is given on the y-axis. Contractions are expressed as percentage of the mean of the five contractions before adding IBMX (trains 1 to 5; A, C & E) or of the five contractions in the presence of IBMX just before adding prucalopride (trains 11 to 15 for fundus and colon; trains 6 to 10 for jejunum; B, D & F). Experiments were performed in the continuous presence of 4 µM guanethidine, 300 µM L-NAME and for colon also 1 µM MRS 2500. Means ± S.E.M. Left panels, last EFS-induced contraction in the presence of PDE inhibitor before adding prucalopride: ° $P < 0.05$ versus control (unpaired *t*-test). Right panels, last EFS-induced contraction: ns not significant, * $P < 0.05$, *** $P < 0.001$ (one-way ANOVA with Bonferroni corrected *t*-test for three comparisons i.e. prucalopride versus control and both concentrations of IBMX versus prucalopride).

Table VI.2 - Overview of reported IC_{50} values for the PDE inhibitors studied (in bold for PDE subtype, wherefore the inhibitor is considered selective).

PDE inhibitor	IC_{50} (µM)					Reference (see below)
	PDE1	PDE2	PDE3	PDE4	No subtype identified	
IBMX	12	20	2	5		1 #
	A: 0.8 C: 23	27	11	21		2 #
	7.1	18.6	7.1	11.4		3 #
vinpocetine	**23.2**	ND	ND	ND		4 *
	32.0	69.2	> 100	41.3		3 #
EHNA	ND	**9.2**	ND	ND		4 *
	> 100	**0.8 (human)** **2 (pig)**	> 100	> 100		5 #
	> 50	**3.5**	> 50	> 50		6 #
	> 400	**4.3**	> 400	310		7 #
cilostamide	> 300	12.5	**A: 0.027** **B: 0.05**	88.8		4 *
	221	48	**0.13**	99		7 #
					0.063	8 °
rolipram	ND	ND	ND	**0.45**		4 *
	ND	ND	ND	**D1: 0.05** **D2: 0.05** **D3: 0.14/0.32** **D4: 0.06/0.05** **D5: 0.08/0.59**		9 *
	ND	0.5	0.18	**0.4**		10 *
	ND	ND	ND	**A: 0.0011** **B: 0.0009** **C: 0.3246** **D: 0.0611**		11 *
	A: 50 C: > 100	> 100	> 100	**0.6**		2 #
	> 100	> 100	> 100	**0.72**		3 #
	ND	ND	ND	**0.065**		6 #
					1	12 °

ND: Not determined.

IC_{50} values obtained in paradigms:

* with specific PDE expression;

where PDE subtypes were purified and/or identified on the basis of their regulatory and kinetic properties;

° where no subtype identification was done,

as specified below.

References Table VI.2:

1 # - Coste and Grondin, 1995: cAMP (PDE1, 3 and 4) or cGMP (PDE2) hydrolysis by purified PDE1 and 3 from bovine aorta and human recombinant PDE2 and 4.;
2 # - Torphy and Cieslinski, 1990: cAMP (PDE1) and cGMP (PDE2, 3 and 4) hydrolysis by chromatography separated PDE isozymes in canine trachealis.
3 # - Saeki and Saito, 1993: cAMP or cGMP hydrolysis by PDE1 to 5 isozymes, separated by chromatography and identified on the basis of their regulatory and kinetic properties (type 1 preferentially hydrolyzed cGMP and was stimulated by Ca^{2+}-calmodulin; type 2 was stimulated by cGMP and hydrolyzed cAMP and cGMP; type 3 was inhibited by cGMP and preferentially hydrolyzed cAMP; type 4 was cAMP-specific; type 5 was cGMP specific and its activity did not depend on calmodulin), from the supernatant of pig aortic smooth muscle homogenates.
4 * - Sudo *et al.*, 2000: cAMP (PDE2, 3 and 4) or cGMP (PDE1) hydrolysis by recombinant PDE isozymes expressed in insect (Sf9) cells using baculovirus expression system; cDNAs from bovine PDE1B1 and PDE2A1, human PDE3A1, and rat PDE3B1 and PDE4B2.
5 # - Podzuweit *et al.*, 1995: cAMP (PDE3 and 4) or cGMP (PDE1 and 2) hydrolysis by PDE1 to 4 isozymes, separated by chromatography and identified on the basis of their regulatory and kinetic properties (PDE1 was stimulated by Ca^{2+}-calmodulin and PDE2 by cGMP; PDE3 had a high affinity for cAMP and was inhibited by cGMP; PDE4 was cAMP specific), from human and porcine myocardium.
6 # - Michie *et al.*, 1996: cAMP hydrolysis by murine thymocyte PDE1, 2, 3 and 4, separated by chromatography.
7 # - O'Grady *et al.*, 2002: cAMP hydrolysis by PDE1, 2, 3 and 4 purified from T_{84} human colonic epithelial cell line or bovine cardiac muscle; the IC_{50} value of cilostamide for PDE3 was tested on Cl secretion in T_{84} cells.
8 ° - Zacher and Carey, 1999: cAMP hydrolysis by phosphodiesterases in swine adipocyte microsomal membrane fraction.
9 * - Bolger *et al.*, 1997: cAMP hydrolysis by monkey COS-7 cells expressing cDNA of human PDE4D isoenzymes.
10 * - Bolger *et al.*, 1993: cAMP hydrolysis by yeast (*Saccharomyces cerevisiae*) extracts expressing cDNA clones for human PDE2 through 4.
11 * - Wang *et al.*, 1997: cAMP hydrolysis by purified human PDE4A, B, C and D expressed in insect (Sf9) cells using baculovirus expression system.
12 ° - Schwabe *et al.*, 1976: cAMP hydrolysis in rat brain slices.

The results obtained with the selective PDE inhibitors in fundus strips are given in Figure VI.9 and Figure VI.10. In the left panels, responses are expressed as percentage of the mean of the five contractions before adding the PDE inhibitor, allowing to judge the effect of the PDE inhibitor on EFS-induced contractions. In the right panels, responses are expressed as percentage of the mean of the five contractions just before adding prucalopride in the presence of PDE inhibitor; values given below for the facilitating effect of prucalopride refer to this way of expression. As judged from the 10^{th} EFS-induced contraction in its presence, EHNA at 10 µM had no influence on EFS-induced submaximal cholinergic contractions when compared to the two parallel strip groups i.e. the pure time controls and the strips going to receive prucalopride in the absence of the PDE inhibitor (Figure VI.9 A). Prucalopride, 0.003 µM, significantly enhanced the contractions to 149 ± 6% ($P < 0.001$ versus time controls); the facilitating effect of prucalopride was not enhanced but significantly decreased in the presence of 10 µM EHNA to a value of 121 ± 11% ($P < 0.05$ versus strips only receiving prucalopride; Figure VI.9 B). Vinpocetine, 3 µM, did not influence the EFS-induced submaximal cholinergic contractions (Figure VI.9 C), but 10 µM significantly increased them to 130 ± 19% ($P < 0.05$ versus parallel time control strips; Figure VI.9 E). The facilitating effect of 0.003 µM prucalopride on EFS-induced contractions (to 165 ± 13%, $P < 0.001$, in the series with 3 µM vinpocetine, and to 146 ± 10%, $P < 0.01$, in the series with 10 µM vinpocetine) was not influenced by 3 or 10 µM vinpocetine (Figure VI.9 D and F). Cilostamide, 0.01 µM, did not influence the EFS-induced submaximal cholinergic contractions (Figure VI.10 A) but 1 µM reduced them to 74 ± 6% ($P < 0.05$ versus parallel strips going to receive prucalopride in the absence of cilostamide; Figure VI.10 C). Neither concentration of cilostamide influenced the facilitating effect of 0.003 µM prucalopride on the EFS-induced contractions (to 140 ± 10%, $P < 0.01$, in the series with 0.01

µM cilostamide, Figure VI.10 B; to 132 ± 8%, $P < 0.01$, in the series with 1 µM cilostamide, $P < 0.01$, Figure VI.10 D). Rolipram in a concentration of 1 and 3 µM did not influence the EFS-induced submaximal cholinergic contractions (Figure VI.10 E and G). Also rolipram did not enhance the facilitating effect of 0.003 µM prucalopride on the EFS-induced contractions (to 140 ± 10%, $P < 0.01$, in the series with 1 µM rolipram, Figure VI.10 F; to 143 ± 8%, $P < 0.001$, in the series with 3 µM rolipram, Figure VI.10 H).

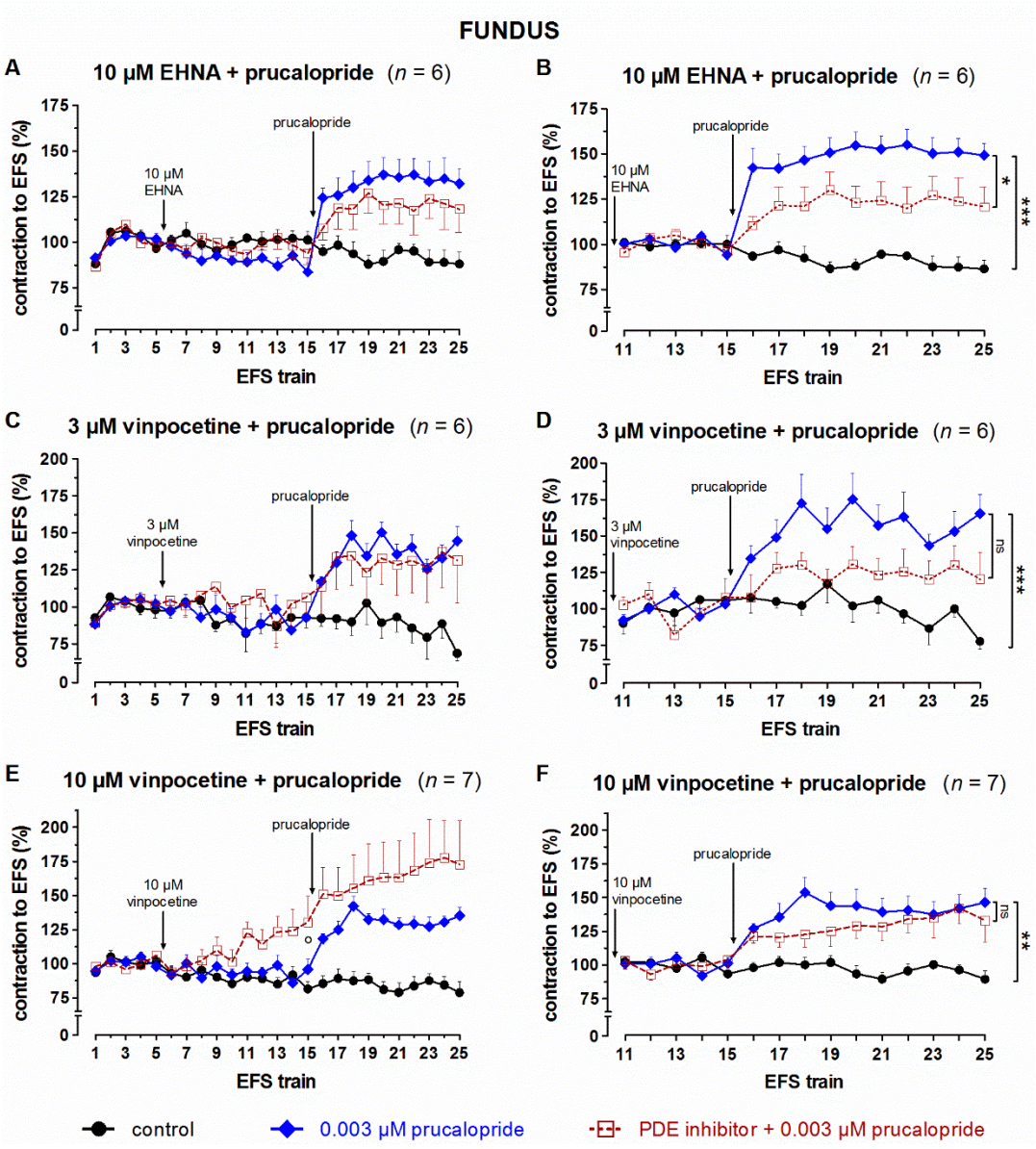


Figure VI.9 - Influence of 10 µM EHNA (A & B), 3 (C & D) and 10 (E & F) µM vinpocetine on the facilitating effect of 0.003 µM prucalopride on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 4 Hz, 0.5 ms, interval of 5 min) in murine fundus circular smooth muscle strips. The number of the consecutive stimulation trains is given on the y-axis. Contractions are expressed as percentage of the mean of the five contractions before addition of the PDE inhibitor (trains 1 to 5; A, C & E) or of the five contractions in the presence of the PDE inhibitor just before adding prucalopride (trains 11 to 15; B, D & F). Experiments were performed in the continuous presence of 4 µM guanethidine and 300 µM L-NAME. Means ± S.E.M. Left panels, last EFS-induced contraction before adding prucalopride: ° $P < 0.05$ versus control (one-way ANOVA with Bonferroni corrected *t*-test for two comparisons i.e. PDE inhibitor versus control and versus prucalopride). Right panels, last EFS-induced contraction: ns not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA with Bonferroni corrected *t*-test for two comparisons i.e. prucalopride versus control and versus prucalopride in the presence of the PDE inhibitor).

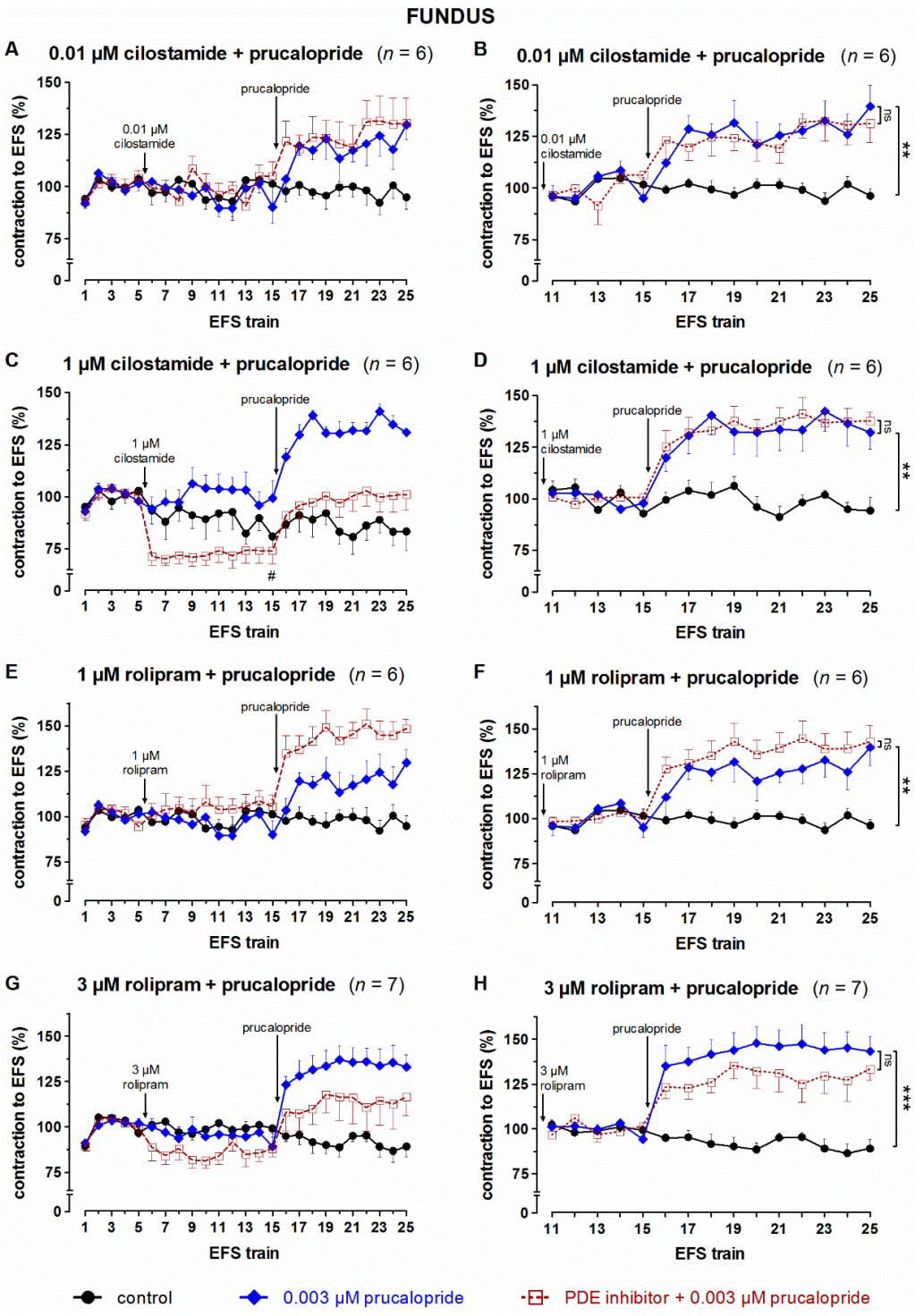


Figure VI.10 - Influence of 0.01 (A & B) and 1 (C & D) µM cilostamide, 1 (E & F) and 3 (G & H) µM rolipram on the facilitating effect of 0.003 µM prucalopride on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 4 Hz, 0.5 ms, interval of 5 min) in murine fundus circular smooth muscle strips. The number of the consecutive stimulation trains is given on the y-axis. Contractions are expressed as percentage of the mean of the five contractions before addition of the PDE inhibitor (trains 1 to 5; A, C, E & G) or of the five contractions in the presence of the PDE inhibitor just before adding prucalopride (trains 11 to 15; B, D, F & H). Experiments were performed in the continuous presence of 4 µM guanethidine and 300 µM L-NAME. Means ± S.E.M. Left panels, last EFS-induced contraction before adding prucalopride: $^{\#}$ $P < 0.05$ versus prucalopride (one-way ANOVA with Bonferroni corrected *t*-test for two comparisons i.e. PDE inhibitor versus control and versus prucalopride). Right panels, last EFS-induced contraction: ns not significant, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA with Bonferroni corrected *t*-test for two comparisons i.e. prucalopride versus control and versus prucalopride in the presence of the PDE inhibitor).

The results for the jejunum are shown in Figure VI.11 and Figure VI.12. Prucalopride, 0.003 µM, significantly enhanced the EFS-induced submaximal cholinergic contractions in all series. None of the PDE inhibitors enhanced the facilitating effect of prucalopride. The last EFS-induced contraction in the presence of 1 µM cilostamide and prucalopride was significantly higher than that in the presence of prucalopride alone (Figure VI.12 D). However, 1 µM cilostamide per se reduced EFS-induced contractions by 80% (Figure VI.12 C); a mild increase of these small contractions by prucalopride thus already induces a pronounced percentual increase when expressing results as percentage of the mean of the five contractions before adding prucalopride. The results for the colon are given in Figure VI.13 and Figure VI.14. The facilitating effect of 0.003 µM prucalopride on EFS-induced contractions was significantly enhanced by 1 µM cilostamide (Figure VI.14 D) and 3 µM rolipram (Figure VI.14 H). But again, these concentrations of PDE inhibitor per se decreased EFS-induced contractions in a pronounced way (by 84% for 1 µM cilostamide, Figure VI.14 C and by 34% for 3 µM rolipram, Figure VI.14 G). Mild to moderate facilitation by prucalopride of these small contractions leads to a pronounced increase when expressing results as percentage of the mean of the five contractions before adding prucalopride, but from the expression as percentage of the mean of five contractions, before adding the PDE inhibitor, it is clear that EFS-induced contractions in the presence of 1 µM cilostamide plus prucalopride (Figure VI.14 C) or 3 µM rolipram plus prucalopride (Figure VI.14 G) are smaller than those in the presence of prucalopride so that no clear-cut evidence for synergy is obtained.

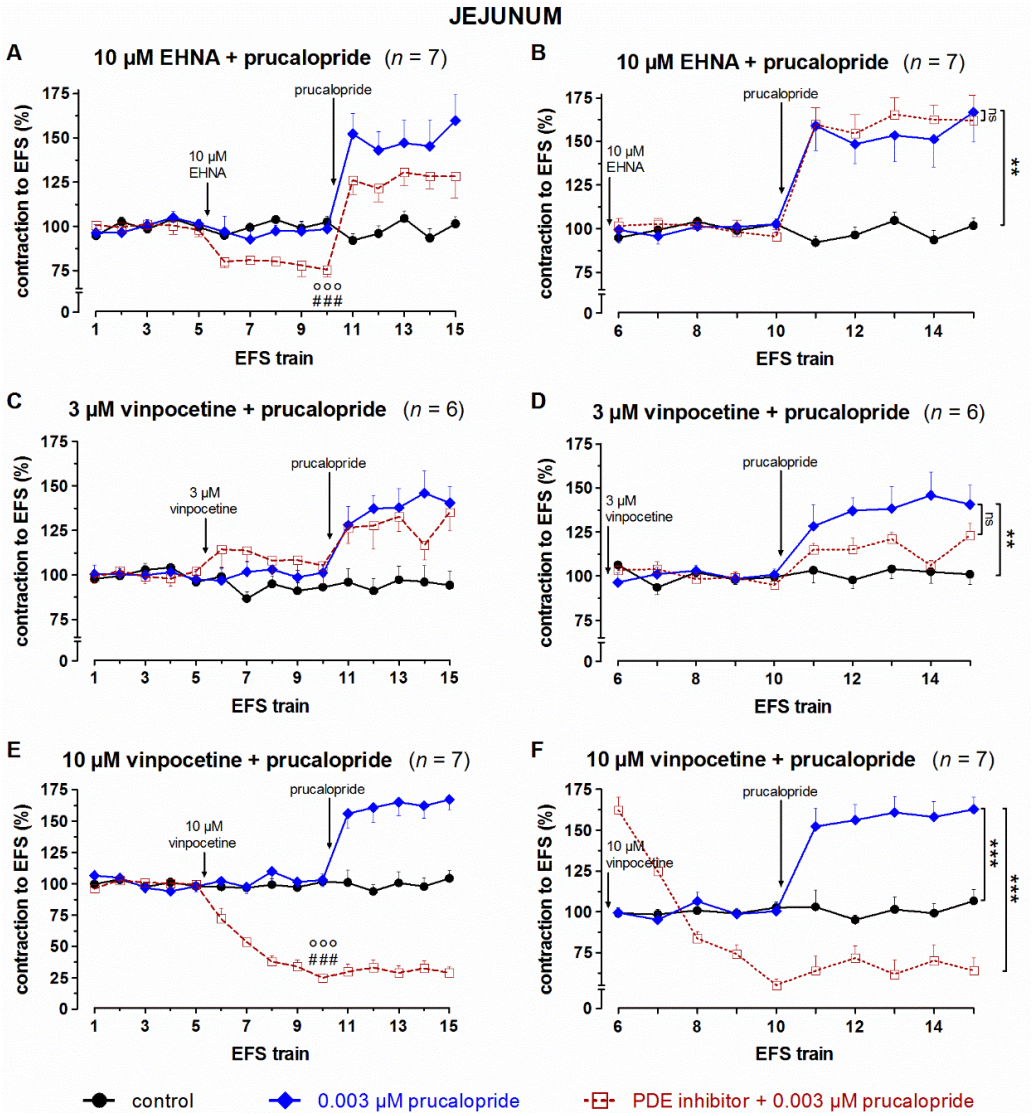


Figure VI.11 - Influence of 10 µM EHNA (A & B), 3 (C & D) and 10 (E & F) µM vinpocetine on the facilitating effect of 0.003 µM prucalopride on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 8 Hz, 0.5 ms, interval of 10 min) in murine jejunum circular smooth muscle strips. The number of the consecutive stimulation trains is given on the y-axis. Contractions are expressed as percentage of the mean of the five contractions before addition of the PDE inhibitor (trains 1-5; A, C & E) or of the five contractions in the presence of the PDE inhibitor just before adding prucalopride (trains 6-10; B, D & F). Experiments were performed in the continuous presence of 4 µM guanethidine and 300 µM L-NAME. Means ± S.E.M. Left panels, last EFS-induced contraction before adding prucalopride: ^{ooo} $P < 0.001$ versus control and $^{###}$ $P < 0.001$ versus prucalopride (one-way ANOVA with Bonferroni corrected *t*-test for two comparisons i.e., PDE inhibitor versus control and versus prucalopride). Right panels, last EFS-induced contraction: ns not significant, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA with Bonferroni corrected *t*-test for two comparisons i.e., prucalopride versus control and versus prucalopride in the presence of the PDE inhibitor).

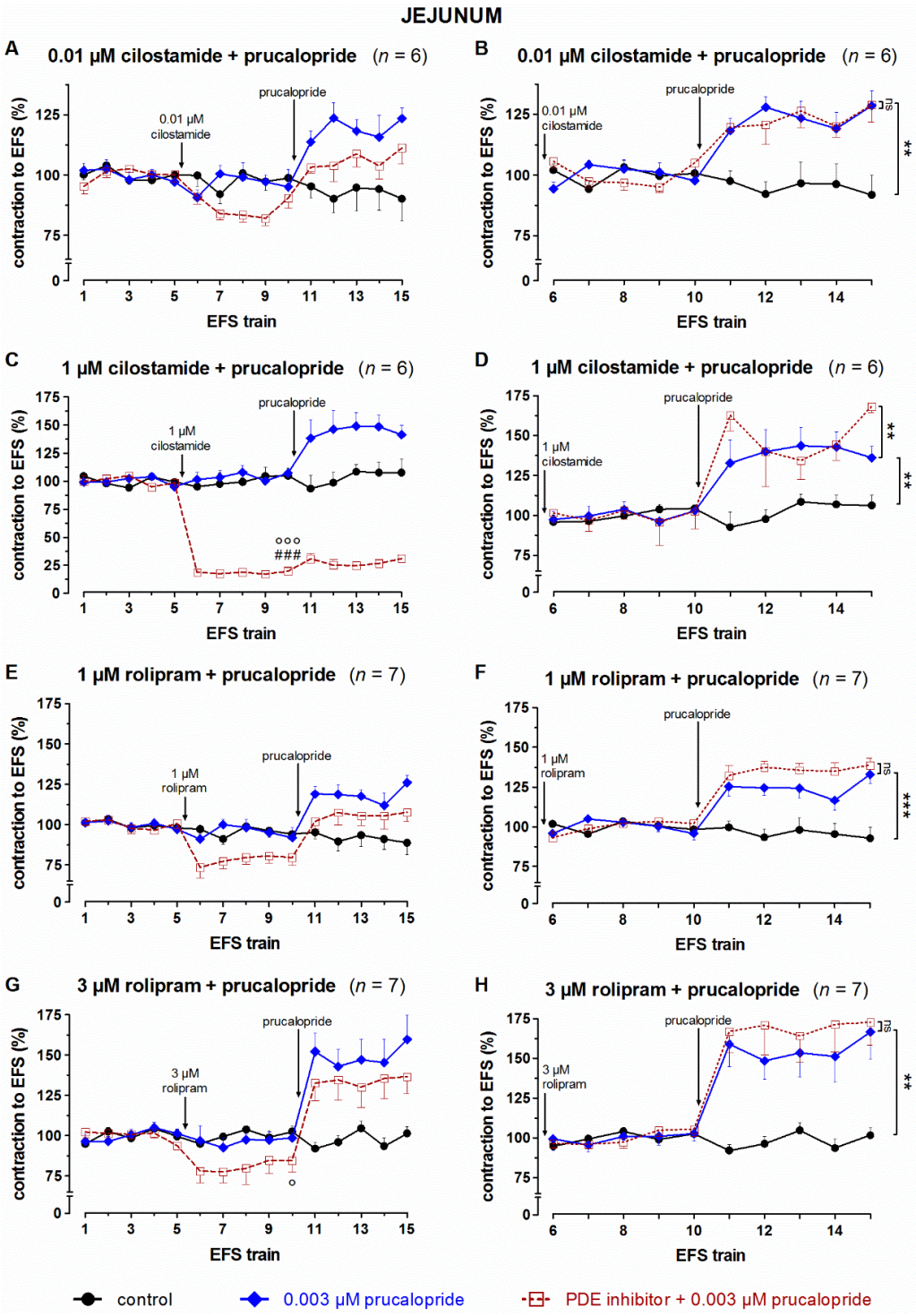


Figure VI.12 - Influence of 0.01 (A & B) and 1 (C & D) µM cilostamide, 1 (E & F) and 3 (G & H) µM rolipram on the facilitating effect of 0.003 µM prucalopride on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 8 Hz, 0.5 ms, interval of 10 min) in murine jejunum circular smooth muscle strips. The number of the consecutive stimulation trains is given on the y-axis. Contractions are expressed as percentage of the mean of the five contractions before addition of the PDE inhibitor (trains 1-5; A, C, E & G) or of the five contractions in the presence of the PDE inhibitor just before adding prucalopride (trains 6-10; B, D, F & H). Experiments were performed in the continuous presence of 4 µM guanethidine and 300 µM L-NAME. Means ± S.E.M. Left panels, last EFS-induced contraction before adding prucalopride: ° $P < 0.05$, °°° $P < 0.001$ versus control and ### $P < 0.001$ versus prucalopride (one-way ANOVA with Bonferroni corrected *t*-test for two comparisons i.e., PDE inhibitor versus control and versus prucalopride). Right panels, last EFS-induced contraction: ns not significant, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA with Bonferroni corrected *t*-test for two comparisons i.e., prucalopride versus control and versus prucalopride in the presence of the PDE inhibitor).

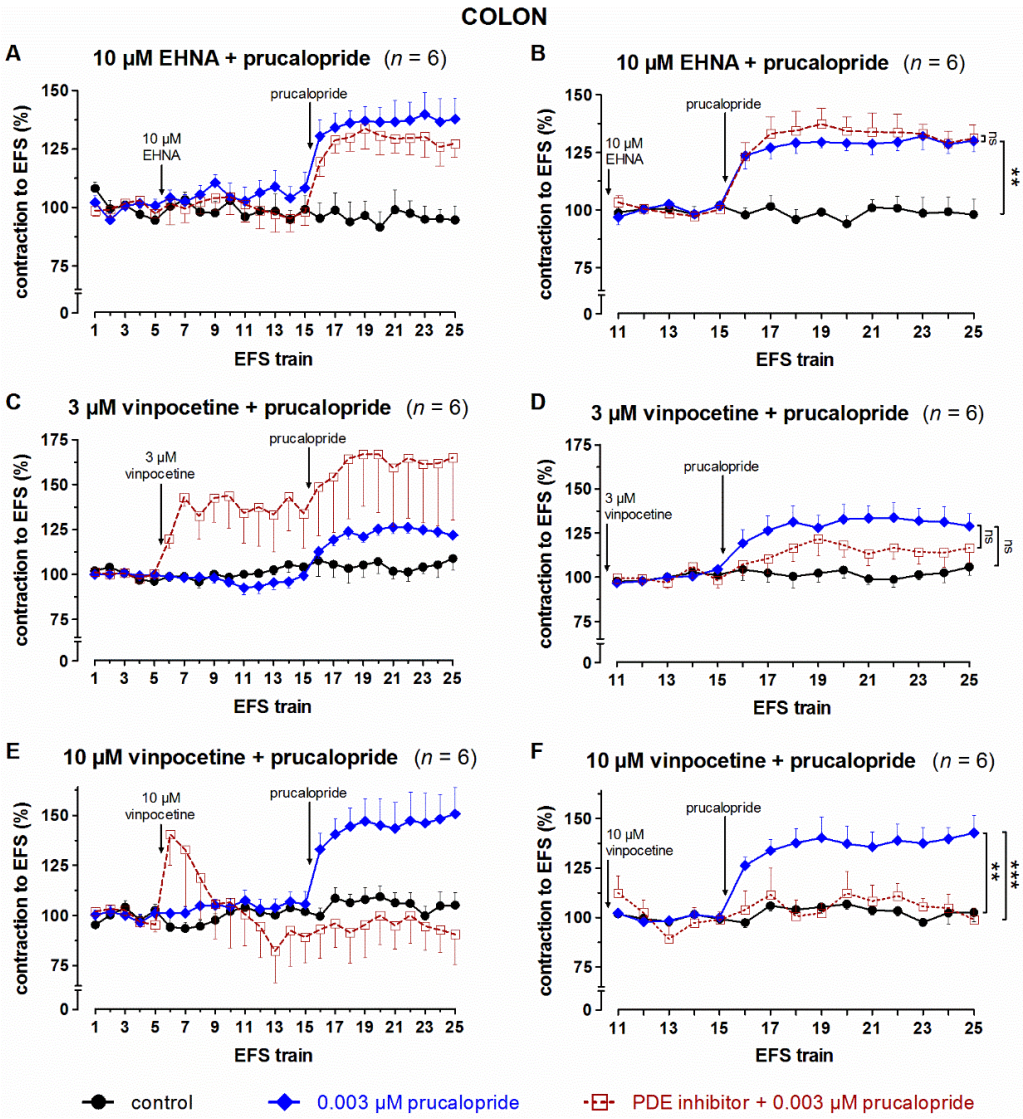


Figure VI.13 - Influence of 10 µM EHNA (A & B), 3 (C & D) and 10 (E & F) µM vinpocetine on the facilitating effect of 0.003 µM prucalopride on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 8 Hz, 0.5 ms, interval of 5 min) in murine colon circular smooth muscle strips. The number of the consecutive stimulation trains is given on the y-axis. Contractions are expressed as percentage of the mean of the five contractions before addition of the PDE inhibitor (trains 1-5; A, C & E) or of the five contractions in the presence of the PDE inhibitor just before adding prucalopride (trains 11-15; B, D & F). Experiments were performed in the continuous presence of 4 µM guanethidine, 300 µM L-NAME, and 1 µM MRS 2500. Means ± S.E.M. Left panels, last EFS-induced contraction before adding prucalopride: one-way ANOVA with Bonferroni corrected *t*-test for two comparisons i.e., PDE inhibitor versus control and versus prucalopride did not reach significance. Right panels, last EFS-induced contraction: ns not significant, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA with Bonferroni corrected *t*-test for two comparisons i.e., prucalopride versus control and versus prucalopride in the presence of the PDE inhibitor).

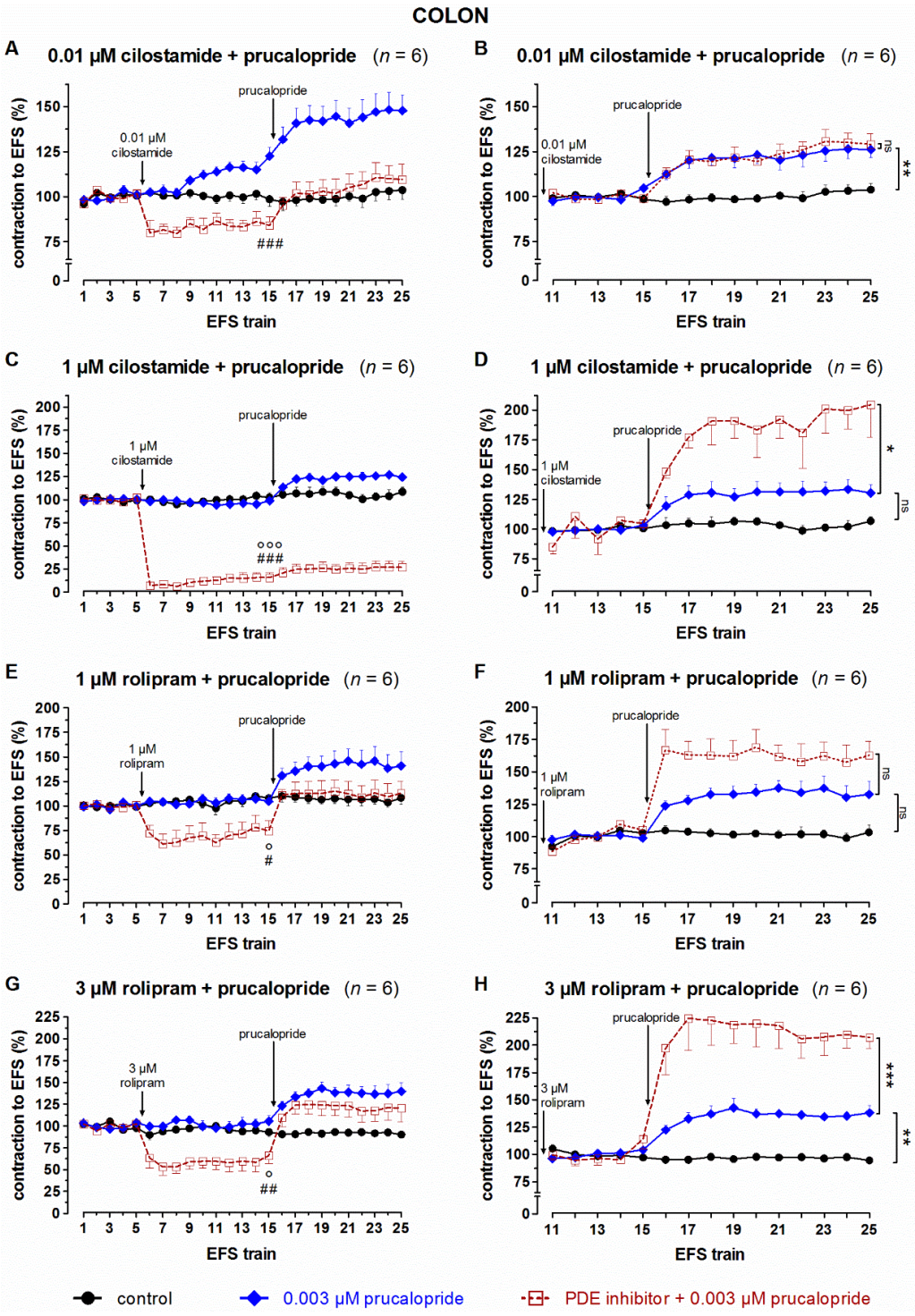


Figure VI.14 - Influence of 0.01 (A & B) and 1 (C & D) µM cilostamide, 1 (E & F) and 3 (G & H) µM rolipram on the facilitating effect of 0.003 µM prucalopride on submaximal electrically induced cholinergic contractions at $V_{50\%}$ (10 s trains at 8 Hz, 0.5 ms, interval of 5 min) in murine colon circular smooth muscle strips. The number of the consecutive stimulation trains is given on the y-axis. Contractions are expressed as percentage of the mean of the five contractions before addition of the PDE inhibitor (trains 1-5; A, C, E & G) or of the five contractions in the presence of the PDE inhibitor just before adding prucalopride (trains 11-15; B, D, F & H). Experiments were performed in the continuous presence of 4 µM guanethidine, 300 µM L-NAME, and 1 µM MRS 2500. Means ± S.E.M. Left panels, last EFS-induced contraction before adding prucalopride: ° $P < 0.05$, °°° $P < 0.001$ versus control and $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$ versus prucalopride (one-way ANOVA with Bonferroni corrected *t*-test for two comparisons i.e., PDE inhibitor versus control and versus prucalopride). Right panels, last EFS-induced contraction: ns not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA with Bonferroni corrected *t*-test for two comparisons i.e., prucalopride versus control and versus prucalopride in the presence of the PDE inhibitor).

VI.5 Discussion

The aim of this study was to investigate whether the intraneuronal pathway of 5-HT_4 receptors, enhancing cholinergic neurotransmission toward the circular muscle layer of murine gastric fundus, jejunum and colon is regulated by PDEs and possibly more particular by PDE4, in view of its role in the signal transduction pathway of facilitating 5-HT_4 receptors in circular muscle of porcine stomach and colon, and human large intestine (Priem *et al.*, 2012; Priem *et al.*, 2013; Lefebvre *et al.*, 2016; Pauwelyn *et al.*, 2018). 5-HT_4 receptors signal via cAMP; the classic PDEs 1, 2 and 3 are able to metabolize cAMP as well as cGMP, while PDE4 is cAMP specific (Maurice *et al.*, 2003; Lugnier, 2006). These PDEs were therefore assessed by use of selective inhibitors, in addition to the non-selective PDE inhibitor IBMX. It would be optimal to study the influence of PDE inhibition on basal and 5-HT_4 receptor-stimulated acetylcholine release by measuring acetylcholine release directly. But a set of preliminary experiments showed that our method, applied to measure tritiated acetylcholine release in porcine and human GI tissue (Lefebvre *et al.*, 2016; Pauwelyn *et al.*, 2018), was not able to pick up consistent values in murine GI tissues. When studying then the influence of PDE inhibitors in a functional model where electrically induced cholinergic muscle contractions are enhanced with a 5-HT_4 receptor agonist, one has to take in account their possible influence at the muscular level. If a given PDE is involved in the breakdown of cAMP (and/or of cGMP for PDE1, 2 and 3) in the murine GI smooth muscle cells, the corresponding inhibitor will induce relaxation by the increase of cAMP and/or cGMP as shown before in the GI tract of other species (Tomkinson and Raeburn, 1996; Jones *et al.*, 2002). The influence of IBMX and the selective PDE1, 2, 3 and 4 inhibitors was therefore first investigated on submaximal cholinergic activity, induced by exogenous carbachol or by endogenous acetylcholine released through electrical field stimulation.

VI.5.1 Influence of PDE inhibitors on carbachol- and EFS-induced submaximal contractions

In the three GI regions, the concentration range of IBMX (1 to 30 µM) inhibiting carbachol-induced submaximal contractions is in agreement with the IC_{50} values of IBMX reported for PDE1-4 (Table VI.2) suggesting the involvement of one or more PDE subtypes in the regulation of murine GI smooth muscle cell activity. To identify the PDE subtype(s) involved, the selective PDE inhibitors were investigated. The selective PDE3 inhibitor cilostamide and the selective PDE4 inhibitor rolipram concentration-dependently decreased the contractions over a large concentration range, the maximal decrease with rolipram being smaller than with cilostamide in the three GI regions. This suggests a role of PDE3 and PDE4 in the regulation of cyclic nucleotide content, with predominance of PDE3. As in contrast to PDE4, PDE3 is also able to metabolize to some extent cGMP in addition to cAMP (Maurice *et al.*, 2003; Lugnier, 2006), some increase in cGMP content might also play a role in the more pronounced inhibitory effect of cilostamide on carbachol-induced contractions than with rolipram. The PDE2 inhibitor EHNA and the PDE1 inhibitor vinpocetine only decreased carbachol-induced contractions in the highest or two highest concentrations suggesting either a very minor involvement of PDE1 and/or 2 in the control of muscular cyclic nucleotide content or a non-specific action at PDE3 and/or 4.

Similar effects were observed with the PDE inhibitors when tested on EFS-induced submaximal cholinergic contractions. However, in fundus and jejunum, but not in the colon, cilostamide and rolipram reduced EFS-induced contractions less extensively than carbachol-induced contractions; in the fundus rolipram even did not significantly decrease EFS-induced contractions. While carbachol-induced contraction is a purely postsynaptic phenomenon, presynaptic release of acetylcholine and postsynaptic acetylcholine-induced contraction are involved in EFS-induced contraction so that presynaptic influences of PDE inhibitors can play a role. cAMP-mediated facilitation of acetylcholine release was shown in equine airway cholinergic neurons (Zhang *et al.*, 1996) as well as in guinea pig small intestine (Reese and Cooper, 1984; Yau *et al.*, 1987), where increasing the amount of cAMP by adenylyl cyclase activation with forskolin or PDE inhibition with IBMX, as well as adding cAMP analogues stimulates the release of acetylcholine. If such a presynaptic effect of cilostamide and rolipram occurs in the electrically activated cholinergic neurons of murine fundus and jejunum, the resulting increase in acetylcholine release can be expected to counteract the postsynaptic relaxing effect; this could explain the more pronounced relaxing effects of cilostamide and rolipram on carbachol-induced contractions where presynaptic effects of PDE inhibitors cannot be relevant.

The observation that PDE3 is the main functionally important cyclic nucleotide degrading PDE in murine GI smooth muscle, with a supportive role for PDE4 is in agreement with our previous observations in porcine stomach where PDE3 and 4 have a redundant role, PDE3 being predominant (Priem *et al.*, 2012) and in porcine colon descendens, where PDE3 even seems the sole regulator (Priem *et al.*, 2013). But it contrasts to canine colonic smooth muscle, where PDE4 is functionally the most important PDE in the regulation of smooth muscle contraction with a less pronounced role of PDE3 (Barnette *et al.*, 1993) and the guinea-pig and rat ileum, where next to PDE3 and 4, also PDE5 and possibly PDE1 modulate GI smooth muscle contractility (Tomkinson and Raeburn, 1996).

VI.5.2 Influence of PDE inhibitors on the effect of prucalopride on EFS-induced submaximal cholinergic contractions

In order to be able to observe a possible facilitating influence of a PDE inhibitor on the facilitating effect of prucalopride, a submaximal concentration of the selective 5-HT_4 receptor agonist prucalopride was used. In our previous study in the murine GI tract (Pauwelyn and Lefebvre, 2017), cumulative concentration-response curves showed that 0.003 µM prucalopride produced less than 50% of the maximal facilitation of electrically induced submaximal cholinergic contractions. In the actual study, 0.003 µM prucalopride systematically enhanced EFS-induced submaximal cholinergic contractions in the murine fundus, jejunum and colon but with a quite variable degree of facilitation from series to series. Interseries variability in the facilitating effect of prucalopride was also seen in our previous study for a 10-fold higher concentration, 0.03 µM: 141 to 204% in fundus, 130 to 176% in jejunum and 124 to 174% in colon (Pauwelyn and Lefebvre, 2017). The highest degree of facilitation with 0.003 µM prucalopride in the actual study never reached the degree of facilitation observable with 0.03 µM, so that further enhancement of the facilitation was possible.

IBMX did not significantly enhance the facilitating effect of prucalopride, but in the fundus a trend for an increase by IBMX was observed. Also, as IBMX was tested versus prucalopride in concentrations reducing EFS-induced contractions at most by 20% (see VI.4.2), there cannot be excluded that IBMX in this concentration (3 µM in fundus, 0.3 µM in jejunum and 1 µM in colon) is insufficiently inhibiting a particular PDE that might be important in facilitating the effect of prucalopride. Reported IC_{50} values at specific PDEs indeed range from 0.8 to 23 µM (Table VI.2). Selective PDE inhibitors were therefore also studied; they were in principle tested in the highest concentration that per se did not influence EFS-induced contractions to avoid difficulties in interpreting results (when the PDE inhibitor clearly reduces EFS-induced contractions the action of prucalopride on these reduced contractions is not really comparable to the action of prucalopride on more pronounced control contractions). However when this concentration of the PDE inhibitor was low in comparison to reported IC_{50} values, also a higher concentration was tested even if decreasing the EFS-induced contractions in one or more of the GI regions.

The influence of the PDE inhibitors on the EFS-induced contractions upon single administration (see VI.4.3) was similar to the effect observed with this concentration when part of the cumulatively administered concentration series (see VI.4.2) with exception of 10 µM vinpocetine in fundus and colon. In the cumulative series, 10 µM vinpocetine did not influence and decreased the EFS-induced contractions in fundus and colon respectively, while single administration increased the contractions in fundus, and showed a biphasic effect, increase followed by decrease, in colon; we have no explanation for these effects.

Neither the PDE1 inhibitor vinpocetine (3 and 10 µM) nor the PDE2 inhibitor EHNA (10 µM) enhanced the effect of prucalopride, suggesting that neither PDE1 nor PDE2 is regulating the signal transduction of the facilitating 5-HT_4 receptors on cholinergic neurons contracting murine GI circular muscle. The PDE3 inhibitor cilostamide at 0.01 µM did not influence EFS-induced contractions in fundus and jejunum but moderately though significantly decreased them in colon; in this concentration that is lower than most reported IC_{50} values, cilostamide did not enhance the effect of prucalopride. When testing 1 µM, cilostamide significantly enhanced the effect of prucalopride in jejunum and colon, but the interpretation of these results is not reliable due to the very pronounced relaxing effect of the PDE inhibitor as such. The enhancement of the effect of prucalopride is apparent, related to prucalopride acting on contractions with very small amplitude in the presence of cilostamide in comparison to the amplitude of contractions in control tissues. The same is true for the results with 3 µM of the PDE4 inhibitor rolipram in the colon. These data in the murine GI tract of C57Bl/6J mice provide thus no evidence for a PDE-mediated control of the signaling pathway of 5-HT_4 receptors on cholinergic nerves innervating the circular smooth muscle layer. It cannot be excluded that a possible enhancing effect of PDE3 or PDE4 inhibition on the 5-HT_4 receptor-mediated facilitation of acetylcholine release is counteracted by the relaxing effect at the muscular level. But obviously, in C57Bl/6J murine GI circular muscle, neither cilostamide nor rolipram allows to obtain more pronounced electrically induced cholinergic contractions with prucalopride. This contrasts to the situation in porcine stomach and colon, and human large intestine where the PDE4 inhibitors rolipram and roflumilast enhance the effect of

prucalopride on electrically induced cholinergic contractions and/or acetylcholine release (Priem *et al.*, 2012; Priem *et al.*, 2013; Lefebvre *et al.*, 2016; Pauwelyn *et al.*, 2018) and points to important species differences. It cannot be excluded that the investigation of other mouse strains might lead to different results. But the actual study indicates that the murine C57Bl/6J model, where facilitation of GI cholinergic neurotransmission by 5-HT_4 receptor agonists is documented, is not suitable for *in vivo* testing of the possible synergistic gastroprokinetic effects of a 5-HT_4 receptor agonist combined with a selective PDE4 inhibitor.

VI.6 Conclusion

In agreement with the porcine GI tract, PDE3 is the main regulator of the cyclic nucleotide turnover in fundus, jejunum and colon circular smooth muscle of C57Bl/6J mice, with contribution of PDE4. Although 5-HT_4 receptors are present on cholinergic neurons innervating the circular smooth muscle layer in fundus, jejunum and colon, the facilitation of electrically induced cholinergic contractions by stimulation of these 5-HT_4 receptors cannot be enhanced by specific PDE inhibition in contrast to the porcine GI tract. This means that the murine C57Bl/6J model is unsuitable for *in vivo* testing of the possible synergistic gastroprokinetic effects of a 5-HT_4 receptor agonist combined with a selective PDE inhibitor.

VI.7 Ethics statement

This study was carried out in accordance with the recommendations of the most recent national legislation (Belgian Royal Decree, 29/05/2013) and European Directive (Directive 2010/63/EU). The protocol was approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University (ECD 14/22).

VI.8 Acknowledgments

The authors thank Mrs. Els Van Deynse for technical support.

VI.9 References

Afzal F., Andressen K.W., Mork H.K., Aronsen J.M., Sjaastad I., Dahl C.P., Skomedal T., Levy F.O., Osnes J.B., and Qvigstad E. (2008). 5-HT4-elicited positive inotropic response is mediated by cAMP and regulated by PDE3 in failing rat and human cardiac ventricles. *Br J Pharmacol* 155, 1005-1014. DOI: 10.1038/bjp.2008.339

Barnette M.S., Manning C.D., Price W.J., and Barone F.C. (1993). Initial biochemical and functional characterization of cyclic nucleotide phosphodiesterase isozymes in canine colonic smooth muscle. *J Pharmacol Exp Ther* 264, 801-812.

Bolger G., Michaeli T., Martins T., St John T., Steiner B., Rodgers L., Riggs M., Wigler M., and Ferguson K. (1993). A family of human phosphodiesterases homologous to the dunce learning and memory gene product of Drosophila melanogaster are potential targets for antidepressant drugs. *Mol Cell Biol* 13, 6558-6571.

Bolger G.B., Erdogan S., Jones R.E., Loughney K., Scotland G., Hoffmann R., Wilkinson I., Farrell C., and Houslay M.D. (1997). Characterization of five different proteins produced by alternatively spliced mRNAs from the human cAMP-specific phosphodiesterase PDE4D gene. *Biochem J* 328, 539-548. DOI: 10.1042/bj3280539

Brattelid T., Qvigstad E., Lynham J.A., Molenaar P., Aass H., Geiran O., Skomedal T., Osnes J.B., Levy F.O., and Kaumann A.J. (2004). Functional serotonin 5-HT4 receptors in porcine and human ventricular myocardium with increased 5-HT4 mRNA in heart failure. *Naunyn Schmiedebergs Arch Pharmacol* 370, 157-166. DOI: 10.1007/s00210-004-0963-0

Cellek S., John A.K., Thangiah R., Dass N.B., Bassil A.K., Jarvie E.M., Lalude O., Vivekanandan S., and Sanger G.J. (2006). 5-HT4 receptor agonists enhance both cholinergic and nitrergic activities in human isolated colon circular muscle. *Neurogastroenterol Motil* 18, 853-861. DOI: 10.1111/j.1365-2982.2006.00810.x

Cellek S., Thangiah R., Jarvie E.M., Vivekanandan S., Lalude O., and Sanger G.J. (2008). Synergy between 5-HT4 receptor activation and acetylcholinesterase inhibition in human colon and rat forestomach. *Neurogastroenterol Motil* 20, 539-545. DOI: 10.1111/j.1365-2982.2007.01062.x

Coste H., and Grondin P. (1995). Characterization of a Novel Potent and Specific Inhibitor of Type-V Phosphodiesterase. *Biochem Pharmacol* 50, 1577-1585. DOI: 10.1016/0006-2952(95)02031-4

Craig D.A., and Clarke D.E. (1990). Pharmacological characterization of a neuronal receptor for 5-hydroxytryptamine in guinea-pig ileum with properties similar to the 5-hydroxytryptamine-4 receptor. *J Pharmacol Exp Ther* 252, 1378-1386.

De Maeyer J.H., Straetemans R., Schuurkes J.a.J., and Lefebvre R.A. (2006). Porcine left atrial and sinoatrial 5-HT4 receptor-induced responses: fading of the response and influence of development. *Br J Pharmacol* 147, 140-157. DOI: 10.1038/sj.bjp.0706497

Galindo-Tovar A., Vargas M.L., Escudero E., and Kaumann A.J. (2009). Ontogenic changes of the control by phosphodiesterase-3 and -4 of 5-HT responses in porcine heart and relevance to human atrial 5-HT4 receptors. *Br J Pharmacol* 156, 237-249. DOI: 10.1111/j.1476-5381.2008.00023.x

Galligan J.J., and Vanner S. (2005). Basic and clinical pharmacology of new motility promoting agents. *Neurogastroenterol Motil* 17, 643-653. DOI: 10.1111/j.1365-2982.2005.00675.x

Gershon M.D., and Tack J. (2007). The serotonin signaling system: From basic understanding to drug development-for functional GI disorders. *Gastroenterology* 132, 397-414. DOI: 10.1053/j.gastro.2006.11.002

Jones O.M., Brading A.F., and J. M.M.N. (2002). Phosphodiesterase inhibitors cause relaxation of the internal anal sphincter in vitro. *Dis Colon Rectum* 45, 530-536. DOI: 10.1007/s10350-004-6235-8

Kaneda T., Shimizu K., Urakawa N., and Nakajyo S. (2004). Effects of various selective phosphodiesterase inhibitors on carbachol-induced contraction and cyclic nucleotide contents in guinea pig taenia coli. *J Vet Med Sci* 66, 1047-1052. DOI: 10.1292/jvms.66.1047

Leclere P.G., and Lefebvre R.A. (2002). Presynaptic modulation of cholinergic neurotransmission in the human proximal stomach. *Br J Pharmacol* 135, 135-142. DOI: 10.1038/sj.bjp.0704471

Leclere P.G., Prins N.H., Schuurkes J.a.J., and Lefebvre R.A. (2005). 5-HT4 receptors located on cholinergic nerves in human colon circular muscle. *Neurogastroenterol Motil* 17, 366-375. DOI: 10.1111/j.1365-2982.2005.00621.x

Lefebvre R.A., Van Colen I., Pauwelyn V., and De Maeyer J.H. (2016). Synergistic effect between 5-HT4 receptor agonist and phosphodiesterase 4-inhibitor in releasing acetylcholine in pig gastric circular muscle in vitro. *Eur J Pharmacol* 781, 76-82. DOI: 10.1016/j.ejphar.2016.04.003

Lugnier C. (2006). Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. *Pharmacol Ther* 109, 366-398. DOI: 10.1016/j.pharmthera.2005.07.003

Maurice D.H., Palmer D., Tilley D.G., Dunkerley H.A., Netherton S.J., Raymond D.R., Elbatarny H.S., and Jimmo S.L. (2003). Cyclic nucleotide phosphodiesterase activity, expression, and targeting in cells of the cardiovascular system. *Mol Pharmacol* 64, 533-546. DOI: 10.1124/mol.64.3.533

Michie A.M., Lobban M., Muller T., Harnett M.M., and Houslay M.D. (1996). Rapid regulation of PDE-2 and PDE-4 cyclic AMP phosphodiesterase activity following ligation of the T cell antigen receptor on thymocytes: analysis using the selective inhibitors erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) and rolipram. *Cell Signal* 8, 97-110. DOI: 10.1016/0898-6568(95)02032-2

O'grady S.M., Jiang X., Maniak P.J., Birmachu W., Scribner L.R., Bulbulian B., and Gullikson G.W. (2002). Cyclic AMP-dependent Cl secretion is regulated by multiple phosphodiesterase subtypes in human colonic epithelial cells. *J Membrane Biol* 185, 137-144. DOI 10.1007/s00232-001-0120-3

Parker S.G., Taylor E.M., Hamburger S.A., Vimal M., and Kaumann A.J. (1995). Blockade of human and porcine myocardial 5-HT4 receptors by SB 203186. *Naunyn Schmiedebergs Arch Pharmacol* 353, 28-35. DOI: 10.1007/BF00168912

Pauwelyn V., and Lefebvre R.A. (2017). 5-HT4 receptors facilitate cholinergic neurotransmission throughout the murine gastrointestinal tract. *Neurogastroenterol Motil* 00:e13064. DOI: 10.1111/nmo.13064

Pauwelyn V., Ceelen W., and Lefebvre R.A. (2018). Synergy between 5-HT4 receptor stimulation and phosphodiesterase 4 inhibition in facilitating acetylcholine release in human large intestinal circular muscle. *Neurogastroenterol Motil* 30, e13162. DOI: 10.1111/nmo.13162

Podzuweit T., Nennstiel P., and Muller A. (1995). Isozyme selective inhibition of cGMP-stimulated cyclic nucleotide phosphodiesterases by erythro-9-(2-hydroxy-3-nonyl) adenine. *Cell Signal* 7, 733-738. DOI: 10.1016/0898-6568(95)00042-N

Priem E.K.V., and Lefebvre R.A. (2011). Investigation of neurogenic excitatory and inhibitory motor responses and their control by 5-HT4 receptors in circular smooth muscle of pig descending colon. *Eur J Pharmacol* 667, 365-374. DOI: 10.1016/j.ejphar.2011.06.021

Priem E.K.V., Van Colen I., De Maeyer J.H., and Lefebvre R.A. (2012). The facilitating effect of prucalopride on cholinergic neurotransmission in pig gastric circular muscle is regulated by phosphodiesterase 4. *Neuropharmacology* 62, 2126-2135. DOI: 10.1016/j.neuropharm.2011.12.020

Priem E.K.V., De Maeyer J.H., and Lefebvre R.A. (2013). Influence of phosphodiesterases on basal and 5-HT4 receptor facilitated cholinergic contractility in pig descending colon. *Eur J Pharmacol* 705, 156-163. DOI: 10.1016/j.ejphar.2013.02.011

Prins N.H., Akkermans L.M.A., Lefebvre R.A., and Schuurkes J.a.J. (2000). 5-HT4 receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. *Br J Pharmacol* 131, 927-932. DOI: 10.1038/sj.bjp.0703615

Prins N.H., Van Der Grijn A., Lefebvre R.A., Akkermans L.M.A., and Schuurkes J.a.J. (2001). 5-HT4 receptors mediating enhancement of contractility in canine stomach; an in vitro and in vivo study. *Br J Pharmacol* 132, 1941-1947. DOI: 10.1038/sj.bjp.0703985

Raymond J.R., Mukhin Y.V., Gelasco A., Turner J., Collinsworth G., Gettys T.W., Grewal J.S., and Garnovskaya M.N. (2001). Multiplicity of mechanisms of serotonin receptor signal transduction. *Pharmacol Ther* 92, 179-212. DOI: 10.1016/S0163-7258(01)00169-3

Reese J.H., and Cooper J.R. (1984). Stimulation of Acetylcholine-Release from Guinea-Pig Ileal Synaptosomes by Cyclic-Nucleotides and Forskolin. *Biochem Pharmacol* 33, 3007-3011. DOI: 10.1016/0006-2952(84)90601-4

Saeki T., and Saito I. (1993). Isolation of cyclic nucleotide phosphodiesterase isozymes from pig aorta. *Biochem Pharmacol* 46, 833-839.

Schwabe U., Miyake M., Ohga Y., and Daly J.W. (1976). 4-(3-Cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (ZK 62711): a potent inhibitor of adenosine cyclic 3',5'-monophosphate phosphodiesterases in homogenates and tissue slices from rat brain. *Mol Pharmacol* 12, 900-910.

Sudo T., Tachibana K., Toga K., Tochizawa S., Inoue Y., Kimura Y., and Hidaka H. (2000). Potent effects of novel anti-platelet aggregatory cilostamide analogues on recombinant cyclic nucleotide phosphodiesterase isozyme activity. *Biochem Pharmacol* 59, 347-356.

Tomkinson A., and Raeburn D. (1996). The effect of isoenzyme-selective PDE inhibitors on methacholine-induced contraction of guinea-pig and rat ileum. *Br J Pharmacol* 118, 2131-2139. DOI: 10.1111/j.1476-5381.1996.tb15653.x

Tonini M., Candura S.M., Onori L., Coccini T., Manzo L., and Rizzi C.A. (1992). 5-Hydroxytryptamine4 receptor agonists facilitate cholinergic transmission in the circular muscle of guinea-pig ileum: antagonism by tropisetron and DAU-6285. *Life Sci* 50, Pl173-Pl178. DOI: 10.1016/0024-3205(92)90453-V

Torphy T.J., and Cieslinski L.B. (1990). Characterization and Selective-Inhibition of Cyclic-Nucleotide Phosphodiesterase Isozymes in Canine Tracheal Smooth-Muscle. *Mol Pharmacol* 37, 206-214.

Wang P., Myers J.G., Wu P., Cheewatrakoolpong B., Egan R.W., and Billah M.M. (1997). Expression, purification, and characterization of human cAMP-specific phosphodiesterase (PDE4) subtypes A, B, C, and D. *Biochem Biophys Res Commun* 234, 320-324. DOI: 10.1006/bbrc.1997.6636

Weninger S., De Maeyer J.H., and Lefebvre R.A. (2012). Study of the regulation of the inotropic response to 5-HT4 receptor activation via phosphodiesterases and its cross-talk with C-type natriuretic peptide in porcine left atrium. *Naunyn Schmiedebergs Arch Pharmacol* 385, 565-577. DOI: 10.1007/s00210-012-0746-y

Yau W.M., Dorsett J.A., and Youther M.L. (1987). Stimulation of acetylcholine-release from myenteric neurons of guinea-pig small-intestine by forskolin and cyclic AMP. *J Pharmacol Exp Ther* 243, 507-510.

Zacher L.A., and Carey G.B. (1999). Cyclic AMP metabolism by swine adipocyte microsomal and plasma membranes. *Comp Biochem Physiol B Biochem Mol Biol* 124, 61-71. DOI: 10.1016/S0305-0491(99)00098-X

Zhang X.Y., Robinson N.E., and Zhu F.X. (1996). Potentiation of acetylcholine release from tracheal parasympathetic nerves by cAMP. *Am J Physiol Lung Cell Mol Physiol* 270, L541-L546. DOI: 10.1152/ajplung.1996.270.4.L541

CHAPTER VII

General discussion and future perspectives

CHAPTER VII - General discussion and future perspectives

Although 5-HT is best known for its role in the brain, 95% of 5-HT in the human body is found in the GI tract, mainly synthetized in EC cells and to a small extent in serotoninergic neurons of the myenteric plexus (Gershon and Tack, 2007), where it is among other involved in GI motility (Gershon, 2013; Kendig and Grider, 2015). In several GI disorders, such as constipation, irritable bowel syndrome, gastro-esophageal reflux, gastroparesis and functional dyspepsia, GI dysmotility is one of the underlying factors. Gastroprokinetic drugs, stimulating GI motility, were therefore developed by the pharmaceutical industry. One class of gastroprokinetics are the 5-HT_4 receptor agonists. 5-HT_4 receptors are GPCRs, signaling by generation of cAMP via activation of adenylyl cyclase. They are present in the brain, heart, adrenal cortex and GI tract of man. In the human GI tract, three locations of 5-HT_4 receptors influencing motility have been described:

1. on smooth muscle cells, inducing relaxation (McLean and Coupar, 1996; Prins *et al.*, 2000b);
2. on inhibitory nitrergic neurons, inducing nitric oxide release counteracting contraction (Cellek *et al.*, 2006);
3. on myenteric excitatory cholinergic neurons toward the smooth muscle layer, enhancing acetylcholine release and smooth muscle contraction. This location is by far most extensively established and was clearly shown *in vitro* in human stomach (Leclere and Lefebvre, 2002) and colon (Prins *et al.*, 2000a; Leclere *et al.*, 2005; Cellek *et al.*, 2006; Cellek *et al.*, 2008). Stimulation of these 5-HT_4 receptors on cholinergic neurons is thought to be the underlying mechanism for the gastroprokinetic properties of 5-HT_4 receptor agonists.

Cisapride, which is an agonist at 5-HT_4 receptors but an antagonist at 5-HT_2 and 5-HT_3 receptors (De Maeyer *et al.*, 2008), was frequently used for gastro-esophageal reflux, gastroparesis and some forms of constipation in the nineties. However it was withdrawn from the market in 2000 because of serious cardiac arrhythmias (Barbey *et al.*, 2002), caused by interaction with hERG potassium channels (Mohammad *et al.*, 1997). Tegaserod, approved for constipation-predominant irritable bowel syndrome in 2002 was withdrawn in 2007 because of an increased risk for atherosclerotic events (Pasricha, 2007). Tegaserod is a 5-HT_4 receptor agonist, but also an agonist and antagonist at respectively 5-HT_1 and 5-HT_2 receptors (De Maeyer *et al.*, 2008). The mechanism of the cardiovascular events with tegaserod were not definitely established; its interaction with 5-HT_1 and/or 5-HT_2 receptors might be involved (Beattie and Smith, 2008; De Maeyer *et al.*, 2008). Although the cardiovascular side effects of cisapride and tegaserod were not related to 5-HT_4 receptor agonism, these withdrawals slowed down the further clinical development of the highly selective 5-HT_4 receptor agonist prucalopride. Prucalopride had been shown to accelerate gastric emptying, stimulate colonic transit, increase stool frequency and decrease stool consistency in healthy volunteers (Emmanuel *et al.*, 1998; Bouras *et al.*, 1999; Poen *et al.*, 1999; Kessing *et al.*, 2014) as well as in patients with constipation (Bouras *et al.*, 2001; Emmanuel *et al.*, 2002; Sloots *et al.*, 2002; Coremans *et al.*, 2003). It was also known to induce positive chronotropic and inotropic effects in porcine and human atria via atrial 5-HT_4 receptors (Krobert *et al.*, 2005). Subsequent

research showed that the transient and weak amplitude of the inotropic responses was due to the tight control of cardiac 5-HT_4 receptors by PDEs (De Maeyer *et al.*, 2006b): in porcine heart by a redundant action of PDE3 plus PDE4 (Galindo-Tovar *et al.*, 2009; Weninger *et al.*, 2012) and in human heart by PDE3 (Afzal *et al.*, 2008). These PDEs quickly degrade cAMP induced by stimulation of cardiac 5-HT_4 receptors to inactive 5'-AMP, thus limiting the response. In contrast to the transient effect of prucalopride at the level of the heart, the facilitation of cholinergic neurotransmission with prucalopride in the GI tract is pronounced and sustained (Prins *et al.*, 2000a; De Maeyer *et al.*, 2006a; Broad *et al.*, 2014a; Broad *et al.*, 2014b). Still this effect could be enhanced by selective PDE4 inhibition in porcine stomach (Priem *et al.*, 2012) and colon (Priem *et al.*, 2013), which suggests a PDE4-mediated control of the signaling pathway of 5-HT_4 receptors in myenteric cholinergic neurons.

Prucalopride was marketed for chronic idiopathic constipation in 2009. Subsequent surveys of its use in patients reported it to be cardiovascularly safe (Manabe *et al.*, 2010; Tack *et al.*, 2012; Keating, 2013). Still some concern for possible cardiac effects of prokinetic compounds remains (Giudicessi *et al.*, 2018). The Commented Drug Repertory 2017 of the Belgian Centre for Pharmacotherapeutic Information reports under Special Precautions: "At this moment there are no indications that prucalopride causes prolongation of the QT-interval or torsades de pointes. Because of the chemical similarity with cisapride, known to induce these disturbances of cardiac rhythm, caution remains recommended in older patients with polypharmacy and other patients with risk factors for torsades de pointes".

A combination therapy, allowing to lower the dose and thus the plasma concentrations of prucalopride while the prokinetic effect is maintained, would still further reduce the risk of cardiac adverse events. A first possibility might be to combine a 5-HT_4 receptor agonist and an acetylcholinesterase inhibitor. Indeed, combination of tegaserod with the acetylcholinesterase inhibitor neostigmine had a synergistic effect on *in vivo* colonic transit time in rat (Campbell-Dittmeyer *et al.*, 2009). Also *in vitro* in human colon, synergy between prucalopride and an acetylcholinesterase inhibitor, either neostigmine (Cellek *et al.*, 2008) or donepezil (Broad *et al.*, 2013), for facilitation of electrically induced cholinergic activity was reported.

The observation that the selective PDE4 inhibitor rolipram *in vitro* enhances the facilitating effect of submaximal concentrations of prucalopride on electrically induced cholinergic contractions in porcine stomach and colon (Priem *et al.*, 2012; Priem *et al.*, 2013) opens the possibility of combining a 5-HT_4 receptor agonist with a selective PDE4 inhibitor. The aim of this thesis was to further investigate this possibility:

- by corroborating the control by PDE4 of the signaling pathway of 5-HT_4 receptors on cholinergic neurons in the porcine GI tract, studying other compounds than prucalopride and rolipram;
- by studying whether this controlling mechanism is also present in the human GI tract;
- by investigating whether the mouse, an easy and accessible laboratory animal, might be a suitable model to test the combination of a 5-HT_4 receptor agonist with a selective PDE4 inhibitor for GI prokinetic activity *in vivo*. First, the presence of 5-HT_4 receptors on murine myenteric cholinergic neurons was documented. As facilitating 5-HT_4 receptors were found throughout the

murine GI tract, the influence of PDE inhibitors on GI contractions and on the effect of 5-HT_4 receptor stimulation was investigated.

VII.1 Confirmation of PDE4-mediated control of 5-HT_4 receptors in porcine stomach: synergy between 5-HT_4 receptor agonist and PDE4 inhibitor (CHAPTER III)

Previous evidence suggested the presence of 5-HT_4 receptors on cholinergic neurons toward circular muscle in the porcine stomach. Stimulation with the selective 5-HT_4 receptor agonist prucalopride enhanced electrically induced cholinergic contractions and this effect was antagonized by the 5-HT_4 receptor antagonist GR 113808 (Priem *et al.*, 2012). The facilitation with a submaximal concentration of prucalopride was enhanced by the selective PDE4 inhibitor rolipram, while selective PDE1, 2 and 3 inhibitors had no influence (Priem *et al.*, 2012). This suggests a PDE4-mediated control of the intraneuronal signal transduction pathway of 5-HT_4 receptors present on myenteric cholinergic neurons in porcine stomach. Again by studying electrically induced cholinergic contractions, evidence for this controlling mechanism was also obtained in porcine colon descendens (Priem *et al.*, 2013). For the porcine stomach, the mechanism was confirmed by measuring acetylcholine release directly. Prucalopride concentration-dependently increased electrically induced acetylcholine release in a GR 113808-sensitive way; the effect of a minimally effective concentration of prucalopride was enhanced by rolipram in a synergistic way (Priem *et al.*, 2012). The technique with direct measurement of acetylcholine release from cholinergic neurons allows to fully exclude the possible interference of the PDE inhibitor under study at muscular level, which can occur when measuring electrically induced cholinergic contractions. As our group had optimized and used the technique of acetylcholine measurement for porcine stomach, this paradigm was now also used for an extension study with another 5-HT_4 receptor agonist than prucalopride and another PDE4 inhibitor than rolipram. The additional PDE4 inhibitor used was roflumilast, marketed to suppress exacerbations in patients with COPD (Rabe, 2011; Wan *et al.*, 2015). Velusetrag was used as another highly selective 5-HT_4 receptor agonist; it belongs to another chemical class than prucalopride (Smith *et al.*, 2008; Long *et al.*, 2012a; Long *et al.*, 2012b) and is actually under clinical development (phase 2 completed) for constipation (Goldberg *et al.*, 2010; Manini *et al.*, 2010) and gastroparesis (Ahn *et al.*, 2015).

Roflumilast per se induced an increase of electrically induced acetylcholine release as previously found with rolipram per se, suggesting that the basal content of cAMP in porcine gastric cholinergic neurons is constitutively regulated by PDE4. Combination of roflumilast with a submaximal concentration of prucalopride had a synergistic effect, as roflumilast enhanced the prucalopride-evoked acetylcholine release to a higher level than the sum of their individual effects. Like prucalopride, velusetrag concentration-dependently increased electrically induced acetylcholine release. However the maximal effect of velusetrag observed with the highest concentration tested, was less pronounced than with maximally effective concentrations of prucalopride. This might correlate with its lower maximal agonist effect compared with prucalopride reported for the guinea pig colon (Beattie *et al.*, 2011). Mild facilitation of electrically induced acetylcholine release with a low concentration of velusetrag was enhanced by rolipram, again in a synergistic way. The effects of roflumilast plus prucalopride, and of

rolipram plus velusetrag, corroborated that PDE4 regulates the intraneuronal transduction pathway of facilitatory 5-HT_4 receptors in myenteric cholinergic neurons in porcine stomach. The next step was therefore to study whether this controlling mechanism could also be demonstrated in the human GI tract.

VII.2 PDE4-mediated control of 5-HT_4 receptors in human large intestine: synergy between 5-HT_4 receptor agonist and PDE4 inhibitor (CHAPTER IV)

The presence of 5-HT_4 receptors on cholinergic neurons innervating smooth muscle, was already shown in human stomach circular muscle (Leclere and Lefebvre, 2002), and colon circular (Leclere *et al.*, 2005; Cellek *et al.*, 2006; Cellek *et al.*, 2008) as well as longitudinal muscle (Prins *et al.*, 2000a). Electrically induced cholinergic contractions were facilitated by 5-HT_4 receptor agonism, which is prevented by the 5-HT_4 receptor antagonists SB204070 and GR 113808, confirming interaction with 5-HT_4 receptors. However, a PDE4-mediated control of the intraneuronal signaling pathway of these receptors, as shown in porcine stomach (Priem *et al.*, 2012) and colon (Priem *et al.*, 2013) with rolipram and prucalopride and now also confirmed with roflumilast and velusetrag (Lefebvre *et al.*, 2016), was not yet investigated in man. Confirmation of this in man is required to further consider the possible usefulness of combining a 5-HT_4 receptor agonist and a PDE4 inhibitor for gastroprokinetic purposes. Our group has previously studied prucalopride, using the technique directly measuring electrically induced acetylcholine release, to establish the presence of 5-HT_4 receptors on myenteric cholinergic neurons toward circular muscle in both human stomach (Leclere and Lefebvre, 2002) and colon (Leclere *et al.*, 2005). As this technique excludes muscular interference of PDE inhibitors, it was now used again to study the influence of PDE inhibitors on the facilitating effect of prucalopride on electrically induced acetylcholine release in human large intestine circular smooth muscle. The human large intestine was selected as prucalopride is currently marketed for chronic constipation.

Prucalopride was used in a concentration only leading to a small (non-significant) increase of electrically induced acetylcholine release, to leave room for further enhancement by PDE inhibition. When combined with the non-selective PDE inhibitor IBMX, that as such had no influence on electrically induced acetylcholine release, the prucalopride-induced facilitation was significantly enhanced to a pronounced level suggesting a PDE-mediated control of the 5-HT_4 receptor. As cAMP is the cyclic nucleotide involved in the signaling pathway of 5-HT_4 receptors, the classic cAMP degrading PDE subtypes (PDE1 to 4) were investigated with the selective PDE inhibitors vinpocetine (PDE1), EHNA (PDE2), cilostamide (PDE3), and rolipram and roflumilast (PDE4) to identify the responsible PDE subtype. Rolipram or roflumilast per se did not influence the acetylcholine release, thus no evidence for PDE4-mediated regulation of the basal cAMP content in the myenteric cholinergic neurons of the human large intestine was obtained, in contrast to the porcine stomach (Priem *et al.*, 2012; Lefebvre *et al.*, 2016). Combination of prucalopride with either rolipram or roflumilast enhanced the effect of prucalopride to the same magnitude as observed with IBMX, while vinpocetine, EHNA and cilostamide did not influence it. This suggests that, similar to the porcine GI tract, PDE4 controls facilitating 5-HT_4 receptors on cholinergic neurons in the human large intestine; this might be an explanation for the poor coupling of

5-HT_4 receptors to acetylcholine release as previously suggested in a functional study in human colon (Cellek *et al.*, 2008).

The PDE4-mediated control of facilitating 5-HT_4 receptors on cholinergic neurons in the human large intestine supports the idea of combining a 5-HT_4 receptor agonist with a PDE4 inhibitor to obtain pronounced prokinetic effects with a lower dose of the 5-HT_4 receptor agonist. One should consider other locations of 5-HT_4 receptors and PDE4:

- The effect of the 5-HT_4 receptor agonist at cardiac 5-HT_4 receptors in man will not be influenced by the concomitant treatment with a PDE4 inhibitor as cardiac 5-HT_4 receptors are solely controlled by PDE3 in the human heart (Afzal *et al.*, 2008).
- PDEs contribute to the regulation of the basal cAMP content in smooth muscle cells. If the involved PDE subtype in human large intestine is PDE4, PDE4 inhibition might increase the cAMP content leading to smooth muscle relaxation (Barnette *et al.*, 1993; Kaneda *et al.*, 2004), which could counteract the cholinergic facilitation at the neuronal level. In porcine colon circular muscle, PDE3 is the predominant PDE (Priem *et al.*, 2013). In human rectum circular muscle a role for PDE1 to 4 was suggested (Jones *et al.*, 2002); in human colon circular muscle a reduction of the basal tone was observed with IBMX and rolipram, with rolipram having a smaller maximal effect (McLean and Coupar, 1996). These results suggest that PDE4 is not the sole PDE involved in cAMP regulation at the muscular level, in contrast to its predominant role in the control of 5-HT_4 receptors at the neuronal level. To clarify in detail the muscular role of PDE subtypes in human large intestine, further investigation is needed.
- In human colon, relaxant 5-HT_4 receptors have been described on circular smooth muscle (McLean and Coupar, 1996; Prins *et al.*, 2000b). In addition, PDE4 inhibition with rolipram facilitated the relaxant effect of 5-HT via these receptors (McLean and Coupar, 1996). The possible contribution of relaxant 5-HT_4 receptors on GI smooth muscle to the clinical effect of 5-HT_4 receptor agonists, that are *in vivo* clear-cut prokinetic, is not well understood. The prokinetic effects of 5-HT_4 receptor agonists might depend on a fine balance between their contractile and relaxing effects; facilitation of both effects by a PDE4 inhibitor might then also be beneficial. Still the presence of relaxing 5-HT_4 receptors on smooth muscle could not be systematically confirmed in human colon (Cellek *et al.*, 2006); these receptors could also not be demonstrated in porcine colon (Priem and Lefebvre, 2011).

Thus the *in vitro* data in human large intestine support the possible usefulness of the combination therapy for GI motility disorders. To further develop this idea, the synergistic GI prokinetic effect of a 5-HT_4 receptor agonist and a PDE4 inhibitor should be confirmed *in vivo*. A good animal model for *in vivo* testing is thus required. Therefore the murine GI tract was investigated *in vitro* to know whether the mouse might be a suitable *in vivo* model.

VII.3 Evaluation of the mouse as possible model for *in vivo* testing of the prokinetic effect of combining a 5-HT$_4$ receptor agonist with a PDE4 inhibitor (CHAPTER V and VI)

The pig is considered a good model for GI functions in view of the similar morphology and physiology of the GI tract. Although relaxing 5-HT$_4$ receptors, that are suggested to be present on smooth muscle and nitrergic neurons in human large intestine (McLean and Coupar, 1996; Cellek *et al.*, 2006), could not be found in the pig, the pig is a good model for gastric and colonic 5-HT$_4$ receptors located on cholinergic neurons (Priem and Lefebvre, 2011) and their PDE4-mediated control (Priem *et al.*, 2012; Priem *et al.*, 2013; Lefebvre *et al.*, 2016). However, this species is expensive, relatively difficult to handle and techniques to study GI transit *in vivo* in pigs are rare. The mouse is a classic, easier to handle and easily accessible laboratory animal with regard to *in vivo* investigations. Hence, the presence of 5-HT$_4$ receptors on cholinergic neurons toward murine GI circular smooth muscle and their possible PDE-mediated control, were investigated.

At RNA and protein level 5-HT$_4$ receptors were shown in the submucosal and myenteric plexus in the murine GI tract (Liu *et al.*, 2005) and some evidence exists for 5-HT$_4$ receptors on cholinergic neurons toward macrophages mediating anti-inflammatory effects upon stimulation with prucalopride (Gomez-Pinilla *et al.*, 2014). *In vivo* data support an influence of 5-HT$_4$ receptors on GI motility in mice. On the one hand, 5-HT$_4$ receptor knockout mice exhibit delayed gastric emptying and small intestinal transit when compared to wild-type mice (Liu *et al.*, 2009). On the other hand, 5-HT$_4$ receptor agonists have GI prokinetic effects in mice as RS67506 shortens whole gut transit (Nagakura *et al.*, 1997) and DA-6886 shortens colonic transit in normal as well as in constipated (loperamide-induced) mice (Lee *et al.*, 2014). 5-HT was shown to activate myenteric cholinergic neurons and to induce defecation, both effects being absent when 5-HT$_4$ receptors are selectively antagonized (Wang *et al.*, 2007). In gastric antrum muscle rings from healthy and diabetic mice, tegaserod enhanced electrically induced primarily cholinergic contractions (James *et al.*, 2004). A systematic study whether 5-HT$_4$ receptor agonists are able to enhance acetylcholine release from cholinergic neurons innervating the smooth muscle in the murine GI tract, using the paradigm of electrically induced cholinergic contractions, as used in several species including man and pig, was not yet performed.

VII.3.1 Presence of 5-HT$_4$ receptors on cholinergic neurons in the murine GI tract (CHAPTER V)

In murine fundus, jejunum and colon circular smooth muscle strips, optimal conditions were delineated to obtain electrically induced cholinergic contractions. The effect of the nicotinic receptor blocker mecamylamine suggested a contribution of preganglionic neurons to the electrically induced cholinergic contractions in the three tissues, as it reduced these contractions. Just as in rat (Cellek *et al.*, 2008), canine (Prins *et al.*, 2001), porcine (De Maeyer *et al.*, 2008; Priem *et al.*, 2012) and human stomach (Broad *et al.*, 2014b), and in human colon (Cellek *et al.*, 2006), prucalopride was capable to enhance submaximal cholinergic contractions in the murine GI tract. The facilitation with prucalopride was abolished by GR 113808, which confirms the interaction with 5-HT$_4$ receptors. The degree of maximal facilitation was similar to that obtained in the stomach of rat (Cellek *et al.*, 2008), dog (Prins *et al.*, 2001), pig (De Maeyer *et al.*, 2006a; Priem *et al.*, 2012) and man (Broad *et al.*, 2014b), and in human colon

(Cellek *et al.*, 2006). However in mice, this maximum was reached with lower concentrations as no concentration-dependency was found when investigating the same concentration range as previously tested in porcine (Priem *et al.*, 2012) and canine stomach (Prins *et al.*, 2001). A trend for concentration-dependency was observed with tenfold lower concentrations of prucalopride, suggesting a higher number and/or more effective coupling of the 5-HT_4 receptors on myenteric cholinergic neurons in mice. The facilitating effect of prucalopride was reduced by mecamylamine in the fundus but not in jejunum and colon, which suggests that in murine fundus preganglionic 5-HT_4 receptors contribute to the prucalopride-induced facilitation. When other 5-HT receptors than 5-HT_4 were antagonized, the enhancement of cholinergic contractions was confirmed with 5-HT; the effect of 5-HT was abolished (fundus and jejunum) or greatly reduced (colon) in the presence of GR 113808. Thus the facilitation of cholinergic contractions upon 5-HT_4 receptor activation was clearly shown throughout the murine GI tract, illustrating the presence of 5-HT_4 receptors on myenteric cholinergic neurons innervating circular smooth muscle. This justifies further investigation of a possible PDE-mediated control of these receptors.

VII.3.2 Evidence for a role of PDE3 with contribution of PDE4 in the cyclic nucleotide turnover at the level of smooth muscle cells in the murine GI tract (CHAPTER VI)

As preliminary experiments showed that the method to measure tritiated acetylcholine release in porcine and human GI tissue (Lefebvre *et al.*, 2016; Pauwelyn *et al.*, 2018) was not applicable in murine GI tissues, this study also used the paradigm of electrically induced cholinergic contractions where effects at neuronal but also at smooth muscle level are picked up. As already discussed above, PDE inhibition in smooth muscle, increases the basal level of cyclic nucleotides inducing relaxation (Barnette *et al.*, 1993; Kaneda *et al.*, 2004). When studying the influence of PDE inhibitors in a functional model, one has thus to take in account their possible influence at muscular level. Therefore, the influence of the PDE inhibitors was first investigated on submaximal cholinergic contractions, induced with the exogenous muscarinic receptor agonist carbachol and induced by endogenous acetylcholine released in response to electrical stimulation.

The inhibition of direct smooth muscle contractions induced by carbachol with the non-selective PDE inhibitor IBMX suggested the involvement of one or more PDE subtypes in the regulation of murine GI smooth muscle activity. The experiments with the selective PDE inhibitors suggested a role for PDE3 and PDE4, with predominance of PDE3, since concentration-dependent decreases of the contractions over a large concentration range were found with the selective PDE3 inhibitor cilostamide and to a lesser extent with the selective PDE4 inhibitor rolipram; this was not observed with the PDE1 inhibitor vinpocetine nor with the PDE2 inhibitor EHNA. The more pronounced effects of cilostamide could be due to inhibition of cGMP metabolism, as PDE3 is also able to metabolize to some extent cGMP in addition to cAMP, while PDE4 is cAMP specific (Maurice *et al.*, 2003; Lugnier, 2006). Similar effects with cilostamide and rolipram were observed on electrically induced submaximal cholinergic contractions, but in fundus and jejunum the reduction was less pronounced than on carbachol-induced contractions. A possible explanation for this difference is presynaptic influences of the PDE inhibitors increasing

acetylcholine release, which is expected to counteract the postsynaptic relaxing effect. In equine airway cholinergic neurons (Zhang *et al.*, 1996) as well as in guinea pig small intestine (Reese and Cooper, 1984; Yau *et al.*, 1987) cAMP-mediated facilitation of acetylcholine release was indeed observed.

VII.3.3 Lack of evidence for a PDE-mediated control of 5-HT$_4$ receptors on cholinergic neurons in the murine GI tract (CHAPTER VI)

Once the muscular effect of the PDE inhibitors was known, their effect on submaximal prucalopride-induced facilitation of electrically induced cholinergic contractions was studied to evaluate whether the intracellular pathway of the 5-HT$_4$ receptors on cholinergic neurons innervating circular muscle in the murine GI tract is also controlled by a PDE. The selective PDE inhibitors were tested in the highest concentration that did not reduce electrically induced contractions in the three GI regions. If this concentration was below the known IC_{50} values at the PDE wherefore the inhibitor is considered selective, a higher concentration was also investigated, although the reduction by the PDE inhibitor of the electrically induced contractions before adding prucalopride made interpretation of the results then difficult. IBMX was not able to enhance the effect of prucalopride in jejunum and colon, and in the fundus only a trend for enhancement was found. Still the selective PDE inhibitors were tested as insufficient inhibition of a particular PDE that might be important to facilitate the effect of prucalopride, cannot be excluded with the non-selective PDE inhibitor IBMX. Our elaborated study with the selective PDE inhibitors, showed that none of them was able to clearly enhance the prucalopride-induced facilitation of cholinergic contractions in the three tested murine GI regions. It cannot be excluded that a possible enhancing effect of selective PDE3 or PDE4 inhibition is counteracted by its relaxing effect at the muscular level. But clearly, none of the PDE inhibitors allowed to enhance the effect of prucalopride on the electrically induced smooth muscle contractions, in contrast to rolipram in porcine stomach and colon (Priem *et al.*, 2012; Priem *et al.*, 2013). The mouse is thus unsuitable for *in vivo* testing of the possible synergistic gastroprokinetic effects of a 5-HT$_4$ receptor agonist combined with a selective PDE4 inhibitor (or another PDE inhibitor if we had discovered a controlling function for another PDE at neuronal 5-HT$_4$ receptors in the murine GI tract).

VII.4 Conclusion

The PDE4-mediated control of the intraneuronal signal transduction of 5-HT$_4$ receptors in cholinergic neurons in porcine stomach and colon (Priem *et al.*, 2012; Priem *et al.*, 2013) was confirmed in porcine stomach (Lefebvre *et al.*, 2016) with velusetrag, a 5-HT$_4$ receptor agonist belonging to another chemical class than prucalopride, and roflumilast, a PDE4 inhibitor clinically used to suppress exacerbations of COPD. The controlling mechanism is also present in human large intestinal circular muscle, as shown by the synergistic effect of prucalopride with either rolipram or roflumilast on electrically induced acetylcholine release (Pauwelyn *et al.*, 2018). If confirmed *in vivo*, combination therapy of a low dose of a 5-HT$_4$ receptor agonist with a PDE4 inhibitor might be considered in man to obtain pronounced prokinetic effects without influencing cardiac 5-HT$_4$ receptors, which are under sole control of PDE3. Throughout the murine GI tract, the presence of 5-HT$_4$ receptors enhancing acetylcholine release from

cholinergic neurons innervating circular smooth muscle was clearly established (Pauwelyn and Lefebvre, 2017); these 5-HT_4 receptors might be present in a higher number and/or might be more effectively coupled in comparison to other species. However, none of the selective PDE inhibitors tested was able to enhance the facilitating effect of a submaximal concentration of prucalopride (Pauwelyn and Lefebvre, 2018). Mouse GI preparations can thus be used for testing the potency and efficiency of newly developed 5-HT_4 receptor agonists, but the mouse is not suitable for *in vivo* investigation of the GI prokinetic effect of combination therapy of a 5-HT_4 receptor agonist with a PDE4 inhibitor.

VII.5 Future perspectives

- The pig is expensive and more difficult to handle *in vivo* than rodents but seems currently the only appropriate animal model to test the GI prokinetic effect of combining a 5-HT_4 receptor agonist with a PDE4 inhibitor *in vivo*, as it is the only species except for man where a PDE4-mediated control of facilitating 5-HT_4 receptors on cholinergic neurons toward GI smooth muscle was demonstrated. The pig also possesses cardiac 5-HT_4 receptors which corresponds to the human situation, while rodents have no functional cardiac 5-HT_4 receptors. Methods to measure GI transit in pigs are scarce. Intragastric administration of markers is not reliable because of a very variable retention time in the stomach (Davis *et al.*, 2001; Moore *et al.*, 2005). Markers can be inserted distally to the stomach via laparotomy and small incision of the GI tract, and their intestinal distribution can then be studied after a time interval. Vilz et al. (2013) placed radio-opaque glass beads in the duodenum of pigs via a small duodenotomy, and another set of slightly larger radio-opaque beads in the cecum, and studied their small intestinal respectively colonic distribution 24 h later. The feasibility of this technique to study the prokinetic effect of prucalopride in the absence and presence of roflumilast in pigs can be evaluated.
- Our study in human large intestine showed that PDE4 inhibitors enhance the facilitating effect of prucalopride on electrically induced acetylcholine release. The human large intestinal specimens obtained for that project, generally only allowed to prepare 4 circular muscle strips for parallel investigation of acetylcholine release, excluding functional experiments. A functional follow-up study can be performed to evaluate whether PDE4 inhibitors similarly enhance the facilitating effect of submaximal concentrations of prucalopride on electrically induced cholinergic contractions. The influence of selective PDE1, 2, 3 and 4 inhibitors per se on the electrically induced cholinergic contractions can then also be studied to determine which PDEs contribute to the control of the cAMP level in the smooth muscle cells of the human large intestine.
- Prucalopride is currently only used for constipation, but 5-HT_4 receptor agonists were also used for gastroparesis and velusetrag is under clinical development for this indication. An *in vitro* study on human gastric tissue investigating the influence of PDE inhibition on the facilitating effect of 5-HT_4 receptor agonists on neuron-induced cholinergic activity, can reveal whether the PDE4-mediated control of 5-HT_4 receptors on cholinergic neurons toward the smooth muscle layer is also present in human stomach. A study directly measuring acetylcholine release, as performed for the human large

intestine, can be set up; if human gastric specimens obtained from the department of abdominal surgery are sufficiently large to prepare 8 or 12 muscle strips, functional experiments could run in parallel with those where acetylcholine is measured.

- 5-HT_4 receptor agonists have also been shown to enhance the vagal cholinergic anti-inflammatory pathway. Mosapride and prucalopride were reported to prevent postoperative ileus in respectively rats (Tsuchida *et al.*, 2011) and mice (Gomez-Pinilla *et al.*, 2014) via this pathway. The 5-HT_4 receptor agonists were proposed to enhance acetylcholine release from cholinergic nerves, innervating residential macrophages in the muscularis that become pro-inflammatory under surgical stress; released acetylcholine is dampening the inflammatory action of the macrophages via nicotinic receptors. PDE4 inhibitors have anti-inflammatory effects in the GI tract, as they generate elevated intracellular levels of cAMP that down-regulate the release of pro-inflammatory cytokines in the mucosa of inflammatory bowel disease patients (Spadaccini *et al.*, 2017). It might thus be interesting to investigate whether this mucosal anti-inflammatory effect of PDE4 inhibitors can also be shown at muscular level by studying the effect of roflumilast in the mouse model of postoperative ileus currently used in our laboratory. If so, the combination of prucalopride with roflumilast can then be studied in the same model to evaluate whether their anti-inflammatory effect is additive or even synergistic.

VII.6 References

Afzal F., Andressen K.W., Mork H.K., Aronsen J.M., Sjaastad I., Dahl C.P., Skomedal T., Levy F.O., Osnes J.B., and Qvigstad E. (2008). 5-HT4-elicited positive inotropic response is mediated by cAMP and regulated by PDE3 in failing rat and human cardiac ventricles. *Br J Pharmacol* 155, 1005-1014. DOI: 10.1038/bjp.2008.339

Ahn A., Barnes C., Shaywitz D., Grimaldi M., and Canafax D.M. (2015). Velusetrag Improves Gastric Emptying Time in Subjects With Diabetic or Idiopathic Gastroparesis. *Gastroenterology* 148, S507-S507.

Barbey J.T., Lazzara R., and Zipes D.P. (2002). Spontaneous adverse event reports of serious ventricular arrhythmias, QT prolongation, syncope, and sudden death in patients treated with cisapride. *J Cardiovasc Pharmacol Ther* 7, 65-76. DOI: 10.1177/107424840200700202

Barnette M.S., Manning C.D., Price W.J., and Barone F.C. (1993). Initial biochemical and functional characterization of cyclic nucleotide phosphodiesterase isozymes in canine colonic smooth muscle. *J Pharmacol Exp Ther* 264, 801-812.

Beattie D.T., and Smith J.A. (2008). Serotonin pharmacology in the gastrointestinal tract: a review. *Naunyn Schmiedebergs Arch Pharmacol* 377, 181-203. DOI: 10.1007/s00210-008-0276-9

Beattie D.T., Armstrong S.R., Vickery R.G., Tsuruda P.R., Campbell C.B., Richardson C., Mccullough J.L., Daniels O., Kersey K., Li Y.P*., et al.* (2011). The Pharmacology of TD-8954, a Potent and Selective 5-HT(4) Receptor Agonist with Gastrointestinal Prokinetic Properties. *Front Pharmacol* 2, 25. DOI: 10.3389/fphar.2011.00025

Bouras E.P., Camilleri M., Burton D.D., and Mckinzie S. (1999). Selective stimulation of colonic transit by the benzofuran 5HT(4) agonist, prucalopride, in healthy humans. *Gut* 44, 682-686. DOI: 10.1136/gut.44.5.682

Bouras E.P., Camilleri M., Burton D.D., Thomforde G., Mckinzie S., and Zinsmeister A.R. (2001). Prucalopride accelerates gastrointestinal and colonic transit in patients with constipation without a rectal evacuation disorder. *Gastroenterology* 120, 354-360. DOI: 10.1053/gast.2001.21166

Broad J., Kung V.W., Boundouki G., Aziz Q., De Maeyer J.H., Knowles C.H., and Sanger G.J. (2013). Cholinergic interactions between donepezil and prucalopride in human colon: potential to treat severe intestinal dysmotility. *Br J Pharmacol* 170, 1253-1261. DOI: 10.1111/bph.12397

Broad J., Goralczyk A., Mannur K., Dukes G.E., and Sanger G.J. (2014a). Drugs acting at 5-HT4, D-2, motilin, and ghrelin receptors differ markedly in how they affect neuromuscular functions in human isolated stomach. *Neurogastroenterol Motil* 26, 851-861. DOI: 10.1111/nmo.12338

Broad J., Hughes F., Chin-Aleong J., Sifrim D., and Sanger G.J. (2014b). Regionally dependent neuromuscular functions of motilin and 5-HT4 receptors in human isolated esophageal body and gastric fundus. *Neurogastroenterol Motil* 26, 1311-1322. DOI: 10.1111/nmo.12394

Campbell-Dittmeyer K., Hicks G.A., Earnest D.L., and Greenwood-Van Meerveld B. (2009). Increased colonic transit in rats produced by a combination of a cholinesterase inhibitor with a 5-HT4 receptor agonist. *Neurogastroenterol Motil* 21, 1197-e1108. DOI: 10.1111/j.1365-2982.2008.01238.x

Cellek S., John A.K., Thangiah R., Dass N.B., Bassil A.K., Jarvie E.M., Lalude O., Vivekanandan S., and Sanger G.J. (2006). 5-HT4 receptor agonists enhance both cholinergic and nitrergic activities in human isolated colon circular muscle. *Neurogastroenterol Motil* 18, 853-861. DOI: 10.1111/j.1365-2982.2006.00810.x

Cellek S., Thangiah R., Jarvie E.M., Vivekanandan S., Lalude O., and Sanger G.J. (2008). Synergy between 5-HT4 receptor activation and acetylcholinesterase inhibition in human colon and rat forestomach. *Neurogastroenterol Motil* 20, 539-545. DOI: 10.1111/j.1365-2982.2007.01062.x

Coremans G., Kerstens R., De Pauw M., and Stevens M. (2003). Prucalopride is effective in patients with severe chronic constipation in whom laxatives fail to provide adequate relief - Results of a double-blind, placebo-controlled clinical trial. *Digestion* 67, 82-89. DOI: 10.1159/000070202

Davis S.S., Illum L., and Hinchcliffe M. (2001). Gastrointestinal transit of dosage forms in the pig. *J Pharm Pharmacol* 53, 33-39. DOI: 10.1211/0022357011775163

De Maeyer J.H., Prins N.H., Schuurkes J.a.J., and Lefebvre R.A. (2006a). Differential effects of 5-hydroxytryptamine4 receptor agonists at gastric versus cardiac receptors: an operational framework to explain and quantify organ-specific behavior. *J Pharmacol Exp Ther* 317, 955-964. DOI: 10.1124/jpet.106.101329

De Maeyer J.H., Straetemans R., Schuurkes J.a.J., and Lefebvre R.A. (2006b). Porcine left atrial and sinoatrial 5-HT4 receptor-induced responses: fading of the response and influence of development. *Br J Pharmacol* 147, 140-157. DOI: 10.1038/sj.bjp.0706497

De Maeyer J.H., Lefebvre R.A., and Schuurkes J.a.J. (2008). 5-HT4 receptor agonists: similar but not the same. *Neurogastroenterol Motil* 20, 99-112. DOI: 10.1111/j.1365-2982.2007.01059.x

Emmanuel A.V., Kamm M.A., Roy A.J., and Antonelli K. (1998). Effect of a novel prokinetic drug, R093877, on gastrointestinal transit in healthy volunteers. *Gut* 42, 511-516. DOI: 10.1136/gut.42.4.511

Emmanuel A.V., Roy A.J., Nicholls T.J., and Kamm M.A. (2002). Prucalopride, a systemic enterokinetic, for the treatment of constipation. *Aliment Pharmacol Ther* 16, 1347-1356. DOI: 10.1046/j.1365-2036.2002.01272.x

Galindo-Tovar A., Vargas M.L., Escudero E., and Kaumann A.J. (2009). Ontogenic changes of the control by phosphodiesterase-3 and -4 of 5-HT responses in porcine heart and relevance to human atrial 5-HT4 receptors. *Br J Pharmacol* 156, 237-249. DOI: 10.1111/j.1476-5381.2008.00023.x

Gershon M.D., and Tack J. (2007). The serotonin signaling system: From basic understanding to drug development-for functional GI disorders. *Gastroenterology* 132, 397-414. DOI: 10.1053/j.gastro.2006.11.002

Gershon M.D. (2013). 5-Hydroxytryptamine (serotonin) in the gastrointestinal tract. *Curr Opin Endocrinol Diabetes Obes* 20, 14-21. DOI: 10.1097/MED.0b013e32835bc703

Giudicessi J.R., Ackerman M.J., and Camilleri M. (2018). Cardiovascular safety of prokinetic agents: A focus on drug-induced arrhythmias. *Neurogastroenterol Motil*. DOI: 10.1111/nmo.13302

Goldberg M., Li Y.P., Johanson J.F., Mangel A.W., Kitt M., Beattie D.T., Kersey K., and Daniels O. (2010). Clinical trial: the efficacy and tolerability of velusetrag, a selective 5-HT4 agonist with high intrinsic activity, in chronic idiopathic constipation - a 4-week, randomized, double-blind, placebo-controlled, dose-response study. *Aliment Pharmacol Ther* 32, 1102-1112. DOI: 10.1111/j.1365-2036.2010.04456.x

Gomez-Pinilla P.J., Di Giovangiulio M., Nemethova A., Stakenborg N., Farro G., Bosmans G., Matteoli G., and Boeckxstaens G.E. (2014). Prucalopride Activates the Intestinal Cholinergic Anti-Inflammatory Pathway and Prevents Postoperative Ileus (Abstract). *Gastroenterology* 146, S89-S89.

James A.N., Ryan J.P., Crowell M.D., and Parkman H.P. (2004). Regional gastric contractility alterations in a diabetic gastroparesis mouse model: effects of cholinergic and serotoninergic stimulation. *Am J Physiol Gastrointest Liver Physiol* 287, G612-G619. DOI: 10.1152/ajpgi.00431.2003

Jones O.M., Brading A.F., and Mc C.M.N.J. (2002). Phosphodiesterase inhibitors cause relaxation of the internal anal sphincter in vitro. *Dis Colon Rectum* 45, 530-536.

Kaneda T., Shimizu K., Urakawa N., and Nakajyo S. (2004). Effects of various selective phosphodiesterase inhibitors on carbachol-induced contraction and cyclic nucleotide contents in guinea pig taenia coli. *J Vet Med Sci* 66, 1047-1052. DOI: 10.1292/jvms.66.1047

Keating G.M. (2013). Prucalopride: A Review of Its Use in the Management of Chronic Constipation. *Drugs* 73, 1935-1950. DOI: 10.1007/s40265-013-0140-1

Kendig D.M., and Grider J.R. (2015). Serotonin and colonic motility. *Neurogastroenterol Motil* 27, 899-905. DOI: 10.1111/nmo.12617

Kessing B.F., Smout A.J., Bennink R.J., Kraaijpoel N., Oors J.M., and Bredenoord A.J. (2014). Prucalopride decreases esophageal acid exposure and accelerates gastric emptying in healthy subjects. *Neurogastroenterol Motil* 26, 1079-1086. DOI: 10.1111/nmo.12359

Krobert K.A., Brattelid T., Levy F.O., and Kaumann A.J. (2005). Prucalopride is a partial agonist through human and porcine atrial 5-HT4 receptors: comparison with recombinant human 5-HT4 splice variants. *Naunyn Schmiedebergs Arch Pharmacol* 371, 473-479. DOI: 10.1007/s00210-005-1068-0

Leclere P.G., and Lefebvre R.A. (2002). Presynaptic modulation of cholinergic neurotransmission in the human proximal stomach. *Br J Pharmacol* 135, 135-142. DOI: 10.1038/sj.bjp.0704471

Leclere P.G., Prins N.H., Schuurkes J.a.J., and Lefebvre R.A. (2005). 5-HT4 receptors located on cholinergic nerves in human colon circular muscle. *Neurogastroenterol Motil* 17, 366-375. DOI: 10.1111/j.1365-2982.2005.00621.x

Lee M.J., Cho K.H., Park H.M., Sung H.J., Choi S., and Im W. (2014). Pharmacological profile of DA-6886, a novel 5-HT4 receptor agonist to accelerate colonic motor activity in mice. *Eur J Pharmacol* 735, 115-122. DOI: 10.1016/j.ejphar.2014.03.061

Lefebvre R.A., Van Colen I., Pauwelyn V., and De Maeyer J.H. (2016). Synergistic effect between 5-HT4 receptor agonist and phosphodiesterase 4-inhibitor in releasing acetylcholine in pig gastric circular muscle in vitro. *Eur J Pharmacol* 781, 76-82. DOI: 10.1016/j.ejphar.2016.04.003

Liu M., Geddis M.S., Wen Y., Setlik W., and Gershon M.D. (2005). Expression and function of 5-HT4 receptors in the mouse enteric nervous system. *Am J Physiol Gastrointest Liver Physiol* 289, G1148-1163. DOI: 10.1152/ajpgi.00245.2005

Liu M.T., Kuan Y.H., Wang J.W., Hen R., and Gershon M.D. (2009). 5-HT4 Receptor-Mediated Neuroprotection and Neurogenesis in the Enteric Nervous System of Adult Mice. *J Neurosci* 29, 9683-9699. DOI: 10.1523/Jneurosci.1145-09.2009

Long D.D., Armstrong S.R., Beattie D.T., Choi S.K., Fatheree P.R., Gendron R.A., Goldblum A.A., Humphrey P.P., Marquess D.G., Shaw J.P.*, et al.* (2012a). Discovery, oral pharmacokinetics and in vivo efficacy of a highly selective 5-HT4 receptor agonist: clinical compound TD-2749. *Bioorg Med Chem Lett* 22, 4849-4853. DOI: 10.1016/j.bmcl.2012.05.034

Long D.D., Armstrong S.R., Beattie D.T., Choi S.K., Fatheree P.R., Gendron R.a.L., Genov D., Goldblum A.A., Humphrey P.P., Jiang L.*, et al.* (2012b). Discovery, oral pharmacokinetics and in vivo efficacy of velusetrag, a highly selective 5-HT4 receptor agonist that has achieved proof-of-concept in patients with chronic idiopathic constipation. *Bioorg Med Chem Lett* 22, 6048-6052. DOI: 10.1016/j.bmcl.2012.08.051

Lugnier C. (2006). Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. *Pharmacol Ther* 109, 366-398. DOI: 10.1016/j.pharmthera.2005.07.003

Manabe N., Wong B.S., and Camilleri M. (2010). New-generation 5-HT4 receptor agonists: potential for treatment of gastrointestinal motility disorders. *Expert Opin Investig Drugs* 19, 765-775. DOI: 10.1517/13543784.2010.482927

Manini M.L., Camilleri M., Goldberg M., Sweetser S., Mckinzie S., Burton D., Wong S., Kitt M.M., Li Y.P., and Zinsmeister A.R. (2010). Effects of Velusetrag (TD-5108) on gastrointestinal transit and bowel function in health and

pharmacokinetics in health and constipation. *Neurogastroenterol Motil* 22, 42-49, e47-48. DOI: 10.1111/j.1365-2982.2009.01378.x

Maurice D.H., Palmer D., Tilley D.G., Dunkerley H.A., Netherton S.J., Raymond D.R., Elbatarny H.S., and Jimmo S.L. (2003). Cyclic nucleotide phosphodiesterase activity, expression, and targeting in cells of the cardiovascular system. *Mol Pharmacol* 64, 533-546. DOI: 10.1124/mol.64.3.533

Mclean P.G., and Coupar I.M. (1996). Further investigation into the signal transduction mechanism of the 5-HT4-like receptor in the circular smooth muscle of human colon. *Br J Pharmacol* 118, 1058-1064. DOI: 10.1111/j.1476-5381.1996.tb15506.x

Mohammad S., Zhou Z.F., Gong Q.M., and January C.T. (1997). Blockage of the HERG human cardiac K+ channel by the gastrointestinal prokinetic agent cisapride. *Am J Physiol Heart Circ Physiol* 273, H2534-H2538. DOI: 10.1152/ajpheart.1997.273.5.H2534

Moore B.A., Overhaus M., Whitcomb J., Ifedigbo E., Choi A.M., Otterbein L.E., and Bauer A.J. (2005). Brief inhalation of low-dose carbon monoxide protects rodents and swine from postoperative ileus. *Crit Care Med* 33, 1317-1326. DOI: 10.1097/01.CCM.0000166349.76514.40

Nagakura Y., Ito H., Kiso T., Naitoh Y., and Miyata K. (1997). The selective 5-hydroxytryptamine (5-HT)(4)-receptor agonist RS67506 enhances lower intestinal propulsion in mice. *Jpn J Pharmacol* 74, 209-212. DOI: 10.1254/jjp.74.209

Pasricha P.J. (2007). Desperately seeking serotonin... A commentary on the withdrawal of tegaserod and the state of drug development for functional and motility disorders. *Gastroenterology* 132, 2287-2290. DOI: 10.1053/j.gastro.2007.04.057

Pauwelyn V., and Lefebvre R.A. (2017). 5-HT4 receptors facilitate cholinergic neurotransmission throughout the murine gastrointestinal tract. *Neurogastroenterol Motil* 00:e13064. DOI: 10.1111/nmo.13064

Pauwelyn V., Ceelen W., and Lefebvre R.A. (2018). Synergy between 5-HT4 receptor stimulation and phosphodiesterase 4 inhibition in facilitating acetylcholine release in human large intestinal circular muscle. *Neurogastroenterol Motil* 30, e13162. DOI: 10.1111/nmo.13162

Pauwelyn V., and Lefebvre R.A. (2018). cAMP Catalyzing Phosphodiesterases Control Cholinergic Muscular Activity But Their Inhibition Does Not Enhance 5-HT4 Receptor-Mediated Facilitation of Cholinergic Contractions in the Murine Gastrointestinal Tract. *Front Pharmacol* 9. DOI: 10.3389/fphar.2018.00171

Poen A.C., Felt-Bersma R.J., Van Dongen P.A., and Meuwissen S.G. (1999). Effect of prucalopride, a new enterokinetic agent, on gastrointestinal transit and anorectal function in healthy volunteers. *Aliment Pharmacol Ther* 13, 1493-1497. DOI: 10.1046/j.1365-2036.1999.00629.x

Priem E.K.V., and Lefebvre R.A. (2011). Investigation of neurogenic excitatory and inhibitory motor responses and their control by 5-HT4 receptors in circular smooth muscle of pig descending colon. *Eur J Pharmacol* 667, 365-374. DOI: 10.1016/j.ejphar.2011.06.021

Priem E.K.V., Van Colen I., De Maeyer J.H., and Lefebvre R.A. (2012). The facilitating effect of prucalopride on cholinergic neurotransmission in pig gastric circular muscle is regulated by phosphodiesterase 4. *Neuropharmacology* 62, 2126-2135. DOI: 10.1016/j.neuropharm.2011.12.020

Priem E.K.V., De Maeyer J.H., and Lefebvre R.A. (2013). Influence of phosphodiesterases on basal and 5-HT4 receptor facilitated cholinergic contractility in pig descending colon. *Eur J Pharmacol* 705, 156-163. DOI: 10.1016/j.ejphar.2013.02.011

Prins N.H., Akkermans L.M.A., Lefebvre R.A., and Schuurkes J.a.J. (2000a). 5-HT4 receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. *Br J Pharmacol* 131, 927-932. DOI: 10.1038/sj.bjp.0703615

Prins N.H., Shankley N.P., Welsh N.J., Briejer M.R., Lefebvre R.A., Akkermans L.M.A., and Schuurkes J.a.J. (2000b). An improved in vitro bioassay for the study of 5-HT4 receptors in the human isolated large intestinal circular muscle. *Br J Pharmacol* 129, 1601-1608. DOI: 10.1038/sj.bjp.0703254

Prins N.H., Van Der Grijn A., Lefebvre R.A., Akkermans L.M.A., and Schuurkes J.a.J. (2001). 5-HT4 receptors mediating enhancement of contractility in canine stomach; an in vitro and in vivo study. *Br J Pharmacol* 132, 1941-1947. DOI: 10.1038/sj.bjp.0703985

Rabe K.F. (2011). Update on roflumilast, a phosphodiesterase 4 inhibitor for the treatment of chronic obstructive pulmonary disease. *Br J Pharmacol* 163, 53-67. DOI: 10.1111/j.1476-5381.2011.01218.x

Reese J.H., and Cooper J.R. (1984). Stimulation of Acetylcholine-Release from Guinea-Pig Ileal Synaptosomes by Cyclic-Nucleotides and Forskolin. *Biochem Pharmacol* 33, 3007-3011. DOI: 10.1016/0006-2952(84)90601-4

Sloots C.E.J., Poen A.C., Kerstens R., Stevens M., De Pauw M., Van Oene J.C., Meuwissen S.G.M., and Felt-Bersma R.J.F. (2002). Effects of prucalopride on colonic transit, anorectal function and bowel habits in patients with chronic constipation. *Aliment Pharmacol Ther* 16, 759-767. DOI: 10.1046/j.1365-2036.2002.01210.x

Smith J.A., Beattie D.T., Marquess D., Shaw J.P., Vickery R.G., and Humphrey P.P. (2008). The in vitro pharmacological profile of TD-5108, a selective 5-HT(4) receptor agonist with high intrinsic activity. *Naunyn Schmiedebergs Arch Pharmacol* 378, 125-137. DOI: 10.1007/s00210-008-0282-y

Spadaccini M., D'alessio S., Peyrin-Biroulet L., and Danese S. (2017). PDE4 Inhibition and Inflammatory Bowel Disease: A Novel Therapeutic Avenue. *Int J Mol Sci* 18. DOI: 10.3390/ijms18061276

Tack J., Camilleri M., Chang L., Chey W.D., Galligan J.J., Lacy B.E., Muller-Lissner S., Quigley E.M., Schuurkes J., De Maeyer J.H., *et al.* (2012). Systematic review: cardiovascular safety profile of 5-HT(4) agonists developed for gastrointestinal disorders. *Aliment Pharmacol Ther* 35, 745-767. DOI: 10.1111/j.1365-2036.2012.05011.x

Tsuchida Y., Hatao F., Fujisawa M., Murata T., Kaminishi M., Seto Y., Hori M., and Ozaki H. (2011). Neuronal stimulation with 5-hydroxytryptamine 4 receptor induces anti-inflammatory actions via alpha7nACh receptors on muscularis macrophages associated with postoperative ileus. *Gut* 60, 638-647. DOI: 10.1136/gut.2010.227546

Vilz T.O., Sommer N., Kahl P., Pantelis D., Kalff J.C., and Wehner S. (2013). Oral CPSI-2364 Treatment Prevents Postoperative Ileus in Swine without Impairment of Anastomotic Healing. *Cell Physiol Biochem* 32, 1362-1373. DOI: 10.1159/000356575

Wan Y., Sun S.X., Corman S., Huang X., Gao X., and Shorr A.F. (2015). A longitudinal, retrospective cohort study on the impact of roflumilast on exacerbations and economic burden among chronic obstructive pulmonary disease patients in the real world. *Int J Chron Obstruct Pulmon Dis* 10, 2127-2136. DOI: 10.2147/COPD.S80106

Wang L., Martinez V., Kimura H., and Tache Y. (2007). 5-Hydroxytryptophan activates colonic myenteric neurons and propulsive motor function through 5-HT4 receptors in conscious mice. *Am J Physiol Gastrointest Liver Physiol* 292, G419-428. DOI: 10.1152/ajpgi.00289.2006

Weninger S., De Maeyer J.H., and Lefebvre R.A. (2012). Study of the regulation of the inotropic response to 5-HT4 receptor activation via phosphodiesterases and its cross-talk with C-type natriuretic peptide in porcine left atrium. *Naunyn Schmiedebergs Arch Pharmacol* 385, 565-577. DOI: 10.1007/s00210-012-0746-y

Yau W.M., Dorsett J.A., and Youther M.L. (1987). Stimulation of acetylcholine-release from myenteric neurons of guinea-pig small-intestine by forskolin and cyclic AMP. *J Pharmacol Exp Ther* 243, 507-510.

Zhang X.Y., Robinson N.E., and Zhu F.X. (1996). Potentiation of acetylcholine release from tracheal parasympathetic nerves by cAMP. *Am J Physiol Lung Cell Mol Physiol* 270**,** L541-L546. DOI: 10.1152/ajplung.1996.270.4.L541

Summary

Summary

Gastrointestinal (GI) dysmotility is one of the underlying factors in GI disorders like constipation and gastroparesis, explaining the search for gastroprokinetic drugs stimulating GI motility in the pharmaceutical industry. The majority of serotonin (5-hydrotryptamine, 5-HT) in the human body is present in the GI tract, where it is involved in the regulation of motility. An important class of gastroprokinetic drugs are 5-HT_4 receptor agonists such as cisapride, tegaserod and prucalopride. 5-HT_4 receptors are G protein-coupled receptors signaling via activation of adenylyl cyclase and the generation of cAMP. In the human GI tract, 5-HT_4 receptors influencing GI motility have been described on smooth muscle cells and inhibitory nitrergic neurons, both leading to relaxant effects, and on myenteric excitatory cholinergic neurons toward the smooth muscle layer. Stimulation of these 5-HT_4 receptors on cholinergic neurons enhances acetylcholine release and smooth muscle contraction, and is thought to be the underlying mechanism for the gastroprokinetic properties of 5-HT_4 receptor agonists. Cisapride was frequently used for GI hypomotility disorders, but was withdrawn in 2000 because of the risk for serious cardiac arrhythmias. Tegaserod was approved for treatment of constipation-predominant irritable bowel syndrome in 2002 but was soon associated with an increased risk for atherosclerotic obstructive events, leading to its withdrawal in 2007. Although these cardiovascular side effects were due to non-selectivity of these agonists and were not related to interaction with 5-HT_4 receptors, these withdrawals delayed the clinical development of highly selective 5-HT_4 receptor agonists; prucalopride was marketed for chronic idiopathic constipation in 2009. Prucalopride *in vitro* stimulates GI cholinergic neurotransmission toward the muscle layer and has *in vivo* gastroprokinetic properties. Via cardiac 5-HT_4 receptors, it induces positive chronotropic and inotropic effects. The inotropic responses are transient and weak in amplitude due to the tight control of cardiac 5-HT_4 receptors by phosphodiesterases (PDEs): in pigs by a redundant action of PDE3 plus PDE4 and in man by PDE3. The cAMP generated upon 5-HT_4 receptor stimulation in the heart, is quickly degraded by the PDEs which limits the response. In contrast to the cardiac responses of prucalopride, its GI effects on cholinergic neurotransmission are sustained and pronounced. Still this effect can be enhanced *in vitro* by selective PDE4 inhibition in porcine stomach and colon, which suggests a PDE4-mediated control of the signaling pathway of GI 5-HT_4 receptors in cholinergic neurons.

Although prucalopride was found to be cardiovascularly safe till now, some concern for possible cardiac effects remains. The risk of cardiac side effects could be further reduced by combination therapy, where the prokinetic effect is maintained with a lower dose and thus plasma concentrations of prucalopride. A first possibility might be combination of a 5-HT_4 receptor agonist with an acetylcholinesterase inhibitor, as a synergistic effect of this combination on *in vitro* cholinergic activity in human colon and on *in vivo* colonic transit time in rat was reported. Combining the 5-HT_4 receptor agonist prucalopride with the selective PDE4 inhibitor rolipram enhances electrically induced acetylcholine release and cholinergic contractions in a synergistic way in the porcine GI tract. If the *in vitro* synergy between a 5-HT_4 receptor agonist and a PDE4 inhibitor can also be confirmed in humans, and it can be shown that

this also translates to synergistic gastroprokinetic effects *in vivo*, this combination therapy might also be considered. To further investigate the synergy of this combination, the **aims** of this thesis were:

- to corroborate the control by PDE4 of the signaling pathway of 5-HT_4 receptors on cholinergic neurons in the porcine GI tract with other substances than prucalopride and rolipram (**CHAPTER III**);
- to study whether this PDE4-mediated control of 5-HT_4 receptors is also present in the human GI tract (**CHAPTER IV**);
- to evaluate the mouse as possible model for *in vivo* testing of the synergistic prokinetic effect of a 5-HT_4 receptor agonist with a PDE4 inhibitor (**CHAPTER V** and **VI**).

In **CHAPTER III** the synergy between 5-HT_4 receptor agonism and PDE4 inhibition was corroborated in circular muscle of porcine stomach with roflumilast and velusetrag by direct measurement of electrically induced acetylcholine release. Roflumilast is a PDE4 inhibitor, marketed to suppress exacerbations in patients with COPD; velusetrag is a highly selective 5-HT_4 receptor agonist belonging to another chemical class than prucalopride and is actually under clinical development for constipation and gastroparesis. Roflumilast per se increased electrically induced acetylcholine release and thus confirmed the regulatory role for PDE4 in the basal cAMP content in porcine gastric cholinergic neurons as previously discovered with rolipram. The facilitation of acetylcholine release by a submaximal concentration of prucalopride was enhanced by roflumilast in a synergistic way. Just as prucalopride, velusetrag concentration-dependently increased the electrically induced acetylcholine release, but the maximal observed effect of velusetrag was less pronounced. This might correlate with a lower maximal agonist effect of velusetrag. Submaximal facilitation of electrically induced acetylcholine release by velusetrag was enhanced by rolipram, again in a synergistic way. The synergistic effect of roflumilast plus prucalopride, and of rolipram plus velusetrag, corroborated that PDE4 regulates the signal transduction of facilitatory 5-HT_4 receptors in cholinergic neurons in porcine stomach, as previously shown with rolipram and prucalopride.

In **CHAPTER IV** this controlling mechanism was investigated in circular muscle of the human large intestine using the same technique with direct measurement of acetylcholine release. The facilitation of electrically induced acetylcholine release by a submaximal concentration of prucalopride was enhanced by the non-selective PDE inhibitor IBMX. This suggests a PDE-mediated control of the 5-HT_4 receptors on cholinergic myenteric neurons and the responsible PDE subtype was further identified by investigation of selective inhibitors of the classic cAMP degrading PDE subtypes (PDE1 to 4). In contrast to the porcine stomach, rolipram or roflumilast per se did not influence acetylcholine release; in the human large intestine there is thus no evidence for a PDE4-mediated regulation of the basal cAMP content in myenteric cholinergic neurons. As well rolipram as roflumilast enhanced the effect of prucalopride to the same magnitude as observed with IBMX; while selective PDE1, 2 and 3 inhibitors had no influence. This suggests that also in the human large intestine PDE4 controls facilitating 5-HT_4

receptors on cholinergic neurons, which might explain the previously suggested poor coupling of 5-HT_4 receptors to acetylcholine release in a functional study of human colon. The idea of combining a 5-HT_4 receptor agonist with a PDE4 inhibitor to obtain pronounced GI prokinetic effects with a low dose of the 5-HT_4 receptor agonist is thus supported, but other locations of 5-HT_4 receptors and PDE4 should be considered. Cardiac 5-HT_4 receptors are no concern as they are controlled by PDE3 in man; the effect of their stimulation will thus not be influenced by concomitant treatment with a PDE4 inhibitor. But the prokinetic effect by enhanced acetylcholine release from the myenteric neurons when combining a 5-HT_4 receptor agonist with a PDE4 inhibitor might be influenced by relaxant 5-HT_4 receptors suggested to be present on smooth muscle and by PDE4 regulating the basal cAMP turnover in smooth muscle cells in human large intestine; this requires further investigation.

Finally, in **CHAPTER V** and **VI**, it was evaluated whether the mouse might be suitable for *in vivo* testing of the prokinetic effect of combining a 5-HT_4 receptor agonist with a PDE4 inhibitor. In **CHAPTER V** the presence of 5-HT_4 receptors on murine myenteric cholinergic neurons was documented. Prucalopride concentration-dependently enhanced electrically induced submaximal cholinergic contractions in the murine fundus, jejunum and colon and this effect was abolished by a 5-HT_4 receptor antagonist. This illustrates the presence of 5-HT_4 receptors on myenteric cholinergic neurons innervating circular smooth muscle, as also confirmed by use of 5-HT. The maximal degree of facilitation with prucalopride was similar to that obtained in other species but was reached with lower concentrations suggesting a higher number and/or more effective coupling of 5-HT_4 receptors on myenteric cholinergic neurons in mice. Reduction of the prucalopride-induced facilitation by the nicotinic receptor blocker mecamylamine in fundus, but not in jejunum and colon, points to the contribution of preganglionic 5-HT_4 receptors in the upper stomach.
As the presence of 5-HT_4 receptors on murine myenteric cholinergic neurons toward circular smooth muscle was clearly shown, their possible PDE-mediated control was studied in **CHAPTER VI**. The method directly measuring acetylcholine release was not applicable in murine GI tissues, so the paradigm of electrically induced cholinergic contractions where effects at neuronal as well as at smooth muscle level are picked up was used. First, the influence of the PDE inhibitors on carbachol- and electrically induced submaximal cholinergic contractions was investigated. The non-selective PDE inhibitor IBMX inhibited the carbachol-induced contractions illustrating the involvement of one or more PDE subtypes in the regulation of murine GI smooth muscle activity. The selective PDE3 inhibitor cilostamide and to a lesser extent the selective PDE4 inhibitor rolipram concentration-dependently decreased the carbachol-induced contractions over a large concentration range; the PDE1 inhibitor vinpocetine and PDE2 inhibitor EHNA did not. This indicates a role for PDE3 and PDE4, with predominance of PDE3, in the control of the cyclic nucleotide turnover in murine GI smooth muscle. The more pronounced effects of cilostamide in comparison to rolipram might be due to inhibition of cGMP metabolism because PDE3 metabolizes cAMP and cGMP, while PDE4 is cAMP specific. The involvement of PDE3 and PDE4 was confirmed when studying electrically induced submaximal cholinergic contractions where similar effects of the PDE inhibitors were found, but the effects were less pronounced than on carbachol-induced contractions. The latter

might be explained by presynaptic actions of PDE inhibitors as presynaptic cAMP-mediated facilitation of acetylcholine release, expected to counteract the postsynaptic relaxation, is possible.
The effect of the PDE inhibitors on submaximal prucalopride-induced facilitation of electrically induced cholinergic contractions was then studied. Neither IBMX nor the selective PDE1 to 4 inhibitors were able to enhance the effect of prucalopride on electrically induced cholinergic contractions in murine fundus, jejunum and colon. In contrast to the porcine stomach and colon, also the PDE4 inhibitor rolipram did not enhance the effect of prucalopride in the murine GI tract. The mouse is thus unsuitable for *in vivo* testing of the possible synergistic gastroprokinetic effects of a 5-HT_4 receptor agonist combined with a selective PDE4 inhibitor.

In **conclusion**, the PDE4-mediated control of 5-HT_4 receptors on myenteric cholinergic neurons toward smooth muscle was confirmed in porcine stomach with roflumilast and velusetrag. In human large intestine circular muscle, this controlling mechanism is also present as synergy between prucalopride and either rolipram or roflumilast on electrically induced myenteric acetylcholine release was obtained. If confirmed *in vivo*, combination therapy of a low dose of a 5-HT_4 receptor agonist with a PDE4 inhibitor might thus be considered in man to obtain pronounced prokinetic effects without influencing cardiac 5-HT_4 receptors, which are under sole control of PDE3. Throughout the murine GI tract the presence of 5-HT_4 receptors on cholinergic neurons innervating circular smooth muscle was clearly established and these 5-HT_4 receptors might be present in a higher number and/or might be more effectively coupled in comparison to other species. However, none of the selective PDE inhibitors tested was able to enhance the facilitating effect of a submaximal concentration of prucalopride on electrically induced submaximal cholinergic contractions. Mouse GI preparations can thus be used for testing the potency and efficiency of newly developed 5-HT_4 receptor agonists, but the mouse is not suitable for *in vivo* investigation of the GI prokinetic effect of combination therapy of a 5-HT_4 receptor agonist with a PDE4 inhibitor.

Samenvatting

Samenvatting

Een verstoorde gastro-intestinale (GI) motiliteit is één van de onderliggende factoren bij GI aandoeningen zoals constipatie en gastroparese. Dit verklaart de zoektocht van de farmaceutische industrie naar gastroprokinetische farmaca die de GI motiliteit stimuleren. Het grootste deel van serotonine (5-hydrotryptamine, 5-HT) in het menselijk lichaam bevindt zich in de GI tractus, waar het betrokken is in de regulatie van GI motiliteit. Een belangrijke klasse van gastroprokinetische farmaca zijn 5-HT_4-receptoragonisten zoals cisapride, tegaserod en prucalopride. 5-HT_4-receptoren zijn G-proteïne-gekoppelde receptoren die adenylylcyclase activeren en cAMP genereren. In de GI tractus van de mens werden reeds 5-HT_4-receptoren beschreven, die de GI motiliteit beïnvloeden, namelijk op gladde spiercellen en op inhiberende nitrerge neuronen, beide leidend tot relaxatie, alsook op myenterische exciterende cholinerge neuronen naar de gladde spierlaag. Stimulatie van deze 5-HT_4-receptoren op cholinerge neuronen verhoogt de vrijstelling van acetylcholine en de spiercontracties en dit wordt verondersteld het onderliggende mechanisme te zijn voor de gastroprokinetische eigenschappen van 5-HT_4-receptoragonisten. Cisapride werd vaak gebruikt voor GI hypomotiliteits-stoornissen, maar werd in 2000 uit de handel gehaald vanwege het risico op ernstige hartritme-stoornissen. Tegaserod werd goedgekeurd als behandeling van constipatie-predominant prikkelbare darm syndroom in 2002, maar werd al snel geassocieerd met een verhoogd risico op ischemische incidenten, met zijn terugtrekking tot gevolg in 2007. Hoewel deze cardiovasculaire bijwerkingen te wijten zijn aan niet-selectieve acties van deze agonisten en niet gerelateerd zijn aan interactie met 5-HT_4-receptoren, werd door deze terugtrekkingen de klinische ontwikkeling van zeer selectieve 5-HT_4-receptoragonisten vertraagd. Prucalopride kwam op de markt in 2009 als behandeling van chronische constipatie. Prucalopride stimuleert *in vitro* de GI cholinerge neurotransmissie naar de gladde spierlaag en heeft *in vivo* een gastroprokinetische werking. Via cardiale 5-HT_4-receptoren worden positieve chrono- en inotrope effecten uitgelokt. De inotrope antwoorden zijn kortdurend en hebben een lage amplitude door een strakke controle van cardiale 5-HT_4-receptoren door fosfodiësterasen (PDEs): bij het varken door PDE3 plus PDE4 en bij de mens door PDE3. cAMP gegenereerd bij stimulatie van de 5-HT_4-receptoren in het hart wordt snel afgebroken door PDEs, wat het antwoord sterk beperkt. In tegenstelling tot de cardiale effecten van prucalopride, zijn de GI effecten op cholinerge neurotransmissie blijvend en uitgesproken. Toch kan dit effect *in vitro* versterkt worden door selectieve PDE4-inhibitie in de maag en het colon van het varken. Dit suggereert een PDE4-gemedieerde controle van de signaaltransductie van 5-HT_4-receptoren in cholinerge neuronen.

Hoewel prucalopride tot op heden cardiovasculair veilig werd bevonden, blijft enige bezorgdheid voor cardiale bijwerkingen bestaan. Het risico op cardiale bijwerkingen kan verder worden gereduceerd met combinatietherapie, waarbij het prokinetisch effect behouden blijft met een lagere dosis en dus lagere plasmaconcentratie van prucalopride. Een eerste mogelijkheid is combinatie van een 5-HT_4-receptoragonist met een acetylcholine-esterase inhibitor, want met deze combinatie werd een synergistisch effect *in vitro* op cholinerge activiteit in het colon van de mens en *in vivo* op de transittijd

van de dikke darm bij de rat gerapporteerd. Combinatie van de 5-HT_4-receptoragonist prucalopride met de selectieve PDE4-inhibitor rolipram verhoogde elektrisch geïnduceerde vrijstelling van acetylcholine en cholinerge contracties op synergistische wijze in de GI tractus van het varken. Indien deze *in vitro* synergie tussen een 5-HT_4-receptoragonist en een PDE4-inhibitor bevestigd kan worden bij de mens, en het eveneens *in vivo* kan worden aangetoond, kan deze combinatietherapie worden overwogen. Om de synergie van deze combinatie verder te onderzoeken, waren de **doelstellingen** van dit proefschrift:

- bevestiging van de PDE4-gemedieerde controle van de signaaltransductie van 5-HT_4-receptoren op cholinerge neuronen in de GI tractus van het varken met andere farmaca dan prucalopride en rolipram (**hoofdstuk III**);
- nagaan of deze PDE4-gemedieerde controle van 5-HT_4-receptoren ook aanwezig is in de GI tractus van de mens (**hoofdstuk IV**);
- evaluatie van de muis als mogelijk model voor *in vivo* studie van het synergetische prokinetisch effect van een 5-HT_4-receptoragonist met een PDE4-inhibitor (**hoofdstuk V** en **VI**).

In **hoofdstuk III** werd de synergie tussen 5-HT_4-receptoragonisme en PDE4-inhibitie bevestigd in circulaire spieren van de maag van het varken met roflumilast en velusetrag door directe meting van elektrisch geïnduceerde vrijstelling van acetylcholine. Roflumilast is een PDE4-inhibitor, op de markt gebracht om exacerbaties bij COPD-patiënten te onderdrukken; velusetrag is een zeer selectieve 5-HT_4-receptoragonist die behoort tot een andere chemische klasse dan prucalopride en zich momenteel in klinische fase van ontwikkeling bevindt voor constipatie en gastroparese. Roflumilast op zich verhoogde de elektrisch geïnduceerde vrijstelling van acetylcholine, wat de regulerende rol van PDE4 in de basale hoeveelheid cAMP in cholinerge neuronen in de maag van het varken bevestigt, zoals eerder werd ontdekt met rolipram. De facilitering van de vrijstelling van acetylcholine door een submaximale concentratie van prucalopride werd verhoogd door roflumilast op synergistische wijze. Net als prucalopride, verhoogde velusetrag de elektrisch geïnduceerde vrijstelling van acetylcholine op concentratie-afhankelijke wijze, maar het maximaal waargenomen effect met velusetrag was minder uitgesproken. Dit kan correleren met een lager maximaal agonistisch effect van velusetrag. Submaximale facilitatie van elektrisch geïnduceerde vrijstelling van acetylcholine door velusetrag werd versterkt door rolipram, eveneens op synergistische wijze. Het synergistisch effect van roflumilast plus prucalopride, en van rolipram plus velusetrag, bevestigen dat PDE4 de signaaltransductie van faciliterende 5-HT_4-receptoren in cholinerge neuronen reguleert in de maag van het varken, zoals eerder aangetoond met rolipram en prucalopride.

In **hoofdstuk IV** werd dit controlerend mechanisme onderzocht in de circulaire spierlaag van de dikke darm van de mens; hierbij werd opnieuw gebruik gemaakt van de techniek met directe meting van de vrijstelling van acetylcholine. De facilitering van elektrisch geïnduceerde vrijstelling van acetylcholine door een submaximale concentratie van prucalopride werd verhoogd met de niet-selectieve PDE-inhibitor IBMX. Dit suggereert een PDE-gemedieerde controle van de 5-HT_4-receptoren op myenterische

cholinerge neuronen en het hiervoor verantwoordelijke PDE-subtype werd verder geïdentificeerd door studie van selectieve inhibitoren voor de klassieke PDE-subtypes (PDE1 tot 4). In tegenstelling tot de maag van het varken, hadden rolipram of roflumilast op zich geen effect op de vrijstelling van acetylcholine; in de dikke darm van de mens is er dus geen evidentie voor een PDE4-gemedieerde regulatie van de basale hoeveelheid cAMP in myenterische cholinerge neuronen. Zowel rolipram als roflumilast verhoogden het effect van prucalopride tot dezelfde grootteorde als IBMX; selectieve PDE1-, 2- en 3-inhibitoren hadden geen effect. Dit geeft aan dat ook in de dikke darm van de mens de faciliterende 5-HT_4-receptoren op cholinerge neuronen onder controle staan van PDE4, wat een mogelijke verklaring is voor de eerder gesuggereerde zwakke koppeling tussen 5-HT_4-receptoren en de vrijstelling van acetylcholine in een functioneel onderzoek van het colon van de mens. Het idee om een 5-HT_4-receptoragonist te combineren met een PDE4-inhibitor om uitgesproken GI prokinetische effecten te bekomen met een lage dosis 5-HT_4-receptoragonist, wordt dus ondersteund, maar ook andere locaties van 5-HT_4-receptoren en PDE4 moeten worden beschouwd. Cardiale 5-HT_4-receptoren vormen geen probleem aangezien deze onder controle staan van PDE3 bij de mens; het effect van hun stimulatie zal dus niet worden beïnvloed door gelijktijdige behandeling met een PDE4-inhibitor. Het prokinetisch effect bij verhoogde vrijstelling van acetylcholine uit myenterische neuronen als gevolg van een 5-HT_4-receptoragonist in combinatie met een PDE4-inhibitor kan echter wel beïnvloed worden door relaxerende 5-HT_4-receptoren aanwezig op gladde spiercellen en door PDE4 dat de basale hoeveelheid cAMP in gladde spiercellen regelt in de menselijke dikke darm; dit vereist verder onderzoek.

Tot slot werd in **hoofdstuk V** en **VI** de muis geëvalueerd als mogelijk model voor *in vivo* onderzoek van het prokinetisch effect van de combinatie 5-HT_4-receptoragonist plus PDE4-inhibitor. In **hoofdstuk V** werd de aanwezigheid van 5-HT_4-receptoren op myenterische cholinerge neuronen bij de muis gedocumenteerd. Prucalopride verhoogde de elektrisch geïnduceerde submaximale cholinerge contracties op concentratie-afhankelijke wijze in fundus, jejunum en colon van de muis; dit effect werd geblokkeerd door een 5-HT_4-receptorantagonist. Dit illustreert de aanwezigheid van 5-HT_4-receptoren op myenterische cholinerge neuronen naar circulaire gladde spiercellen, wat werd bevestigd met 5-HT. De maximale facilitatiegraad met prucalopride was vergelijkbaar met deze bekomen bij andere species, maar werd bereikt met lagere concentraties wat wijst op een hoger aantal en/of efficiënter gekoppelde 5-HT_4-receptoren op myenterische cholinerge neuronen bij muizen. Vermindering van de met prucalopride bekomen facilitatie door de nicotinereceptor-blokker mecamylamine in de fundus, maar niet in jejunum en colon, wijst op een bijdrage van preganglionaire 5-HT_4-receptoren in het bovenste deel van de maag.
Gezien bij de muis de aanwezigheid van 5-HT_4-receptoren op myenterische cholinerge neuronen naar de circulaire gladde spierlaag duidelijk werd aangetoond, werd hun mogelijke PDE-gemedieerde controle bestudeerd in **hoofdstuk VI**. De methode met directe vrijstellingsmeting van acetylcholine was niet toepasbaar in GI weefsel van de muis, vandaar dat de methode van elektrisch geïnduceerde cholinerge contracties werd gebruikt, waarbij zowel effecten op neuronaal als op spiercelniveau opgepikt worden. Eerst werd de invloed van PDE-inhibitoren op carbachol- en elektrisch geïnduceerde

submaximale cholinerge contracties nagegaan. De niet-selectieve PDE-inhibitor IBMX inhibeerde de carbachol-geïnduceerde contracties. Dit illustreert dat één of meerdere PDE-subtypes betrokken zijn bij de regulatie van GI gladde spieractiviteit bij de muis. De selectieve PDE3-inhibitor cilostamide en in mindere mate de selectieve PDE4-inhibitor rolipram verlaagden de carbachol-geïnduceerde contracties op concentratie-afhankelijke wijze over een breed concentratiegebied; met de PDE1-inhibitor vinpocetine en PDE2-inhibitor EHNA was dit niet het geval. Dit wijst op een rol voor PDE3 en PDE4, waarbij PDE3 belangrijkst is, in de controle van cyclische nucleotiden in GI gladde spiercellen bij de muis. De meer uitgesproken effecten van cilostamide in vergelijking met rolipram kunnen te wijten zijn aan inhibitie van het cGMP-metabolisme aangezien PDE3 zowel cAMP als cGMP metaboliseert, terwijl PDE4 cAMP-specifiek is. De betrokkenheid van PDE3 en PDE4 werd bevestigd bij studie van de elektrisch geïnduceerde submaximale cholinerge contracties waar gelijkaardige effecten werden gevonden die weliswaar minder uitgesproken waren dan bij carbachol-geïnduceerde contracties. Dit laatste zou kunnen verklaard worden door presynaptische acties van de PDE-inhibitoren, vermits presynaptische cAMP-gemedieerde facilitatie van de vrijstelling van acetylcholine mogelijk is; hiervan kan een tegenwerkende postsynaptische relaxatie worden verwacht.
Het effect van PDE-inhibitoren op submaximale prucalopride-geïnduceerde facilitatie van elektrisch uitgelokte contracties werd hierna bestudeerd. Noch IBMX, noch de selectieve PDE1- tot 4-inhibitoren waren in staat het effect van prucalopride op elektrisch uitgelokte cholinerge contracties te verhogen in fundus, jejunum en colon van de muis. In tegenstelling tot de maag en het colon van het varken kon bij de muis het effect van prucalopride niet verhoogd worden door de PDE4-inhibitor rolipram. De muis is dus niet geschikt om *in vivo* mogelijke synergistische gastroprokinetische effecten van een 5-HT_4-receptoragonist in combinatie met een selectieve PDE4-inhibitor te testen.

De algemene **conclusie** is als volgt: De PDE4-gemedieerde controle van 5-HT_4-receptoren op myenterische cholinerge neuronen naar gladde spiercellen werd in de maag van het varken bevestigd met roflumilast en velusetrag. In de circulaire gladde spierlaag van de menselijke dikke darm is dit controlerend mechanisme eveneens aanwezig, aangezien synergie tussen prucalopride en rolipram of roflumilast op elektrisch geïnduceerde myenterische vrijstelling van acetylcholine werd waargenomen. Indien bevestigd *in vivo*, kan combinatietherapie van een lage dosis 5-HT_4-receptoragonist met een PDE4-inhibitor bij de mens overwogen worden om uitgesproken prokinetische effecten te bekomen zonder de cardiale 5-HT_4-receptoren, die uitsluitend onder controle staan van PDE3, te beïnvloeden. Doorheen de GI tractus van de muis werd de aanwezigheid van 5-HT_4-receptoren op cholinerge neuronen naar circulaire gladde spiercellen duidelijk vastgesteld, met evidentie voor een hoger aantal en/of efficiënter gekoppelde receptoren in vergelijking met andere species. Niettemin kon geen enkele selectieve PDE-inhibitor het faciliterend effect van een submaximale concentratie van prucalopride op elektrisch uitgelokte submaximale cholinerge contracties verhogen. GI preparaten van de muis zijn dus bruikbaar voor het testen van de potentie en efficiëntie van nieuw ontwikkelde 5-HT_4-receptoragonisten, maar de muis kan niet gebruikt worden om *in vivo* mogelijke synergistische gastroprokinetische effecten van combinatietherapie van een 5-HT_4-receptoragonist met een selectieve PDE4-inhibitor te testen.

Scientific *Curriculum Vitae*

Scientific *Curriculum Vitae*

PARTICULARS

Name	Vicky Pauwelyn
Date of birth	December 14th, 1990
Place of birth	Roeselare, Belgium
Nationality	Belgian

EDUCATION

2013 – 2018	Assistant / PhD candidate in Health Sciences Department of Pharmacology – Heymans Institute (Research group Gastro-intestinal Neuropharmacology) Ghent University, Ghent, Belgium *Dissertation: 'Phosphodiesterase-mediated regulation of 5-HT_4 receptors, facilitating cholinergic neurotransmission, in the gastrointestinal tract'* Promotor: Prof. dr. Romain A. Lefebvre
2011 - 2013	Master of Science in Biomedical Sciences – *magna cum laude* (Major Neurosciences) Ghent University, Ghent, Belgium *Dissertation: 'Effect van hippocampale neurostimulatie op angst en geheugen bij de rat'* Promotor: Prof. dr. Robrecht Raedt
2008 - 2011	Bachelor of Science in de Biomedical Sciences – *magna cum laude* Ghent University, Ghent, Belgium
2002 - 2008	Science-Mathematics Sint-Jozefsinstituut-College, Torhout, Belgium

TRAINING COURSES

2017	Embase: basis en geavanceerd zoeken voor onderzoekers Kenniscentrum voor de Gezondheidszorg Gent - Ghent University
2014-2017	Seminarie Welzijn & Milieu: Veilig werken met ioniserende straling (Annual rehearsal session) Ghent University
2016	Recognition and prevention of pain, suffering and distress in laboratory animals Ghent University
2015	Analysis of Variance (Specialist course) Institute for Continuing Education in Science - Ghent University
2015	Introductory statistics. Basics of statistical inference (Specialist course) Institute for Continuing Education in Science - Ghent University

2013	Seminar in transferable skills: Schrijven van niet-technische fiches van dierproeven Ghent University
2013	Certificate Good Clinical Practice - Profess Basic ICH GCP Qualification Training Course and Examination Ghent University in association with Profess Academy
2011	Laboratory Animal Science - FELASA type C (Federation of European Laboratory Animal Science Associations, 80 h); Principle investigator in conducting animal research Ghent University

LIST OF PUBLICATIONS (https://biblio.ugent.be/person/000080267496)

A1 publications (peer-reviewed)

- Lefebvre RA, Van Colen I, **Pauwelyn V**, De Maeyer JH. Synergistic effect between 5-HT_4 receptor agonist and phosphodiesterase 4-inhibitor in releasing acetylcholine in pig gastric circular muscle in vitro. *European Journal of Pharmacology* 2016; 781: 76–82.
  DOI: 10.1016/j.ejphar.2016.04.003
  https://doi.org/10.1016/j.ejphar.2016.04.003

- **Pauwelyn V**, Lefebvre RA. 5-HT_4 receptors facilitate cholinergic neurotransmission throughout the murine gastrointestinal tract. Neurogastroenterology *and Motility* 2017; 29(8): e13064.
  DOI: 10.1111/nmo.13064
  https://doi.org/10.1111/nmo.13064

- **Pauwelyn V**, Ceelen W, Lefebvre RA. Synergy between 5-HT_4 receptor stimulation and phosphodiesterase 4 inhibition in facilitating acetylcholine release in human large intestinal circular muscle. *Neurogastroenterology and Motility* 2018; 30(2): e13162.
  DOI: 10.1111/nmo.13162
  https://doi.org/10.1111/nmo.13162

- **Pauwelyn V**, Lefebvre RA. cAMP catalyzing phosphodiesterases control cholinergic muscular activity but their inhibition does not enhance 5-HT_4 receptor-mediated facilitation of cholinergic contractions in the murine gastrointestinal tract. *Frontiers in Pharmacology* 2018; 9: 171.
  DOI: 10.3389/fphar.2018.00171
  https://doi.org/10.3389/fphar.2018.00171

C3 publications (published abstracts)

- **Pauwelyn V**, Van Deynse E, Lefebvre RA. Facilitation of cholinergic neurotransmission via 5-HT_4 receptors in the murine gastrointestinal tract. *Acta Gastroenterologica Belgica* 2015; 78(1): B08.
  http://www.bwge.be/wp-content/themes/Evento/pdf/2015_BW_Abstracts.pdf
  Communication (oral) at 27th Belgian Week of Gastroenterology, Brussels, Belgium (25-28/02/2015).

- **Pauwelyn V**, Van Deynse E, Lefebvre RA. Facilitation of murine enteric cholinergic neurotransmission by 5-HT_4 receptor activation: control by phosphodiesterases. *Neurogastroenterology and Motility* 2015; 27(S2): 91.
  http://onlinelibrary.wiley.com/doi/10.1111/nmo.12598/abstract

Communication (poster) at NeuroGastro 2015, Meeting of the European Society of Neurogastroenterology and Motility, Istanbul (04-06/06/2015).

- **Pauwelyn V**, Van Deynse E, Lefebvre RA. Facilitation of cholinergic neurotransmission via 5-HT_4 receptors in murine stomach, jejunum and colon. *Proceedings Autumn Meeting of Belgian Society of Physiology and Pharmacology* 2015; 7: 0-12.
  http://users.ugent.be/~jvdvoord/physiology&pharmacology/2015%20November%206%20abstractbook.pdf
  Communication (oral) at the Autumn Meeting 2015 of the Belgian Society of Physiology and Pharmacology; Brussels (06/11/2015).

- **Pauwelyn V**, Van Colen I, Ceelen W, Lefebvre RA. Control of the signalling pathway of 5-HT_4 receptors in cholinergic neurons of human colon by phosphodiesterases. *E-journal of the British Pharmacological Society* (pA2 online) 2015; 13(3): 083P.
  http://www.pa2online.org/abstracts/vol13issue3abst083p.pdf
  Communication (poster) at Pharmacology 2015, Winter Meeting of the British Pharmacological Society, London (15-17/12/2015).

- **Pauwelyn V**, Van Colen I, Ceelen W, Lefebvre RA. The signaling pathway of 5-HT_4 receptors in cholinergic neurons of human colon is controlled by phosphodiesterases. *Acta Gastroenterologica Belgica* 2016; 79(1): B02.
  http://www.bwge.be/wp-content/themes/Evento/pdf/2016_BW_Abstracts.pdf
  Communication (oral) at 28th Belgian Week of Gastroenterology, Brussels (18-20/02/2016).

- **Pauwelyn V**, Van Deynse E, Lefebvre RA. Influence of phosphodiesterases on cholinergic contractility and its 5-HT_4 receptor-mediated modulation in the murine gastrointestinal tract. *Proceedings Autumn Meeting of Belgian Society of Physiology and Pharmacology* 2016; 8: 0-07.
  http://users.ugent.be/~jvdvoord/physiology&pharmacology/2016%20Autumn%20Meeting%20abstractbook.pdf
  Communication (oral) at the Autumn Meeting 2016 of the Belgian Society of Physiology and Pharmacology, Brussels (21/10/2016).

- **Pauwelyn V**, Van Deynse E, Lefebvre RA. Influence of phosphodiesterases on basal and 5-HT_4 receptor-facilitated cholinergic contractility in the murine gastrointestinal tract. *Acta Gastroenterologica Belgica* 2017; 80(1): B08.
  http://www.bwge.be/wp-content/themes/Evento/pdf/2017_BW_Abstracts.pdf
  Communication (oral) at 29th Belgian week of Gastroenterology, Antwerp (09-11/02/2017).

SUPERVISION

Supervision Master Thesis - 1st and 2nd Master of Medicine

2014-2016	*'Diepe hersenstimulatie als behandeling van de ziekte van Parkinson'* Giel Van Vanschoenbeek
2016-2018	*'Probiotica ter behandeling van constipatie'* Casper De Bock

Supervision 'Z-lijn paper' (2013-2018) - 2nd Bachelor of Medicine

Acknowledgements

Acknowledgements

Mijn doctoraat was een geweldig avontuur met hoogte- en dieptepunten, een onvergetelijke ervaring waartoe heel wat mensen, elk op hun eigen manier, hebben bijgedragen. Dit werk zou nooit tot stand gekomen zijn zonder hun hulp, bijdrage en ondersteuning!

Hier verdient uiteraard mijn **promotor prof. dr. Romain Lefebvre** een bijzondere bedanking. Zijn constructieve input, wetenschappelijke ervaring, grenzeloze kennis, geduld, motivatie, precisie en zo veel meer, hebben een onmisbare rol gespeeld voor het slagen van dit doctoraat. Steevast opvolgen van de experimenten, bespreken van protocollen en resultaten, het nalezen van mijn schrijfsels,… Niets was u te veel en uw deur stond steeds open voor uw doctoraatsstudenten. Een welgemeende dankjewel voor alles!

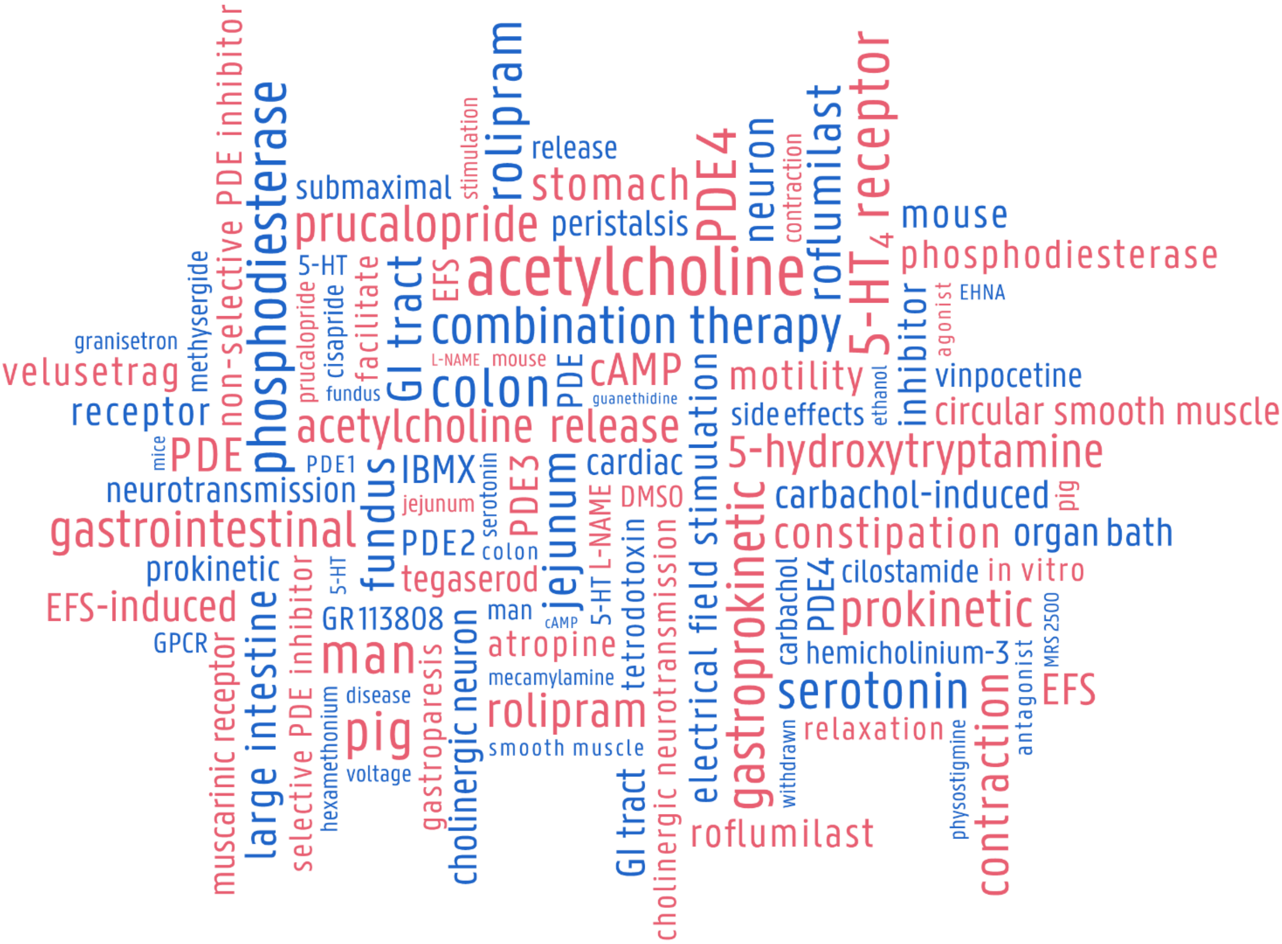
Ook mijn **collega's** en in het bijzonder Inge, Els en Danielle, wil ik bedanken voor hun praktische hulp, zij waren steeds bereid hun kennis en ervaring te delen en te helpen waar mogelijk. Samen met Sarah, Jonas, Dinesh, Tom, Sam, Valère en alle andere (ex-)collega's die ik tijdens mijn doctoraat leerde kennen, dragen zij bij tot de fijne werksfeer in blok B en de ontspannende middagpauzes. Collega's, dankjulliewel voor deze fijne tijd!

Daarnaast wil ik prof. dr. Wim Ceelen en de **dienst Gastro-intestinale Heelkunde** bedanken voor de samenwerking. In het bijzonder de studieverpleegkundigen Saskia, Inge en Eva voor het afnemen van 'informed consent' en om mij via ontelbaar veel mails en telefoontjes op de hoogte te houden van goed en minder goed nieuws vanuit het operatiekwartier. Ook alle patiënten die ondanks de moeilijke tijd die ze doorstaan toch bereid waren mee te werken aan dit onderzoek, bedankt, uw deelname werd erg geapprecieerd!

I would like to thank the **members of the examination committee** for their time and effort spent on reading and evaluating this dissertation. I really appreciated their constructive feedback and the nice discussion during the internal defense.

Een belangrijke plaats in dit dankwoord is ook weggelegd voor mijn **familie en vrienden**. Hoewel zij vaak geen flauw idee hadden waar dit alles over ging, hebben zij steeds geluisterd wanneer nodig en me onvoorwaardelijk gesteund op de meest uiteenlopende manieren. Bedankt dat ik steeds op jullie kon rekenen om mijn gedachten te verzetten! In het bijzonder wil ik mijn ouders en zus graag bedanken om er altijd te zijn voor mij. En dit niet alleen tijdens mijn doctoraat, maar ook al die jaren daarvoor, een oprechte merci voor alles! Last but not least… Hendrik, bedankt voor alles! Om er altijd voor mij te zijn, om geduldig te luisteren, in mij te geloven, mij te steunen, te begrijpen, op te beuren of gewoon te laten doen. Woorden schieten tekort om te omschrijven wat jouw dagelijkse aanwezigheid voor mij betekent. Ik kijk al uit naar wat de toekomst ons gezinnetje brengt!

Iedereen die, al dan niet onbewust, heeft bijgedragen tot dit werk, **BEDANKT**,
zonder jullie was dit niet mogelijk geweest!



non-selective PDE inhibitor
phosphodiesterase
rolipram
release
submaximal
stimulation
stomach
prucalopride
peristalsis
PDE4
neuron
contraction
roflumilast
5-HT4 receptor
mouse
phosphodiesterase
methysergide
5-HT
prucalopride
cisapride
facilitate
GI tract
EFS
acetylcholine
EHNA
agonist
granisetron
combination therapy
inhibitor
velusetrag
L-NAME
mouse
fundus
colon
PDE
cAMP
guanethidine
motility
ethanol
vinpocetine
receptor
acetylcholine release
side effects
circular smooth muscle
mice
PDE
PDE1
fundus
IBMX
serotonin
PDE3
jejunum
cardiac
stimulation
5-hydroxytryptamine
neurotransmission
jejunum
L-NAME
DMSO
carbachol-induced
pig
gastrointestinal
PDE2
colon
tetrodotoxin
gastroprokinetic
constipation
organ bath
prokinetic
selective PDE inhibitor
5-HT
tegaserod
cholinergic neurotransmission
electrical field stimulation
carbachol
PDE4
cilostamide
in vitro
EFS-induced
large intestine
GR113808
man
cAMP
5-HT
prokinetic
MRS2500
GPCR
man
atropine
hemicholinium-3
muscarinic receptor
hexamethonium
disease
gastroparesis
cholinergic neuron
mecamylamine
serotonin
antagonist
EFS
pig
rolipram
withdrawn
relaxation
physostigmine
contraction
voltage
smooth muscle
GI tract
roflumilast