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Sensory and secretory responses to intestinal distension; implications for the pathophysiology of the irritable bowel syndrome

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ABSTRACT

Sensory and secretory responses to intestinal distension; implications for the pathophysiology of the irritable bowel syndrome

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Irritable bowel syndrome (IBS) is a common gut disorder, characterized by abdominal pain and/or discomfort associated with disturbed bowel habits. The pathophysiology of IBS is complex and still largely unknown, although visceral hypersensitivity is frequently associated with the disease. The aim of the present thesis was to test alternative pathophysiological mechanisms involved in IBS and to establish relevant animal models.

In the first two papers, the role of intestinal secretomotor neurons was evaluated. The relation between intestinal pressure and transmural potential difference (PD) was used as a marker for activation of mechanosensitive secretomotor neurons. The pressure-PD relationship was studied by modified multilumen manometry in humans or by distension of an isolated duodenal segment in rats and mice. In the last two reports, a colorectal distension (CRD) model in mice was developed, and the effect of dextran-sodium sulphate (DSS)-induced colitis on visceral sensitivity was studied.

IBS patients had an increased propagation speed of the phase III of the migrating motor complex. Maximal PD during motor activity was elevated in both duodenum and jejunum and the return of PD to baseline levels at the end of phase III was prolonged in IBS patients. In anaesthetized rats and mice, the PD response to distension was biphasic, with an initial rapid phase followed by a sustained phase. Tetrodotoxin, a nerve-blocking agent, reduced both responses, implying that they are at least partially neurally mediated. The amplitude and rate of rise of the rapid response were reduced by ganglionic blockade with hexamethonium, by serosal lidocaine and by tachykinin receptor blockade (NK1). The sustained response was reduced by tachykinin receptor blockade (NK1 and NK3) and by blockade of the VIP-sensitive VPAC receptor. Electromyographic (EMG) recordings in mice correlated linearly with intracolonic balloon pressures between 10 and 80 mmHg. The response to CRD was reduced by μ- and κ-opioid receptor agonists, but was not affected by DSS-induced inflammation.

Conclusions: The data suggest an abnormal response of secretomotor neurons to phase III contractions in IBS patients. The complex time course and pharmacology seen in the animal experiments may reflect network behaviour of intrinsic primary afferent neurons. Most of the data can be explained by an equivalent circuit consisting of at least two parallel-coupled networks operating via tachykinin- and VPAC receptors. The sensory response to CRD can be readily monitored in conscious mice. However, DSS-evoked colitis does not appear to alter colorectal mechanosensitivity.

Key words: irritable bowel syndrome, secretion, transmucosal potential difference, migrating motor complex, colorectal distension, visceral sensitivity, colitis, enteric nervous system, rat, mouse

LIST OF PAPERS

This thesis is based on the following papers, which are referred to by their Roman numerals in the text.

I. Larsson M.H, Simrén M, Thomas E.A, Bornstein J.C, Lindström E & Sjövall H
    Elevated motility-related transmucosal potential difference in the upper small intestine in the irritable bowel syndrome.
    Neurogastroenterol. Motil. 2007; In Press.

II. Larsson M.H, Sapnara M, Thomas E.A, Bornstein J.C, Svensson D.J, Lindström E & Sjövall H
     Pharmacological analysis of components of the change in transmural potential difference evoked by distension of rat proximal small intestine in vivo.
     Manuscript.

III. Larsson M.H, Arvidsson S, Ekman C & Bayati A.
      A model for chronic quantitative studies of colorectal sensitivity using balloon distension in conscious mice - effects of opioid receptor agonists.
      Neurogastroenterol. Motil. 2003; **15**: 371-81.

IV. Larsson M.H, Rapp L & Lindström E.
     Effect of DSS-induced colitis on visceral sensitivity to colorectal distension in mice.
     Neurogastroenterol. Motil. 2006; **18**: 144-152.

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ABBREVIATIONS

Ach	acetylcholine
AH	designation of neurons having slow after-hyperpolarizing potentials
AHP	after-hyperpolarizing potential
AMP	adenosine monophosphate
ATP	adenosine triphosphate
AUC	area under the curve
Ca^{2+}	calcium ion
CFTR	cystic fibrosis transmembrane conductance regulator
CGRP	calcitonin gene-related peptide
Cl^-	chloride ion
CNS	central nervous system
CRD	colorectal distension
DRG	dorsal root ganglion
DSS	dextran sodium sulphate
EMG	electromyogram
ENS	enteric nervous system
EPAN	extrinsic primary afferent
EPSP	excitatory post-synaptic potential
5-HT	5-hydroxytryptamine (serotonin)
GI	gastro-intestinal
IBS	irritable bowel syndrome
d-IBS	diarrhea predominant IBS
c-IBS	constipation predominant IBS
IGLE	intraganglionic laminar ending
IPAN	intrinsic primary afferent neuron
IPSP	inhibitory post-synaptic potential
K^+	potassium ion
MMC	migrating motor complex
MP	myenteric plexus
MPO	myeloperoxidase
Na^+	sodium ion
NG	nodose ganglion
NK	neurokinin
NO	nitric oxide
NPY	neuropeptide Y
P_2X	purine receptor 2X
PACAP	pituitary adenylyl cyclase activating peptide
PD	potential difference
SAC	stretch activated channel
SEM	standard error of the mean
SMP	submucosal plexus
SP	substance P
TTX	tetrodotoxin
VIP	vasoactive intestinal peptide
VMR	visceromotor response
VPAC	vasoactive intestinal peptide receptor

BACKGROUND

The gastrointestinal tract is needed to receive food, digest it into small molecules, absorb the nutrients and to eliminate indigestible leftovers. The integration of all of these events is exerted by multiple and exceedingly complex regulatory systems, which monitor the events within the gastrointestinal tract. Neural control plays a key role. The information from the gastrointestinal tract is processed both locally in the enteric nervous system (ENS) and part of it is also conveyed to the central nervous system (CNS) where appropriate commands to increase or decrease activities are given to fulfill the digestive process. Since there are many components involved in this regulatory system and because the gastrointestinal system is exposed to the external environment, there are several mechanisms that can be disturbed and cause disease.

Against this background, it is not surprising that disturbances of gut function and pain/discomfort attributed to gut dysfunction are exceedingly common. This syndrome, i.e. abdominal pain/discomfort relieved by defecation and accompanied by a disturbed stool evacuation pattern, is named the irritable bowel syndrome (IBS). IBS is common, with a prevalence of 10-20% in both Western and third world populations (Camillieri and Choi, 1997; Chang and Lu, 2007; Hungin et al., 2005; Hungin et al., 2003). Both the etiology and pathophysiology are largely unknown, although there are a large number of explanatory models ranging from visceral hyperalgesia to brain-gut dysfunction and undiagnosed inflammatory processes. The most prevalent current model is visceral hypersensitivity, i.e. an exaggerated response to sensory signals originating from the gut. However, this phenomenon has been demonstrated only in a subpopulation of IBS patients (Kuiken et al., 2005; Mayer et al., 2001; Mertz et al., 1995).

The general aim of the current thesis was to develop new models useful for studying the pathophysiology of the IBS. Distension of the gut is an established way of activating pain fibres that transmit signals to the CNS. However, distension also activates intramural sensory neurons (intrinsic primary afferents, IPANs). IPANs are connected in networks, which in turn project to the mucosa eliciting local secretory reflexes generating a transmucosal potential difference (PD) that can be readily measured in real time. Monitoring the PD response to distension might therefore be a new way to study network behaviour of intramural sensory systems and their response to distension. A similar secretory response is also activated by

intestinal contractions. Studying the relation between sustained contractions and PD may therefore be a potential way to indirectly monitor the behaviour of these networks during spontaneous motor activity in awake humans. The mouse is an important species for mechanistic experiments, since sensory transmission can then be studied in various knockout models. It is therefore of great value to develop a model for quantifying sensory responses to intestinal distension in awake mice. It is also of interest to study the effect of inflammation on the response to distension in mice, since low-degree of inflammation has been reported to be present in some subgroups of IBS patients (Törnblom et al., 2002). In addition, an infectious gastroenteritis seems to increase the probability of developing IBS (Gwee et al., 1999; Marshall et al., 2006; Parry et al., 2003; Spiller, 2003). An animal model for post-inflammatory IBS would thus have a great value for drug development.

Before describing the sets of experiments performed to address these issues, the essential features of the innervation of the gastrointestinal tract, the sensory transmission systems involved and current views regarding the pathophysiology of IBS will be summarized.

INTRODUCTION

1. Irritable bowel syndrome

The irritable bowel syndrome is one of the most common functional gastrointestinal disorders seen in both primary (Thompson et al., 2000) and secondary-tertiary care (Harvey et al., 1983). IBS occurs worldwide and affects people of all ages and both sexes. In the Western community, the prevalence of IBS ranges between 10-20%, with a higher prevalence in females (Camillieri and Choi, 1997; Hungin et al., 2005; Hungin et al., 2003; Thompson et al., 2000; Wilson et al., 2004). It is not only a Western disease, since in Asian countries, the prevalence is within 5-10% regardless of age and gender (Chang and Lu, 2007; Han et al., 2006; Kwan et al., 2002). Although IBS has gained increased attention in the last 20 years, it has actually been recognized for more than a century, with different names over the years. In the late 19th century it was called membranous enteritis (Da Costa, 1871), while in the 20th century it has been termed mucous colitis (Poppel et al., 1955; White and Jones, 1940), the irritable colon syndrome (Lumsden et al., 1963), the spastic colon (Lechin et al., 1977) and nowadays the irritable bowel syndrome. The most common symptoms of IBS include lower abdominal pain or discomfort, disturbed defecation (diarrhea and/or constipation) and bloating (Drossman, 1999). These symptoms occur in the absence of (known) structural, biochemical or pathophysiological abnormalities that might otherwise explain these symptoms (Drossman, 1999). Therefore various diagnostic criteria have been implemented for the diagnosis of IBS. Efforts to define symptom-based criteria began in the 1970s, resulting in the Manning criteria (Manning et al., 1978), which was the first set of criteria to identify individuals with IBS. The Manning criteria have since been modified by the ROME I (Thompson et al., 1989), the ROME II (Table 1) (Thompson et al., 1999) and recently the ROME III criteria (Drossman, 2006). The main difference between the ROME II and ROME III criteria is that the demands regarding symptom duration have been reduced in the ROME III criteria. Instead of having symptoms over the last 12 months, symptoms over the last 6 months are now considered to be sufficient to be diagnosed with IBS.

Table 1. The ROME II criteria (Thompson et al., 1999).

Continuous or recurrent symptoms for more than 3 months in the preceding 12 months of abdominal pain or discomfort that has two of three features.   1. Relieved with defecation; and/or   2. Onset associated with a change in frequency of stool; and/or   3. Onset associated with a change in form (appearance) of stool
With the following supportive symptoms to subgroup the IBS patients into diarrhea or constipation predominant.   • Abnormal stool frequency (> 3/day or < 3/week)   • Abnormal stool form (loose/watery or lumpy/hard)   • Abnormal stool passage (straining, urgency or feeling of incomplete evacuation)   • Passage of mucus   • Bloating or feeling of abdominal distension.

1.1 Pathophysiology

Despite numerous studies, the pathophysiology of IBS is still largely unknown. Over the years, motor abnormalities (Kellow et al., 1988; Lind, 1991), sensory abnormalities (Mertz et al., 1995; Ritchie, 1973; Whitehead et al., 1990) and brain-gut abnormalities (Hobson and Aziz, 2004; Naliboff et al., 2001) have been proposed to play a causative role.

IBS was for long considered a gastrointestinal motility disorder. Indeed, a number of different patterns of abnormal gastrointestinal myoelectric and/or motor patterns (e.g. colonic myoelectric activity, transit times and number of MMCs) have been described in IBS patients. However, there are also studies that have not been able to confirm these findings, hence there are a lot of discrepancies in the data, for reviews see (Camilleri et al., 2002; Drossman, 1999; Drossman et al., 1997; Posserud et al., 2006). Furthermore, abnormal motility has been difficult to relate to symptoms, particularly abdominal pain (McKee and Quigley, 1993a; McKee and Quigley, 1993b). Since the beginning of the 1970s, visceral hypersensitivity has emerged as being an important factor associated with IBS. Ritchie was the first to demonstrate that IBS patients were more sensitive to colorectal balloon distension than normal controls (Ritchie, 1973). Since then, a number of different studies have confirmed the results obtained by Ritchie (Whitehead et al., 1990; Mertz et al., 1995). Visceral sensitivity is now regarded as a biomarker of IBS (Drossman, 2006; Mertz et al., 1995; Thompson et al., 1999), although visceral perception is not abnormal in all patients with IBS (Kuiken et al., 2005; Mayer et al., 2001). In addition, the brain-gut-axis has fairly

recently been implicated in the pathophysiology of IBS. Brain imaging studies have shown that pain processing areas such as the anterior cingulate, prefrontal cortex, insula and thalamus are activated to a different extent and pattern in IBS patients compared to controls (Hobson and Aziz, 2004; Mertz, 2002). Compared to healthy volunteers, IBS patients also show increased activities in brain areas involved in attention, arousal and autonomic responses (Naliboff et al., 2001). Taken together, these findings suggest that several factors, including dysregulation of the enteric nervous system, dysregulation of the brain-gut axis and abnormal attention to sensations from the gut may contribute to the generation of IBS symptoms.

1.2. Etiology

The etiology of IBS is largely unknown. However, it has been reported that stressful life events, such as a history of physical or sexual abuse during childhood, death of close relatives, divorce or other major trauma, frequently precede the onset of IBS symptoms (Drossman et al., 1990). In addition, an infectious gastroenteritis is believed to increase the probability to develop IBS (Gwee et al., 1999; Marshall et al., 2006; Parry et al., 2003; Spiller, 2003). Genetic factors are also likely to contribute to the development of IBS, since IBS tends to run in families and because it has been shown that concordance for IBS is significantly greater in monozygotic twins compared to dizygotic twins (Levy et al., 2001; Morris-Yates et al., 1998). However, it is not know which genes that are involved.

1.3. Current animal models

Today, visceral hypersensitivity is generally regarded as a valid biomarker for IBS and is considered as the cornerstone of the definition of IBS (Drossman, 2006; Thompson et al., 1999). The most extensively used model to study visceral sensitivity in both humans and animals is colorectal distension (CRD). The methods of measuring visceral sensitivity induced by colorectal distension in humans range from using the subject's subjective assessment of pain by the visual analogue scale, to different types of brain imaging studies. However, animals obviously cannot verbally report their assessment of the visceral nociception induced by CRD. Instead, in conscious animals, CRD results in a series of

stereotypic behavioral and autonomic responses, including passive avoidance, increases in arterial pressure and heart rate and a visceromotor response (VMR) in the form of contractions of the abdominal wall muscles (Al-Chaer et al., 2000; Gebhart and Ness, 1991; Larsson et al., 2003; Ness and Gebhart, 1988; Ness et al., 1991).

Experimental animal models are required to gain further insight into pathways, transmitters and mechanisms involved in mechanical hypersensitivity. Animal experiments may also assist in the drug development process. In addition to the *in vivo* approach of using CRD in awake animals, electrophysiology has also been performed both *in situ* in anaesthetized animals and *in vitro* in order to map the different neurons and transmitters involved in neurotransmission (Brierley et al., 2004; Cervero, 1994; Cervero and Sharkey, 1988; Gebhart and Sengupta, 1995; Su and Gebhart, 1998). With these experimental models, there have been subsequent attempts to develop disease models reflecting visceral hypersensitivity. This has been achieved by the use of different stressors, such as psychological stress (Bradesi et al., 2005; Coutinho et al., 2002), mechanical stress and chemical stress (Bercik et al., 2004; Burton, 1995; Coutinho et al., 1996; Coutinho et al., 2000; Gschossmann et al., 2002; Gschossmann et al., 2004). However, it is difficult to predict how the different animal models represent the mechanism/s involved in hyperalgesia in IBS patients.

2. Innervation of the gastrointestinal tract

Extrinsic and intrinsic innervation work together to control and coordinate gastrointestinal functions. The extrinsic innervation consists of the sympathetic and the parasympathetic pathways, while the enteric nervous system (ENS) constitutes the intrinsic innervation. Both extrinsic and intrinsic innervation belongs to the autonomic nervous system (Langley, 1921). The parasympathetic innervation is supplied mainly by the vagus and pelvic nerves, while the sympathetic innervation is supplied by the splanchnic nerves (arising from the thoraco-lumbar region in the spinal cord) (Fig. 1). Norepinephrine is the main transmitter in the post-ganglionic efferent sympathetic neurons, while acetylcholine is the main transmitter in the parasympathetic efferent neurons. Neuropeptides (e.g. NPY in the sympathetic neurons, VIP in the parasympathetic neurons) are also co-released and involved in neurotransmission. Stimulation of the sympathetic efferent fibres inhibits activation of enteric neurons, which subsequently leads to decreased motility and secretion. Activation of the parasympathetic

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efferent fibres has given conflicting results in animals, showing both excitation (de Groat and Krier, 1976; de Groat and Krier, 1978) and inhibition (Fasth et al., 1980) of contractility in the colon. A rich extrinsic afferent innervation (about 90% of the vagal nerve consists of afferents) conveys information from the gut to the CNS, while in comparison, the extrinsic efferent innervation which modulates the activity of the ENS is relatively sparse, with about 1 efferent fibre innervating 300 enteric neurons.

It is well documented that the ENS contains reflex pathways for regulation of motility and secretion and that these reflexes can function independently of the CNS. The extrinsic innervation is considered to be of importance to convey information about the state of the GI tract and to modulate intrinsic enteric reflexes.
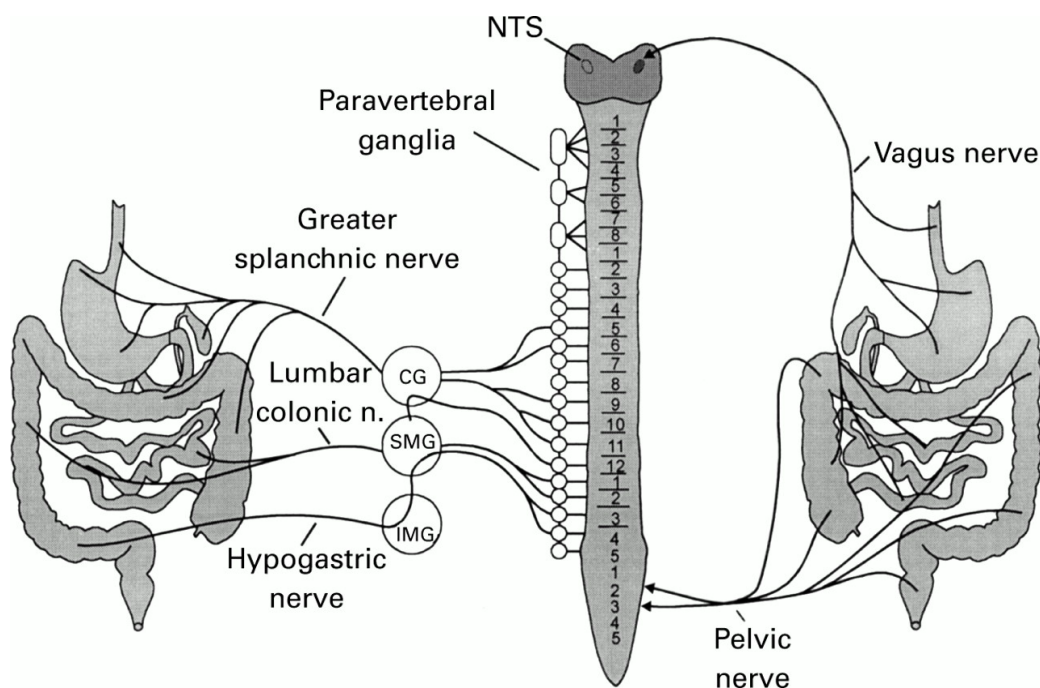
Fig. 1. Representation of the extrinsic nerves that innervate the gastrointestinal tract. The sensory innervation that is part of the sympathetic spinal nervous system is shown on the left hand side of the spinal cord. The sensory innervation that is part of the parasympathetic nervous system is shown on the right. CG: celiac ganglion; SMG: superior mesenteric ganglion; IMG: inferior mesenteric ganglion. From (Gebhart, 2000c), used with permission.

2.1 The enteric nervous system (ENS)

The ability of the ENS to sustain local reflex activity independently of the CNS system was shown already in 1899 by Bayliss and Starling (Bayliss and Sterling, 1899). The ENS is entirely located within the gut wall and is able to regulate several gastrointestinal functions, such as motility, absorption and secretion without input from the central nervous system. The ENS contains approximately 10^8 neurons and about ten or more distinct neuron populations have been distinguished on electrical, pharmacological, histochemical, biochemical and ultrastructural grounds (Furness and Costa, 1980).

Since most of the characterizations of the constituent neurons in the ENS have been performed in guinea-pig ileum, this segment is mainly referred to when describing these neurons.

2.1.1 Structure of the ENS

The enteric nervous system is organised in two main ganglionated plexuses that are located within the gut wall. The myenteric plexus is distributed along the entire gastrointestinal tract and is localised between the longitudinal and circular muscle layers. A distinct submucous plexus is found only in the small and large intestine where it is localised within the submucosa (between the circular muscle layer and the mucosa) (Furness and Costa, 1980) (Fig. 2). The shape of the myenteric meshwork is generally conserved throughout the gastrointestinal tract and between species, although the shape, size and orientation of the myenteric ganglia varies (Gabella, 1981). The submucosal plexus is finer and the ganglia are smaller compared to the myenteric plexus (Timmermans et al., 2001). Small animals, such as the guinea-pig, usually have a single layer of submucosal plexus, while larger animals have two layers, where the inner layer resembles that of smaller animals (Furness et al., 2003b; Timmermans et al., 2001). The two plexuses are connected to each other by numerous fibre bundles that run almost perpendicular to the circular muscle layer (Brehmer et al., 1997; Furness et al., 1990a). It is generally considered that the myenteric plexus mainly regulates motility, while the submucosal plexus mainly regulates secretion.

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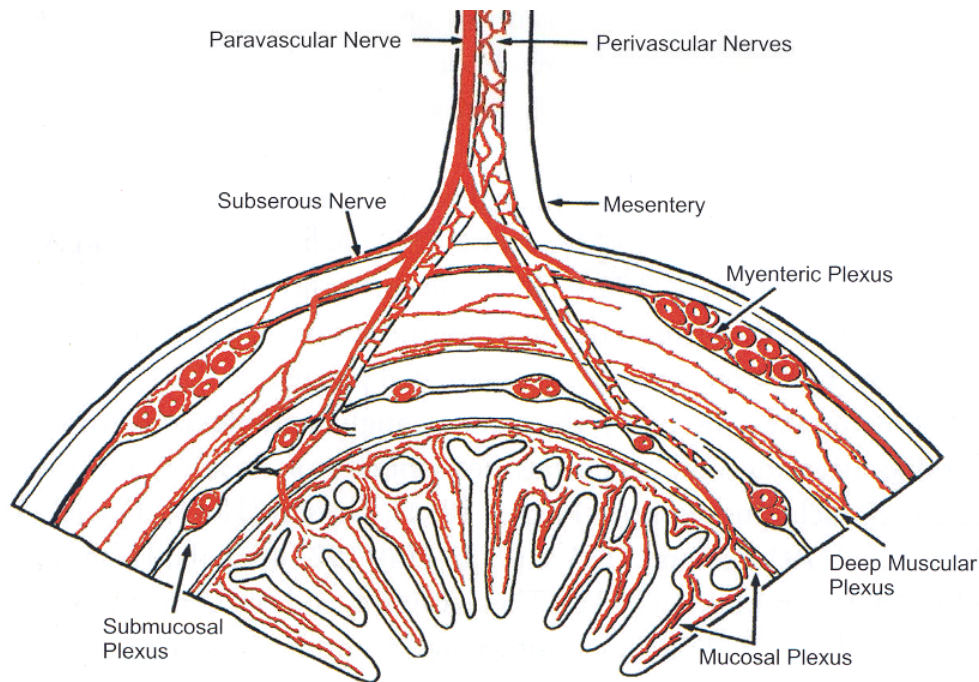
Fig. 2. Illustration of the enteric plexuses in the small intestine in guinea pig, as it is seen in transverse. From (Furness, 2006), used with permission.

2.1.2 Basic neurophysiology of the neuro-neuronal synapses

There are four main types of neuronal transmission that occur between neuronal synapses in the ENS; fast excitatory post-synaptic potentials (fEPSP), slow excitatory post-synaptic potentials (sEPSP), slow inhibitory post-synaptic potentials (sIPSP) and pre-synaptic inhibition.

The dominant part of fast transmission in the ENS is cholinergic, involving nicotinic cholinergic receptors (Hirst and McKirdy, 1974; Nishi and North, 1973). ATP acting at purinergic P_2X receptors and 5-HT (serotonin) acting through $5\text{-}HT_3$ receptors have also been shown to be involved (Galligan, 2002). The slow EPSPs are primarily due to reduction in resting K^+ conductance. The primary transmitters involved include acetylcholine acting at muscarinic receptors and tachykinins binding to tachykinin receptors (NK1 and NK3). It has also been suggested that 5-HT may induce sEPSPs, but the data are inconsistent (Bornstein et al., 2002). Slow IPSPs are observed in myenteric neurons, but are more common in submucosal neurons (Hirst and McKirdy, 1975; Wood and Mayer, 1978). Slow IPSPs are primarily due to increased K^+ conductance and are mediated by the release of norepinephrine

from sympathetic noradrenergic nerves and can be blocked by α_2-adrenergic antagonists (North and Surprenant, 1985). There is also a non-adrenergic sIPSP that possibly involves somatostatin (Shen and Surprenant, 1993). Neurotransmitters can in addition reduce transmitter release at excitatory synapses, a process called pre-synaptic inhibition. Acetylcholine, binding to pre-synaptic muscarinic receptors, decreases the release of transmitters. This mechanism plays an important role for local modulation of transmitter release. Norepinephrine and opioids may also bind pre-synaptically to reduce the release of acetylcholine.

2.1.3 Physiological classification of the constituent neurons in the ENS

Dogiel was the first to morphological characterize the enteric neurons based on their different shapes (Dogiel, 1895; Dogiel, 1899). Three types of neurons were described, nowadays generally referred to as Dogiel types I, II and III. Dogiel type I neurons consist of a single long axon and 4 to 20 or more short, broad and flat dendrites. This type of morphology is not unique to a single functional class of neurons. Both inhibitory and excitatory motor neurons and interneurons have Dogiel type I morphology. These types of neurons usually exhibit S/type 1 electrophysiological characteristics (Hirst and McKirdy, 1974; Nishi and North, 1973), consisting of action potentials of short duration followed by only a brief afterhyperpolarization (<100 ms). The action potential is blocked by TTX, i.e. they are mediated by opening of rapid Na^+ channels. Electrical stimulation of these neurons leads to fast excitatory postsynaptic potentials (fEPSP) that are blocked by hexamethonium. The transmission thus involves acetylcholine binding to nicotinic acetylcholine receptors.

Dogiel type II neurons have large round or oval cell bodies with multiple long axon processes (Clerc et al., 1997; Hendriks et al., 1990) with primarily circumferential projections (Furness et al., 1990b). Dogiel type II neurons are found both in the myenteric and submucosal ganglia and they supply terminals that innervate neurons in adjacent ganglia (Dogiel, 1899; Lomax et al., 2001; Reed and Vanner, 2001). Both myenteric and submucosal Dogiel type II neurons project to the mucosa (Furness et al., 1990b; Lomax and Furness, 2000). About 80-90% of Dogiel type II neurons are immunoreactive for the calcium binding protein calbindin (Furness et al., 1990b; Iyer et al., 1988). Electrophysiologically these neurons are characterized as AH/type 2 neurons (Hirst and McKirdy, 1974; Nishi and North, 1973),

exhibiting a more long lasting action potential than S/type neurons and having an inflection on the falling phase. The action potential is characteristically followed by a late prolonged afterhyperpolarising (AHP) current that can last between 2 to about 30 s. The late AHP is due to opening of Ca^{2+} dependent K^+ channels with intermediate conductance (IK channels). The excitability of the AH-neurons is critically dependent on the degree of the activity of the late AHP, which can be suppressed by sEPSPs, for a review see (Furness et al., 2004). Both TTX-sensitive Na^+ currents and TTX-insensitive Ca^{2+} currents (North, 1973; Rugiero et al., 2003) and TTX-resistant Na^+ currents, involving the NaV 1.9 subunit, have been demonstrated in these neurons (Rugiero et al., 2003). They operate mainly via slow synaptic transmission. Fast synaptic transmissions can be evoked rarely, but in that case they are of low amplitude, generally less than 5 mV (Brookes et al., 1988; Hirst and McKirdy, 1974; Iyer et al., 1988).

2.1.4 Major functional classification of enteric neurons in the small intestine

The enteric neurons are also classified into different groups according to their main functional properties. An overview is illustrated in Fig. 3.
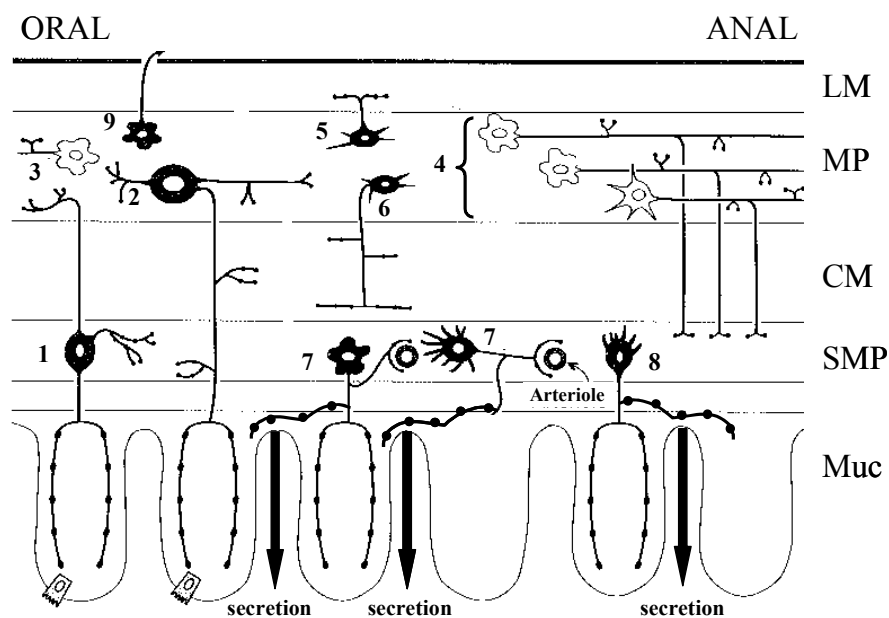
Fig. 3. Schematic illustration of the main functional neurons in the small intestine of guinea pig ENS. 1, submucosal IPAN; 2, myenteric IPAN; 3, ascending interneuron; 4, descending interneurons; 5-6, motor neurons; 7, secretomotor and vasodilator neurons; 8, secretomotor neurons; 9, intestinofugal neurons. LM: longitudinal muscle; MP: myenteric plexus; CM: circular muscle; SMP: submucosal plexus; Muc: mucosa. Modified from (Furness, 2006), with permission.

Motor neurons

Motor neurons are electrophysiologically characterized as S/Type 1 neurons with Dogiel type I morphology. They generate both fast and slow EPSPs and have their cell bodies in the myenteric plexus. Both excitatory and inhibitory motor neurons exist. The excitatory motor neurons project orally about 6-12 mm and their primary transmitters are acetylcholine and tachykinins. The inhibitory motor neurons project in the anal direction for distances of about 3-25 mm and their primary transmitters are ATP, NO, VIP and PACAP. Activation of excitatory motor neurons elicits a depolarization (i.e. EPSP) in circular smooth muscle and results in contraction, whereas activation of the inhibitory motor neurons elicits a hyperpolarization (i.e. IPSP) leading to relaxation of the smooth muscle (Bornstein et al., 2004).

Secretomotor and vasomotor neurons

Secretomotor and vasomotor neurons are electrophysiologically characterized as S/Type 1 neurons with Dogiel type I histology and they have their cell bodies in the submucous plexus. They project to the mucosa where they induce active secretion, or, in the case of vasomotor neurons, to the small arterioles where they induce vasodilatation. There are three types of enteric secretomotor/vasodilator neurons: non-cholinergic VIP-containing and cholinergic/calretinin-containing secretomotor/vasodilator neurons, and cholinergic/NPY-containing secretomotor neurons (Furness et al., 2003b). They receive both fast and slow EPSPs and slow IPSPs (Bornstein and Furness, 1988). The inhibitory inputs come mainly from sympathetic noradrenergic nerves involving α_2-receptors, but also somatostatin (Mihara et al., 1987; Shen and Surprenant, 1993) may induce IPSPs. The fast and slow excitatory inputs probably arise from neurons located in both myenteric and submucosal plexuses. The fast EPSPs originate from acteylcholine acting at nicotinic receptors and also from the release of 5-HT acting at 5-HT$_3$ receptors. The sEPSPs are mediated by VIP, SP and 5-HT (Bornstein and Furness, 1988).

Interneurons

There are three classes of descending interneurons and one class of ascending interneurons. These neurons are involved in the conduction of the sensory signal from the afferents to the motor neurons. Most of the interneurons have Dogiel type I morphology. The ascending

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interneurons are excitatory and contain acetylcholine and tachykinins. They make up functional chains that are linked by cholinergic nicotinic synapses (Brookes et al., 1997). The descending pathways are inhibitory and include neurons that contain acetylcholine, somatostatin, 5-HT, VIP and NO. They connect via cholinergic and non-cholinergic pathways. The non-cholinergic pathways probably involve ATP acting on P_2X receptors (Bian et al., 2000; Galligan and Bertrand, 1994).

Intestinofugal neurons

Intestinofugal neurons have their cell bodies within the gut and their processes project to the prevertebral ganglia where they synapse with post-ganglionic sympathetic ganglia (Szurszewski and Miller, 1994). The sympathetic neurons that are innervated by intestinofugal neurons inhibit intrinsic motility and secretomotor neurons. Most of these neurons have Dogiel type I morphology and are immunoreactive for acetylcholine and VIP (Mann et al., 1995).

Sensory neurons

The sensory neurons of the gut are referred to as the primary afferent neurons. Two broad classes of primary afferent neurons are associated with the gut, the intrinsic primary afferent neurons (IPANs) and the extrinsic primary afferent neurons (EPANs). IPANs are located within the gut wall and have cell bodies in either the submucosal plexus or the myenteric plexus (Furness et al., 2004; Pan and Gershon, 2000). The EPANs consist of the spinal afferent neurons with their cell bodies in dorsal root ganglia (DRG) and the vagal afferent neurons which have their cell bodies in the nodose ganglia (Cervero and Sharkey, 1988) (Fig. 4). Sensory innervation of the GI-tract involves all layers of the intestine (mucosa, muscle and serosa) and both intrinsic and extrinsic visceral afferents exhibit chemosensitivity, thermosensitivity and mechanosensitivity.
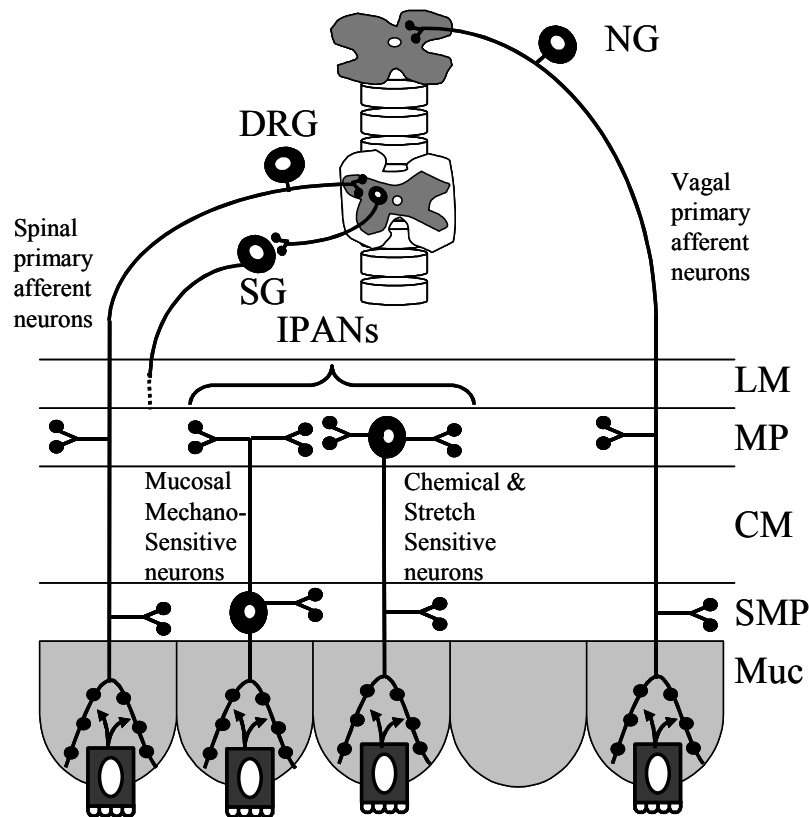
Fig. 4. Schematic illustration of the afferent neurons and the spinal efferent ganglia (SG) in the gut. Spinal afferent neurons have their cell bodies in DRGs, while vagal afferent neurons have their cell bodies in NGs. LM: longitudinal muscle; MP: myenteric plexus; CM: circular muscle; SMP: submucosal plexus; Muc: mucosa; IPAN: intrinsic primary afferent; DRG: dorsal root ganglia; NG: nodose ganglia; SG: spinal ganglia.

Intrinsic primary afferent neurons (IPANS)

Definitive identification of IPANs was first established during the late 1980s by Kirchgessner and Gershon (Kirchgessner and Gershon, 1988). IPANs are found both in the myenteric (MP) and submucous plexus (SMP) (Furness et al., 2004; Pan and Gershon, 2000) and have common characteristics independent of their location. These neurons are electrophysiologically characterized as AH neurons with Dogiel type II morphology (Furness et al., 2004). Both myenteric and submucosal IPANs have one or more processes that innervate the mucosa, just beneath the epithelial cells (Furness et al., 1990b; Lomax and Furness, 2000). The myenteric IPANs connect to several types of myenteric nerve cells (i.e. other myenteric IPANs, interneurons and motor neurons) and also to submucosal IPANs. The submucosal IPANs connects to other submucosal IPANs and to myenteric IPANs (Lomax et al., 2001; Reed and Vanner, 2001). The myenteric IPANs are sensitive to chemical stimuli in the lumen and also possesses mechanosensitive ion channels that are sensitive to stretch

(Kunze et al., 1999), while mucosal IPANs respond to mucosal distortion (Furness et al., 2004; Pan and Gershon, 2000). Distension stimuli can thus activate both mucosal and myenteric IPANs. IPANs operate mainly via slow synaptic transmission, and their neurochemical characterisation indicates that the main transmitters involved are acetylcholine and tachykinins (Bornstein and Furness, 1988; Costa et al., 1996). The responses of IPANs are graded with stimulus strength (Kunze et al., 1998), and this is true also for the reflexes evoked by their activation. It has also been suggested that submucosal primary afferents may participate in axonal reflexes. This means that action potentials can be propagated antidromically along collateral fibres near crypt cells without passing the cell body.

2.2 Extrinsic sensory innervation

2.2.1 Anatomical and functional properties

Extrinsic afferent neurons (EPANs) convey information from the gastrointestinal tract to the central nervous system (CNS), giving rise to conscious sensations and coordination of reflex functions in the GI-tract (e.g. motility, secretion and blood flow). The CNS also integrates sensory transmission with behavioural responses (e.g. food intake, interpretation of pain and medical seeking behaviour). The extrinsic sensory transmission can be anatomically distinguished into three different subtypes of nerves; 1) the parasympathetic vagal nerve, which innervates the upper GI-tract, from the stomach to the proximal colon with decreasing density, 2) the sympathetic thoraco-lumbar spinal nerves, (splanchnic and hypogastric nerves), which innervate most of the GI-tract and 3) the parasympathetic sacral pelvic nerve, which innervates the distal colon. As mentioned above, the spinal afferent neurons have their cell bodies in dorsal root ganglia (DRG), while the vagal afferent neurons have their cell bodies in the nodose ganglia (Cervero and Sharkey, 1988)

Functionally, three distinct afferent terminal endings can be identified within the gut wall. One population of afferent endings can be found in the serosa and in the mesenteric connections. Other populations form endings in the muscle layers or the myenteric plexus, while a third population has afferent nerve endings in the mucosal lamina propria (Berthoud et al., 2004; Berthoud et al., 1995; Brierley et al., 2004). The different afferents respond to different types of stimuli. The nerve terminals in the serosa and mesentery display pressure

and chemosensitivity with rapid adaptation to circumferential stretch and high threshold of activation (Blumberg et al., 1983; Brierley et al., 2005; Haupt et al., 1983; Longhurst et al., 1984; Lynn and Blackshaw, 1999). The muscular afferents respond to both pressure and circumferential stretch, but have lower thresholds for activation than serosal afferents, thus making them more likely to respond to physiological stimuli (Blumberg et al., 1983; Haupt et al., 1983; Lynn and Blackshaw, 1999). Vagal and pelvic muscular afferents show sustained responses to pressure, whereas splanchnic muscular afferents are more rapidly adapting (Brierley et al., 2004; Lynn and Blackshaw, 1999). The mucosal afferents do not respond to circumferential stretch, but respond to mechanical pressure and are also chemosensitive (Lynn and Blackshaw, 1999; Page and Blackshaw, 1998).

The vagus has two types of endings in the outer layers of the upper gut. Nerve terminals residing between the muscle layers consist of straight axons running in parallel to the respective layers. They are referred to as "intramuscular arrays" and have been suggested to be in-series tension receptors possibly responding to passive stretch and active contraction of the muscle (Berthoud and Powley, 1992; Fox et al., 2000; Iggo, 1955; Wang and Powley, 2000). The second type of endings, intraganglionic laminar endings (IGLEs) are located in the myenteric plexus and consist of flattened branching axonal endings (Berthoud et al., 1997; Wang and Powley, 2000). It has been shown that IGLEs are associated with "hot spots" (localized sensory areas) by probing special areas with calibrated von Frey hairs (Zagorodnyuk and Brookes, 2000; Zagorodnyuk et al., 2001). Similar endings have been found in the rectum, where they are referred to as rectal intraganglionic laminar endings (rIGLEs) (Lynn et al., 2005; Lynn et al., 2003). The IGLEs are characterised as low threshold slowly adapting mechanoreceptors (Lynn et al., 2003; Zagorodnyuk et al., 2001) and the mechanotransduction is probably due to the IGLEs being squeezed between the muscle layers (Lynn et al., 2003).

The mucosal terminals of the upper gut form networks of branching varicose endings within the lamina propria of crypts and villi (Berthoud et al., 1995; Berthoud and Patterson, 1996). They do not seem to penetrate the mucosa and are in this position ideally located to detect substances and mediators that either have penetrated the epithelial cell line from the lumen or have been released by the epithelial cells. They respond to low threshold stimuli, such as stroking and probing with von Frey hairs and they are also chemosensitive (Blumberg et al., 1983; Davison, 1972; Haupt et al., 1983; Lynn and Blackshaw, 1999)

The splanchnic and hypogastric nerves have their endings in the serosa or closely associated with the arteries of the gut wall (Floyd et al., 1976; Morrison, 1973). These endings have been shown to be activated by distension (Blumberg et al., 1983) or probing of the serosa, where all the fibres demonstrated high threshold responses (Lynn and Blackshaw, 1999). They have also been shown to be sensitized by ischemia (Haupt et al., 1983; Longhurst and Dittman, 1987) and inflammatory mediators such as bradykinin and capsaicin (Blumberg et al., 1983; Brierley et al., 2005; Lynn and Blackshaw, 1999).

In summary, the pattern that emerges from the mapping of the different receptor populations is that vagal afferents are mainly involved in physiological regulation of the different processes ongoing in the gastrointestinal system, splanchnic afferents mediate mainly nociception from the gut, while pelvic afferents are involved in both physiological regulation and nociception.

3. Integrated ENS physiology

3.1 Secretomotor reflexes

The intestinal epithelium is the barrier between the external environment and the inside of the body. The intestinal crypt cells secrete anions, in the proximal duodenum mainly bicarbonate and in the rest of the small intestine mainly chloride. The physiological role of the jejunal secretion is probably to lubricate the luminal contents, to act as a vector for substances released from the crypts and to eliminate potentially noxious agents. Distension, mechanical stimulation of the mucosa and chemicals applied to the mucosa represent mechanisms that evoke secretomotor reflexes.

The secretomotor reflexes persist after extrinsic denervation and are blocked by TTX, thus demonstrating that they are mediated through intrinsic nerve circuits (Cooke et al., 1983a; Frieling et al., 1992; Greenwood and Davison, 1985b; Greenwood et al., 1986; Itasaka et al., 1992). However, they are most likely also modulated by extrinsic nerves, since it has been shown that α_2-adrenergic receptor agonists are potent inhibitors of secretion (Lam et al., 2003). There are three types of secretomotor neurons in the small intestine (two cholinergic and one non-cholinergic/VIP containing). Two have collaterals that innervate the arterioles,

indicating that secretion and vasodilation most likely occur at the same time. The secretomotor reflex pathways stimulated by toxins (e.g. cholera) most likely pass through the myenteric plexus, since it has been shown that ablation of this plexus prevents toxin-evoked secretion (Jodal et al., 1993). In contrast, secretion induced by mechanical stimulation of the mucosa or by distension has been proposed to be mediated entirely through the submucosal plexus, since these reflexes can be recorded in preparations where the myenteric plexus has been ablated (Cooke et al., 1997*a;* Frieling et al., 1992; Itasaka et al., 1992; See et al., 1990). However, two recent studies by Reed and Vanner also propose that mucosal stimulation can activate secretomotor neurons (Reed and Vanner, 2007) and vasodilator reflexes (Reed and Vanner, 2003) via long myenteric pathways. The cumulated evidence suggests that both the myenteric and submucous plexuses, working either independently or in an integrated fashion, contribute to the control of mucosal secretion. In addition, Weber et al have shown that both extrinsic and intrinsic primary afferents are involved in distension-induced secretion (Weber et al., 2001), indicating that extrinsic components may have modulatory effects.

3.1.1 Distension-induced secretomotor circuits

The reflex pathway includes stimulation of sensory neurons, transmission through interneurons, and activation of secretomotor neurons that finally induces secretion by the epithelial cells. Initiation of the reflex requires a sensory cell that responds to the stimuli. This sensory cell has not yet been clearly defined, but Weber et al have shown that both extrinsic and intrinsic primary afferents are probably involved in distension-induced secretion (Weber et al., 2001). The initiating stimulus has been proposed to either directly or indirectly stimulate IPANs with nerve terminals in the mucosa, the SMP or the MP, and with immunoreactivity for SP and acetylcholine (Bornstein and Furness, 1988; Costa et al., 1996). It has also been suggested that axonal reflexes can be induced in IPANs. Stimulation of IPANs may thus induce action potentials that travel through the axons and stimulate the release of SP and Ach. These transmitters can then act directly on epithelial cells by inducing secretion via NK1 or muscarinic receptors localised on the enterocytes. It has also been proposed that enterochromaffin cells release serotonin (5-HT) in response to mechanical or tactile stimuli and distension (Cooke et al., 1997*c;* Racke et al., 1995) which subsequently activates the IPANs.

Distension will also induce stretch of the intestinal muscle layers. The IPANs can thus be activated either by direct distortion of their processes or through an indirect mechanism where stretch opens stretch-activated channels (SACs) in the muscle membrane. The muscle then contracts and distorts the IPAN processes (Kunze et al., 1999; Kunze et al., 1998). However the role of the myenteric plexus in distension-induced secretion is unclear, since distension-induced secretion can be recorded in preparations where the myenteric plexus has been ablated (Cooke et al., 1997a; Frieling et al., 1992; Itasaka et al., 1992; See et al., 1990).

There are two types of cholinergic IPANs distinguished by their different combinations of transmitters, Ach/CGRP-containing and Ach/SP-containing. The Ach/SP-containing afferents most likely make direct synapses with the cholinergic and VIP containing secretomotor neurons, where SP stimulates NK1 or NK3 receptors (Cooke, 2000; Cooke, 1998) and Ach most likely stimulates nAch receptors. Moore et al have accordingly shown that the neurotransmission between myenteric IPANs and submucosal S neurons occurs through hexamethonium-sensitive fast synaptic transmission (Moore and Vanner, 2000). Additionally, hexamethonium was not able to block the distension-induced secretory response in studies performed *in vitro* with only the SMP present (Frieling et al., 1992; Sidhu and Cooke, 1995), thus indirectly supporting a role of nicotinic transmission as a mediator of the connection between the two plexuses. Stimulation of the secretomotor neurons then cause release of Ach or VIP at the epithelial cells, where acetylcholine binds to muscarinic receptors (M_3) and stimulates secretion via a Ca^{2+} dependent pathway, while VIP binds to VPAC receptors and induces secretion via elevation of cAMP and opening of the cystic fibrosis transmembrane conductance regulator (CFTR) (Barrett and Keely, 2000).

3.2 The migrating motor complex and its relationship to intestinal secretion

The migrating motor complex (MMC) is a complex motor pattern activated during the fasting state. Its main function is to sweep undigested food through the small intestine and to prevent bacterial overgrowth. The MMC is a cyclic motor pattern consisting of three distinct phases, which in humans are repeated approximately every 90 minutes (although with a large variation in cycle duration): phase I with motor quiescence (40 min), phase II with irregular motor activity (40 min) and phase III with regular motor activity (10 min).

The most characteristic feature of the MMC, the phase III, is associated with activation of intestinal secretion measured as transmural potential difference (PD) (Mellander et al., 2000; Read et al., 1977; Read, 1980). The mechanisms leading to secretion during the phase III are not completely known. It has been shown that spontaneous and induced motor activity is always associated with increased secretion, provided that the mucosa is intact (Greenwood and Davison, 1985b; Greenwood et al., 1986; Greenwood et al., 1990). Furthermore, extrinsic denervation did not eliminate this relationship, while both TTX and vagotomy abolished both responses simultaneously (Greenwood and Davison, 1985b; Greenwood et al., 1986; Greenwood et al., 1990). These results imply that the mechanically evoked secretory reflex is neuronally mediated and intrinsic to the ENS. Another study performed by See et al showed that ablation of the myenteric plexus did not affect the correlation between contractile activity and epithelial secretion (See et al., 1990), hence they proposed that the submucosal plexus alone integrates the motor-evoked secretion. However, as stated above, mucosal stimulation can activate secretomotor neurons also via long myenteric pathways (Reed and Vanner, 2007). This again suggests that both the myenteric and submucous plexuses, working either independently or in an integrated fashion, contribute to the control of mucosal secretion.

A link between motility and secretion clearly exists, and most of the evidence suggests a cause-and-effect relationship, with motor activity/distension activating secretomotor neurons. The possibility of parallel and independent phenomena can however not be entirely excluded.

4. Network behaviour of enteric neurons - a potential mechanism involved in secretomotor reflex circuits

Both myenteric and submucosal AH/Dogiel type II neurons supply terminals that innervate neurons in adjacent ganglia (Dogiel, 1899; Lomax et al., 2001; Reed and Vanner, 2001). The innervation is supplied by dense varicose terminals that surround the nerve cell bodies within their own and adjacent ganglia (Bornstein et al., 1991; Furness et al., 1990b; Pompolo and Furness, 1988). Both physiological and structural analyses support that AH/Dogiel type II neurons synapse with each other, with other AH/Dogiel type II neurons in respective plexus and with interneurons and motor neurons (Furness et al., 2003b; Kirchgessner and Gershon, 1988; Pompolo and Furness, 1988). In addition, electrophysiological experiments have

shown that action potentials evoked by a stimulus to one AH/Dogiel type II neurons induced sEPSPs in an adjacent AH/Dogiel type II neurons (Kunze et al., 1993). It has also been shown that chemical stimuli of the mucosa activate AH/Dogiel type II neurons both directly and indirectly via sEPSPs, while S cells are only activated indirectly (Bertrand et al., 1997). Altogether, this suggests that AH/Dogiel type II neurons form interconnected networks which can be self-reinforcing, due to excitatory recurrent feedback (Bertrand et al., 1997; Thomas and Bornstein, 2003).

In two recent computer modelling studies (Chambers et al., 2005; Thomas et al., 2004), it was shown that the magnitude of the AHP in AH/Dogiel type II neurons plays a key role for the overall function of enteric networks. In the myenteric plexus, lack of AHP will lead to either quiescence or uncontrolled firing of the network, i.e. it will be unable to respond quantitatively to sensory stimuli. In the submucous plexus, changes in the feedback between sensory neurons and secretomotor neurons may lead to prolonged firing on termination of the stimulus, or even uncontrolled firing. The AHP consequently seems to be a "vulnerable spot" in network behaviour. It has also been shown that the excitability of AH/Dogiel type II neurons is increased in a model of colonic inflammation and that this increased excitability was due to a decrease of the late AHP (Linden et al., 2003). The late AHP of the IPANs may thus work as a gate and control the degree of activation of the network.

There is also emerging evidence that VIP-secretomotor neurons may be able to form recurrent networks within the submucous plexus. It has been shown that AH/Dogiel type II neurons communicate to VIP-containing S neurons via fEPSPs (Bertrand et al., 1997; Kunze et al., 1995) and that a VIP neuron can cause sEPSPs in a nearby VIP neuron within the submucous plexus (Reed and Vanner, 2001). When the VIP neurons pass through adjacent ganglia they have been shown to have axonal varicosities and occasional varicose collaterals (Evans et al., 1994; Reed and Vanner, 2001). In addition, one study postulates that $VPAC_1$ (vasoactive intestinal peptide receptor) is likely to function as an autoreceptor, facilitating the release of VIP (Schulz et al., 2004). Thus structural and physiological evidence suggests that VIP secretomotor neurons may also form recurrent networks within the submucous plexus.

5. Mechanisms of visceral hypersensitivity, modulation and transmitters involved

Visceral sensations from the gut are mediated via spinal afferents to the brain, where the nociceptive signals are perceived. The brain interprets the signals and modulates the efferent response back to the intestine. Pain processing is thus an active and plastic process that involves sensory afferents and emotional and cognitive experiences. Using positron emission tomography (PET) or functional magnetic resonance imaging (fMRI), it has been shown that the brain signal elicited in response to colorectal distension is larger and altered in IBS patients compared to controls, for a review (Jones et al., 2006). This enhanced brain response may be due to increased signalling from the gut (i.e. peripheral sensitization). A normal signal may also be amplified at the level of prevertebral ganglia (DRG), the spinal cord or in the brain itself and result in hypersensitivity (i.e. central sensitization). There could also be defects in the descending inhibitory pathways that modulate the pain transmission from the periphery to the brain.

5.1 Peripheral sensitization

Most visceral stimuli are not consciously perceived. The stimuli that give rise to conscious visceral sensation (e.g. fullness, bloating, discomfort and pain) originate mostly from stimuli of mechanical character, such as distension and stretch, which activates mechano-sensitive afferents in the gut. Therefore, visceral sensory afferents are traditionally classified according to their ability to respond to mechanical stimuli, although it is well known that visceral sensory neurons also exhibit chemosensitivity (including changes in pH, ischemia, hypoxia and inflammatory mediators) and thermosensitivity. Visceral sensory neurons have also been shown to have polymodal characteristics, i.e. they can respond to more than one stimulus. For example mechanosensitive afferent neurons have in addition been shown to respond to chemical and/or thermal stimuli (Cervero and Sharkey, 1988; Su and Gebhart, 1998). There are three classes of mechanosensitive afferents that innervate the gut and contribute to conscious sensory nociception from the gut. The first class is the mechanosensitive fibres that have low thresholds to mechanical stimuli. These fibres (about 75%) have thresholds in the physiological range and start to respond to mechanical stimuli between 1-5 mmHg. In

addition, these afferents also respond to noxious stimuli and as a group they have responses of greater magnitude than high-threshold fibres (Coutinho et al., 2000; Gebhart, 2000b). The second group is the high-threshold fibres (about 25%) that respond only to pressures above 25-30 mmHg, which is in the magnitude of the threshold for pain perception in humans. These afferents probably represent the nociceptors of the gut. The third class consists of visceral afferents that normally are insensitive to mechanical stimuli, but become activated and acquire spontaneous activity and mechanosensitivity by tissue injury or inflammation. These afferents are called "silent nociceptors" or "sleeping nociceptors". For reviews, see (Cervero, 1994; Gebhart, 2000b).

Peripheral sensitization represents an increase in nociceptor excitability, i.e. an increase in the magnitude of the response (i.e. hyperalgesia), or a decrease in threshold for activation (i.e. allodynia). The sensitization of peripheral nociceptors is characterized by two main changes in their response properties. First, the appearance of spontaneous resting activity and second a decrease in threshold where a normally non-noxious stimulus now activates the nociceptors and becomes noxious. Both the low- and high threshold fibres have the ability to sensitize and contribute to peripheral sensitization (Coutinho et al., 2000; Gebhart, 2000b; Sengupta et al., 1996; Su et al., 1997). Sensitization is also believed to involve recruitments of "silent nociceptors".

Gastrointestinal inflammation (Sengupta and Gebhart, 1994) and tissue injury are generally believed to cause sensitization of afferent nerves and to induce behavioural manifestations of visceral hypersensitivity in animals. Different chemical irritants, e.g. trinitrobenzene sulphonic acid (TNBS) (Gschossmann et al., 2002; Morteau et al., 1994), turpentine (Ide, 1997), zymosan (Coutinho et al., 1996; Coutinho et al., 2000) or acetic acid (Burton, 1995) have been shown to sensitize visceral afferents and to induce acute visceral hypersensitivity to CRD in animals. Sensitization induced by inflammation most likely involves the release of inflammatory mediators (i.e. ATP, bradykinin, prostaglandins, serotonin and histamine) and changes in local pH (Woolf and Salter, 2000). These inflammatory mediators reduce the threshold for activation of primary afferents and most likely activate previously silent nociceptors (Woolf and Salter, 2000). The ion channels/receptors involved in the increased excitability of these primary afferents most likely include: voltage gated sodium channels (Beyak and Vanner, 2005; Cervero and Laird, 2003; Laird et al., 2002), the vanilloid receptor (transient receptor potential vanilloid type 1, TRPV1) (Jones et al., 2005; Winston et al.,

2007), members of the acid sensing ion channel family (ASIC) (Jones et al., 2005; Page et al., 2005), ATP-gated ion channels (Anand et al., 2007) and tachykinins acting at the NK1 and/or NK2 receptors (Laird et al., 2001; Laird et al., 2000; Toulouse et al., 2000). For reviews, see (Anand et al., 2007; Cervero and Laird, 2004).

5.2 Central sensitization

Interestingly, although afferent nerve fibres have a transient short time-course of sensitization, it has been shown that the hypersensitivity to CRD may persist for several days and even several weeks after induction of inflammation (Bercik et al., 2004; Coutinho et al., 2000; Gschossmann et al., 2004; Verma-Gandhu et al., 2007). This suggests that a central mechanism is involved in the maintenance of visceral hyperalgesia.

Neuroplastic changes have been shown to occur also at the level of the spinal cord in response to inflammation and tissue injury. Central sensitization can occur by enhanced nociceptor input that activates intracellular signalling cascades within the spinal dorsal horn neurons, which then leads to amplified responses to both noxious and innocuous stimuli. Neurotransmitters or neuromodulators involved in this facilitation include glutamate (which activates ligand gated ion channels, such as NMDA and AMPA receptors and G-protein coupled metabotropic receptors), substance P (activating neurokinin receptors), brain-derived neurotrophic factors (BDNF, acting on tyrosine kinase receptors) and prostaglandins (Anand et al., 2007). There may also be activity dependent changes in transcription in DRG neuronal genes, which lead both to an increase and modification of constitutively expressed genes, as well as recruitment of novel genes (Neumann et al., 1996; Woolf and Costigan, 1999). For example, inflammation has been shown to induce non-nociceptive afferents to produce SP and BDNF, which resulted in allodynia (Neumann et al., 1996; Woolf and Costigan, 1999). Depression of spinal inhibitory mechanisms, which requires NMDA receptor activation, is also important for central sensitization (Woolf and Salter, 2000). The involvement of NMDA receptors in visceral nociception is demonstrated by the inhibition of CRD induced visceromotor responses using NMDA receptor antagonists (Traub et al., 2002).

IBS symptoms have also been reported to be preceded by stressful life events, such as a history of physical or sexual abuse during childhood, death of close relations, divorce or

other major trauma (Drossman et al., 1990), suggesting that stress may also induce sensitization. Indeed, several animal models, including maternal separation (Coutinho et al., 2002) and water avoidance stress (Bradesi et al., 2005) have been shown to induce visceral hypersensistivity to CRD in animals. Corticotropin-releasing factor (CRF) has been suggested to be involved in stress-induced visceral hyperalgesia and cause central sensitisation (Tache et al., 2004; Tache et al., 2005), since it is released by increased limbic activity and induces increased levels of cortisol, which may have a role in facilitating intestinal sensitivity and in increasing general arousal (Lechner et al., 1997; Lembo et al., 1996). Indeed, CRF receptor antagonists have been shown to decrease visceral sensitivity to CRD in rats (Schwetz et al., 2004; Schwetz et al., 2005). In addition, there is clinical evidence that a CRF receptor antagonist reduced visceral pain and anxiety in IBS patients (Sagami et al., 2004).

5.3 Endogenous modulation

Pain processing in the spinal cord is also modulated by descending modulatory systems originating in the brain. Descending modulatory pathways seems to originate at almost all brain levels in the brain-gut-axis (Jones et al., 2006). There are both descending facilitatory and inhibitory inputs in the spinal visceral nociception (Zhuo and Gebhart, 2002) and the balance in this system is important for pain modulation. Enhanced visceral perception, due to stress or inflammation, may be due to enhanced signalling in the descending facilitatory pathways or decreased signalling in the inhibitory pathways. It has also been suggested that inhibitory efferent fibres containing serotonin or noradrenaline presynaptically inhibit the afferent pain signals at the level of the dorsal horn (Jones et al., 2006).

It has been shown that stimulation of the periaqueductal grey (PAG) significantly reduces the visceromotor response to CRD in rats (Gebhart and Ness, 1991), suggesting that the PAG is important in the descending inhibitory pathway. There is considerable evidence that the opioid mechanisms are involved in the descending control of pain (Fields, 2004). It has been shown that both μ- and κ- opiod agonists significantly reduces visceral nociception induced by CRD in rats and mice (Burton and Gebhart, 1998; Larsson et al., 2003; Sengupta et al., 1996), suggesting a role for opioids in the modulation of visceral nociception. Agonists for κ-opioid receptors have been proposed to have a peripheral mode of action (Gebhart et al.,

2000a; Sengupta et al., 1996), while the μ-opiod receptors are believed to have a more central mode of action (Danzebrink et al., 1995; Sengupta et al., 1996).

AIMS OF THE STUDY

It is clear from this summary of the literature that there is a large gap between the extensive information about enteric neurophysiology and the lack of knowledge about the pathophysiology of IBS. Distension, particularly of the colon, is commonly used to activate sensory neurons in both humans and animals. Distension and sustained smooth muscle contractions are likely to activate AH/Dogiel type II networks and, at least with high pressures, extrinsic afferents. The response to distension is therefore a highly relevant model to understand the interactions between these systems. The visceromotor (pain) response to high-pressure distension has been studied in rats, but these studies have not been performed in mice. There is also very little information regarding neural mechanisms behind the secretory response to distension, particularly so in IBS patients.

Therefore, the specific aims of this thesis were:

- To evaluate the linkage between intestinal motor activity and secretion in patients with IBS and to compare this with controls, using spontaneously occurring repeated contractions (MMC phase III) as a triggering signal.

- To pharmacologically characterize the neural mechanisms involved in a distension-induced increase in mucosal secretion in proximal rat small intestine *in vivo*.

- To develop a model for assessing visceral sensitivity in mice, using colorectal distension as the sensory stimulus.

- To test if DSS-evoked inflammation induced visceral hypersensitivity to colorectal distension in mice and to evaluate if this represents a model of post-inflammatory visceral hypersensitivity.

METHODS AND METHODOLOGICAL COMMENTS

1. Materials and ethics

1.1 Humans (paper I)

Experiments were performed in healthy volunteers and in patients diagnosed with IBS or celiac disease. The IBS patients were included on the basis of the ROME II criteria (Thompson et al., 1999). Organic gastrointestinal diseases were excluded using appropriate investigations based on the presenting symptoms, as judged proper by the referring gastroenterologist. Patients with diarrhoeal symptoms were accordingly evaluated by both gastroscopy and colonoscopy, with normal results. None of the patients had had previous abdominal surgery, with the exception of appendectomy. Symptomatic pharmacological treatments (e.g. laxatives, fibres, loperamide) were withdrawn for at least a week prior to the experimental day. None of the patients were on anti-depressants.

The patients with celiac disease were diagnosed on the basis of suspected malabsorption and/or anaemia and in some cases irregular bowel habits. Only one of them had moderate dominating diarrhoeal symptoms. At the time of measurements, five of them were on a normal, gluten-containing diet. The sixth patient (a male) had remaining villus atrophy despite a gluten-free diet. All the individuals gave informed consent and the ethics committee at the University of Göteborg approved the study.

Comments: The composition of the patient population is always crucial in clinical studies. The patients included in this study were recruited from those referred to the outpatient GI clinic at Sahlgren´s University hospital. When getting the clinical diagnosis of IBS, they were offered to participate in the study. The patients were not consecutive and some patients were unwilling to participate in the experiments. It is therefore questionable if this population reflects a common IBS population in general practice.

Another important issue is that the patients were divided into two "extreme" groups, i.e. diarrhoea-dominant IBS and constipation-dominant IBS. Many IBS patients are also of the alternating type and those patients were excluded from the study. The division into diarrhoea- and constipation-dominant patients was done to tentatively evaluate the degree of linkage between dominating symptom and possible disturbances observed in enteric nerve function.

1.2 Animals (papers II, III, IV)

Animal experiments were performed in mice (papers II, III and IV) and rats (paper II). Two different strains of mice (C57BL/6 males and females and Balb/c males) were used. In paper II, male Sprague-Dawley rats were used. The animals were housed in plastic cages, male mice alone, female mice up to 4 animals per cage and rats in groups of at least two, in a temperature-controlled environment (19-23°C) with humidity of 25-70% and a light and dark cycle of 12 h. They were given standard food (R3, Lactamin AB, Sweden) and drinking water *ad libitum*. The animals were kept in the animal quarters at least 7 days prior to the experiments. In papers III and IV, experiments started at the earliest 7 days after surgery. All the experiments were approved by the animal ethics review committee in Göteborg, Sweden.

Comments: The reason for choosing C57BL/6 mice in papers II and III is that this strain is the most common one used when constructing transgenic mice. In paper IV intestinal inflammation was induced using dextran sodium sulphate (DSS), we therefore used Balb/c mice, which have been used extensively with respect to DSS-evoked colitis, both in the literature and in-house. We chose the C57BL/6 mice for strain comparison, since this strain has been used in our earlier CRD studies and we have therefore generated a large amount of information regarding their normal visceromotor responses to CRD.

The choice of male Sprague-Dawley rats was based on the extensive information we have on enteric neurophysiology *in vivo* in this species (Lundgren, 2002).

1.2.1 Anaesthesia (papers II, III and IV)

For the shorter surgical procedures in mice (implantation of chronic fistulas containing EMG electrodes) a mixture of Dormicum® (midazolam 5 mg/ml, Roche, Stockholm, Sweden), Hypnorm® (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml, Janssen Animal Health, Beerse, Belgium) and sterile water (1:1:2) (10 ml/kg i.p) were used (paper III and IV). Isoflurane anaesthesia (Forene, Abbott Scandinavia AB, Sweden) was used for longer experiments (PD recordings) (paper II) and for insertion of the balloon for CRD experiments (paper III and VI) in mice. In rats, anaesthesia was induced by pentobarbital sodium (60 mg/kg i.p) and maintained with infusion of chloralose (3.6 mg/ml, i.a) (paper II).

Comments: Isoflurane is generally considered to be the best option for mouse anaesthesia (Coupar, 1985). The depth of the anaesthesia is easily controlled and can rapidly be altered. Isoflurane also produces rapid induction and recovery from anaesthesia. In addition, the interference with neural control systems is considered to be small. Isoflurane was therefore used for PD recordings in mice and for insertion of the balloon for CRD experiments. It was not used for implantation of EMG electrodes, since it is difficult to keep the mice in a fixed position (as is needed for inhalation anaesthesia) with this type of surgery. Instead a combination of an analgesic (fentanyl, Hypnorm®) and a sedative and muscle relaxant drug (midazolam, Dormicum®) was used.

Chloralose was used for the maintenance of anaesthesia in rats, since it produces minimal cardiovascular and respiratory system depression (Flecknell, 1996). In addition, secretion has been shown to be maintained with this anaesthetic (Coupar, 1985).

2. Recording of transmural potential difference in humans (paper I)

The general experimental setup and recording methods are described in more detail in paper I. Briefly, after an overnight fast, the subjects were intubated transnasally with a multilumen polyvinyl tube containing eight separate channels, six of which were used in the experiment. At two sites (the middle recording point in the proximal duodenum and the jejunal recording point at the end of the tube), transmural potential difference (PD) and luminal pressure were simultaneously recorded, using infusion of isotonic saline (instead of water) as a flowing electrode (salt bridge). PD was measured between the gut lumen and a common reference electrode connected to a subcutaneous infusion of saline into the left forearm. The saline electrodes were connected to calomel half-cells (Radiometer, Copenhagen, Denmark) and PD was continuously recorded

Comments: Flowing salt electrodes have been used to measure intestinal transmural PD for many years and this is considered to be a standard technique in humans (Gutske et al., 1981; Read et al., 1977; Wingate, 1973). It has been shown that the PD between mucosa and serosa is the same as between mucosa and peripheral venous blood (Geall et al., 1970). Accordingly, there is no electrical PD between the serosal surface and a subcutaneous compartment (Wingate, 1973). Intestinal transmural PD can therefore be recorded accurately by placing

one electrode in the intestinal lumen while placing the reference electrode in either the bloodstream (Gutske et al., 1981) or in subcutaneous tissue (Mellander et al., 2000; Wingate, 1973).

Since the aim of the study was to relate motility and PD, it was crucial to measure the two signals at the same site. The flowing electrode technique was therefore the obvious method of choice. With this method one in addition ensures adequate contact between the infused fluid and the luminal contents, and a constant ion composition at the measuring site. A theoretical drawback with this technique is that electrochemical potentials can be generated if the ion composition of the luminal contents changes very markedly, while being unchanged in the subcutaneous compartment. Our lab has previously evaluated the magnitude of this potential problem in bench-side experiments, and found that the magnitude of this source of error is marginal with moderate changes in ion composition changes. An example of a situation where this source of error is much larger is when measuring in an acid environment, since hydrogen ions have a much higher mobility and electrical affinity than sodium ions (Read and Fordtran, 1979). It seems very unlikely that changes in the ion composition at the recording site explain our PD patterns, but this source of error cannot be totally excluded.

3. Recording of transmural potential difference in animals (paper II)

3.1 Surgical preparation and experimental setup

The general surgery procedure and experimental setup is described in more detail in paper II. Briefly, after fasting overnight, the abdomen was opened via a midline incision and the intestinal segment was localised (proximal duodenum or colon). The segment was isolated between ligatures with intact vascular suuply and the remaining intestine was put back into the abdominal cavity. A double-lumen catheter, containing one polyethylene tube for fluid administration and pressure measurement and one polyethylene tube filled with saline-agar (4%) for PD recordings was inserted into the proximal end of the segment. The distal end of the intestinal segment was cannulated with a plastic tube, which was clamped during distensions and kept open between distensions. The fluid administration catheter was connected to a pressure transducer (for pressure recordings) and the agar bridge was immersed in a beaker containing 1M KCl and a calomel half cell (REF 401, Radiometer,

Copenhagen, Danmark). Another agar bridge was placed in the abdominal cavity with the other end immersed in 1M KCl containing a similar calomel half-cell. A metal grounding electrode was placed in the abdominal cavity. The pressure transducers and calomel half-cells were connected to bridge amplifiers and the signals were collected in digital form and sampled at 4 Hz using a Labview programme.

Comments: Rats were chosen for the pharmacological experiments for two reasons: first, we already have extensive information about effects of neural antagonists on intestinal secretion in this species (Lundgren, 2002) and second, blood pressure is more easily monitored in rats, which is a great advantage when using vasoactive drugs like TTX and hexamethonium. Anaesthesia and laparotomy always represent problems and need to be carefully considered when studying the enteric nervous system. We chose chloralose as an anaesthetic agent and tried to minimize the surgical trauma. We also left all extrinsic nerves intact and recorded systemic blood pressure as an indicator of the vital conditions of the animal. The PD recording is technically easy and the signal is stable over time (if there is no physiological variation). The alternative, direct recording of fluid secretion, is technically much more difficult and involves several sources of error. If one uses a gravimetric technique (Cassuto et al., 1983), a much more extensive preparation is needed. If one uses dilution of a volume marker (e.g. PEG), one loses the real-time signal, and there is also a high risk for problems with mucus, sub-total recovery of volume marker etc. The evidence that the PD signal in the small intestine reflects chloride secretion is also quite strong (Frieling et al., 1992; Itasaka et al., 1992; Kuwahara et al., 1987), provided that there is no substrate for electrogenic sodium transport. The choice of segment for pharmacological interventions (distal duodenum) was based on data from this particular segment in awake humans (Larsson et al., 2007; Mellander et al., 1995; Mellander et al., 2000). In one of these studies, we also showed that the PD signal correlated quantitatively with fluid secretion (measured with PEG 4000) (Mellander et al., 1995).

3.2 Experimental procedure

After finishing the abdominal surgery, the segment was allowed to recover for at least 15 minutes while a stable baseline PD was attained. Distensions were performed by injecting a bolus of warm (37°C) saline intraluminally via the perfusion catheter. Fluid was emptied from the intestinal segment between distensions, except for a small amount, sufficient to maintain a PD signal. Graded distensions at pressures of 5, 10 and 20 mmHg were performed with 10 minute intervals between distensions. The distensions lasted for 5 minutes in all sets of experiments but one, where 30 s distensions were used.

Comments: The studied segment has an intact extrinsic (and intrinsic) innervation, which is in contrast to *in vitro* setups where one in addition usually has to strip off the myenteric plexus to ensure proper oxygenation. The standard *in vitro* setup is therefore essentially a mucosa-submucous plexus preparation only, which has to be kept in mind when comparing results.

The distending pressures used (5, 10 and 20 mmHg) are within the same range as those seen during MMC phase III contractions in humans (paper I). The 5 minute distension period roughly corresponds to that of physiological phase III activity. 10 minutes between distensions was considered to be sufficient for the system to return to control levels, as evaluated in initial pilot experiments. We have also verified that the response to a distension of this type is reproducible within the same animal and that a repetition of distension at 5, 10 and 20 mmHg gives a similar result during the first and second series (data not shown).

4. CRD and EMG recordings in mice (papers III and IV)

4.1 Surgical procedures

Implantation of chronic fistulas containing electrodes for EMG recordings was performed according to surgical procedures described in more detail in papers III and IV. Briefly, the abdomen was opened via a midline incision and a fistula containing one bipolar and one monopolar bioelectric electrode was chronically implanted in the right abdominal muscle. About 1 cm of the bipolar electrodes was implanted in the left external abdominal oblique muscle 2 cm lateral to the midline incision with a distance of approximately 2-3 mm between

the electrodes, and sealed with tissue adhesive. The monopolar electrode was placed in the abdominal cavity and used as reference. Experiments started at the earliest 7 days after surgery.

Comments: The mice tolerated the fistula and electrodes well and they were used in multiple experiments for up to 6-8 months. Experiments were not performed more often than twice a week, in order for the mice to recover between experiments. An important finding was that mice with fistulas had to be housed together. Otherwise, non-operated mice often harassed fistula-implanted mice. The electrodes can also be externalized at the neck (Kamp et al., 2003), but using abdominal fistulas is more convenient, since the mice are not disturbed by the fistulas, can move freely and be kept together in the same cage. They can also be used over a longer time period.

Bipolar electrodes were used for EMG recordings for two reasons. First, bipolar recordings reduce the risk of recording background electrical activity associated with power generation and transmission. Second, if one electrode is lost, EMG can still be measured between the second electrode and the reference electrode.

4.2 Experimental procedure

The visceromotor response evoked by CRD was quantified using EMG recordings of the external abdominal oblique muscle activity as described in more detail in papers III and IV. Briefly, the balloon was gently inserted into the colon under isoflurane anaesthesia, and in the experiments where different compounds were administered (paper III), a polyethylene catheter (PE25) was placed under the skin, behind the neck of the animal for subcutaneous administration. A cable was connected to the fistula and the mouse was placed in a Bollmann cage and allowed to recover from the anaesthesia. The catheter and the cable were respectively connected to the barostat and EMG acquisition system (developed at AstraZeneca, Mölndal, Sweden). A computer, running on customized software (Labview, National Instruments), controlled the barostat and recorded balloon pressure and EMG signals. CRD experiments were performed in four mice simultaneously and started approximately 15 minutes after the last mouse was placed in the Bollmann cage. After the experiments, the balloon and the connecting cable were removed under isoflurane anaesthesia, and the animals were returned to their home cage.

Three types of distension protocols were used in the studies, two increasing phasic paradigms (10, 25, 40, 65 and 80 mmHg) and (10, 30, 40 and 60 mmHg) with three distensions at each pressure, and one isobaric phasic paradigm (12x55 mmHg). All distensions lasted for 10 s with 5 min intervals between them.

Comments: Before being included in any experiments, all mice were placed in Bollman cages at least three times, 30 min each time, to become familiar with the environment. In order to get used to the experimental conditions and to test if the electrodes were functioning, the mice went through a preliminary test using an increasing (10-80 mmHg) and an isobaric (12x55 mmHg) phasic CRD paradigm. All mice with electrodes recording interfering 50 Hz signals from the power supply were discarded. In addition, when using the isobaric paradigm, the average response to CRD during pulses 1-3 needed to be at least 60% higher than baseline recordings in order to be considered as a consistent VMR (for calculations and definition of threshold, the reader is referred to paper III). The mice not reaching a 60% response above baseline were excluded from that particular experiment and when they had failed three times in a row, they were sacrificed, since the electrodes were probably not working correctly. However, it cannot be excluded that lower VMRs recorded in some mice are due to individual variation in sensitivity to CRD.

4.3 The barostat and EMG recordings

For a more detailed description, the reader is referred to paper III. The barostat, which regulates the inflation of the balloons is controlled by a computer program (Acquire 5.0, designed by Alfred Bayati using Labview software, National Instruments). The same computer program is used for continuously monitoring, and recording of EMG activity (amplified 5000x or 10 000x and filtered with a 3 Hz high-pass filter and a 1000 Hz low-pass filter) and balloon pressure (with a data-sampling rate of 200 Hz) for subsequent analyses.

Comments: The main power spectrum of the EMG signal is contained within the frequency band of DC-500Hz. In order to enhance this signal, it is useful to filter away signals caused by motion artefacts and background electrical activity as much as possible. The high pass filter is used to eliminate signals produced from movements of the electrodes. These

movements are usually slow with frequencies below 10 Hz. A low pass filter is also used, since the signals above 1000 Hz are most likely due to high-frequency background noise. The presence of a 50 Hz signal in the recording was manually checked when analyzing the data.

General comments: Our group and others (Martinez et al., 1999) have tried to visually count the number of abdominal contractions during CRD in mice. However, the characteristic abdominal contractions in response to CRD seen in rats were absent in mice. This might be due to the small size of mice, making contractions more difficult to visually detect. Clearly, more sensitive techniques, such as EMG recordings, are required to detect changes in muscle activity in this species.

Interestingly, in rats the VMR to CRD lasts as long as the distension persists. In contrast, in mice, the VMR to CRD lasts for only approximately 5 s independently of the distension time used. The reason for this rapid adaptation in mice is not known, but this might be one of the explanations why abdominal contractions are not visually detectable in mice.

Another model that has been used to evaluate visceral nociception in mice is the "writhing test" (Elmer et al., 1998; Martinez et al., 1999; Mogil et al., 1996b). However, this is a mixed somatic and visceral pain model with ethical problems, since the noxious stimulus is long lasting and cannot be directly manipulated once it is initiated. The balloon distension model is ethically much more attractive and signs of general distress have not been observed in the animals during distensions. In addition, it is also similar to the procedure that is used to assess visceral pain in humans.

5. Inflammation (paper IV)

5.1 Induction of inflammation and collection of tissue

In paper IV, Balb/c mice were exposed to drinking water containing 4% DSS for 6 or 7 days, while C57BL/6J mice were treated with 4% DSS for 5 days only. Thereafter, the DSS-containing drinking water was replaced with regular drinking water. Fresh DSS solutions were prepared daily.

For collection of tissues, the animals were sacrificed, the abdominal cavity was opened and the colon (excluding caecum) was excised. The colon was divided into three parts of equal size: proximal, middle and distal. For histological examination, a 0.5 cm-length segment of

each colonic part was fixed in 4% buffered formaldehyde. The remaining tissue was washed in saline, weighed and placed in Eppendorf tubes, which were snap frozen in liquid nitrogen and then stored at -70°C until assayed for MPO activity.

Comments: The intention of the study was to evoke a mild mucosal inflammation. We therefore allowed mice to respond with diarrhea, but blood in the stools was not considered acceptable. Due to appearance of bloody stools after 7 days in a few Balb/c mice in the histology group, the DSS-treatment in the subsequent CRD study was shortened to 6 days. C57BL/6 mice were treated with 4% DSS for 5 days only (due to the appearance of bloody stools after 6 days in pilot experiments). Control groups received regular drinking water.

DSS was chosen as an inducer of inflammation since the intention was to evoke a relatively mild inflammation that is claimed to resolve in 3-4 weeks. The reason for this was that we wanted to develop a post-inflammatory disease model with some characteristics similar to post-infectious IBS (PI-IBS). There are several means of inducing inflammation in the colon. Agents like acetic acid, ethanol and mustard oil will most likely induce inflammation by severely damaging the mucosa. Dinitrobenzenesulfonic acid (DNBS) is another agent that has been used in mice (Qiu et al., 1999). A drawback with DNBS is that the inflammation severity is hard to control and the degree of inflammation varies considerably between mice. Another possibility is to induce inflammation by infection with nematodes, e.g. *Trichinella Spiralis* (Bercik et al., 2004). Thus, intestinal inflammation can be caused in several ways in experimental animals. However, it is difficult to predict which of the models that would most resemble the inflammatory mechanism in PI-IBS, which is crucial for a good model in drug development.

5.2 Evaluation of inflammation

To follow the course of inflammation, the mice were weighed and examined for clinical symptoms (e.g. bloody stool and general appearance) once a day during the first 10 days after induction of inflammation. Thereafter, they were weighed every second day.

At necropsy, the colon was visually inspected, measured and weighed. Thereafter it was divided into three parts of equal size for histological examination and measurement of MPO activity (for details se paper IV).

Comments: For histological examination, the colon samples were subjected to standard procedures for haematoxylin/eosin staining. To avoid bias in the inflammatory scoring, the slides were coded and two persons, blinded to the treatment, independently examined the sections and scored for inflammation-related histopathological changes.

6. Data analysis and interpretation of data

6.1 Transmural potential difference (papers I and II)

The PD signal is relative easy to measure *in vivo*, while interpreting the acquired data is quite complex. An elevated lumen-negative transmural PD may be due to increased electrogenic absorption of cations (mainly sodium), to increased mucosal electrical resistance or to increased intestinal secretion of anions (chloride and/or bicarbonate). The active secretion of anions generates a current (I) which together with the electrical resistance of the epithelium (R) generates a potential difference (PD) over the mucosa. The magnitude of the PD is then calculated according to Ohm's law:

$$PD = R \times I$$

The most important electrogenic transport mechanisms in the upper small intestine are sodium-coupled solute transport (e.g. glucose and amino acids) and electrogenic chloride secretion. In the fasting state, there are no substrates for glucose or amino acid transport, implying that the contribution of sodium-coupled transport to the PD signal is minimal (Barrett and Keely, 2000; Baxter et al., 1989). The change in PD might theoretically be due to modifications in paracellular resistance, i.e. tight junctions or transcellular resistance. However, patients with a defective cystic fibrosis transmembrane conductance regulator (CFTR) mechanism have a normal motility pattern, but do not exhibit changes in PD during phase III contractions (Baxter et al., 1989). If the PD response during phase III was due to increased electrical resistance, one would expect an intact PD response in CFTR-deficient patients. Thus, it is likely that the PD response measured in the current study mainly reflects activation of electrogenic chloride secretion via the CFTR. In addition, several studies have shown that removal of Cl⁻-ions from the solution used in Ussing chamber experiments or

addition of bumetanide (which blocks the sodium-chloride-transporter (NKCC) on the basolateral membrane and prevents chloride secretion) reduces distension-induced transmural PD (Frieling et al., 1992; Itasaka et al., 1992; Kuwahara et al., 1987). In contrast, removal of HCO_3^- ions is without effect (Kuwahara et al., 1987). Conceivably, the major part of the distension-induced PD response is due to chloride ion secretion.

6.2 EMG recordings (papers III and IV)

For details, the reader is referred to paper III. The extracted EMG raw data were analyzed using customized software (Grafview 5.0, designed by Alfred Bayati using Labview software, National instruments). First, the computer program extracted the EMG data during 10 sec prior to and during each distension. A data reduction (200 sampling points/s was reduced to a single value/s) was then performed on the extracted raw EMG. The resting EMG (basal activity) was calculated as the average area under the curve (AUC/s) of the data 10 seconds before each distension. For each distension, the VMR was calculated as the average area under the curve (AUC/s) of the data over the first 0-5 seconds following the start of distension. Then, the average value of the data was calculated for three consecutive distensions, i.e. distensions 1-3, 4-6, 7-9 and 10-12 in the isobaric phasic paradigm and for each pressure in the increasing phasic paradigm. In most of the analyses, the average of the VMR (AUC/s) was divided by the average of the basal activity (AUC/s) to obtain a relative VMR (i.e. if the ratio of the VMR to basal activity equals 1, then there is no response to distension).

For drug effects, the VMR is presented as %VMR remaining after dose, in which the average of the relative VMR of pulses 1-3 was set to 100% and compared to the average of the relative VMR of pulses 4-12. This normalization of data allows comparison of drug effects between sexes.

Comments: Since the VMR to CRD was virtually absent after about 5 s, regardless of the distension time (10 s or 5 min), the VMR was calculated only for the first 0-5 seconds following the start of distension. In all the analyses the mean value of the data was calculated for three consecutive distensions. The reason for this is to minimize the influence of possible movements of the mice in the Bollman cage, which can give rise to an inaccurate baseline EMG activity or VMR.

7. Drugs

Table. 2. Drugs used in papers II and III.

Drugs	Dose	Administration	Company	References
TTX	0.3 µg/10 cm	serosal	Alomone	(Cassuto et al., 1983)
Lidocaine	0.5 mg/10 cm	serosal	Sigma A.	(Cassuto et al., 1983)
SR 140333 (NK1-antag.)	3 µmol/kg	i.v	AstraZeneca	(Lecci et al., 1997)
SR 48968 (NK2-antag.)	3 µmol/kg	i.v	AstraZeneca	(Lecci et al., 1997)
SB 223412 (NK3-antag.)	3 µmol/kg	i.v	AstraZeneca	In house data
Hexamethonium	10 mg/kg	i.v	Sigma A.	(Cassuto et al., 1983)
[4Cl-D-Phe^6, Leu^{17}]-VIP	2 µg/kg*min^{-1}	i.v (infusion)	Sigma A.	(Kordasti et al., 2006)
Atropine	0.5 mg/kg	i.v	Sigma A.	(Sun et al., 2000)
Granisetron (5-HT$_3$ receptor antagonist)	40 µg/kg	i.v	Roche AB	(Sjöqvist et al., 1992)
Fentanyl (µ-opioid receptor agonist)	0.005 - 0.25 mg/kg	s.c	Janssen P	
U-69593 (κ-opioid receptor agonist)	0.2 - 25.0 mg/kg	s.c	RBI	

Comments: The doses of the drugs were chosen according to effects shown in earlier studies in the literature (Table 2).

Tetrodotoxin was used since it is an established blocker of rapid sodium channels, and is frequently used as a "golden standard" to evaluate the contribution of neurons in a response. However, one has to keep in mind that TTX-resistant Na$^+$-currents (Rugiero et al., 2003) also are present in enteric neurons (e.g. some types of IPANs). An issue with TTX is that it is extremely toxic, i.e. it is difficult to administer without side effects. Giving it locally on the serosa, as described previously (Cassuto et al., 1983; Kordasti et al., 2006) circumvented this.

Lidocaine is a local anaesthetic agent, which mainly acts on sensory neurons. It is poorly lipid soluble, i.e. it diffuses relatively slowly through the tissue. With the current dose, it only reaches high concentrations in the external muscle layer (Cassuto et al., 1983), but it can not be excluded that the submucous plexus was affected. The tachykinin substance P (SP) is found in a large number of enteric neurons, including myenteric and submucosal AH/Dogiel

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type II neurons (Bornstein et al., 1987; Furness et al., 2004; Lomax and Furness, 2000; Moore and Vanner, 2000). Selective **tachykinin receptor antagonists** were used to evaluate the contribution of each tachykinin receptor subtype to distension-evoked secretion. **Hexamethonium** is a nicotinic blocking agent that blocks nicotinic fast transmission in both the ENS and in parasympathetic and sympathetic ganglia. It is therefore exceedingly unlikely that effects remaining after hexamethonium are mediated by extrinsic efferent neurons, in particular sympathetic ones. The effect of the drug on systemic pressure fades within less than an hour in rats, i.e. administration has to be repeated at regular intervals (in our study 45 minutes). **[4Cl-D-Phe[6], Leu[17]]-VIP** is a peptide antagonist that is not very well pharmacologically characterized. The biological effects of vasoactive intestinal peptide (VIP) are mediated through two receptors, designated vasoactive intestinal peptide receptor (VPAC)$_1$ and VPAC$_2$. The VPAC$_1$ receptor is most abundant in the mucosa and myenteric neurons whereas the VPAC$_2$ receptor is localised to neuroendocrine cells, blood vessels and smooth muscle (Schulz et al., 2004). Although the VPAC antagonist [4Cl-D-Phe[6], Leu[17]]-VIP used in the present study is not selective for any of the VPAC subtypes, it has been shown to have partial antagonistics effects on VIP-induced secretion in a colonic tumour cell line (Pandol et al., 1986) and to reduce distension-induced secretions in rats (Kordasti et al., 2006). It also blocked cholera toxin-induced secretion in anaesthetized rats (Kordasti et al., 2006), a pattern that we interpreted as an action on VIP neuron networks. However, this interpretation was complicated by the observation that it also blocked the effects on an intravenous VIP infusion on PD. **Granisetron**, finally, blocks 5-HT$_3$ receptors which exist in both dendrites of primary afferents and in intestinal ganglia. It has been reported that granisetron prevents cholera toxin induced secretion (Sjöqvist et al., 1992; Turvill et al., 2000; Turvill et al., 1998) and it was therefore considered to be a relevant tool for studying the pharmacology of the distension response.

8. Statistics

For descriptive purposes, in general all data are expressed as mean values ± SEM (papers I-IV). In paper I, data are also expressed as box plots with median, upper and lower quartiles and range or as scatter plots with a mean value. Statistical significance was evaluated using one-way ANOVA (papers I, III and IV) or ANCOVA (paper III) followed by the non-parametric Mann-Whitney U-test (paper I), Dunnett's post hoc test (paper IV) or an unpaired Student´s t-test (paper III) when appropriate in order to test for individual differences. In paper II, the statistical test consisted of a standard linear repeated measure model with (ARH (1)) within-subject correlation structure, using a t contrast model to test group differences. Student's unpaired t-test was used to evaluate differences between treatments in paper IV. In paper I, a significant difference between the incidences of high PD values (defined as above 95th percentile) in different groups was tested by Fischer's exact test. A p value less than 0.05 were regarded as statistically significant.

RESULTS AND COMMENTS

1. MMC-related upper small intestinal secretion (paper I)

The aim of the first paper was to evaluate the linkage between intestinal motor activity and secretion in controls and patients with IBS, using spontaneously occurring repeated contractions (MMC phase III) as a triggering signal.

1.1 Dynamic behaviour of the PD signal in association with MMC phase III

A similar dynamic behaviour of the PD signal was seen in controls and patients. At the onset of phase III, PD started to increase and eventually reached a peak that was reasonably well coordinated in time with the pressure signal. Both the pressure and PD signal tended to adapt after the initial increase in both the duodenum and jejunum, and returned towards baseline levels (Fig. 5).
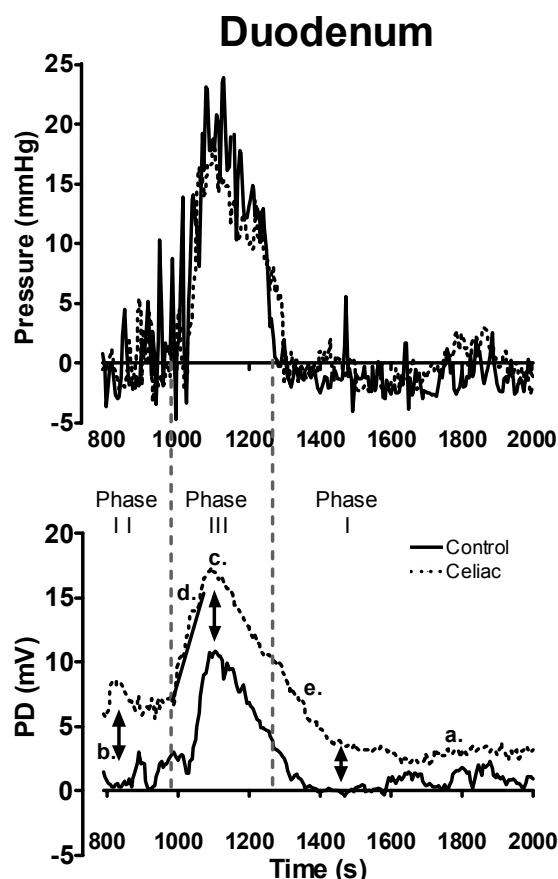

Fig. 5. A representative MMC phase III pressure signal and the corresponding changes in the transmucosal potential difference (PD). The average value from three healthy volunteers and three patients with celiac disease, all with similar phase III duration, are shown.

1.2. Definition and quantification of key variables reflecting the shape of the PD signal.

There was no significant difference in motor phase III pressure amplitude between healthy controls and patients with celiac disease. However, the PD in celiac disease was elevated before onset of phase III, rose more rapidly, reached a higher maximal level and remained at a higher level after phase III was completed (Fig 5, $p<0.05$). The corresponding parameters (Mean PD before and after phase III, PD rate of rise and maximal PD during phase III) were chosen for subsequent analysis in IBS patients.

Comments: In the initial analysis, we characterized the relationship between phase III motor activity and PD in controls and celiac disease patients. Celiac disease patients were included in the study since we have previously demonstrated up-regulation of motility-associated duodenal PD, associated with an increased net fluid secretion in this patient group (Mellander et al., 1995; Mellander et al., 2000). Based on the differences between controls and celiacs a number of parameters reflecting the shape of the PD curve were defined. These parameters were then used to investigate the linkage between phase III motor activity and PD in patients with IBS. In addition, we were able to compare the magnitude and shape of the motility-related PD between IBS- and celiac disease patients, thus enabling us to better discuss potential mechanisms involved in IBS patients.

1.3 Pressure-PD linkage in patients with IBS

1.3.1 MMC phase I, II & III and corresponding PD values

The next step was to characterize PD and its corresponding changes in relation to the different phases of the MMC cycle in IBS patients. The data were analyzed during a 5 min period preceding the phase III contraction (phase II) and during a 15 min period after the end of phase III (post phase III), in analogy with the control and celiac disease data.

We found an elevated maximal PD during MMC phase III, both in the duodenum and jejunum, in patients with IBS (Fig. 6, $p<0.05$). The maximal PD was increased in IBS patients despite similar mean pressure increases during phase III compared to healthy controls. The PD elevation was present in both duodenum and jejunum in d-IBS patients, but

only in the jejunum in the c-IBS patients. There were no significant differences, neither in duodenum nor jejunum, in mean PD during late phase II (i.e. phase II in the figure) between controls and IBS patients. The post phase III PD was however significantly elevated in c-IBS patients (Fig. 6, p<0.05).
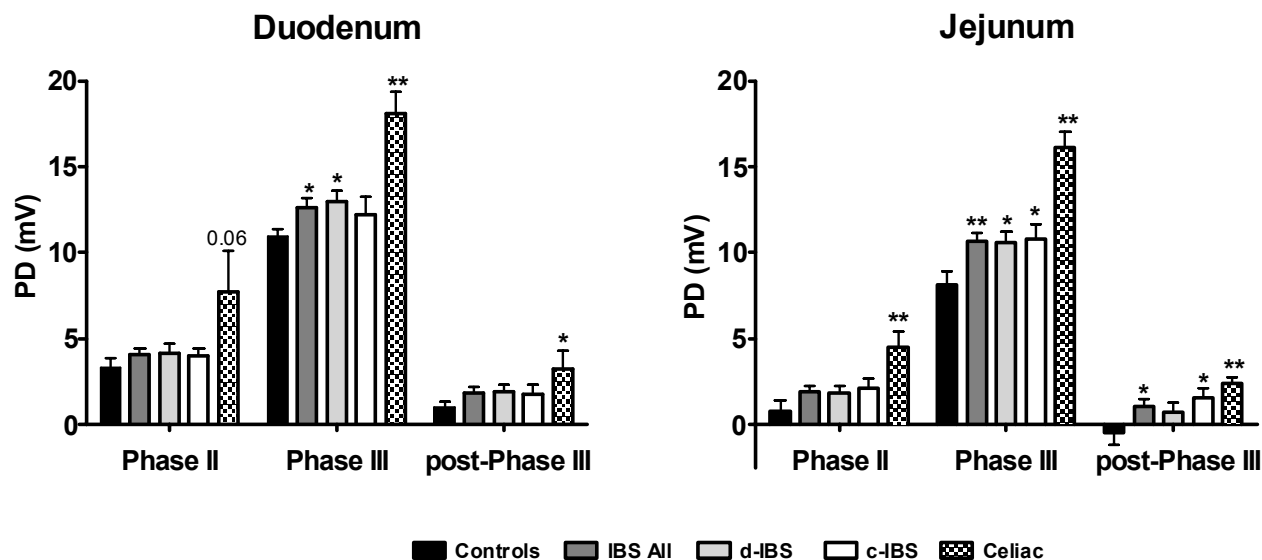

Fig. 6. A summary of transmural PD, as related to late phase II (phase II), phase III and post phase III of the MMC cycle in healthy controls and patients with IBS or celiac disease. Phase II PD corresponds to the mean PD during the five minutes preceding phase III, phase III PD represents maximum value and post-phase III corresponds to the mean PD during the five minute period starting 10 min after end of phase III. * p < 0.05, ** p < 0.01, n = 4-22.

Comments: The finding that c-IBS patients had increased maximal PD values in the jejunum may seem contradictory, since one would assume an increased PD to be associated with diarrhea. However, phase III mainly occurs at night (i.e. during prolonged fasting), and the amount of fluid generated during this period can be readily absorbed in the colon, provided that its function is intact and that transit time is not dramatically reduced. The importance of the colon in relation to bowel habits is illustrated by the fact that constipation occasionally occurs also in patients with celiac disease (Schulzke et al., 1995b). A concomitant disturbance in colonic mucosal function may indeed occur in d-IBS, since Pienkowski et al (Pienkowski et al., 1989) have shown that a group of d-IBS patients had a decreased colonic PD compared to controls, a pattern similar to that in ulcerative colitis (Edmonds and Pilcher,

1973; Rask-Madsen and Dalmark, 1973), although of lower magnitude. When interpreting the current data, one should regard the motility-related PD signal as a marker for submucous plexus activity at the measuring site only.

1.3.2 Decline time of the PD - from peak PD to post-phase III PD

The time required to reach a stable post phase III PD was significantly prolonged in both the duodenum and jejunum of d-IBS patients, but only in the jejunum of c-IBS patients (Fig. 7, $p<0.05$).

Decline time
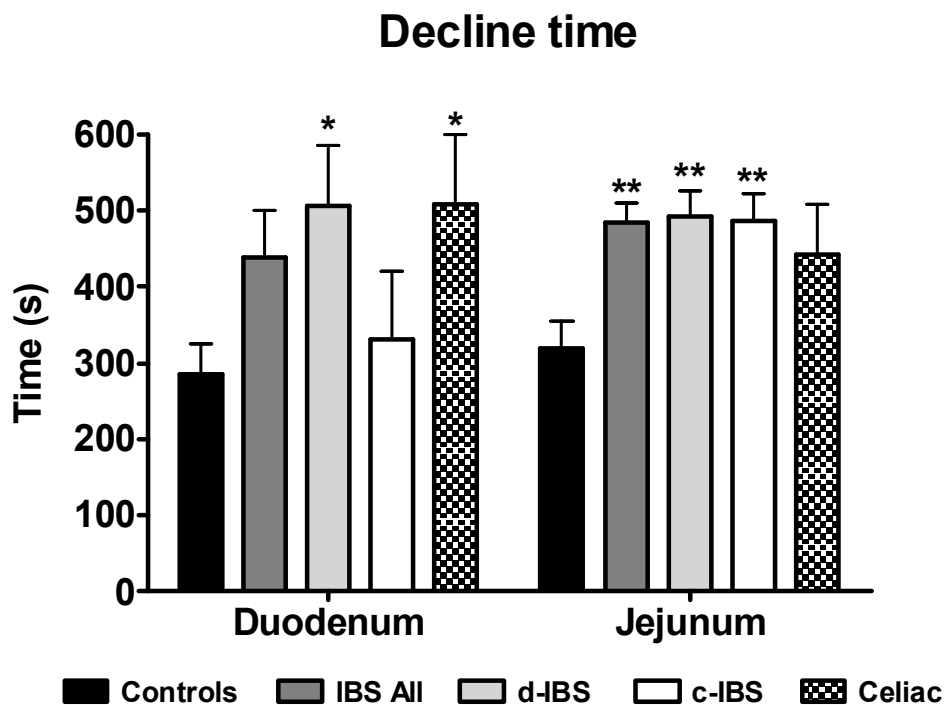
Fig. 7. The time required reaching the post phase III PD (i.e. the plateau where the PD starts to stabilize) in the duodenum or jejunum after a MMC phase III period in healthy controls and patients diagnosed with celiac disease or IBS. * $p < 0.05$, ** $p < 0.01$, n = 4-22.

Comments: The complex shape of the PD response in association with motor phase III is compatible with network behaviour of submucous neurons. If the submucous sensory networks are hyperactive, they will be able to sustain network activity when a mechanical stimulus is terminated (Chambers et al., 2005; Thomas and Bornstein, 2003), a pattern that might account for the slow decay of the PD signal in some IBS patients. The decline to a

stable post phase III plateau was chosen for calculation, rather than the slope of the curve, since the return of the PD to a stable plateau value consisted of at least two phases. The first phase seems to be linear, then the PD-decline slows down and gradually returns to stable values. We believe that it is the second phase that is the most important to reflect sustained activity in intramural neurons and considered that the best way to interpret this was to calculate the time to stable values rather than the slope.

1.3.3 Propagation velocity of MMCs in IBS

Since one of our hypotheses was that network behaviour of AH/Dogiel type II neurons is disturbed in IBS patients, we also tested another prediction based on our previous modelling work. That is, if there is increased synaptic efficacy in AH/Dogiel type II networks, the propagation speed of MMC should be increased. Indeed, we found that the MMC propagation velocity was significantly faster in d-IBS patients (9.8 ± 1.3 cm/min, $p<0.05$), but not in c-IBS patients (8.4 ± 1.4 cm/min) compared to the propagation speed observed in healthy controls (6.5 ± 1.3 cm/min).

Comments: The propagation speed of motor phase III was calculated as the time for the end of phase III to travel from the duodenal recording point to the jejunal recording point. The propagation speed of motor phase III in healthy controls is in the same range reported by other studies (Aytug et al., 2001; Soffer et al., 1998; Wilmer et al., 1997). That, d-IBS patients, but not c-IBS patients, had an increased propagation speed of phase III might reflect the different bowel habits in these two populations.

According to computer modelling studies by Thomas et al, the increased propagation speed of motor phase III in IBS patients may be due to increased synaptic efficacy in myenteric sensory neuron networks (Thomas and Bornstein, 2003; Thomas et al., 2004).

1.4 Discriminative power of the PD signal

As shown by the contingency analysis, approximately 50% of the IBS patients had a duodenal PD that was significantly above the normal range. While, approximately 30-40% of

the IBS patients had a jejunal PD that was above the normal range, although this was not statistically significant (Table 3).

	Controls	IBS All	d-IBS	c-IBS
PD Duodenum (paper I)	7 %	50 % *	55 % *	43 %
PD Jejunum (paper I)	8 %	41 % (p=0.06)	46 % (p=0.07)	33 %
Visceral Hypersensitivity (Kuiken et al., 2005)		44 %	43 %	44 %

Table 3. The percentage (%) of subjects in each group (controls and IBS patients with sub-groups) outside "normal range" (defined as above the 95th percentile) of maximal PD during MMC phase III in the duodenum. Results from a study of visceral hypersensitivity is also included in the table (Kuiken et al., 2005). * $p < 0.05$.

Comments: In our analysis, we used one of the statistical tests (above the 95th percentile) on which prevalence values for visceral hypersensitivity is based (Kuiken et al., 2005). Incidentally, the prevalence of this particular disturbance, measured as an elevated sensory response to colorectal distension, was also found to be about 50% in IBS patients (Kuiken et al., 2005; Mayer et al., 2001; Mertz et al., 1995). Whether PD-positive and visceral hyperalgesia-positive IBS patients represent separate pathophysiological entities, with different clinical profiles, is an intriguing question that needs to be addressed by further research.

2. Mechanisms behind the pressure-PD linkage *in vivo*: neuropharmacology of the distension response in anaesthetized rats (paper II)

The aim of paper II was to use relevant pharmacological tools to delineate the neural mechanisms behind distension-induced secretion in rat proximal small intestine *in vivo*.

2.1 Time course of the PD response to distension

Luminal distension of the duodenum for 5 min induced a biphasic PD response, consisting of an initial PD increase leading to a transient PD peak (defined as the rapid response). After the rapid response, PD levels decreased and reached a stable plateau, lasting until the end of the distension period (referred to as the sustained response) (Fig. 8). Distending the duodenum with an increasing stimulus (5, 10 and 20 mmHg) demonstrated that both the rapid and sustained responses were pressure dependent.

Reducing the duration of the pressure increase from 5 minutes to 30 seconds did not affect the amplitude or the rate of rise of the rapid response at any pressure applied. Moreover, the sustained response was not seen during 30s distensions, and the time to reach baseline levels after distensions were similar using 5 min or 30s distensions.
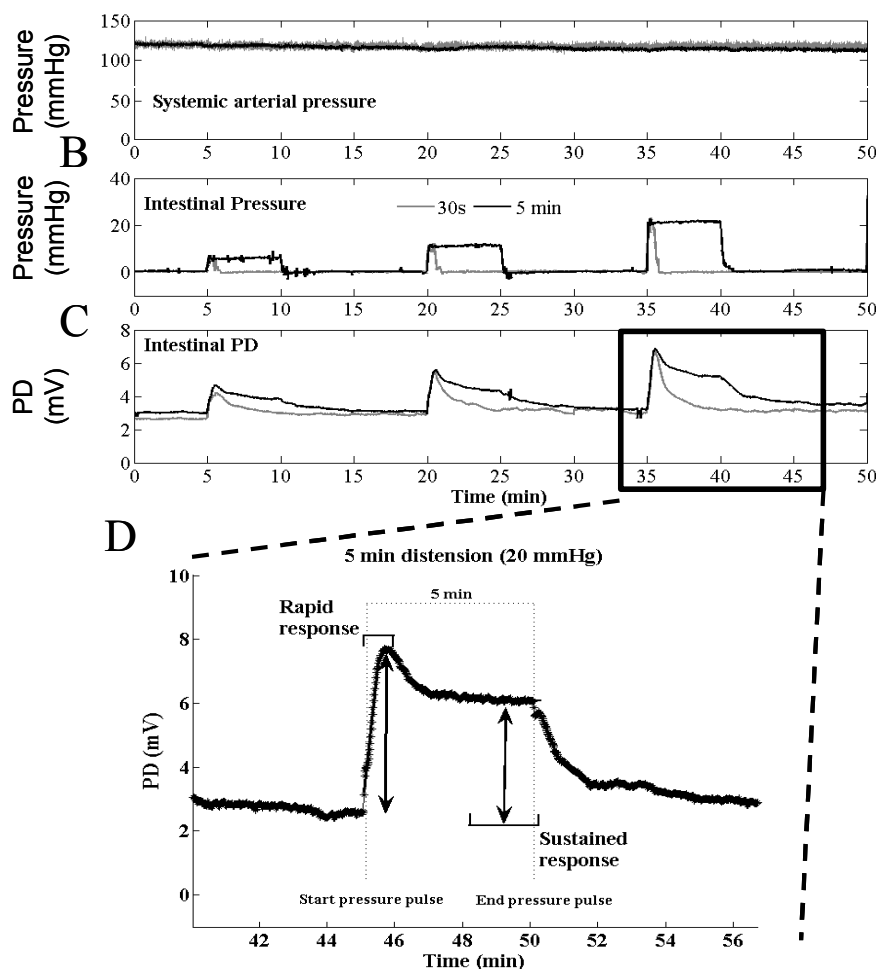

Fig. 8. Time course of changes in blood pressure (A), intestinal pressure (B) and intestinal PD (C) when performing 5 min and 30 s distensions at 5, 10 and 20 mmHg in rat duodenum. An enlargement of the dynamic behaviour of the PD signal in association with increased intraluminal pressure during 5 min is shown in (D).

Comments: The biphasic response obtained in the present study is also seen during distension of both guinea pig and rat colon *in vitro* (Frieling et al., 1992; Itasaka et al., 1992). However, the fact that a grossly similar response occurs *in vitro* does not necessarily imply that the submucosal plexus is the sole mediator of the response *in vivo*.

The shorter distension period was tested in order to evaluate if this time was enough to induce a secretomotor response similar to the long-lasting stimuli. Accordingly, the data support that the shorter distension time (30 s) is sufficient to activate enteric networks, since it evoked a similar rapid response and since the time to reach baseline levels after distensions was similar using 5 min or 30 s distensions.

2.2 Intestinal segment- and species-related PD responses to distension

The PD response to distension in mouse duodenum and rat colon was biphasic with a similar shape to that in rat duodenum. The rate of rise was pressure-dependent and almost 5-fold higher in mice and approximately 2-fold higher in rat colon, compared to rat duodenum. Furthermore, baseline PD and the magnitude of the rapid and sustained responses were all significantly elevated at all pressures applied in rat colon and at 5 and 10 mmHg in mice duodenum compared to rat duodenum (Fig. 9).
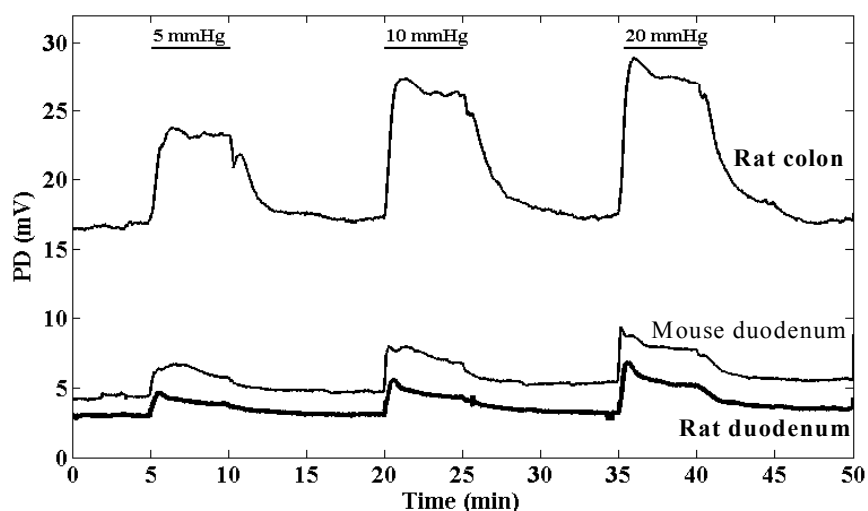

Fig. 9. Comparison between distension induced PD signals in mouse and rat duodenum and rat colon. The average value of the raw data from each entire group is shown (Rat duodenum n = 26; Rat colon n = 8; Mouse duodenum n = 8).

Comments: The overall profile of the pressure induced PD-response seems to be similar between different species and segments. This is in agreement with earlier studies where a similar profile has been obtained in *in vitro* studies of distension induced secretion in both rat and guinea-pig colon (Frieling et al., 1992; Itasaka et al., 1992) as well as in *in vivo* studies in the jejunum (See et al., 1990) and distal duodenum (Kordasti et al., 2006) in rats. A similar PD profile has also been obtained in response to electrical field stimulation (Cooke, 1984; Cooke et al., 1983b; Cooke et al., 1983a).

The rate of rise of the rapid response was 4-6 times faster in mice than in rat duodenum. Nurgali et al (Nurgali et al., 2004) have shown that in mouse distal colon, neurons with Dogiel type II morphology often do not exhibit a pronounced after-hyperpolarizing potential (AHP). Two recent computer modelling studies (Chambers et al., 2005; Thomas et al., 2004), have shown that suppression of the AHP and/or increased synaptic efficacy in submucous sensory networks will lead to enhanced responses of VIP secretomotor neurons to sensory stimulation (Chambers et al., 2005). Thus, a potential explanation for the increased rate of rise in mice could be a reduced AHP in AH/Dogiel type II neurons rendering them more sensitive to for instance mechanical stimuli. One may therefore speculate that the magnitude of the late AHP in sensory neurons is involved in determining the reactivity of the secretory response.

2.3 Neuropharmacology of the distension-induced PD response

The magnitude of the rapid response was significantly reduced by the neuronal blockers TTX and lidocaine, by the ganglionic blocker hexamethonium and also by the NK1 receptor antagonist (SR 140333). The VPAC receptor antagonist [4Cl-D-Phe^6, Leu^{17}]-VIP, the muscarinic receptor antagonist atropine, the 5-HT$_3$ receptor antagonist granisetron, the NK2 receptor antagonist (SR 48968) and the NK3 receptor antagonist talnetant (SB 223412) were without effect (Fig. 10A). Lidocaine, SR 140333, SR 48968 and hexamethonium also significantly reduced the rate of rise of the rapid response (Fig. 10C). The sustained response was significantly reduced by TTX, SR 140333, talnetant and [4Cl-D-Phe^6, Leu^{17}]-VIP (Fig. 10B).
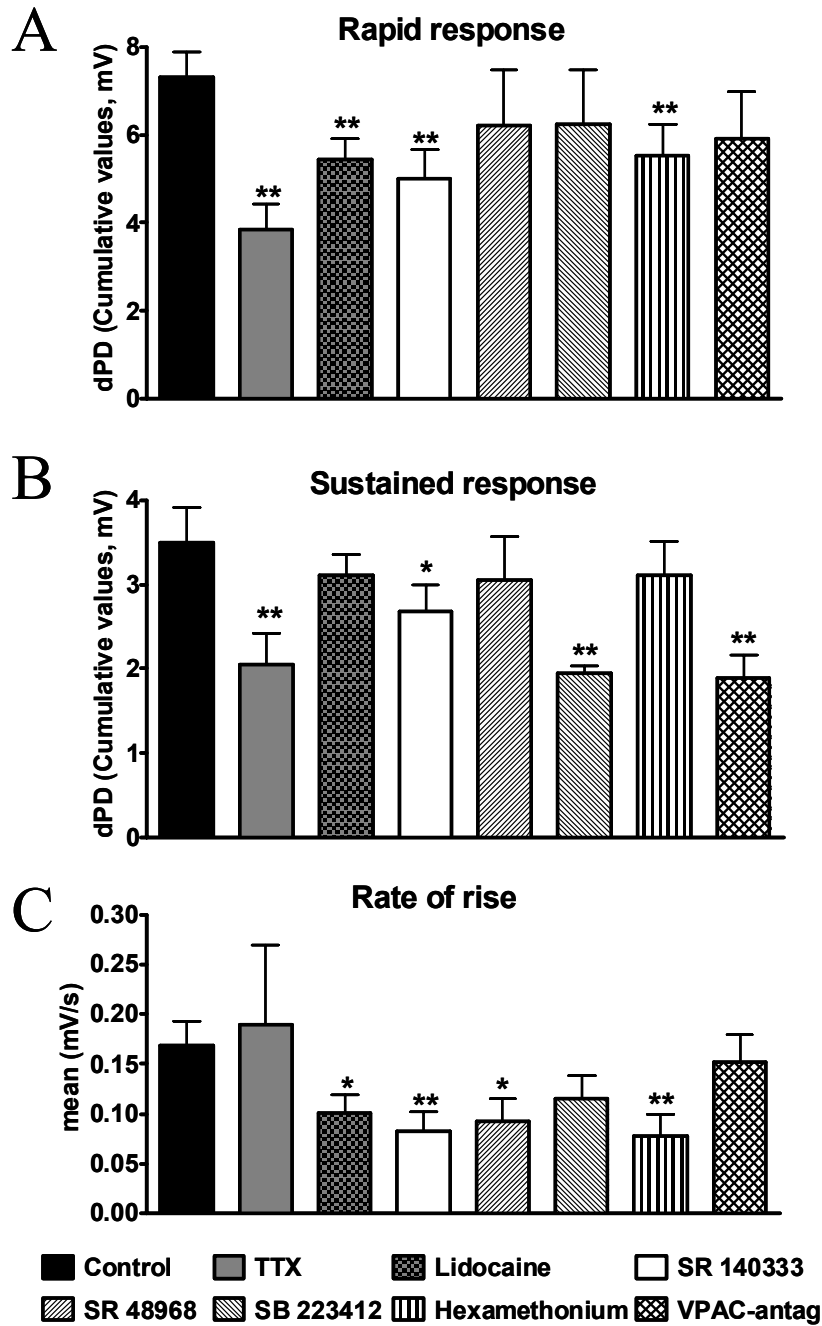
Fig. 10. Effects of the different antagonists on the magnitude of the rapid response (A), the sustained response (B) and the rate of rise of the rapid response (C). Only the compounds that had effect in any of the measured variables are shown. Data are presented as means ± SEM (n = 4-26). * $p < 0.05$, ** $p < 0.01$ compared with control experiments.

Comments: Both the rapid and sustained responses were reduced by approximately 40-50% by TTX, implying that they are largely neurally mediated. Since the dose of TTX that can be given *in vivo* is restricted, it cannot be ascertained whether the partial response was a dosage phenomenon, depended on TTX-resistant currents or was due to non-neural mechanisms.

Although TTX-sensitive currents dominate in most enteric neurons, TTX-resistant currents, involving the NaV 1.9 subunit, have been demonstrated in AH/Dogiel type II neurons (Rugiero et al., 2003). These neurons may thus be active also in the presence of TTX. A lower dose of TTX, administered in the same way, does inhibit cholera toxin induced secretion (Cassuto et al., 1983), thus, it is less likely that the dose was too low to inhibit TTX-dependent neural transmission. However, it cannot be ruled out that total TTX-sensitive ion channel blockade was not accomplished. The partial effects of TTX are in agreement with data obtained by previous reports in guinea pig colon (Frieling et al., 1992; Weber et al., 2001) and rat duodenum (Kordasti et al., 2006).

Hexamethonium and lidocaine reduced both the magnitude and the rate of rise of the rapid response, but had no effect on the sustained response. It has been shown that myenteric AH/Dogiel type II neurons contain acetylcholine which activates submucous nicotinic receptors via fast transmission (Moore and Vanner, 2000). In addition, hexamethonium was not able to block the distension-induced secretory response in studies performed *in vitro* with only the SMP present (Frieling et al., 1992; Sidhu and Cooke, 1995), thus supporting a role of nicotinic transmission as a mediator of the connection between the two plexuses.

Lidocaine was locally applied on the serosal side, in order to block the myenteric plexus only. Although it has previously been shown that serosally applied lidocaine only reaches high concentrations in the external muscle layer (Cassuto et al., 1983), it can not be excluded that the submucous plexus has been affected by the drug.

The VPAC receptor antagonist [4Cl-D-Phe[6], Leu[17]]-VIP only reduced the sustained response. The exact pharmacological profile of this peptide antagonist is not well defined. It has e.g. been reported that it does not block VIP-mediated secretion in isolated intestine in the Ussing chamber (Cox and Cuthbert, 1989). On the other hand, it clearly blocked VIP-induced secretion in rat distal duodenum *in vivo* (Kordasti et al., 2006).

Earlier studies have shown that endogenous tachykinins such as SP evoke secretion via both NK1 and NK3 receptors (Frieling et al., 1999; MacNaughton et al., 1997) and that a combination of NK1 and NK3 receptor antagonists attenuates distension-evoked secretion in guinea pig ileum *in vitro* (Weber et al., 2001). Different effects were obtained with the different tachykinin receptor antagonists. SR 140333 (NK1 receptor antagonist) reduced the

magnitude of both the rapid and the sustained responses, as well as the rate of rise of the rapid response, SR 48968 (NK2 receptor antagonist) reduced only the rate of rise of the rapid response and SB 223412 (NK3 receptor antagonist) reduced only the sustained response. This complex pattern might be due to the different locations of the receptors. NK1 and NK3 receptors are localised on enteric neurons (Grady et al., 1996; Mann et al., 1997; Sternini et al., 1995), while NK2 receptors are localised on the muscle cells (Grady et al., 1996).

The negative results with the muscarinic receptor antagonist atropine were somewhat surprising, since several studies in the guinea pig, using both electrical field stimulation and distension to induce secretion, have revealed muscarinic components of the respective responses (Cooke, 1984; Cooke et al., 1983b; Frieling et al., 1992; Kuwahara et al., 1987). However, this observation is not unique to our system. Schulzke et al were likewise unable to demonstrate involvement of muscarinic receptors in distension-induced secretion in the rat colon (Schulzke et al., 1995a), whereas Itasaka et al demonstrated a 20% reduction of the response to distension in rat colon by atropine (Itasaka et al., 1992), thus part of the discrepancy may be species-dependent.

In our hands, granisetron did not affect the rapid or sustained responses. This is in agreement with the *in vitro* studies performed by (Frieling et al., 1992) and (Engelmann et al., 2006), who did not show any effect with a 5-HT$_3$ receptor antagonist. However, effects of 5-HT on other receptors (e.g. 5HT$_4$) cannot be excluded. In addition, 5-HT may be relatively more important in activating neural pathways when secretion is induced by mucosal distortion (Cooke et al., 1997b; Cooke et al., 1997c).

3. The colonic sensory response to distension and effects of mucosal inflammation (Papers III & IV)

3.1 EMG response profile

CRD induced a prompt EMG response (reflecting both an increase in amplitude and frequency) following the increase in colonic pressure. However, unlike the maintained response normally seen in rats, EMG-activity faded away and returned to baseline levels approximately 5 seconds after the start of distension, despite the fact that the balloon pressure was kept constant during the whole distension period (Fig. 11).
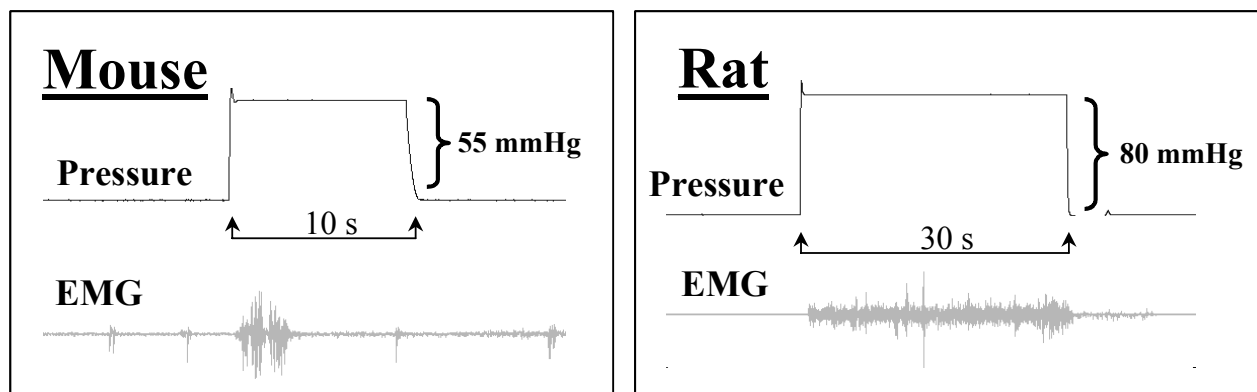

Fig. 11. A representative EMG tracing of the abdominal oblique muscle during a phasic distension of the colon in mouse (55 mmHg for 10 s) and rat (80 mmHg for 30 s).

Comments: This transient EMG response occurred even when using longer tonic distensions (up to 5 minutes) at pressures of 25, 40 or 60 mmHg (in-house data). Transient EMG responses occurring in mice has also been confirmed in other studies (Kamp et al., 2003). In addition, we have recently shown that also the mechanical response to CRD is transient in mice (Arvidsson et al., 2006). The reason for this transient response is still unknown. One possible explanation could be the fear-related freezing behaviour (i.e. the mice are completely still when the threatening stimulus is applied) seen in mice. However, we were not able to detect freezing behaviour in mice during the experiments. On the contrary, they tended to move instead. Another possible explanation, accounting for the transient response, could be activation of endogenous pain inhibitory systems that reduce pain perception in connection to stressful situations and painful stimuli, so-called stress-induced analgesia

(Kelly, 1986). This has been demonstrated in mice (Mogil et al., 1996a; Mogil and Belknap, 1997), however psychological stress is also claimed to increase sensitivity to CRD in rodents, see (Fioramonti and Gebhart, 2007) for review. More detailed studies in mice are required to resolve this mechanism. An additional possible explanation could be that pathways involving the periaqueductal grey (PAG)-mediated analgesia system are activated (Bandler and Keay, 1996; Keay and Bandler, 1993; Lanteri-Minet et al., 1993). In a separate study (not published) we evaluated if descending inhibiting opioid pathways were involved in the attenuation of the response. However, administration of the opioid receptor antagonist naloxone (0.3 - 30 mg/kg) did not affect the magnitude or duration of the visceromotor response.

3.2 Relation between intracolonic pressure and sensitivity

One important aspect of a CRD model is the linearity of the VMR related to different intracolonic pressures as has been demonstrated in the rat previously by others (Gebhart and Sengupta, 1995; Ness and Gebhart, 1988; Ness et al., 1991). To be able to study acute visceral nociception from the colon and compare different series of experiments it is also important that the VMR to CRD is reproducible over time.

It is clearly demonstrated that the VMR to CRD is linearly correlated to the intracolonic pressure at a pressure range between 10 and 80 mmHg in both female and male mice. In addition, it is shown that male mice had a higher VMR at all the distension pressures used above 10 mmHg (Fig. 12A). When repeating the same type of experiments 8 months later in new series of female and male mice, a similar VMR to CRD was obtained. This confirms that the VMR is a reproducible physiological response in mice and that different groups of mice can be used for comparison of different treatments in larger studies.

Using the repeated isobaric phasic distension paradigm (12x55 mmHg), male mice responded with progressively larger responses despite stimulated with constant pressure, whereas female mice had a stable response throughout the twelve distensions (Fig. 12B). These data further support that male C57BL/6 mice are more susceptible to mechanical sensitization than female C57BL/6 mice.
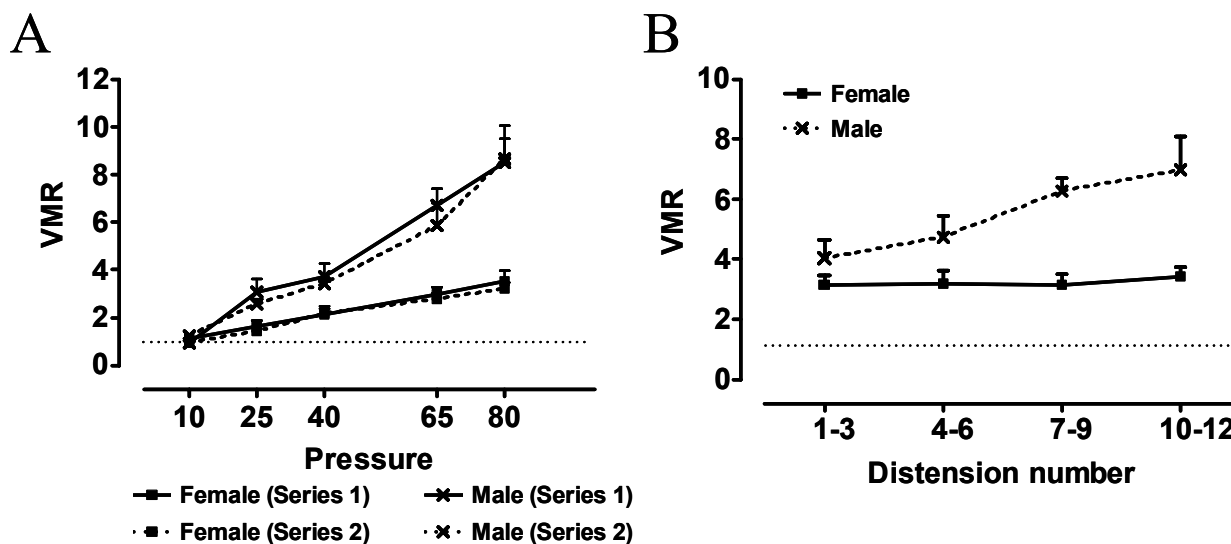
64

Fig. 12. The relation between intracolonic pressure and VMR in female and male C57BL/6 mice in an increasing (A) and a repeated (B) phasic paradigm (n = 7-18).

Comments: The present study establishes that the VMR is a reproducible physiologic response in conscious mice and hence can be used to study acute visceral nociception from the colon. This has subsequently been confirmed by other studies (Arvidsson et al., 2006; Bercik et al., 2004; Kamp et al., 2003). However, this is in contrast with other models of visceral pain in mice, for example the writhing test, which have significant limitations due to lack of specificity (mixed viscero-somatic model) and reproducibility (Hendershot and Forsaith, 1959).

Male C57BL/6 mice appear to be more sensitive to CRD than female C57BL/6 mice. These results agree with a recently published study which also showed that male C57BL/6 mice were more sensitive to CRD than female C57BL/6 mice (Arvidsson et al., 2006). However, they contrast with data showing that female 129S6 mice were more sensitive to CRD than 129S6 males (Kamp et al., 2003). Nonetheless, in the same study by Kamp et al (Kamp et al., 2003), male C57BL/6 mice had responses to CRD of similar magnitude to those observed in 129S6 females; unfortunately, responses in C57BL/6 females were not characterized in that study. These data, together with our observations, suggests the existence of both strain- and sex-related differences in visceral pain sensitivity in mice, which have previously been shown for somatic pain (Craft et al., 2004; Elmer et al., 1998; Mogil et al., 2000).

3.3 Effects of opioid receptor agonists

Treating the animals with either fentanyl (μ-opioid receptor agonist) or U-69593 (κ-opioid receptor agonist) in the isobaric phasic paradigm (12x55 mmHg) resulted in a significant decrease of the VMR in both female and male mice (Fig. 13).
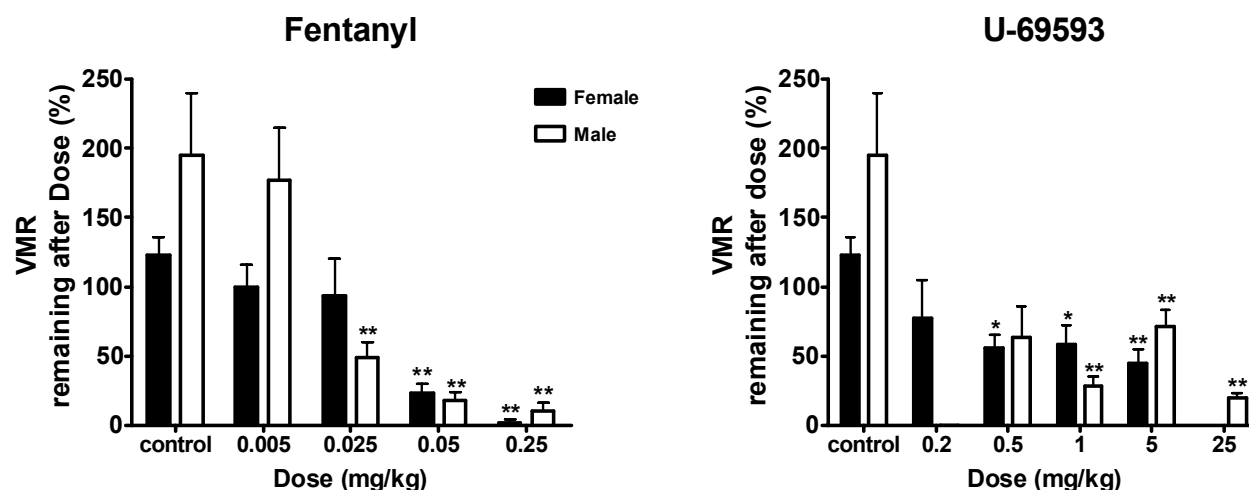
Fig. 13. Effects of Fentanyl and U-69593 on the VMR during repeated colonic distensions in female and male C57BL/6 mice. Drugs were administered s.c 1 min after pulse 3. The average VMR of pulse 1-3 was set to 100%. The average VMR was compared before (pulse 1-3) and after (pulse 4-12) drug administration. Data are presented as means ± SEM (n = 3 - 15). * $p < 0.05$, ** $p < 0.01$ compared with control experiments.

Comments: The analgesic effect of fentanyl is probably mediated by a spinal mechanism, since intrathecal administration of μ-opioid receptor agonists was shown to significantly elevate the visceromotor threshold to CRD, without having effects in peripheral models (Danzebrink et al., 1995; Sengupta et al., 1996). In contrast, U-69593 most likely has both peripheral and central sites of action, depending on the dose administered (Danzebrink et al., 1995; Gebhart et al., 2000a; Sengupta et al., 1996; Su et al., 1997) (unpublished in-house results).

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3.4 Effects of DSS induced inflammation on colonic sensitivity

The aim of paper IV was to characterize the inflammatory status and visceral sensitivity to CRD at several time points after DSS-induced colitis. This rendered comparison of the VMR with inflammatory status of the mice. Both C57BL/6 and Balb/c mice were used to study the possibility of strain differences.

3.4.1 Extent of colonic inflammation

These experiments show that oral treatment with DSS induces colitis in mice, reflected by histopathological changes and increases in MPO activity, in both Balb/c and C57BL/6 mice. In both mouse strains, significant histopathological changes developed between days 2 and 5 of DSS treatment, and persisted until day 12 (Fig. 14, p<0.05). On day 15, inflammatory scores were reduced by about 50%. On day 20, total inflammatory scores continued to decrease in Balb/c mice while C57BL/6 mice had sustained scores on all the following days monitored (i.e. 15, 20 and 30) (Fig. 14).
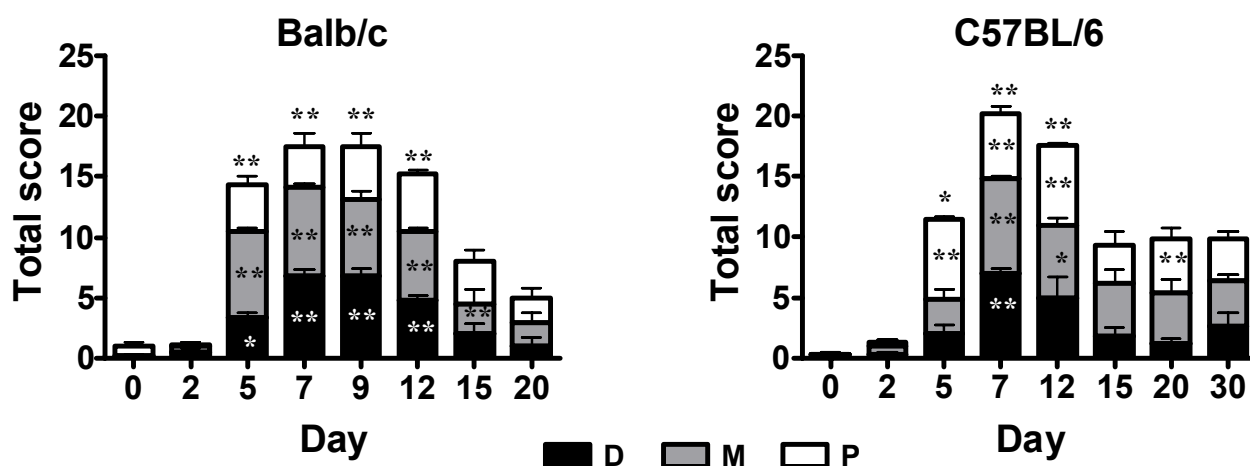
Fig. 14. Extent of inflammation according to histological examination in Balb/c and C57BL/6 mice treated with 4% DSS for 7 and 5 days respectively. Each bar shows a mean value for the total inflammation score in the entire colon. All bars are also sub-divided into three fragments that represent mean values ± SEM of different parts of the colon; D = distal, M = middle, P = proximal. (n = 2-3 for each segment at each day.) * p<0.05, ** p<0.01 vs inflammatory scores at day 0.

Comments: DSS is commonly used to induce colonic inflammation in mice. It is administered in a dose range of 3-10% during 5-10 days, depending on the susceptibility of the mouse strain or the molecular weight of the DSS used (Kitajima et al., 2000; Mahler et al., 1998; Okayasu et al., 1990). The DSS-evoked symptoms include clinical signs (such as reduction of body weight and changes in faecal consistency), while histopathological changes and infiltration of inflammatory cells and increased activity of myeloperoxidase characterize the inflammatory process. The present study reveals that the inflammatory process starts between days 2-5 and was most prominent between days 5-12. This is in agreement with other studies, which reported that inflammatory changes to DSS usually appear around days 3-5 (Cooper et al., 1993; Dieleman et al., 1994; Kitajima et al., 2000; Melgar et al., 2005). The present study also shows that DSS-induced colitis appears to be chronic in C57BL/6 mice, while the Balb/c mice merely develop an acute, transient colitis. These observations agree with those described by Melgar et al (Melgar et al., 2005).

3.4.2 Effects on visceral sensitivity

DSS-induced inflammation did not affect the visceral sensitivity to CRD in either strain at any time point tested (Fig. 15).
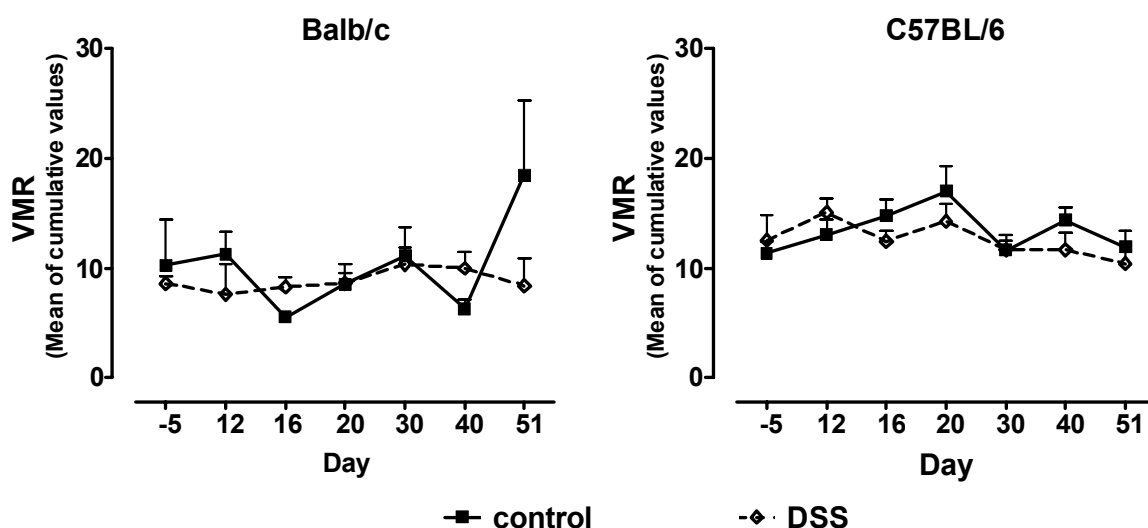
Fig. 15. Cumulative VMR to CRD in control and DSS-treated Balb/c (n = 4-9, except for day 12, where n = 2) and C57BL/6 (n = 15-19) mice. DSS was given for 5 days to C57BL/6 mice and for 6 days to Balb/c mice starting on day 0, thereafter the mice received regular drinking water. Regular drinking water was given to control mice throughout the study. The cumulative VMR to CRD is used to compare the visceral sensitivity in control mice to DSS-treated mice at corresponding days over time. Mean ± SEM.

Comments: The fact that this study could not demonstrate alterations in colonic sensitivity to CRD in DSS-treated mice, despite the fact that the treatment induced colonic inflammation, is in disagreement with several other studies performed in rats (Burton, 1995; Coutinho et al., 1996; Gschossmann et al., 2002; Gschossmann et al., 2004; Morteau et al., 1994) and mice (Bercik et al., 2004; Kamp et al., 2003). There may be several reasons for the discrepancy, e.g. the use of different strains and gender. Kamp et al demonstrated ethanol-induced hypersensitivity in male 129S6 mice (Kamp et al., 2003), whereas Bercik et al demonstrated Trichinella spiralis-increased sensitivity in female NIH Swiss mice (Bercik et al., 2004). However, in the present study, visceral sensitivity was not altered after treatment with DSS in two strains of male mice, C57BL/6 and Balb/c, as well as Balb/c female mice (results not shown). The difference in findings could also be attributed to the different inflammatory stimuli used. Indeed, it has been shown that in the same strain and sex, acute inflammation induced by ethanol, but not acetic acid or mustard oil, caused increased visceral sensitivity in mice (Kamp et al., 2003). In addition, Verma-Gandu et al have shown that chronic DSS-induced colitis is not associated with increased responses to CRD (Verma-Gandhu et al., 2007), which is supportive of the present data.

GENERAL DISCUSSION

1. Possible mechanisms involved in the increased secretory response to intestinal motor activity in patients with IBS

It is now generally accepted that secretion is correlated to the motor activity in the small intestine (Greenwood and Davison, 1987; Mellander et al., 2000; Read et al., 1977). In the present study we used this motilty-related PD signal as a marker for the activity of the enteric neurons involved in this reflex. We found similar alterations between the MMC-associated PD response in celiac disease- and IBS patients, although it was of lower magnitude in IBS patients. For reasons already given, it is difficult to account for this pattern solely on the basis of epithelial factors. An alternative mechanism is dysregulation of sensory neuron networks that might, in turn, be due to neuromodulation by inflammatory mediators. Indeed, others have reported lymphocyte infiltration in the myenteric plexus in patients with severe IBS (Törnblom et al., 2002) or up-regulation of mast cells in patients with IBS (Barbara et al., 2004). It is well established that the autonomic nervous system can reflexively regulate inflammatory responses (Tracey, 2002) and that neuroimmune signaling can affect intestinal ion transport (Cooke, 1994). It has also been shown that the excitability of a sub-population of enteric neurons (AH/Dogiel type II neurons) is increased in a model of colonic inflammation (Linden et al., 2003), and that this increased excitability persists even after the resolution of inflammation (Lomax et al., 2007). There is consequently some support in the literature for interactions between the immune system and enteric sensory network behaviour.

The stretch and mechano-sensitive neurons consist of AH/Dogiel type II neurons characterized by their long lasting afterhyperpolarizing potential (AHP) (Furness et al., 2004). These neurons have been shown to form interconnected networks which can be self-reinforcing, due to excitatory transmission within the networks (Thomas and Bornstein, 2003). The complex shape of the PD response in association with motor phase III is well compatible with network behaviour of submucous neurons and may be involved in the link between motor activity and transmural PD. According to the computer modeling studies of Thomas et al (Thomas and Bornstein, 2003; Thomas et al., 2004), motor phase III itself may be due to maximal coordinated firing of AH/Dogiel type II neurons in the myenteric plexus and an increased synaptic efficacy in myenteric sensory neuron networks will be expected to

increase phase III velocity. If the submucous sensory networks are hyperactive, they will be able to sustain network activity when a mechanical stimulus is terminated (Chambers et al., 2005; Thomas and Bornstein, 2003), and subsequently prolong the decline time of the PD signal.

It is therefore tempting to speculate that an ongoing low-grade inflammation (not routinely detected) or a formerly resolved inflammation may have caused persistent changes in the enteric sensory network in IBS patients. These changes will result in increased excitability of the sensory AH/Dogiel type II-network and enhance the secretory responses to motor activity in IBS patients. If there is an increased excitability in the sensory network this will most likely also increase the extrinsic signaling to the central nervous system and may participate in the induction of visceral sensitization and hence the cause of visceral hypersensitivity as well as symptoms, such as discomfort/pain, seen in IBS patients.

2. Distension induced secretion – possible mechanisms involved

In the animal model used in paper II, we found that the distension induced secretory response was bi-phasic, consisting of an initial rapid response that was followed by a sustained response. This confirms earlier results obtained both *in vitro* (Frieling et al., 1992; Itasaka et al., 1992) and *in vivo* (Kordasti et al., 2006). However, in the present study we extend the former results and show that different transmitter mechanisms are involved in the two responses.

The magnitude and the rate of rise of the rapid response were reduced by hexamethonium and serosal lidocaine, whereas these compounds had no effect on the sustained response. In contrast, (4Cl-D-Phe^6, Leu^{17})-VIP, a VPAC receptor antagonist and talnetant, a NK3 receptor antagonist, left the rapid component unaffected but reduced the sustained component. The NK1 receptor antagonist SR 140333 reduced both the rapid and sustained responses, as well as the rate of rise of the rapid response.

It has been shown that an extensive intramural network of AH/Dogiel type II neurons are found in both the myenteric and submucous plexuses (Furness et al., 2004; Pan and Gershon, 2000). These neurons are synaptically connected to each other via slow EPSPs (i.e. via

connections which are hexamethonium-resistant), and form interconnected networks which can be self-reinforcing due to recurrent excitatory transmission (Bertrand et al., 1997; Kirchgessner et al., 1992; Nurgali et al., 2004; Thomas and Bornstein, 2003). These neurons have also been shown to contain SP (Bornstein et al., 1987; Furness et al., 2004; Lomax and Furness, 2000; Moore and Vanner, 2000) which binds to all NK receptors, but has highest affinity for the NK1 receptors (Maggi, 1995). In addition, NK1 and NK3 receptor immunoreactivity are co-localized in both myenteric and submucosal Dogiel type II neurons of the rat small intestine (Grady et al., 1996; Mann et al., 1997; Sternini et al., 1995).

Sensory AH/Dogiel type II neurons have been shown to communicate with VIP-containing S neurons via fast EPSPs (Bertrand et al., 1997; Kunze et al., 1995). In addition, myenteric AH/Dogiel type II neurons contain acetylcholine which activates submucous nicotinic receptors (Moore and Vanner, 2000), thus supporting a role for nicotinic transmission between sensory neurons and secretomotor neurons. It has been shown that VIP neurons back-project to each other and communicate via slow transmission (Reed and Vanner, 2001) and that VPAC receptors may function as autoreceptors, facilitating the release of VIP (Schulz et al., 2004). This suggests that VIP-secretomotor neurons may form recurrent excitatory networks within the submucous plexus.

With this background in mind we propose a possible conceptual model that accounts for most of the findings in the pharmacological *in vivo* study (Fig. 16). According to this model, there is a rapidly activating tachykinin network(s) (mainly NK1 receptor driven) operating via recurrent feedback, which also requires nicotinic transmission to the secretomotor neurons. This then drives a slowly activating VIP-ergic system, but which is independent of nicotinic transmission. The VIP neuron network can also be activated (but more slowly) via another system operating via both NK1 and NK3 receptors. This slower system is revealed if the more rapid system is blocked by e.g. serosal lidocaine or hexamethonium. A drawback with this model is that one would expect the VPAC antagonist to also block the VPAC receptor at the effector level, i.e. at the level of the secretory enterocyte. If that had been the case, one would have expected blockade also of the rapid component. Neither the rapid nor the slow component was blocked by atropine, i.e. a muscarinic effector mechanism for this component is unlikely. The model thus postulates that the VPAC antagonist used for some reason has a more pronounced antagonistic effect on VPAC network receptors than on VPAC receptors on

enterocytes. To sort out this problem, better antagonists and/or combinations of antagonists will be needed.
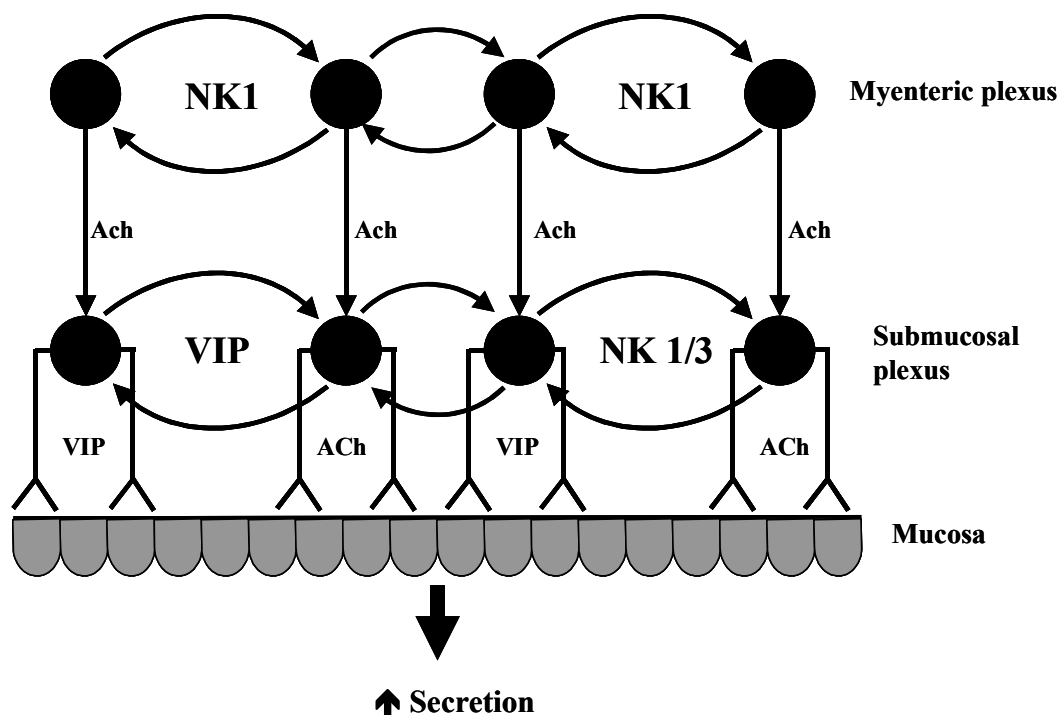

Fig. 16. A possible model for the system generating the complex PD response to distension. According to this model, there is one system of rapidly activating and rapidly adapting tachykininergic neurons operating via a NK1 receptor mechanism, and another slowly activating and slowly adapting system operating via both NK1 and NK3 receptors. The slow system operates via a receptor that is blocked by the peptide antagonist [4Cl-D-Phe[6], Leu[17]]-VIP. In this model, the rapid system is placed in the myenteric plexus and the slow system in the submucous plexus, the reason for this being the effects of hexamethonium and serosal lidocaine. An obvious problem with this model is that if VIP is the final secretomotor transmitter behind the rapid response, one would have expected the VPAC antagonist to attenuate also that component.

3. IBS and animal models

In the present study, alterations in colonic sensitivity to CRD in DSS-inflamed mice were not seen. It is well known that mediators of inflammation have the potential to alter the nociceptive processing both peripherally and centrally in the gastrointestinal DRG neurons (Beyak and Vanner, 2005; Lin and Al-Chaer, 2003; Lin et al., 2004; Sengupta and Gebhart, 1994), but our knowledge about the mechanisms behind the signalling between the inflammatory system and the neural pathways is limited. Recently, the existence of two

spinal neuronal populations (ABRUPT and SUSTAINED) has been demonstrated in rats (Ness and Gebhart, 2000; Ness and Gebhart, 2001). These two neuronal populations were shown to respond to CRD in a different manner after acute inflammation with turpentine. The activity of the SUSTAINED neuronal population was enhanced whereas the ABRUPT was not modified or only slightly enhanced by CRD after turpentine treatment. In addition, Coldwell et al have shown that colonic afferents, isolated from DSS-treated rats, were more sensitive to application of serotonin than controls, while their responsiveness to mechanical stimuli was not affected (Coldwell et al., 2007). These results indicate that inflammatory mediators may not sensitize mechanosensitive neurons, but may sensitize other afferent populations, responding to for instance serotonin.

This assumption is supported by findings in the clinical setting. Clinical studies have shown that patients suffering from inflammatory bowel diseases, such as Crohn´s disease (CD) or ulcerative colitis (UC), experience equal or lower sensitivity to isobaric CRD than healthy volunteers (Bernstein et al., 1996; Chang et al., 2000). This experience was independent of performing rectal or sigmoid distension at the site of mucosal inflammation (Chang et al., 2000) or at a site outside the inflammatory area (Bernstein et al., 1996). Additionally, chronic inflammation of the esophagus and stomach are not associated with visceral mechanical hyperalgesia (Fass et al., 1998; Mertz et al., 1998). Hence, the distension of a visceral organ subjected to inflammation does not necessarily translate into increased sensitivity.

In contrast, a few studies have demonstrated increased sensitivity to CRD in patients with inflammatory bowel disease using isovolumetric distension (Farthing and Lennard-jones, 1978; Loening-Baucke et al., 1989; Rao et al., 1987). This could be due to decreased colonic compliance caused by structural changes of the colon wall, rather than to inflammation-evoked increases in primary afferent nerve excitability. In the current study we did not monitor compliance, which may have been affected by DSS treatment.

CONCLUSIONS

1. In a substantial subgroup of IBS patients, the maximal PD during MMC phase III contractions is significantly elevated, the decay time to baseline conditions is prolonged and the propagation speed of phase III contractions is elevated.

Our interpretation of these data is that there is an abnormal response of secretomotor neurons to phase III contractions in IBS patients, a pattern that is compatible with dysfunction of enteric sensory neuron networks. This dysfunction may be due to neuromodulation by inflammatory mediators, since we found similarities between the MMC-associated PD responses in patients with celiac disease.

2. The *in vivo* PD response to distension in animals was biphasic, consisting of a rapid and a sustained component with different pharmacology.

- The rapid and sustained responses were neurally mediated.
- Hexamethonium, lidocaine and the NK1 antagonist SR 140333 reduced the magnitude and the rate of rise of the rapid response.
- The VPAC antagonist and SR 140333 reduced the sustained response.

Our interpretation of these data is that distension activates two parallel-coupled neural networks, one myenteric tachykinin-driven and one submucosal VPAC-receptor driven, which are connected via a nicotinic receptor mechanism.

3. In mice the sensory response to CRD was pressure dependent and reproducible. We have thus demonstrated that the sensory response to CRD can be readily monitored in conscious mice and hence can be used to study acute visceral nociception from the colon.

4. DSS-evoked colitis did not appear to alter colorectal mechanosensitivity and is therefore not considered useful as a model for visceral hypersensitivity.

FUTURE CONSIDERATIONS

In the current thesis we have shown that IBS patients have an increased secretory response to MMC phase III contractions. However, the study was performed in a rather small population. A substantially larger material is therefore needed to assess the prevalence of this phenomenon before an elevated PD can be regarded as a biomarker for IBS. Whether PD-positive and visceral hypersensitive positive IBS patients represent separate pathophysiological entities, with different clinical profiles, is also an intriguing question that needs to be addressed by further research. In future studies it would therefore be of great value to perform PD recordings and to test the degree of visceral hypersensitivity in the same patients. Ideally, in the same patient, one should also characterize the degree of inflammatory markers present (e.g. mast cells, cytokine production and T-cells) to be able to evaluate the possibility that inflammation is one of the mechanisms involved in the increased PD response and/or visceral hypersensitivity seen in IBS patients. If such a correlation is found, one should further try to delineate the mechanism/s involved by using animal models. It would be interesting to evaluate if inflammation influences any of the phases in the distension induced secretory response in rodents. However, one needs to be careful when choosing the inflammatory stimuli, since different inflammatory models utilize different mechanisms.

Since there is no obvious correlation between the experienced symptom severity and the degree of visceral hypersensitivity (Lembo et al., 1999; Schmulson et al., 2000; Zar et al., 2006), the currently available animal models of visceral hypersensitivity need to be re-evaluated. In order to do this, one needs to perform more studies in humans in order to try to further characterize the mechanism/s contributing to visceral hypersensitivity. Second, more translational studies are required in experimental animals and humans, where the same drugs are evaluated on CRD induced visceral perception in both animals and humans, as well on the relief of symptoms in humans. These bridging studies are required in order to assess the predictability of available animal models and to be able to justify the use of CRD in animals as a relevant model for IBS symptoms. We do not want a drug that is just reducing the CRD induced visceral sensitivity, but one that is actually reducing the general experienced symptom of visceral pain!

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