Gut peptides in gastrointestinal motility and mucosal permeability

MD. ABDUL HALIM
A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)
Gut regulatory peptides, such as neuropeptides and incretins, play important roles in hunger, satiety and gastrointestinal motility, and possibly mucosal permeability. Many peptides secreted by myenteric nerves that regulate motor control are also produced in mucosal epithelial cells. Derangements in motility and mucosal permeability occur in many diseases. Current knowledge is fragmentary regarding gut peptide actions and mechanisms in motility and permeability.

This thesis aimed to 1) develop probes and methods for gut permeability testing, 2) elucidate the role of neuropeptide S (NPS) in motility and permeability, 3) characterize nitrergic muscle relaxation and 4) characterize mechanisms of glucagon-like peptide 1 (GLP-1) and the drug ROSE-010 (GLP-1 analog) in motility inhibition.

A rapid fluorescent permeability test was developed using riboflavin as a transcellular transport probe and the bisboronic acid 4,4'oBBV coupled to the fluorophore HPTS as a sensor for lactulose, a paracellular permeability probe. This yielded a lactulose:riboflavin ratio test. NPS induced muscle relaxation and increased permeability through NO-dependent mechanisms. Organ bath studies revealed that NPS induced NO-dependent muscle relaxation that was tetrodotoxin (TTX) sensitive. In addition to the epithelium, NPS and its receptor NPSR1 localized at myenteric nerves. Circulating NPS was too low to activate NPSR1, indicating NPS uses local autocrine/paracrine mechanisms.

Nitrergic signaling inhibition by nitric oxide synthase inhibitor L-NMMA elicited premature duodenojejunal phase III contractions in migrating motility complex (MMC) in humans. L-NMMA shortened MMC cycle length, suppressed phase I and shifted motility towards phase II. Pre-treatment with atropine extended phase II, while ondansetron had no effect. Intestinal contractions were stimulated by L-NMMA, but not TTX. NOS immunoreactivity was detected in the myenteric plexus but not smooth muscle.

Food-intake increased motility of human antrum, duodenum and jejunum. GLP-1 and ROSE-010 relaxed bethanechol-induced contractions in muscle strips. Relaxation was blocked by GLP-1 receptor antagonist exendin(9-39) amide, L-NMMA, adenylate cyclase inhibitor 2′5′-dideoxyadenosine or TTX. GLP-1R and GLP-2R were expressed in myenteric neurons, but not muscle.

In conclusion, rapid chemistries for permeability were developed while physiological mechanisms of NPS, nitrergic and GLP-1 and ROSE-010 signaling were revealed. In the case of NPS, a tight synchrony between motility and permeability was found.

Keywords: Gut regulatory peptides, Neuropeptides, Gastrointestinal mucosal permeability, Gastrointestinal motility, GLP-1, NPS, ROSE-010
To my family
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


(* these authors contributed equally to the work)

Reprints were made with permission from the respective publishers.
Article not included in this thesis:

**Supervisors**
Associate Professor Dominic-Luc Webb
Department of Medical Sciences, Uppsala University
Uppsala, Sweden

Professor Per M Hellström
Department of Medical Sciences, Uppsala University
Uppsala, Sweden

**Chair**
Professor Hans Törmä
Department of Medical Sciences, Uppsala University
Uppsala, Sweden

**Faculty Opponent**
Professor Lars Fändriks
Department of Gastrointestinal Research and Education,
Göteborgs Universitet
Göteborg, Sweden

**Committee members**
Professor Olof Nylander
Department of Neuroscience, Uppsala University
Uppsala, Sweden

Professor Per-Ola Carlsson
Department of Medical Cell Biology, Uppsala University
Uppsala, Sweden

Professor Susanna Cristobal
Department of Clinical and Experimental Medicine, Linköping University
Linköping, Sweden
# CONTENTS

1. **Introduction** ............................................................................................................. 11
   1.1 Gastrointestinal motility and permeability ......................................................... 11
      1.1.1 Gastrointestinal anatomy and motility ......................................................... 11
      1.1.2 Gut peptides in motility ............................................................................... 12
      1.1.3 Intestinal mucosal permeability ................................................................ 13
      1.1.4 Gut permeability and motility ..................................................................... 15
      1.1.5 Migrating motor complex (MMC) ............................................................... 15
      1.1.6 Gut permeability and motility in health and disease .................................. 16
   1.2 Bisboronic acid-appended viologen (BBV) assay for permeability .................. 17
      1.2.1 Boronic acid and its application ................................................................. 17
      1.2.2 BBV assay for gut permeability .................................................................. 18
   1.3 Neuropeptide S (NPS) ......................................................................................... 18
   1.4 Glucagon-like peptide 1 (GLP-1) ....................................................................... 20

2. **Aims of the thesis** .................................................................................................. 22

3. **Materials** .................................................................................................................. 23
   3.1 Human subjects ..................................................................................................... 23
   3.2 Animals .................................................................................................................. 24

4. **Methodologies** ......................................................................................................... 25
   4.1 Permeability assays ............................................................................................. 25
      4.1.1 Preparation of BBVs .................................................................................... 25
      4.1.2 Mechanism of BBV sugar sensors in permeability ..................................... 25
      4.1.3 Sample collection ....................................................................................... 26
      4.1.4 Riboflavin assay ......................................................................................... 26
      4.1.5 BBV (4,4’oBBV) method for urine lactulose and mannitol ....................... 27
   4.2 Procedures .............................................................................................................. 27
      4.2.1 Surgical procedure in rat ............................................................................. 27
      4.2.2 Gastrointestinal motility *in vivo* in the rat ............................................. 28
      4.2.3 Manometry in rat ......................................................................................... 29
      4.2.4 Gastrointestinal motility *in vivo* in man .................................................. 29
4.2.5 Gastrointestinal motility in vitro in man.............30
4.3 Protein Expression.................................................. 31
  4.3.1 Immunohistochemistry (IHC)............................. 31
  4.3.2 Enzyme-linked immunosorbent assay (ELISA).. 32
  4.3.3 Radioimmunoassay (RIA)................................. 33
5.  Statistics.................................................................33
6.  Results........................................................................35
  6.1 New fluorescence method for permeability (paper I).....35
  6.2 Effects of NPS on motility and permeability (paper II)....35
  6.3 Localization of NPS and its receptor, NPSR1 (paper II)....36
  6.4 Nitric oxide regulation of in vivo MMC in humans (paper III)........................................................................36
  6.5 Localization of nitric oxide synthases nNOS, iNOS and eNOS (paper III)............................................................ 37
  6.6 Involvement of nitric oxide in regulation of in vitro muscle contraction (paper III).................................................. 38
  6.7 Effects of GLP-1 and ROSE-010 on smooth muscles (paper V)........................................................................ 38
  6.8 Localization of GLP-1R and GLP-2R (paper IV).........39
7.  General discussion......................................................40
8.  Conclusions.....................................................................47
9.  Acknowledgements.......................................................49
10. References.....................................................................51
Appendix (paper I-IV)
ABBREVIATIONS

AJ  Adherens junction
ANOVA  Analysis of variance
AP  Alkaline phosphatase
BBV  Bisboronic acid-appended viologen
cGMP  Cyclic guanosine monophosphate
cAMP  Cyclic adenosine monophosphate
DAB  3,3′-Diaminobenzidine
dDA  2′,5′-Dideoxyadenosine
ELISA  Enzyme-linked immunosorbent assay
EDTA  Ethylenediaminetetraacetic acid
EFS  Electrical field stimulation
eNOS  Endothelial nitric oxide synthase
ENS  Enteric nervous system
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
GLP-1  Glucagon-like peptide-1
GLP-1R  Glucagon-like peptide-1 receptor
GLP-2R  Glucagon-like peptide-2 receptor
HEPES  4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HPLC  High-performance liquid chromatography
HPTS  8-Hydroxypyrene-1,3,6-trisulfonic acid
HRP  Horseradish peroxidase
5HT  5-Hydroxytryptamine / Serotonin
IBD  Inflammatory bowel disease
IBS  Irritable bowel syndrome
iNOS  Inducible nitric oxide synthase
IL-1β  Interleukin-1β
[Ca^{2+}]_{i}  Intracellular calcium concentration
L-NMMA  L-N\textsuperscript{G}-monomethyl arginine
L-NAME  L-N\textsuperscript{G}-nitroarginine methyl ester
LLOD  Lower limit of detection
LLOQ  Lower limit of quantification
MBV  Monoboronic acid-appended viologen
MLCK  Myosin light-chain kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMC</td>
<td>Migrating motor complex</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NPS</td>
<td>Neuropeptide S</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NPSR1</td>
<td>Neuropeptide S receptor 1</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide YY</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RFT2</td>
<td>Riboflavin transporter 2</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junction</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

1.1 Gastrointestinal motility and permeability

1.1.1 Gastrointestinal anatomy and motility

The human gastrointestinal (GI) tract is responsible for transportation and digestion of ingested foodstuffs, absorbing nutrients, and excreting waste. The GI tract is divided into upper and lower GI tract (1). The upper GI tract consists of oesophagus, stomach and small intestine (2). Together with the large intestine, these are the main parts of the GI tract. They are separated from each other by special muscles, called sphincters, which regulate the movement of ingested food materials from one part to another. Stomach is further divided into two parts; proximal, consisting of the cardia and fundus and distal, consisting of the corpus and antrum. The small intestine is divided into the duodenum, jejunum and ileum, while the large intestine is subdivided into the cecum, colon, rectum, and anal canal (3, 4). Each part of GI tract can be further divided into mucosa, submucosa, muscular layer and serosa. The enteric nervous system (ENS) is also an important part of GI tract. The main components of the enteric nervous system are the myenteric plexus and submucous plexus, myenteric plexus is located between longitudinal and circular layer of muscle in tunica muscularis and the submucous plexus is located in the submucosa. The function of myenteric plexus is to exert control over digestive tract motility and the functions of submucous plexus are sensing the environment within the lumen, regulating GI blood flow and controlling epithelial cells functions. Three types of neurons are present within two enteric nerve plexuses; 1) afferent or sensory, receive information from sensory receptors in the mucosa and muscle, 2) motor neurons, control GI motility and secretion, and possibly absorption, and 3) interneurons are mainly responsible for integrating information from sensory neurons and providing it to enteric motor neurons. GI motility is defined by the movements of the digestive system, and the transit of contents within it. Motility promotes digestion of food in time and restricts bacteria from growth in the upper GI tract. The major source of contractile activity in the small intestine originates from the muscularis externa, which consists of outer longitudinal and the inner circular muscle layers. GI muscles are innervated by excitatory and inhibitory nerves known as myenteric plexus (5, 6). These two
muscle layers together with ENS enables complex motor patterns such as the peristaltic reflex and segmentation to regulate the tonic contractions (7, 8). A pacemaker zone of interstitial cells of Cajal (ICC) provides spontaneous pacemaker activity in GI muscles and generates slow waves at a high frequency (in man about 3/min in the stomach, 11/min in the small bowel) in the circular layer. GI motility patterns are highly integrated behaviours requiring coordination between smooth muscle cells and utilizing regulatory input from interstitial cells, neurons, endocrine and immune cells. The ionic channels in human GI smooth muscles responsible for excitation-contraction coupling and the specific responses of human muscles to neurotransmitters and other regulatory agents have not been studied in enough depth to clearly describe excitation-contraction coupling mechanisms in human GI smooth muscles. When nerves or muscles in any portion of the digestive tract do not function in networks with their normal strength and coordination, symptoms related to motility problems develop.

Bethanechol is a choline carbamate, a direct-acting muscarinic receptor agonist that has been used as an experimental pro-motility cholinergic agent. Unlike acetylcholine, bethanechol is not hydrolysed by cholinesterase and will therefore have sustained activity. The type 3 muscarinic receptor expressed on smooth muscle, activates phospholipase C, yielding inositol 1,4,5-triphosphate, which liberates Ca\(^{2+}\) from the sarcoplasmic reticulum into the cytosol to induce contraction. Muscarinic receptors are also present in the myenteric plexus, and are involved in both stimulation of contraction and secretion from glands in the GI tract (9).

1.1.2 Gut peptides in motility

The GI tract is the largest endocrine organ in the body. It is a major source of regulatory peptides, such as incretins and neurotransmitters. Most of the regulatory hormones are peptides. They play important roles in feeding, satiety and GI motility. Many peptides found in nerves of the GI tract are also found in mucosal endocrine or paracrine cells. Some of them have an impact on mucosal permeability by affecting secretion of pro- or anti-inflammatory cytokines. Many of them have been shown to affect GI motility. The enteric nervous system (ENS) consists of excitatory and inhibitory neurons. They express neuropeptides that regulate the motor control of GI muscles (10). Peptidergic responses are only elicited at high frequencies of enteric nerve
firing (usually >5 Hz). At low-frequency stimulation, responses can normally be blocked entirely by a combination of M2 and M3 muscarinic receptor blockers, and NO synthase inhibitors (11). However, the same antagonists and inhibitors can block peristaltic reflex responses, receptive or adaptive relaxation, and lower oesophageal sphincter, suggesting that many motility responses depend on small molecule neurotransmitters such as NO, acetylcholine and β-NAD/ATP, while the peptides seem to be reserved for more extreme conditions or possibly when other motor pathways are compromised. Some gut peptides are also neuropeptides with local effects. Neuropeptide S (NPS) is one example. NPS is expressed in neuroendocrine cells. Other peptides act as hormones. Glucagon-like peptide-1 (GLP-1), secreted from intestinal L-cells to circulate with the blood stream and act at distant sites to regulate GI motility. However, little is known about the role of these peptides in mucosal permeability or motility disorders that often accompany inflammatory diseases.

1.1.3 Intestinal mucosal permeability

A physical barrier formed by the epithelial lining prevents direct contact between the external environment and internal intestinal tissues. The GI tract is lined by a continuously secreted mucus layer formed by high molecular mass oligomeric mucin glycoproteins. Mucins are secreted by gastric foveolar mucous cells and intestinal goblet cells that form a barrier that prevents large particles, including most bacteria, from direct contact with the epithelial cells (12). Cell surface mucins are likely to play an important role in immune defence since they serve both as barrier and reporting function. The intestinal epithelium is a single layer of cells lining the gut lumen. In normal healthy gut, this cell lining has two important functions. First, it acts as a barrier to prevent the passage of harmful intraluminal entities, including foreign antigens, microorganisms, and their toxins (13, 14). Second, it acts as a selective filter, allowing the translocation of essential dietary nutrients, electrolytes, and water from the intestinal lumen into the circulation (13-17). The intestinal epithelium mediates selective permeability through two major routes: transcellular (also called transepithelial) and paracellular pathways. The transcellular pathway permits lipophilic molecules to passively diffuse, while non-lipophilic (e.g., many nutrients and macromolecules) are actively transported via membrane channels or transporters. The second pathway, the paracellular route,
is strictly regulated by junctional complexes. Passive transport is determined by concentration gradients for osmotic, electrochemical and electrostatic pressures. The paracellular route is maintained by three components that can be identified at the ultrastructural level. These are desmosomes, adherens junctions (AJs), and tight junctions (TJs) (Fig. 1) (18). The AJ complexes consist of transmembrane proteins that link adjacent cells to the actin cytoskeleton through cytoplasmic scaffolding proteins. The AJs and desmosomes are thought to be more important in the mechanical linkage of adjacent cells (19-21). TJs, on the other hand, are the apical-most junctional complexes and responsible for sealing of the intercellular space and regulating selective paracellular ionic solute transport (21). TJs are multi-protein complexes composed of transmembrane proteins. The AJ and TJ complexes are also important in the regulation of cellular proliferation, polarization, and differentiation (22-24). Both junctions are supported by a dense perijunctional ring of actin and myosin that can regulate the barrier function. The TJ limits solute flux along the paracellular pathway, which is typically more permeable than the transcellular pathway. The TJ is, therefore, the rate-limiting step in trans-epithelial transport and the principal determinant of mucosal permeability. Besides TJ, the enteric nervous system has been shown to involve regulation of the intestinal epithelial barrier permeability (25). Thus, it is important to understand the specific barrier properties of the tight junction. Disruption of the mucin layer (loosely and tightly bound mucin layer) and mucosal layer permit foreign particles, such as bacterial metabolites to pass. Increased paracellular permeability has been shown to be an early event in the progression of different diseases. Patients with irritable bowel syndrome (IBS) display increased intestinal permeability. Mucosal soluble mediators are involved in the pathophysiology of pain in IBS, which has also been shown to affect permeability (26). It is not yet known how these mediators affect IBS symptoms.
1.1.4 Gut permeability and motility

Gut permeability permits small particles (<4 Å or MW ~250 Da) to migrate through the TJ pores (27). A healthy gut barrier prevents large and harmful molecules from passively migrating into the blood circulation (28). The gut microbiota can alter small intestinal and colonic neuromotor function (29) through release of different substances. For example, in 2009, Bar et al. showed a supernatant from *Escherichia coli* Nissle 1917 to increase colonic motility in isolated human muscle strips (30). Depending on the species, intestinal bacteria stimulate or suppress the initiation and aborad migration of the migrating motor complex (MMC) (31). Similarly, motility is one of the most influential determinants in controlling intestinal microbial growth (32). Motility disorders may cause bacterial overgrowth in the intestine that can affect GI motility. Recently, we showed that NPS causes increased permeability *in vivo* in the rat (paper II). However, it has not been extensively studied whether there is cross-talk between gut permeability and motility.

1.1.5 Migrating motor complex

The migrating motor complex (MMC) is a cyclic pattern of electro-mechanical activity observed in GI smooth muscle during the periods...
between meals. The MMC is present in the GI tract of most mammals, including humans. The normal MMC cycle in humans and dogs consists of three phases. Phase I is quiescent, with only rare action potentials and contractions. Phase II consists of intermittent, irregular low-amplitude contractions. Phase III, with short bursts of regular high-amplitude contractions, is the most dramatic feature of the entire MMC cycle (34). The control of the MMC is a complex process. The central, peripheral and enteric nervous systems, hormones and luminal factors are regulatory components of the MMC (35). The interdigestive MMC pattern functions as a housekeeper mechanism that propels chyme, bacteria and cell debris down to the GI tract. This protects the mucosa from damage and counteracts bacterial overgrowth in the small intestine. Transport occurs throughout phase II and phase III of the MMC (36). Absence of the MMC has been associated with motility disorders (e.g., gastroparesis, intestinal pseudo-obstruction) and secondary small intestinal bacterial overgrowth.

1.1.6. Gut motility and permeability in diseases

Inflammatory bowel disease (IBD) is one of the prevailing diseases in the Western society, which seems to be more prone in Europe and America. As many as 1.4 million people in the United States and another 2.2 million in Europe suffer from IBD (37). The onset of IBD is accompanied by, and even preceded by intestinal hyperpermeability and dysmotility that may initially be diagnosed as IBS (38). The main forms of IBD are Crohn’s disease and ulcerative colitis (39, 40). The etiology of IBD is unknown. Different studies show that the disease arises as a result of interactions between environmental and genetic factors. Alterations of enteric bacteria and genetic factors can contribute to IBD (41). So far, 163 susceptibility loci were identified that can increase the susceptibility to IBD (42). These are common to both disorders, suggesting a common mechanism in the pathophysiology (43). The immunopathogenesis of IBD involves three major steps: 1) defects in mucus production and barrier dysfunction that allow luminal contents to penetrate the underlying tissues; 2) inappropriate response of a defective mucosal immune system to the indigenous flora and other luminal antigens; and 3) an immune response leading to production of pro-inflammatory cytokines, which cause increased permeability by re-organizing the TJ proteins (44, 45). The first line of defence of the mucosal immune system is the innate immune system
in the epithelial barrier (46). This barrier is leaky in people with IBD. Several studies have shown that a lowered epithelial resistance and increased permeability of the inflamed and non-inflamed mucosa in Crohn’s disease and ulcerative colitis (47). Furthermore, gut permeability is suspected to be involved in disease symptoms; Parkinson’s disease, Alzheimer’s disease, and other neurodegenerative diseases. Because gut permeability has thus far required expensive and time-consuming methodologies, the first paper of this thesis pursued a more efficient means to study gut permeability.

1.2 Bisboronic acid-appended viologen (BBV) assay for permeability

1.2.1 Boronic acid and its application

Boronic acids are trivalent boron containing organic compounds that contain one alkyl substituent and two hydroxyl groups. It has only six valence electrons, with a deficiency of two electrons in the outer shell. Unlike carboxylic acids, their carbon analogues, boronic acids are not found in nature. The preparation and isolation of a boronic acid was first described by Frankland in 1860 (48). Boronic acids act as mild organic Lewis acids. Their unique properties together with their stability and ease of handling make boronic acids an attractive class of synthetic intermediates. Several types of boronic acid-based fluorescent probes for hydrogen peroxide have been developed that (49-51) that could be used for the detection of the involvement of peroxide in Alzheimer’s and Parkinson’s disease (52, 53). One of the most important applications of boronic acid compounds is in the specific recognition of carbohydrates. A large number of papers have been published on the preparation and use of fluorescent sensors for carbohydrates. However, most of these studies focus on monosaccharide sensing for fundamental chemistry studies instead of biological applications (54, 55). In recent years, there have been increased activities in the preparation of “binders” and sensors for carbohydrate-based biomarkers and other biologically important saccharides and glycosylation products. These binders and sensors have potential in the development of diagnostic and therapeutic agents. The detection of small biomolecules is of central interest in medical diagnostics (56). Boronic acids are known to reversibly bind cis-diols with high affinity to form cyclic boronate esters (57). As a result, many boronic acid-containing mole-
cules have been utilized as chemosensors for the recognition of carbohydrates (58). Specifically, there has been much interest focused on the design of boronic acid-containing fluorescent glucose sensors that operate under physiological conditions (59). If optimized, such sensors can be implantable, and used to continuously monitor glucose concentrations in preterm infants (60), patients in intensive care units (61), and in people suffering from diabetes (62).

1.2.2 Bisboronic acid-appended viologen assay for gut permeability

Over the past several decades there has been a lot of effort to develop simple non-invasive means to test paracellular permeability. Traditionally, small bowel permeability is expressed as the ratio of the fractional excretion of a large molecules to that of a smaller molecules (e.g., the lactulose:mannitol ratio). In recent years, supramolecular analytic chemistry is extensively used in the development of indicator-displacement assays (IDAs) and differential analyte receptors (63). Boronic acid-based fluorescent chemosensors take advantage of the ability of boronic acids to reversibly bind 1,2- and 1,3-diols (64). Much work has been done in using organoboronic acids to quantify sugars (65-67), which have had success in clinics; e.g., eight validated HbA1c assays employ organoboranes (68). However, organoboronic acids are typically more sensitive to lactulose and less sensitive to mannitol. It is therefore important to find a compound that can be used in place of mannitol. Since riboflavin receptor type 2 is expressed at the apical epithelial membranes of the small intestine (69), it could be used as a potential probe for small intestinal permeability. Loss of RFT2 expression results in severe riboflavin deficiency (70, 71). Riboflavin therefore can be used as a marker for poor nutritional absorption.

1.3 Neuropeptide S

Neuropeptide S (NPS) was first described in 2002 (72). NPS was identified as a 20 amino-acid bioactive peptide, whose primary sequence is highly conserved in many species (73, 74). Its receptor, NPSR, is G protein-coupled and exists as two functional isoforms, NPSR1-A and NPSR1-B (Fig. 2) (75), NPS selectively binds and activates an orphan G protein-coupled receptor named the NPS receptor.
NPSR1 (74, 76). NPSR1 is a 7-transmembrane G protein-coupled receptor, which function is poorly characterized. Like its ligand NPS, NPSR1 is mainly expressed in the brain, particularly in regions mediating anxiety and stress responses, such as the amygdaloid complex and the paraventricular hypothalamic nucleus, and in the hippocampus (77). NPS/NPSR1 system is also expressed in the GI tract (78) and leukocytes, suggesting a role for NPS in motility and inflammation. NPSR1 has been shown to be involved in both asthma and IBD (78, 79). NPSR1 activation increases both intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and cAMP levels (73). The receptor potency for NPSR1 is dependent on the Ile107Asn isoform. The Asn107Ile polymorphism results in a gain-of-function characterized by a five- to ten-fold increase in agonist potency at NPSR Ile107 compared to NPSR Asn107 (73). NPS has received much attention for its function in the CNS, mainly in several brain regions (74, 80-82) and its identification as a susceptibility locus for asthma and associated traits (76, 83-87). Recently, NPS is in the limelight for involvement with IBD and IBS. A number of single nucleotide polymorphisms in the NPSR sequence are associated with asthma, elevated serum IgE levels, and bronchial hyper-responsiveness (76, 84, 85). One of these single nucleotide polymorphisms is found in the coding region of the gene and results in mutation of residue 107 from Asn to Ile (80). NPS seems to be related to inflammatory reactions (88, 89) partly because the NPSR1 polymorphism is associated with IBD susceptibility, where NPSR1 mRNA and protein ex-21 expression are relatively high in IBD patients (79, 90). This receptor variant also has been linked with motor and sensory disturbances in the gut, such as hastening colonic transit, pain, gas, and urgency sensations, suggesting the role of NPS in inflammatory and functional GI disorders, which are relevant to IBS and IBD (91).
Figure 2: Schematic diagram of the human NPSR1 protein showing the presumed location of the N107I polymorphism. Also shown are two isoforms NPSR1-A and NPSR1-B. NPSR1-A encodes the shorter protein isoform with a 29 amino-acid long distinct C-terminus. (I= Ile, N=Asn) (Adapted from Pietras et al. 2011) (83).

1.4 Glucagon like peptide-1

Glucagon-like peptide-1 (GLP-1) was first discovered by Graeme Bell and his colleagues in 1983 (92). GLP-1 is a C-terminally amidated 7-36 amino acid peptide. GLP-1 is secreted into the blood stream from L-cells in the ileum and colon (93). Furthermore, endocrine cells that resemble the pancreatic A-cells were reported to be present in the GI mucosa (94). In humans, almost all of the GLP-1 secreted from the gut is amidated (95), whereas in many animals (rodents, pigs), part of the secreted peptide is GLP-1(7-37) (96, 97). The density of L-cells is very high in the ileum in most species (98, 99). A substantial number are present in the colon (100), particularly the distal part. GLP-1 is highly susceptible to the catalytic activity of the enzyme dipeptidyl peptidase IV (101). The catalytic product thus generated GLP-1 9-36 amide or GLP-1 (9-37), is inactive and acts as a competitive antagonist at the GLP-1 receptor (102, 103). GLP-1 secretion is meal related. In the fasting state, the plasma concentrations are very low (10.0 ±0.5 pmol·L⁻¹) (104). Meal intake causes a rapid increase in L-cell secretion, most obvious when measured with carboxyl-terminal assays (105) but often measurable also with assays for the intact hormone
(106). The GLP-1 receptor is a class 2, G protein-coupled receptor (107), meaning it couples to intracellular signalling via a stimulatory G protein to adenylate cyclase (108, 109). GLP-1 has also been shown to have other potent regulatory effects in the GI tract including slowing gastric emptying. Primarily the slowing of gastric emptying after a meal is of major importance for metabolic homeostasis. Since motility effects of GLP-1 seem to be of major importance for its biological actions, focus was set on research in this detail in order to determine whether this might be of clinical use for motility disorders. Full-length GLP-1 is inactive (110, 111). GLP-1 has been proposed as a new therapeutic agent for neurodegenerative diseases, including Alzheimer’s disease (112). In humans, GLP-1 inhibits small intestinal motility in healthy subjects and patients with IBS (113) and reduces pain attacks in IBS (170).
2. AIMS OF THE THESIS

The aims of this doctoral thesis are to improve our knowledge regarding relationships between gut permeability and gut motility in order to clarify the interactions between gut permeability, inflammatory response and GI symptoms, often encountered as a motility problem.

**Paper I:** Evaluate organoboranes to quantify lactulose and mannitol for paracellular permeability and use of riboflavin as an indicator of transcellular absorption.

**Paper II:** Characterize *in vivo* physiology of NPS signalling in the context of gut motility and permeability and explain the association of NPSR to IBD within this context.

**Paper III:** To characterize nitrergic inhibition of antroduodenojejunal motility in man in relation to muscarinic and 5-HT₃ receptor using selective antagonists.

**Paper IV:** To clarify whether infused GLP-1 inhibits *in vivo* prandial motility response and determine the likeliest target cell type and mechanism of action of GLP-1 and its analogue ROSE-010 on motility inhibition using *in vitro* human gut muscle strips.

The overall aim of this thesis was to develop new method for permeability test and to study the importance of nitric oxide for the effect of NPS and GLP-1 in the gut in context of gut motility and permeability.
3. MATERIALS

3.1 Human Subjects

All the studies were approved by Regional Ethics Committee at Uppsala University and/or Karolinska Institutet. All subjects gave informed consent prior to entering the study. Ethics approval numbers; Paper I: Studies were carried out according to ethical approval Dnr 2010/184 held at Uppsala University, Sweden. In this jurisdiction, lactulose, mannitol and riboflavin are available over the counter, Paper II: The experiments were approved by the Regional Ethics Committee at Uppsala University (2010/157 and 2010/184). Ethics approvals were obtained from Uppsala Ethics Committee for Experiments with Animals (C309/10 and C147/13) and Northern Stockholm Animal Ethics Committee (N348/09 and 353/09), Paper III, The investigation was approved as an exploratory study by the regional ethics committees at Karolinska Institutet and Uppsala University (01-313 updated version 2013/965-32), Immunohistochemistry of the study is covered under ethics approval 2010/184 (Uppsala, Sweden), and Paper IV: The experiments were approved by the Regional Ethics Committee at Uppsala University (2010/157 and 2010/184). The study was approved by the Swedish Medical Products Agency and the Ethics Committees of Karolinska Institute and Uppsala University (01-313 updated version 2013/965-32). Informed consent was obtained from all subjects. The study was registered at www.ClinicalTrials.gov with no. NTC02731664.

Paper I
Urine from healthy human subjects was used to measure gut permeability with the number of subjects indicated for each experiment.

Paper II
Organ bath experiments were performed with tissue from patients undergoing elective surgery for non-obstructive colorectal cancer. Smooth muscle specimens were obtained from the middle portion of the greater curvature of the gastric corpus of normal human stomach (n = 10), from the jejunum 70 cm distal to the pylorus (n = 15) in conjunction with gastric bypass surgery, and from the free resection margin in the jejunum 30 cm orally of the ileocecal valve (n = 24) and midportion of the transverse colon within 40 cm distal of the ileocecal valve (n = 24). Paraffin-embedded sections of normal human gastric
corpus, jejunum, ileum, and colon (each n = 3) were immunostained by horseradish peroxidase-diaminobenzidine (HRP-DAB) (mouse primary Abs) or alkaline phosphatase (AP)-Fast red (rabbit primary Abs).

**Paper III**
Antroduodenojejunal motility recordings were performed in healthy subjects given intravenous (i.v.) bolus injection of saline (n = 8), 10 mg/kg L-N\textsuperscript{G}-monomethyl arginine (L-NMMA), or 1 mg atropine or 8 mg ondansetron followed by 10 mg/kg L-NMMA after 10 min (n = 6 in each group).

**Paper IV**
Sixteen healthy male test subjects, 18-55 years of age were studied. The subjects were screened for inclusion in the study by physical examination, BMI 20-25 and normal blood chemistry. On the day before and during the study all test subjects abstained from alcohol, smoking and caffeine. Organ bath experiments were performed with tissue from patients undergoing elective surgery for non-obstructive colorectal cancer. Smooth muscle specimens were obtained from the middle portion of the greater curvature of the gastric corpus of normal human stomach (n = 10), from the jejunum 70 cm distal to the pylorus (n = 15) in conjunction with gastric bypass surgery, and from the free resection margin in the jejunum 30 cm orally of the ileocecal valve (n = 24) and midportion of the transverse colon within 40 cm distal of the ileocecal valve (n = 24). Paraffin-embedded sections of normal human gastric corpus, jejunum, ileum, and colon (each n = 3) were immunostained by HRP-DAB (mouse primary Abs) or (AP)-Fast red (rabbit primary Abs).

**3.2 Animals**

**Paper II**
For studies of small intestinal myoelectric activity in conscious animals, 42 male Sprague-Dawley rats (300–350 g) were purchased from Scanbur (Sollentuna, Sweden). For studies of small and large intestinal motility and mucosal paracellular permeability under anesthesia, 54 male Sprague-Dawley rats (300–350 g) were obtained from Taconic (Ejby, Denmark).
4. METHODOLOGIES

4.1 Permeability assay

4.1.1 Preparation of bisboronic acid-appended viologens

Synthesis of 4,4’oBBV was reported by Camara et al. in 2003 (114). For 4,4’oMBV, 2-bromomethylphenyl boronic acid was reacted with excess 4,4’-bipyridyl in acetone to afford the mono-substituted 4,4’bipyridyl adduct (compound 2) (Fig. 3a). Combining excess compound 2 with benzyl bromide in a solvent mixture of MeCN and MeOH yielded 4,4’oMBV (compound 3) (Fig. 3a) after precipitation from the reaction mixture with acetone. Reagents and conditions were: (i) dimethylformamide, 55 °C, 48 hrs, 90% (compound 1); (ii) acetone, 25 °C, 2 hrs, 70% (compound 2); (iii) MeCN, MeOH, 55 °C, 24 hrs, 86% (compound 3). Chemicals were from Sigma Aldrich (St Louis MO, USA) unless stated otherwise.

![Figure 3a: Synthesis of 4,4’oBBV and 4,4’oMBV](image)

4.1.2. Mechanism of bisboronic acid-appended viologen sugar sensors in permeability

The molecular mechanism behind the BBV-based fluorescent lactulose assay is shown below (Fig. 3b). The sensing ensemble is comprised of an anionic fluorophore, 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) and a boronic acid-appended viologen (4,4’oBBV or 4,4’oMBV). HPTS forms a weak ground state complex with the cationic viologen sugar receptor, quenching its fluorescence. Ground state complex formation between the anionic fluorophore and cationic viologen sugar receptor facilitates an electron transfer from the fluoro-
phore to the viologen, decreasing fluorescence. At pH ~ 7.4, the cationic boronic acid viologen receptor has a high intrinsic affinity for cis-diols, which upon binding, partially neutralizes the charge of the viologen. This is caused by an equilibrium shift from the neutral boronic acid to the anionic boronate ester, lowering its affinity for HPTS, giving increased fluorescence.

Figure 3b: Mechanism behind the BBV-based fluorescent lactulose assay.

4.1.3 Sample collection

Human subjects consumed 0.5 L water the night before and in the morning ~2 hrs prior to urine sample collection. The first morning urine was voided. No food or other beverages were consumed prior to the test. The various permeability probes (i.e. lactulose, mannitol, sucralose, riboflavin) were ingested immediately after baseline urine collection. Doses were: 50 mg riboflavin, 5 g mannitol and 10 g lactulose. Test subjects were permitted to drink water or coffee as desired. Light snacks were permitted after the fourth hr. Urinary volume over a 6-hr period was generally 0.8-1.5 L. For all samples, urine volumes were documented, 50 mL was retained for analysis, although, once assays had been established, 1 mL proved to be plenty.

4.1.4 Riboflavin assay

For riboflavin, 100 µL urine sample or standard (prepared in baseline urine) was immediately diluted in 900 µL 100% EtOH, vortexed, centrifuged and fluorescence read from supernatant in duplicate (40
µL/well, Corning 3694 half area solid black plate) on a Tecan Infinite M200Pro plate reader using Exc/Em 450/580 nm. Baseline reading of urine at time of ingestion was defined as 0 concentration for riboflavin.

4.1.5 Bisboronic acid-appended viologen (4,4’oBBV) method for urine lactulose and mannitol

The bis-boronic acid-appended viologen (4,4’oBBV) was synthesized at the University of California, Santa Cruz (114). Regular 96-well plates (#3694 half area, solid black, Dow Corning, Midland, MI, USA) were prepared by adding 10 µL premix (4X premix buffer: 0.1 M sodium phosphate, 0.1 M 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 0.04% Triton X-100, pH 7.4; and HPTS 16 µM and quencher (1.6 mM 4,4’oBBV or 2.0 mM 4,4’oMBV)) in all wells except blanks. Plates were sealed with PCR plate tape and stored at 4 °C until use. Prior to start of experiments, tape was removed and 30 µL of standards or urine samples were added to wells. The sealing tape was replaced and the plates were put on a plate shaker for 1 hour at room temperature. Plates were then centrifuged at 2500 relative centrifugal force (RCF) for 5 min, plate tape removed and plate placed in a plate reader (Infinite M200 PRO, Tecan, gain 70). The reading height was adjusted to read from the top of the solution (18 mm). Fluorescence was read at 404/535 nm. A Marquardt 4-parameter curve-fit was used.

4.2 Procedure

4.2.1 Surgical procedure in rat

For studies on small bowel barrier function and motility, the surgical and experimental procedures have been described in detail previously (115, 116). Experiments on motility, permeability and water transport as well as bicarbonate secretion in anesthetized rats were started by anesthetizing the animal at 8 am with Inactin®, 120 mg·kg⁻¹ body weight given intraperitoneally. To minimize preoperative stress, anesthesia was performed by experienced personnel at the Animal Department, Biomedical Center, Uppsala, Sweden. In other studies on small intestinal myoelectrical activity in awake animals, implantation of electrodes was first performed in 30 rats un-
der anesthesia with a mixture of midazolam (5 mg·mL⁻¹, Aktavis AB, Stockholm, Sweden) and Hypnorm (fentanyl citrate, 0.315 mg·kg⁻¹ plus fluanisone 10 mg·kg⁻¹; Janssen-Cilag, Oxford, MA) given subcutaneously (s.c.) at a dose of 1.5-2.0 mL·kg⁻¹ body weight. Buprenorphine (0.05 mg·kg⁻¹, Schering-Plough, Stockholm, Sweden) was given s.c. after surgery to avoid post-operative pain. The animals were supplied with three bipolar insulated stainless steel electrodes (SS-5T; Clark Electromedical Instr., Reading, UK) in the wall of the small intestine, 5 (J1), 10 (J2) and 15 (J3) cm distal to the pylorus. All animals were supplied with an i.v. silastic catheter in the external jugular vein for administration of NPS. The electrodes were pierced through the abdominal muscle wall and together with the vein catheter tunneled to the back of the animal’s neck. After surgery, the animals were allowed to recover for 7 days before experiments were started. All animals were monitored daily.

4.2.2 Gastrointestinal motility in vivo in the rat

Duodenal barrier function and motility
In control experiments, duodenal segments were perfused with isotonic saline at a rate ~0.4 mL·min⁻¹, and the rates of duodenal paracellular permeability, duodenal bicarbonate secretion, motor activity, the net fluid-flux as well as the systemic arterial blood pressure and body temperature were recorded at 10-min intervals for about 150 min while for the colon segment experiments were performed for 60 min. In animal groups exposed to i.v. NPS; duodenal segments were challenged with NPS, the experiment protocol was almost same as the control experiment the only difference was, NPS was administered i.v. as bolus injections at 30 min (0.5 nmol·kg⁻¹) and 70 min (5 nmol·kg⁻¹) or in a separate experiment administered as a continuous infusion at 30, 70 and 110 min with a dose of 8, 83, and 833 pmol·kg⁻¹·min⁻¹, respectively. For colonic segments NPS was administered i.v. as a continuous infusion at 30 min with a dose of 833 pmol·kg⁻¹·min⁻¹. For L-NAME exposed control animal group: In control experiment, L-NAME was administered i.v. right after the experiment commenced as a bolus dose 3 mg·kg⁻¹ followed by a continuous infusion of 0.25 mg·mL⁻¹. Animals pretreated with L-NAME and NPS: In duodenal segment L-NAME was administered as same as control group and NPS was continuously administered i.v. at 30, 70 and 110 min with a dose of 8, 83 and 833 pmol·kg⁻¹·min⁻¹, respectively. Paracellular permeability of the duodenal epithelium was assessed by blood-to-lumen
clearance of $^{51}$Cr-EDTA. The clearance of $^{51}$Cr-EDTA from blood-to-lumen was calculated as described previously and is expressed as mL·min$^{-1}$·100 g$^{-1}$ (117).

4.2.3 Myoelectric recordings in the rat

Experiments were carried out in conscious animals after an overnight fast with free access to water. The rats were placed in Bollman cages during the experiments, and the electrodes were connected to electroencephalography preamplifiers (7P5B) operating a Grass Polygraph 7B (Grass Instr., Quincy, MA, USA). The characteristic feature of myoelectrical activity of the small intestine in the fasted state was measured. The MMC cycle length and propagation velocity were calculated. The MMC cycle length was measured at the J1 recording site while the propagation velocity was calculated between the J1 and J2 recording sites. In the control group, a continuous i.v. infusion of saline solution (NaCl 9 g·L$^{-1}$) was given using a microinjection pump (CMA 100; Carnegie Medicine, Stockholm, Sweden) and basal myoelectrical activity was recorded over a period of about 60 min. In NPS-exposed animals, an i.v. infusion of NPS (0.1, 0.3, 1, 2 or 4 nmol·kg$^{-1}$·min$^{-1}$; each dose n = 6) was continued for 60 min, after which the experiment continued until the basal MMC pattern was resumed (within a total experiment time of 6 hrs).

4.2.4 Gastrointestinal motility in vivo in man

Twenty-two healthy volunteers (13 males, 9 females, with a mean age of 27 years, range 22-38 years) were studied. The subjects were studied after an overnight fasting in a comfortable sitting position. A manometry eight-lumen polyethylene tube of 4.8 mm diameter (Cook, Copenhagen, Denmark) was introduced through an anesthetized nostril and passed into the upper jejunum under fluoroscopic guidance. The four aborad measuring points were placed in the horizontal duodenum and at the ligament of Treitz, respectively, spaced 100 mm apart between each measuring point. Water was perfused through the catheter at a constant rate of 0.1 mL·min$^{-1}$ by means of a pneumohydraulic pump (Arndorfer Medical Specialities Inc., Greendale, WI). Pressure changes were measured by applying a transducer (480-AME; Sensonor, Horten, Norway), and the signal was amplified with a PC polygraph (Synmed AB, Stockholm, Sweden).
Basal antroduodenoejunal motility was measured for 4 hrs. Bolus injection i.v. was introduced with either: saline (n = 8), 10 mg·kg⁻¹ L-NMMA (Clinalfa, Bachem GmbH, Weil am Rhein, Germany; n = 6), or 1 mg atropine (Atropin Mylan, Mylan AB, Stockholm, Sweden; n = 6) followed by 10 mg·kg⁻¹ L-NMMA after 10 min, or 8 mg ondansetron (n = 6, Zofran, GlaxoSmithKline, Brentford, UK; n = 6) followed by 10 mg·kg⁻¹ L-NMMA after 10 min. Post infusion, antroduodenoejunal motility was measured for next 4 hrs. Blood pressure was measured every 60 min throughout the experiment. Exhaled and rectal NO was measured as described elsewhere (118).

4.2.5 Gastrointestinal motility in vitro in man

Tissues were collected from patients undergoing surgery at Uppsala University Hospital, Uppsala, Sweden. Excised tissue segments were placed in ice-cold Krebs solution (in mmol L⁻¹: 121.5 NaCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 4.7 KCl, 1.2 MgSO₄, 25 NaHCO₃, 5.6 D-glucose, equilibrated with 5% CO₂ and 95% O₂) within 5-10 min after resection and immediately transported to the laboratory. The mucosa was removed and strips (2-3 mm wide, 12-14 mm long) were cut along the circular axis and soaked in freshly made, oxygenated cold Krebs solution. The strips (2 to 4 strips from each patient, Fig. 4) were mounted between two platinum ring electrodes in organ bath chambers (5 mL, PanLab, ADInstruments, Sydney, Australia) containing Krebs solution, bubbled continuously with 5% CO₂ and 95% O₂ and maintained at 37 °C and pH 7.4. Tension was measured using isometric force transducers (MLT0201, ADInstruments, PanLab, Barcelona, Spain). Data acquisition was performed using PowerLab hardware and LabChart 7 software (ADInstruments). Tissues were equilibrated to a 2 g tension baseline for at least 60 min during which time the bath medium was replaced every 15 min. After equilibration, muscle strips were stimulated with bethanechol 10 µM (Sigma-Aldrich, St. Louis, MO, USA) for 8 min to test tissue viability and as a control of the contractile response. This dose of bethanechol showed submaximal effects corresponding to the EC50 value on the tissue. The effects of NPS (1 nM-1 µM), GLP-1 (1 nM-100 nM) and ROSE-010 (Bachem, Bubendorf, Switzerland) were studied on bethanechol-precontracted tissue strips. To test the possible prejunctional effects of NPS and GLP-1, tissue contraction was evoked by electric field stimulation (EFS) using biphasic square wave pulses of 0.6 ms duration (10 Hz, 50 V, 0.6
train·min\(^{-1}\)) with a GRASS S88 stimulator (Grass Technologies, Astro-Med Inc., West Warwick, RI, USA). For this purpose, NPS, GLP-1 was added on continuous EFS to the colon preparations. The response to NPS was also tested in the presence of tetrodotoxin (TTX) (1 µM) (Sigma-Aldrich), a voltage-dependent Na\(^+\)-channel blocker; and L-NAME (1 µM), an inhibitor of NO synthase (NOS) (Sigma-Aldrich). The response to GLP-1 and ROSE-010 were also tested in presence of L-NMMA (100 µM), TTX (1 µM), exendin(9-39)amide (1 µM) and an adenylate cyclase inhibitor 2',5'-dideoxyadenosine (DDA; 10 µM). Neither TTX nor DDA showed any effect on baseline motility.

Figure 4: Human circular muscle strip in organ bath (A), typical response to beth 10^{-5} M (B) and EFS (10 Hz, 50 V, 0.6 train·min\(^{-1}\)) (C).

4.3 Protein Expression

4.3.1 Immunohistochemistry

Paraffin-embedded sections of normal human gut, including smooth muscle layer, were immunostained using HRP-DAB and mouse primary monoclonal clones 2F10 (GPRA-N) and 7C5 (GPRA-A) from Icosagen, Estonia (119). Immunohistochemical (IHC) analysis was done on samples collected from patients that underwent surgery. The tissues were embedded in paraffin and cut into 4 µM thick tissue sections on glass slides. Paraffin-embedded tissues first went through a deparaffinization step to remove paraffin, then an antigen retrieval step by heating the slides in citrate buffer at 500 W for 10 min in a microwave oven. Sections were incubated overnight at 4 °C with a primary antibody. The antibodies were NPS, NPSR1, eNOS, nNOS, iNOS, GLP-1R, GLP-2R and neu-
ron specific enolase. Primary Abs were mouse monoclonal clone 7C5 against NPSR1 (GPRA-A, COOH-terminal selective, antigen: CREQRSQDSRMTFRERTER from accession number Q6W5P4-1, the canonical isoform 1 sequence, 1:1,000) from Icosagen (Tartu, Estonia), rabbit polyclonal against NPS from Abcam (1:1,000, Cambridge, UK), and rabbit polyclonal against nNOS from Santa Cruz Biotechnology (1:400, NOS1, Dallas, TX). Neuron-specific staining with this nNOS primary Ab was confirmed using rabbit monoclonal primary Ab against neuron-specific enolase from Cell Signalling Technology (Beverly, MA) (1:1,000, clone D20H2). Double-staining was done by using HRP-DAB and AP-Fast red simultaneously on the same sections. Tissues were immunostained by alkaline phosphatase–Fast red method using rabbit polyclonal antibodies against nNOS and epithelial NOS (eNOS) (1:400, NOS1 and NOS3, 200 µg·mL⁻¹; Santa Cruz Biotechnology, Dallas, TX, USA) and inducible NOS (iNOS) (1:400, N-terminal selective, 500 µg·mL⁻¹; Abcam, Cambridge, UK). Human neuronal enolase primary Ab was a rabbit monoclonal kindly donated for validation by Cell Signalling Technology (Danvers, MA, USA). Tissue was immunostained by alkaline phosphatase-Fast red method using goat polyclonal and rabbit polyclonal antibodies against GLP-1 and GLP-2 from (GLP-1 dilution 1:50 and GLP-2 dilution 1:100 Santa Cruz Biotechnology, Dallas, TX, USA). Neuron-specific staining was confirmed using rabbit monoclonal primary antibody against neuron-specific enolase (1:1000, Cell Signaling, Danvers, MA, USA). As secondary antibody, biotinylated horse anti-mouse or biotinylated goat anti rabbit or biotinylated anti-rabbit was used.

4.3.2 Enzyme-linked immunosorbent assay

Pre-coated 96-well microtiter plates (Millipore, Billerica, MA) with rabbit anti-human NPS antibody were used. Prior to start of the experiment each well was washed 3 times with 300 µL of wash buffer 1:2 diluted matrix and assay buffer was added to relevant wells. A 50 µL NPS Standard, QC1, QC2 and unknown samples were added in duplicate to relevant wells. Then, 20 µL of detection antibody was added to all wells and incubated for 2 hours on an orbital microtiter plate shaker and washed 3 times with washing buffer. Unbound antibody was removed by washing 3 times with washing buffer. Pre-titered streptavidin-horseradish peroxidase conjugate specific for biotinylated goat anti-human NPS antibody was then added and incubated for 30 min at room temperature. Antibody binding was visualized by using
3,3′,5,5′-tetramethylbenzidine substrate. The reaction was stopped by addition of 0.3 M HCl, and absorbance was read at 450 nm using an automated microtiter plate reader (Infinite M200 PRO, Tecan, Männedorf, Switzerland). Standard curves were used to determine concentrations using Marquardt 4-parameter curve-fit.

4.3.3 Radioimmunoassay

Blood samples were drawn into cold ethylenediaminetetraacetic acid (EDTA) vacutainer tubes (10 mL). Samples were immediately centrifuged (1500 g, 4 °C, 10 min) and the supernatants were stored at -20 °C until analysis. Before RIA of GLP-1 and GLP-2, the plasma samples were extracted in a final concentration of 75% ethanol to remove unspecific cross-reacting substances. The RIA for determination of plasma concentration of GLP-1 was performed as previously described (120). The lower limit of detection (LLOD) was 7.8 pmol·L⁻¹ and the coefficient of variation (CV) 7%. The RIA for GLP-2 was done as described elsewhere (121). This assay had a detection limit of 5 pmol·L⁻¹ and CV of 5%.

5. Statistical analysis

Results are presented as mean ± standard error of mean (SEM) unless otherwise specified. The significance level was set at P <0.05.

Paper I

The LLOD and lower limit of quantification (LLOQ) were defined as the analyte concentration in the urine sample at which fluorescence intensity in the assay was 3 and 10 standard deviations above the mean baseline fluorescence, respectively.

Paper II

Paired t-test was used when comparing the MMC cycle length, phase III duration and velocity. Statistical difference of duodenal mucosal paracellular permeability and motility was tested by repeat measures ANOVA followed by Tukey post-hoc test to test differences within a group. A two-way repeated measure ANOVA was applied followed by a Bonferroni post-hoc test to test the difference between groups. Repeat measures ANOVA were used for comparing contractility changes to EFS-stimulated colon. The Prism software package 5.0 (GraphPad Software Inc., San Diego, CA, USA) was used for statisti-
cal comparisons, except SigmaPlot software used to analyze in vitro tissue experiment data.

**Paper III**
Paired t-test was used to compare MMC parameters (i.e. duration of phase I, phase III, propagation velocity, amplitude and contraction frequency during phase III) within the same group, and one-way ANOVA with Bonferroni’s multiple comparison test was used to analyze differences between groups. Kruskal-Wallis test with Dunn’s multiple comparison tests was used to evaluate differences in MMC, phase II duration and time to effect of L-NMMA. Changes in blood pressure were tested with paired t-test, while differences in NO production were evaluated with Wilcoxon signed rank test. All graphs and statistical tests were generated using Prism 5 (GraphPad Software Inc., La Jolla, CA, USA).

**Paper IV**
For in vivo recordings, statistical comparisons of motility index between 60 min basal, 30 min preprandial and 60 min prandial at each recording site were carried out employing the non-parametric Kruskal-Wallis test. Then, the motility response to food intake was compared between basal and prandial, as well as between prandial and the two doses of GLP-1 using the Kruskal-Wallis test. For in vitro recordings the student’s t-test was used to compare two groups for all the treatment groups in organ bath. One-way ANOVA was used to compare different doses for dose-response curves.
6. RESULTS

6.1 New fluorescence method for permeability (paper I)

The 4,4´oBBV sensor showed stronger de-quenching with increasing concentrations of both mannitol and lactulose sugar, compared to 4,4´oMBV. We therefore used 4,4´oBBV in gut permeability assays. Standard curves were used to determine the LLOD and LLOQ for lactulose and mannitol, as well as to compare to other sugars (Fig. 2a, paper I). Lactulose absorption was very low in healthy subjects, the lower LLOQ and LLOQ of 4,4´oBBV was also very low (90 µM and 364 µM). For 4,4´oMBV, the LLOD and LLOQ were 108 µM and 704 µM. Our results show that 4,4´oMBV is a less potent quencher than 4,4´oBBV. The temporal appearance of riboflavin and mannitol followed a similar pattern (Fig. 5, right panel). At 6 hours, the urine sample showed no mannitol or riboflavin residues, demonstrating that a 6-hour urine collection is an acceptable cut-off time for studies of small intestinal permeability. In urine of healthy human volunteers, the percent of ingested lactulose measured by using 4,4´oBBV was 0.56 ±0.25% and the lactulose/riboflavin ratio was 0.12 ±0.09, n = 10 volunteers. The enzyme assay, regarded as a gold standard for urine lactulose (135) yielded similar results: 0.76 ±0.21% and 0.10 ±0.03. Hence, the viologen assay is competitive with the gold standard enzyme assay.

6.2 Effects of NPS on motility and permeability (paper II)

NPS at low dose induced irregular myoelectrical spiking in rat. In conscious rat, i.v. administration of NPS increased the MMC cycle length and phase III duration in a dose-dependent manner (Table 1, paper II).

In vitro experiments with NPS (1 nM-1 µM) demonstrated relaxation in bethanechol-precontracted human small intestinal muscle strips in a dose-dependent manner (Fig. 9A, paper II). In small intestinal muscle strips, phasic contractions were modestly reduced by NPS. In colonic muscle strips, NPS (1-1000 nM) also inhibited bethanechol-induced contractions. This effect was, however, sporadic so dose-dependency could not be accurately quantified (n = 6). The inhibitory effects of NPS were abolished when tissues were pretreated with TTX (1 µM) (n = 6, Fig. 9C paper II). Furthermore, the inhibitory effect of NPS on
motility was abolished in human small intestine by pretreatment with L-NAME (1 µM) (n = 6, paper II). Submaximal EFS-induced contractions of colonic muscle strips were dampened to ~61 ±7% of by addition of 1 nM NPS (n = 6) (Fig. 9D, P <0.01, paper II). In vivo studies in the rat, infusion of NPS (0.5, 5.0 and 50 nmol·kg⁻¹·min⁻¹ i.v.) inhibited duodenal motility in a dose-dependent manner (Fig. 2, A and C, paper II). Similar to the in vitro organ bath experiments, L-NAME pretreatment significantly abolished the inhibition of duodenal motility induced by infusion of NPS at doses of 0.5, 5.0 and 50 nmol·kg⁻¹·min⁻¹, respectively (Fig. 3, A and B, paper II). The i.v. infusion of NPS also induced a dose-dependent inhibition of the net reduction of paracellular permeability as observed in control animals (Fig. 2, B and D, paper II).

6.3 Localization of neuropeptide S and its receptor, NPSR1 (paper II)

IHC was done with tissue from human gastric corpus, jejunum, ileum, and colon. All the tissues showed strong immunoreactivity to NPS and NPSR1 (Fig. 5, A and B, paper II). Immunoreactivity to NPS and NPSR1 were mainly observed at neuron within myenteric plexus. No immunoreactivity for NPSR1 was observed at smooth muscle cells. Double-staining of nNOS and NPSR1 verified that NPSR1 co-localizes with nNOS within myenteric neurons (Fig. 6A, paper II). Furthermore, double-staining of NPS and NPSR1 revealed that they reside in separate neurons, speaking in favour of a neurocrine function (Fig. 6B, paper II). Neuron-specific immunoreactivity was confirmed by enolase Ab as a positive control (data not shown). However, some cells stained differentially for either NPSR1 or nNOS.

6.4 Nitric oxide regulation of in vivo MMC in humans (paper III)

L-NMMA elicited a premature phase III of the MMC within 4.2 ±0.6 min in all groups except one subject in atropine group (Fig. 5). No significant changes were seen in phase III of the control group. A premature phase III was seen only in duodenojejunal segment with only 5 of 18 subjects shown an effect in both the antrum and duodeno-jejunal segments. L-NMMA markedly decreased the MMC cycle length from 114.0 ±16.8 min to 50.6 ±16.8 min (P = 0.014). After in-
jection, L-NMMA also shortened the subsequent MMC cycle length with a strong suppression of phase I. On the other hand, pre-treatment with a muscarinic receptor blocker, atropine prolonged the subsequent MMC cycle due to extended phase II while ondansetron, a serotonin receptor blocker attenuated L-NMMA effects on the MMC. The early onset of premature phase was due to blocking NO by L-NMMA. This was supported by measuring systemic arterial blood pressure and NO. L-NMMA caused an increase in systolic and diastolic blood pressure (Fig. 5A, paper III), respectively. Furthermore, involvement of NO was confirmed by measuring exhaled NO, where exhaled NO concentrations decreased in all subjects after L-NMMA administration (Fig. 5B, paper III).

![Figure 5: Direct effect of L-NMMA 10 mg·kg⁻¹ i.v. on induction of premature phase III. L-NMMA caused early onset of phase III in different treatment groups (n = 6–8). Results are given as median with 25–75 percentiles and range. *P = 0.031, **P = 0.002 and ****P = 0.0001.](image)

6.5 Localization of nitric oxide synthases nNOS, iNOS and eNOS

Immunohistochemical staining was studied with the tissue sections from gastric corpus (n = 3), jejunum (n = 3) and ileum (n = 3). Immunoreactivity to neuronal NOS (nNOS), iNOS and eNOS was observed in all tissue sections. Immunoreactivity to nNOS was confined to
nerve cell bodies and fibres of the myenteric plexus. Immunoreactivity to iNOS was also found in myenteric neurons, whereas eNOS immunoreactivity was only found in blood vessels. Neuron-specific enolase immunostaining (n = 3) was used as a positive control to confirm the location of myenteric neuron (Fig. 7a-c, paper III).

6.6 Involvement of nitric oxide in regulation of in vitro muscle contraction

In paper III, involvement of NO in GI motility was tested. Organ bath experiment with colonic smooth muscle strips showed a stable baseline tone with superimposed phasic contractions in control condition. Upon application of L-NMMA to the organ bath invariably caused a tonic contraction of the colonic muscle strips (n = 3) and enhanced bethanechol-induced tonic contractions (n = 6). Small intestine showed similar response to that of the colon (each n = 3). The contractile effect of L-NMMA was unchanged with TTX (n = 6). A likely explanation is that TTX blocked nitricergic neurons, thereby preventing L-NMMA to exert an effect (Fig. 8, paper III).

6.7 Effects of GLP-1 and ROSE-010 on intestinal motility

In vivo experiments, GLP-1 at 0.7 pmol·kg⁻¹·min⁻¹ reduced motor activity throughout the antrum, duodenum and jejunum. Higher concentration showed more pronounced effect on the contraction frequency (Fig. 1, paper IV). In in vitro experiments in organ bath, GLP-1 (1-100 nM) inhibited bethanechol-induced muscle contractions in both small intestine and colon in a dose-dependent manner (Fig. 3A, paper IV, n = 7; *P <0.05, **P <0.005). Like GLP-1, its analogue ROSE-010 (1 nM-1 µM) also reduced bethanechol-induced contractions in colonic tissues in a dose-dependent manner (Fig. 3B, paper IV, * P <0.05, ** P <0.005). The responses obtained at the maximal tested concentrations of GLP-1 (100 nM) and ROSE-010 (1 µM) corresponded to 60% reductions in the amplitude of bethanechol-induced contractions. This effect was completely reversible for all GLP-1 concentrations after washout with Krebs solution. To test the specificity of the effect, the muscle strips were pretreated with exendin(9–39)amide (1 µM), a GLP-1R antagonist. Exendin(9–39)amide markedly reduced the inhibitory effect induced by GLP-1 and ROSE-010 (Fig. 4, A and B, paper IV). To assess the involvement of cAMP in the GLP-1 response tis-
sues were pretreated with DDA. DDA blocked the inhibitory effect of GLP-1 and ROSE-010 (Fig. 4, C and D, paper IV). However, DDA did not affect the continuous phasic contractions significantly. Furthermore, TTX (1 µM) abolished the inhibitory effect of GLP-1 and ROSE-010 (Fig. 5, C and D, paper IV), indicating neuronal involvement of GLP-1 effects. GLP-1 and ROSE-010 effects were also blocked, when tissue pretreated with L-NMMA (100 µM), a blocker of NOS indicating NO dependent effects of GLP-1 (Fig. 5, A and B, paper IV).

6.8 Localization of GLP-1R and GLP-2R

To find out the localization of GLP-1R and GLP-2R IHC was done with tissue from human gastric corpus, jejunum, ileum, and colon. Strong immunoreactivity of GLP-1R and GLP-2R was observed at myenteric neurons (Fig. 2, paper IV). There was no immunoreactivity found in muscle cells. However, epithelial cells showed immunoreactivity to GLP-1R but little or no staining for GLP-2R.
7. GENERAL DISCUSSION

The most important function of the GI tract is digestion and absorption of ingested nutrients. The GI tract acts as a portal of entry to a vast array of foreign antigens in the form of food. The GI mucosal epithelial cells form a continuous lining that acts as a physical barrier between the exterior environment and the body of the host. The muscular barrier is a thin and permeable barrier to the interior of the body. The necessity for permeability of the surface lining at this site creates obvious vulnerability to tissue damage and infection and it is not surprising that the vast majority of infectious agents invade the human body through these routes. Most of the gut is heavily colonized by approximately $10^{-14}$ commensal microorganisms, which live in symbiosis with their host (122). These commensal bacteria provide protection against pathogenic bacteria by occupying the ecological niches for bacteria in the gut. However, defects in mucus production and barrier dysfunction that allow luminal contents to penetrate the underlying tissues and inappropriate response of a defective mucosal immune system to the indigenous flora and other luminal antigens are thought to be one of the reasons in the pathogenesis of IBD (123, 124). The GI tract forms the largest and most important physical barrier against the external environment. The mucosal barrier of the gut is a part of the innate immune system by which it protects our body from the invasion of harmful microbes and substances. GI motility propels the ingested food through the gut for digestion and absorption and excretes the remains and debris from the gut in due time. It restrains the body from bacterial overgrowth in the upper GI tract. The immune system has evolved mechanisms to avoid a vigorous immune response to food antigens on one hand and, on the other, to detect and kill pathogenic organisms gaining entry to the gut.

Elevated paracellular leakage has been implicated in many human disorders with immunological components, including type 1 diabetes mellitus (125), obesity and type 2 diabetes mellitus (126), IBD, celiac disease and IBS (127-129), Parkinson's disease (130), environmental enteropathy (131) and cancer (132). Therefore, gut permeability screening becomes an important parameter in assessing disease symptoms. Typically, two different HPLC configurations (e.g., ion-exchange and reverse-phase) are coupled to expensive detectors, such
as mass spectrometers (133). Alternatively, nicotinamide adenine di-
nucleotide (NADPH)-coupled enzyme assays are used (134, 135), but
require considerable time and cost. Much work has been done in using
organoboronic acids to quantify sugars (136, 137). Focus has been on
glucose-related measurements, and these have had success in clinics;
eight validated HbA1c assays employ organoboranes (138). However,
organoboronic acids are typically more sensitive to fructose moiety of
lactulose compare to galactose.

In this thesis, a new and rapid method has been developed for gut
permeability screening (paper I). NPS effects on motility, contractili-
ty, permeability and biomarker expression (paper II), the NOS inhibi-
tor L-NMMA effects on the MMC in relation to muscarinic and 5-HT₃
receptor blockade in man, (paper III), effects of GLP-1 in vivo prandi-
al motility response and the mechanism of action of GLP-1 and its
analogue ROSE-010 using in vitro human gut muscle strips (paper IV)
were investigated and found to be, at least partly, mediated through
NO.

**Paper I: Riboflavin and bisboronic acid-appended vi-
ologen-based lactulose detection method for gut permea-
bility**

In this paper, we showed that the 4,4´oBBV lactulose sensor com-
bined with riboflavin could be potential alternatives to the existing
methods for gut permeability. Due to its higher water solubility mak-
ing it easier to work with, it was hoped that 4.4´MBV would be as
sensitive and selective as equally well as 4.4´oBBV. However,
4.4´oMBV was found to be a weaker HPTS quencher with less lactu-
lose sensitivity than 4.4´oBBV. This could be due to lack of one bo-
ronic acid compared to 4.4´oBBV. This means that boronic acids fa-
cilitate HPTS quenching, but that facilitation is lost when sugars react
with boronic acid groups. Two boronic acids provide more facilitation
to quenching, and as a result, more capacity to de-quench in the pre-
sence of sugar. Sucrose and sucralose are often used in permeability
tests to assess permeability in the duodenum and colon, respectively.
Since neither sucrose nor sucralose interfere with the BBV-based
method, there are no obstacles to their inclusion in clinical studies.
Riboflavin is confined to uptake through the riboflavin transporter 2
(RFT2) and may more strongly correlate with the condition of the vil-
lus tips of duodenum and jejunum. Because RTF2 transport is down-
regulated in some GI diseases, riboflavin measurements should serve to identify those with RFT2 down-regulation. Riboflavin, reflecting transcellular absorptive capacity of the villi, thus can replace mannitol in gut permeability studies. Riboflavin’s fluorescence (~450/580 nm) suggests a methodological advantage over mannitol, while reflecting absorption of an actual nutrient.

The BBV method can be used to measure paracellular permeability or drug absorption experiment in caco-2 cells. We successfully demonstrated that BBV-based sugar sensors have a potential use in monitoring gut permeability (Fig. 6). In our lab, we used decanoic acid as a positive control to open TJs in caco-2 cells to allow permeability probes such as lactulose or mannitol added to an upper (luminal chamber) to pass more freely through the paracellular route in caco-2 cell monolayers into a lower basolateral chamber (unpublished). Samples from the basolateral side were assayed for lactulose or mannitol using the BBV-based method. Future developments of this method could bring about further improvements in sensors, such as lower detection limits and greater selectivity between sugars, as well as about portable permeability devices similar to glucose meters used by diabetics for plasma glucose tests. Improved sensors are possible because altering placement of boronic acid adducts changes their binding and selectivity (139). Creation of an electronic meter is possible due to the ability to couple BBV-sensing to electron transfer to obtain an electrogenic response.

Figure 6: (A) Permeability test using Millicell® culture plate (single well) and (B) Neuropeptide S caused increased permeability in caco-2 cells (unpublished data). Mannitol was used as permeability marker.
Paper II: NPS affects gut permeability and motility through cAMP-dependent nitric oxide-signalling pathway

The NPS/NPSR1 system previously has been associated with immunity and inflammation as well as the risk of developing IBD (140, 141). We sought to investigate whether challenge with NPS is able to induce permeability and motility effects. In paper II, we demonstrated that NPS reduced duodenal motility and increased duodenal mucosal paracellular leakage consistent with a role in the inflammatory reaction as seen in IBD patients where disturbed gut motility is also noted (142, 143). This data is consistent (144, 145) with studies where intestinal permeability is shown to be higher in IBD patients compared to healthy controls (146, 147). Polymorphisms in NPSR1 have been shown to be associated with IBD and colonic transit. Our data showed that NPS prolonged the MMC cycle length and phase III duration in upper small bowel, suggesting that NPS decreases motility. Decreased motility is seen in both CD (146) and UC (148). In contrast to this, Petrella and co-workers (149) found no effect of NPS on gastric emptying or GI transit. Our in vitro contractility study of NPS showed a clear dose-dependent relaxation of small bowel muscle strips, which is in agreement with the reduced amplitude seen in inflamed intestinal muscle both at rest, after EFS stimulation (150) and after stimulatory modulators (151-153). Central administration of NPS inhibits colonic transit (154), further suggesting a dampening motility effect of NPS. Moreover, the relaxatory response to NPS in our study differed between muscle strips from small intestine and colon where the most consistent effect was seen in the small intestine. This difference in effect could be due to the variable expression of the NPS/NPSR1 system throughout the GI tract, in which higher expression of NPS and NPSR1 is seen in the upper part as opposed to the lower part of the GI tract (155). TTX abolished the inhibitory effects induced by NPS, which suggests that neurons within the myenteric plexus are responsible for the action of this peptide in human small intestine and colonic circular smooth muscle strips. L-NAME was also used to see whether the NPS effects are mediated by NO. The fact that the inhibitory effect of NPS was also abolished in the presence of L-NAME, a blocker of NO synthesis, suggests that NPS is plausibly involved in the activation of NOS generating NO, a molecule that is widely known to inhibit GI motility (156, 157). It is commonly accepted that NPS raises cAMP (155). This inhibitory effect of NPS on motility could be due to
cAMP-induced NO production in a smooth muscle preparation (156). Our IHC data showed that NPSR1 was expressed in the myenteric plexus. Recently, it has been shown that NO is responsible for neuronally-induced relaxations which depend on activation of both eNOS and nNOS (158). We propose that the relaxatory effect of NPS on small intestine muscle is dependent on cAMP to induce NO production by eNOS and/or nNOS. This could be further examined by measuring NO production or nitrate in NPS-treated smooth muscle preparations. We also found increased expression of the inflammatory biomarkers IL-1β and CXCL1 in the infusion part of paper II, showing that an inflammatory response was evoked to exogenously applied NPS. This increased expression of inflammatory markers suggests that NPS may stimulate neutrophil infiltration into the upper GI tract, since CXCL1 is a strong neutrophil attractant (159). These inflammatory cells are known to produce both iNOS and IL-1β (160, 161). Furthermore, it has been shown that activated neutrophils can reduce the contractile response of colonic circular muscle (162), indicating that the inflammatory response to NPS can also be involved in the changed motility patters. An IL-1β-induced increase in intestinal TJ permeability has been postulated to play an important role in promoting intestinal inflammation by allowing increased paracellular permeation of luminal antigens (163). This increased permeability is due to increased expression of myosin light-chain kinase (MLCK). In paper II, we showed that NPS increases IL-1β expression in duodenal tissue. Whether this IL-1β expression was involved in NPS induced increased permeability is unknown. MLCK expression and activity are increased in intestinal epithelial cells of patients with IBD. The degree to which MLCK expression and activity are increased correlates with local disease activity, suggesting that these processes may be regulated by local cytokine signalling in these patients (164). Considering all the evidence, we therefore propose two models for motility and permeability, each initiated by cAMP production following NPS receptor activation; first, NPS dampens GI motility through a NO-signalling pathway, and second, increased paracellular permeability through activating MLCK signalling pathway.

Paper III: Involvement of NO in regulation of gut motility
In paper II, we showed involvement of NO in the regulation of gut motility. NO seems to play a role in the control of the GI interdigestive motility (165). In paper III, we showed that L-NMMA induces a
premature phase III and reduces the cycle length of the subsequent MMC, specifically in phase I. The organ bath data are in agreement with this and showed a direct effect of L-NMMA on smooth muscle contractility. L-NMMA increased the amplitude of bethanechol-induced contractions. Earlier publications showed that i.v. infusions of NOS inhibitors have similar effects in dogs, rats, sheep and chickens (166). The L-NMMA effect on NO production was verified by the changes seen not only in blood pressure but also in breath and rectal NO gas concentrations.

Cholinergic and serotonergic receptor antagonists were used to explore the effect of L-NMMA. Atropine or ondansetron did not affect the induction of phase III when given prior to L-NMMA, showing the involvement of NO per se in the initiation of the activity front. Although Tack et al. reported that possibly also 5-HT_1 receptors are involved in the initiation of phase III of the MMC in man (165), this was not seen in our study (paper III). Transition of the MMC from phase I to phase II on the other hand seems to be under a balanced control between inhibitory nitrergic and excitatory cholinergic and serotonergic pathways. This concept is confirmed by the finding that MKC-733, a 5-HT_3 receptor agonist, reduces the duration of phase I of the MMC (167). In our study, we did not find any changes in plasma concentrations of motilin, somatostatin or ghrelin after injection of L-NMMA. Significantly higher plasma somatostatin has previously been shown during phases III and II than during phase I of the MMC (168). Taken together, the data suggest that NO has direct effects on the regulatory functions of the MMC motility pattern (169).

**Paper IV: GLP-1 and ROSE-010 induced muscle relaxation is cAMP- and NO-dependent**

The GLP-1 agonist ROSE-010 alleviates pain in IBS (170). Novel approaches to stimulate GLP-1R, such as with ROSE-010, are in development to treat functional GI disorders, which arise from abnormalities in gut motility and sensational functions, including the brain-gut axis, or immune activation. In this study, we demonstrated that infused GLP-1 at near physiological concentrations inhibited motor activity in the antrum, duodenum and jejunum after food intake. The contraction frequency and motility index displayed the most reliable indication of the motility response to GLP-1. The results of the study are consistent with previous work in the rat where GLP-1 has been
shown to inhibit motility (171, 172). The present results align with those of others studying different animal species (173-175) and man (176, 177) in whom also the metabolic impact of an inhibited antroduodenal motility was shown (178). Although the plasma concentrations changed at pace with dosing, there was no consistent and corresponding GLP-1 dose-response relationship for \textit{in vivo} motility across the different intestinal segments. However, dose-response relationships of GLP-1 and ROSE-010 were observed in organ bath experiments on circular smooth muscle. The GLP-1 doses used for \textit{in vitro} studies were somewhat high relative to \textit{kd}, ~28 pM (179) which may be due to denervation of the tissue \textit{in vitro}. The inhibitory effect of ROSE-010 is consistent with a previous study where ROSE-010 was shown to delay gastric emptying in IBS-C (180). The inhibitory effects of GLP-1 and ROSE-010 were blocked by exendin(9-39)amide, an antagonist of GLP-1 receptor, indicating a specific receptor-mediated effect of GLP-1. TTX, which inhibits nerve impulses by binding to voltage-gated sodium channels in nerve cell membranes, blocked both GLP-1 and ROSE-010 inhibition indicating a nerve-mediated transmission of the GLP-1 response. Furthermore, L-NMMA blocked the inhibitory effect of GLP-1 and ROSE-010, indicating that GLP-1 inhibition relies on nitrergic neurons. A NO-dependent inhibitory effect of GLP-1 has been shown in other species (181-184). NO may therefore widely be regarded as an important component in the regulation of GI motility (185, 186). This suggests that prejunctional receptors of GLP-1 are expressed in the myenteric plexus. Immunohistochemistry data in study IV revealed presence of GLP-1R in myenteric plexus, indicating the motility control of GLP-1 to take place at pre-junctional sites. In study II & III, we showed presence of nNOS in myenteric neuron. The GLP-1R is coupled to the Gsα subunit, hence agonist binding with the receptor results in activation of adenylate cyclase with consequent production of cAMP (187). Moreover, it is well accepted that cAMP is the main mediator of GLP-1R agonism in beta cells (188). Our further experimentation with DDA confirms the cAMP-medicated effects of GLP-1 and ROSE-010. It has also been demonstrated that cAMP signalling can increase NO production (189, 190). Results of paper IV lead to a mechanistic model in which GLP-1 and ROSE-010 inhibit motility through GLP-1Rs at myenteric neurons, which then act on smooth muscles through both NO and cAMP production.
8. CONCLUSIONS

The use of 4,4´oBBV to quantify lactulose combined with riboflavin in urine, proved to be a rapid and low-cost means to quantify small intestinal permeability. Future developments of organoborane chemistry for this application are ongoing (139). The BBV-based method has potential for future development of sugar sensors that can be used to test gut permeability similar to glucose sensors for diabetes. The BBV method can be used for large scale permeability screening in farm animals as well as humans. Further developments of this method could bring very rapid and easy methods that can be used in industrial applications such as large scale drug absorption studies in caco-2 cells.

NPS is able to inhibit motility in the small intestine and colon, likely through neuronal NO release. NPS also causes an increase in paracellular permeability, making possible a role in the early stages of inflammation. Furthermore, NPS involvement in permeability opens a new door for research regarding gut barrier dysfunction involvement in neuropeptide-induced inflammation and motility disorders in several diseases (neurodegenerative disease, and inflammatory diseases). NPSR1 has potential as a drug target for inflammatory and dysmotility disorders in IBD and IBS.

NO inhibits the MMC by suppressing phase III activity independently of muscarinic and 5-HT3 receptor blockade, and independently of ghrelin or somatostatin. Phase I of the MMC seems to have a neuronal nitrergic component, suggested to be counter-regulated by cholinergic and serotonergic mechanisms. Phase II of the MMC is dependent on atropine-sensitive mechanisms. Amplification of bethanecol-induced contractions in vitro by L-NMMA in an organ bath resistant to TTX further supports a neuronally-dependent effect of NO on GI smooth muscle. NO acts as regulatory inhibitor throughout the MMC, predominantly suppressing the induction of phase III activity and extending phase I duration. NOS inhibitors therefore, have potential use for dysmotility disorder in IBD and IBS.

The inhibitory action of GLP-1 on intestinal motility through receptor-mediated mechanisms can be extended to its analogue ROSE-010. The GLP-1 receptor located on smooth muscle cells mediates action through cAMP signalling transduction, involving a nitric oxide-dependent step. Since it alleviates IBS disease symptoms, GLP-1 and
ROSE-010 could potentially be used to treat IBS. NO is also involved in GLP-1 induced motility effects, demonstrating the diverse roles of NO. Since GLP-1 is the endogenous ligand for GLP-1R, it binds strongly to its receptor compared to ROSE-010. However, due to instability of GLP-1, the stability of ROSE-010 should be advantageous for IBS treatment. It also has advantages over other drugs since it reduces pain (170). ROSE-010 has multiple effects in different diseases. This thesis contributed to the field of gastroenterology by addressing several important questions. The first contribution was development of new methods for gut permeability test. This thesis also opened a new door for the research and application of 4,4´oBBV in large scale drug absorption testing. This thesis also showed involvement of NO in regulation of gut motility and permeability by different gut peptides in this case NPS and GLP-1.
9. ACKNOWLEDGEMENTS

This work was carried out at the Department of Medical Sciences, Gastroenterology and Hepatology, Uppsala University, Uppsala, Sweden. Financial support was provided by the Swedish Research Council and ALF.

I would like to express my sincere gratitude to all the people that have contributed to this work and that have helped me through the years. Associate Prof Dominic-Luc Webb, my main supervisor, thanks for sharing research related knowledge in clinics. The questions that you proposed during preparation for my public defense were really helpful. You are an excellent supervisor. Your scientific knowledge always amazed me a lot. Your way of thinking and depthless of knowledge inspired to walk on the difficult field like research.

Professor Per Hellström, my co-supervisor, thank you for spending countless time and patience, delivering vast knowledge and helpful information during my studies. You led me to the finishing line of this long doctoral research journey. I enjoy the style to work with you in an efficient manner, and admire the spirit that you enjoy helping patients in your endless medical career.

Associate Prof Ahmad Al-Saffar, Thank you very much for your suggestions during my research. Your scientific suggestion in running organ bath helped me a lot in saving time and doing successful experiment. Your comment during our lab meeting always helped me to learn something new.

Dr. Linda Ward, thank you very much. You were an excellent senior PhD to work with. Your scientific collaboration helped me a lot in successfully finishing my PhD. Your nice personality always amazed me.

Dr. Hetzel Diaz, DVM. Anas Al-Saffar. Thank you very much for your time and company during my whole research period. The discussion during lunch always gave me extra mental energy to work properly. We tried to learn few sentences from each other languages that were really nice time. Hetzel your smiling face always kept our lab smiling.

Associate professor Markus Sjöblom and Dr Wan Salman Wan Saudi, Thank you very much for your nice collaboration in study II.
All the graduate students I can remember names of, Carly Scott and Luwam Zewenghiel were really nice students to work with. The fun we had during your project was great. Still I remember both of you as King and Queen. Marcia Steele helped us to start working with urine samples. Caroline Landhage who has collaboration in one of my papers, Gustav Hall, Carl Hampus Meijer, Peter Zarelius and Tim Hagelby Edström you guys were really great, a nice part of team during my PhD time. And Venkata Ram Gannavarapu thank you very much for your help in collecting tissue samples and preparing buffer. All other past and present researcher fellows, students and staff in the Clinical Research Sections 2 and 3, I could not remember names of so many of you in this limited context. I am delighted to work with you under the same “roof”. Thanks for the interesting conversations and the discussions at lunch and party.

Research at Södersjukhuset Mohammad Eweida, I don’t have much word to give you thanks. You are really a nice person. You always encouraged me to walk forward. Your priceless help and suggestion made my research time easy going.

Associate prof Md Shahidul Islam, you led me to the research world in Sweden many years ago before my doctoral studies, during my hard time you used to tell me don’t give up, keep trying and I still trying my best to walk forward. We still have contact now and then. My achievement now is based on what I learned from you.

My tutors, relatives and friends in Bangladesh, I am grateful that you never forget me. I miss you all. Still I remember my school life at Saidpur Govt. Technical School. Whenever I look back to school life I get lot of confidence.

Last but not least, I would like to thank my parents and family for supporting me and understanding me so many years. Special thanks to my newly married wife, your support was so precious to me. Thank you for the company and the love.
10. REFERENCES

14. Podolsky DK. Mucosal immunity and inflammation. V. Innate mechanisms of mucosal defense and repair: the best offense is
70. Subramanian VS, Subramanya SB, Rapp L, et al., Differential expression of human riboflavin transporters -1, -2, and -3 in


105. Ørskov C, Wettergren A, Holst JJ. Secretion of the incretin hormones glucagon-like peptide-1 and gastric inhibitory poly-


183. Andrews CN, Bharucha AE, Camilleri M et al. Nitrergic contribution to gastric relaxation induced by glucagon-like pep-


