LUND UNIVERSITY

Neuroimmune Interactions of Enteric Neurons and Mast Cells: Friends or Foes?

Sand, Elin

2010

Link to publication

Citation for published version (APA):
Sand, E. (2010). *Neuroimmune Interactions of Enteric Neurons and Mast Cells: Friends or Foes?* [Doctoral Thesis (compilation)]. Lund University: Faculty of Medicine.

Total number of authors:
1

General rights
Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Download date: 12. Oct. 2024

Neuroimmune Interactions of Enteric Neurons and Mast Cells: Friends or Foes?

Elin Sand

Neurogastroenterology Unit, Department of
Experimental Medical Science
Lund University

LUND UNIVERSITY
Faculty of Medicine

With approval of the Lund University Faculty of Medicine, this thesis will be defended on Friday the 23th of April 2010 at 9.00 am in Segerfalksalen, BMC, Sölvegatan 19, Lund

Supervisor: Professor **Eva Ekblad**

Faculty opponent: Professor **Per Hellström**, Department of Gastroenterology, Uppsala University Hospital, Uppsala University, Sweden

Cover page: Background image; co-culture of rat ileum myenteric neurons and mast cells. Upper right; mast cell in culture. Lower left; myenteric ganglion in rat large intestine.

Supervisor: Professor Eva Ekblad
Neurogastroenterology Unit
Department of Experimental Medical Science
Lund University, Sweden

ISSN 1652-8220
ISBN 978-91-86443-49-8
Lund University, Faculty of Medicine Doctoral Dissertation Series 2010:34

Printed by Media-Tryck, Lund, Sweden

©Elin Sand, 2010

”Vad du än gör, skaffa dig en utbildning!”

Lena Kristensson

”Till min stora familj”

Table of content

Abstract **7**

Abbreviations **8**

List of papers **9**

Short summary **10**

Introduction **12**

- **Psychological distress and the GI-tract** 12
- **Physical strain and the GI-tract** 13
- **Intestinal ischemia** 14
- **Enteric nervous system** 15
  - *Development* 15
  - *Organisation and function* 16
  - *Cellular morphology and electrophysiological properties* 17
  - *Neurotransmitters* 17
- **Enteric glia** 19
- **Mast cells** 19

Aims of this thesis **21**

Methodology **22**

- **Primary cell culture** 22
- **Immunocyto- and histochemistry** 23
- **Ischemia followed by reperfusion** 24

Experimental design and results **25**

- **Paper I** 25
- **Paper II** 26
- **Paper III** 27
- **Paper IV** 28

Discussion **30**

Conclusions **34**

Populärvetenskaplig sammanfattning **35**

Acknowledgements **37**

References **39**

Appendix (Papers I - IV)

Abstract

Psychological distress or physical strain lead to reduced blood flow in the intestine since other organs are prioritised. One aim of this thesis was to investigate how ischemia followed by reperfusion affects the large intestine and the enteric nervous system (ENS). To do so an experimental ischemia/reperfusion (I/R) model was set up using rat large intestine. In order to study how the ENS reacts to various mediators of stress, primary cultures of myenteric neurons from rat small and large intestine were used to study neuronal survival. The intestinal segments exposed to I/R were structurally well preserved, however, local areas containing numerous mast cells were detected in the muscle layers, the serosa and in and around the myenteric ganglia 4-20 weeks post ischemia. Myenteric ganglionic formations within such mast cell rich areas virtually lacked neurons. Myenteric neurons co-cultured with mast cells showed a markedly reduced neuronal survival. The increased neuronal cell death was largely due to mast cell degranulation. Identified mast cell mediators involved were proteinases acting via proteinase activated receptor 2 (PAR_2), prostaglandin D_2 (PGD_2) and interleukin 6 (IL-6). Immunocytochemical examination of rat small and large intestine, revealed frequent co-localization of corticotropin releasing peptide (CRF), known to induce psychological stress reactions in mammals, and vasoactive intestinal peptide (VIP) in enteric neurons. CRF did not affect the survival of myenteric neurons in culture, but was found to counteract the VIP-induced neuroprotective effect. We also showed that the mast cell-induced increase in cell death of cultured myenteric neurons was not executed via CRF signaling pathways. In conclusion: I/R in rat large intestine attracted mast cells to invade the muscle layers and myenteric ganglia. In mast cell-infiltrated areas, ganglionic formations lacked myenteric neurons. Mast cells reduced neuronal survival when cultured together with myenteric neurons from rat small intestine. The mechanisms behind is through PAR_2 activation and release of PGD_2 and IL-6. Presence of CRF counteracted VIP-induced neuroprotection in cultured myenteric neurons.

Abbreviations

Ach	acetylcholine
ACTH	adrenocorticotropic hormone
ATP	adenosine triphosphate
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CRF	corticotropin releasing peptide
$CRF\text{-}R_1$	corticotropin releasing factor receptor 1
$CRF\text{-}R_2$	corticotropin releasing factor receptor 2
CTMC	connective tissue mast cell
ENS	enteric nervous system
EPSP	excitatory postsynaptic potentials
FITC	fluorescein isothiocyanate
GI	gastrointestinal
IBD	inflammatory bowl disease
IBS	irritable bowl syndrome
ICC	interstitial cells of Cajal
IR	immunoreactive
I/R	ischemia/reperfusion
IL1-β	interleukin 1 beta
IL-6	interleukin 6
IPSP	inhibitory postsynaptic potentials
L-NAME	N-nitro-L-arginine-methyl ester
MMC	mucosal mast cell
MPC-1	monocyte chemoattractor protein 1
NGF	nerve growth factor
NO	nitric oxide
NOS	nitric oxide synthase
NPY	neuropeptide Y
PAR_2	proteinase activated receptor 2
PGD_2	prostaglandin D_2
SCF	stem cell factor
SNAP	S-nitroso-N-acethyl-D, L-penicillamine
SP	substance P
TNFα	tumor necrosis factor alfa
VIP	vasoactive intestinal peptide

List of papers

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Kristensson E, Themner-Persson A, Ekblad E. Survival and neurotransmitter plasticity in cultured rat colonic myenteric neurons. Regulatory Peptides 2007;140:109-116
II. Sand E, Themner-Persson A, Ekblad E. Infiltration of mast cells in rat colon is a consequence of ischemia/reperfusion. Digestive Disease and Science 2008;53:3158-3169
III. Sand E, Themner-Persson A, Ekblad E. Mast cells reduce survival of myenteric neurons in culture. Neuropharmacology 2009;56:522-530
IV. Sand E, Themner-Persson A, Ekblad E. Corticotropin releasing factor – distribution in rat intestine and role in neuroprotection. *In manuscript*

Published papers are reprinted with permission from respective publishers.

Short summary

The aim of this thesis was to study how different stressors affect the enteric nervous system (ENS).

Paper I
Aims: To quantify myenteric neuronal subpopulations expressing calcitonin gene-related peptide (CGRP), galanin, neuropeptide Y (NPY), somatostatin, vasoactive intestinal peptide (VIP) and nitric oxide synthase (NOS) in rat colon *in vivo* and after culturing. Also if culturing in presence of CGRP, galanin, VIP, S-nitroso-N-acetyl-D,L-penicillamine (SNAP, a NO-donor) or N-nitro-L-arginine methyl ester (L-NAME, a NOS-inhibitor) affect neuronal survival.
Methods: Myenteric neurons were dissociated and cultured for 4 days in culture media with or without neuroactive compounds. Intestinal whole wall specimens and neuronal cultures were processed for immunocytochemistry and the distribution, neurotransmitter expression, neuronal plasticity and survival were examined.
Results: After 4 days of culturing the proportions of neurons expressing CGRP, NPY, somatostatin or VIP increased as compared to *in vivo*. Neuronal survival was unaffected by any of the neuroactive compounds.
Conclusion: Cultured rat colonic myenteric neurons increase their expression of CGRP, NPY, somatostatin and VIP, suggesting that these neuropeptides may be of importance for neuronal survival.

Paper II
Aims: To investigate if ischemia followed by reperfusion in rat large intestine affects the intestinal morphology, the presence and survival of enteric neurons, ICC and immune cells.
Methods: Rat colon was subjected to ischemia and reperfused for 1 day up to 20 weeks; sham operated rats were used as controls. Cell counting of neurons and mast cells and morphometry were performed on frozen or paraffin embedded tissues processed for immunocyto- and histochemistry.
Results: No structural remodelling of the intestinal segment was detected after ischemia reperfusion (I/R). Local areas containing numerous mast cells were detected in the muscle layers, the serosa and in and around the myenteric ganglia 4-20 weeks post ischemia. Myenteric ganglionic formations within mast cell rich areas virtually lacked neurons.

Conclusion: I/R in rat colon attracts mast cells and death of myenteric neurons occurs in such locations.

Paper III
Aim: To study if rat mast cells, isolated by peritoneal lavage or selected mast cell mediators affect neuronal survival when cultured with myenteric neurons in primary culture from rat small intestine.
Methods: Myenteric neurons were dissociated and cultured for 4 days before addition of mast cells, various mast cell mediators, protease inhibitors, proteinase-activated receptor 2 (PAR_2) agonist/antagonist, vasoactive intestinal peptide (VIP) or corticosteroid. At day 6 (4+2 days *in vitro*) neuronal survival were studied by immunocytochemistry and neuronal cell counting.
Results: Mast cells induced neuronal cell death via degranulation, PAR_2 activation, prostaglandin D_2 (PGD_2) and/or interleukin 6 (IL-6) release. Corticosteroid and VIP were neuroprotective and able to prevent death of myenteric neurons in co-culture with mast cells.
Conclusion: Mast cells induce cell death when co-cultured with myenteric neurons.

Paper IV
Aims: To reveal the distribution of corticotropin releasing factor (CRF) immunoreactive (IR) neurons and possible co-localisation with VIP in rat small and large intestine. To study a possible interplay between CRF, VIP and/or mast cells with myenteric neurons from rat small intestine in culture.
Methods: Myenteric neurons were dissociated and cultured for 4 days before addition of CRF, urocortin 1, CRF-antagonist, VIP or mast cells. At day 6 (4+2 days *in vitro*) neuronal survival was studied by immunocytochemistry and neuronal cell counting. Cellular distribution of CRF and VIP and possible co-localisation were studied by immunocytochemistry.
Results: Addition of CRF did not change neuronal survival while VIP increased neuronal survival. Mast cells induced a reduced neuronal survival, however, not executed via CRF signalling pathways.
Conclusion: CRF and VIP co-exist to a high degree in rat small and large intestine and CRF counteracts the neuroprotective effect of VIP in cultured myenteric neurons.

Introduction

Psychological distress activates the sympathetic nervous system which directs the blood to the brain and limbs. Physical strain, like exercising, also directs the blood to the limbs to give the muscles the full capacity to work. What happens with the gastrointestinal-tract (GI-tract) when blood is shunted to the rest of the body has not yet been thoroughly clarified. Psychological distress or physical strain have probably both short and long term effects on GI-function. The enteric nervous system (ENS), aligning the entire GI-tract, controls most of the secretory and motor functions in the GI-tract. How does reduced blood flow influence the ENS? We have defined studies attempting to answer key questions that will help us understand the effect of different stressors, e.g. reduced blood flow, on enteric neurons. In the following I will give a brief background to my thesis.

Psychological distress and the GI-tract

Psychological distress has long been suspected to affect the physiology of the human body. It can cause physical discomfort like chest pain, sweating, nausea or abdominal pain. Acute life-threatening events, evoke adaptive responses that serve to defend the stability of the internal environment and to ensure survival of the organism. It increases the activity of sympathetic nervous system and reduces the activity of the parasympathetic nervous system. The net effect is an increase of heart rate and vascular resistance which in turn leads to increased blood pressure. Blood flow is concentrated to the coronary bed of the heart, the brain, the skeletal muscle and blood flow is reduced to organs not necessary in a short aspect of life, like for example the kidneys and the GI-tract (Guyton and Hall, 1996).

Another direct response to psychological distress is the release of corticotropin releasing factor (CRF) from the hypothalamus, stimulating the pituitary to secrete the hormone adrenocorticotropin (ACTH). ACTH is transported via the blood to the adrenal glands, where it stimulates secretion of cortisol, which increases catabolic reactions in energy-supplying organs.

These responses are essential and considered one of the reasons of successful survival during evolution (Guyton and Hall, 1996).

Since distress reduces the activity of the parasympathetic nervous system it impairs visceral functions. Clear evidence of responses in gastric and colonic activity due to emotional distress was reported already in the 1950th (Almy et al., 1949; Cannon et al., 1953). Subsequent studies have also established that emotional distress delays emptying of the stomach and suppresses hunger while it increases colonic motility. The autonomic nervous system provides a peripheral neuronal network by which the effects of central distress can be rapidly imposed on gut function. The effects are mediated through the sympathetic, vagal and pelvic parasympathetic innervations of the ENS. CRF and CRF receptors 1 and 2 (CRF-R_1 and CRF-R_2) were recently found to be widely expressed in peripheral tissues, including the ENS lining the GI-tract. This strongly supports the idea of a local action of CRF influencing GI-function (for review Taché and Bonaz, 2007).

In 2007 a case-report of acute stress-related GI-ischemia was published (Veenstra R et al., 2007). This case report linked a psychological distressing event with increased catecholamine release and subsequent severe symptomatic GI-ischemia. By treating the patient with a vasoactive drug normally used for hypertension, abdominal symptoms were greatly improved. This finding suggests that not only extreme physical activity but also psychological stress can create ischemia causing significant harm to the intestine. GI-ischemia is in acute health care often reported due to thrombosis but also as the result of abdominal surgery, transplantation or septic shock. Intestinal ischemia in these cases is often very severe and may have deadly outcome for the patients (for review Mallick et al., 2004).

Physical strain and the GI-tract

Intense physical strain, like for example extreme physical activity, can reduce intestinal blood flow to 20%. This leads to an ischemia which may be critical for the GI-functions. Most athletes have sometimes had troubling GI-symptoms like abdominal pain, nausea and diarrhoea (Sällstedt and Hellström, 2008). Reduced intestinal blood flow affects motility, secretion and absorption. The reduced blood flow also leads to local release of different GI-hormones and neuropeptides. Vasoactive intestinal peptide

(VIP), which stimulates secretion, is one of them and is suggested to induce the diarrhoea often seen in athletes, especially after long distant run (Sällstedt and Hellström, 2008).

Intestinal ischemia

What happens with the intestine when blood is no longer available or seriously reduced? Lack of oxygen creates damage to metabolic active tissues which starts a series of events like production of oxygen free radicals, release of iron storage, damage to the microvasculature, release of inflammatory cytokines and activation of complement factors. This causes damage to the ischemic site and injury is further enhanced when and if the tissue is reperfused. Reperfusion collects and transports harmful mediators produced and brings them to other organs, this is the reason why ischemia sometimes causes distant organic failure (Haglund and Bergqvist, 1999; for review Mallick et al., 2004).

Ischemia has also been reported in the intestine of patients with inflammatory bowel disease (IBD). These patients posses a lifelong disorder, characterized by chronic inflammation in their GI-tract. Data demonstrating increased perfusion of the mesenteric vasculature have been described in these patients but others has pointed at diminished vascular perfusion of the mucosal surfaces and the arterioles in and below the mucosa (for review Hatoum et al., 2005).

Another group of patients that has got a lot of attention in recent years are patients with irritable bowel syndrome (IBS). The attention is accurate since IBS is one of the most common GI-disorders seen in primary care. IBS is a chronic disorder highly associated with abdominal pain, increased visceral hypersensitivity and diarrhoea and/or constipation. Psychological distress, food hypersensitivity and GI-infections have been suggested to play an important role in the development of IBS, but the cause of the disease is still unsettled (for review Santos et al., 2005).

A low-grade mucosal inflammation has, however, been observed in subgroups of IBS patients. Increased numbers of mast cells (immune cells) have been detected in the mucosa. Both morphological and functional evidence of a cross-talk between enteric nerve fiber and mucosal mast cells (MMC's) in these patients have been provided (Barbara et al., 2006; Santos

et al., 2006; for review Nassauw et al., 2007). IBS patients have also been reported to have increased content of MMC products in both the small and large intestine. Although the pathogenesis of IBS patients is poorly understood, mast cells are being considered as therapeutic targets for IBS and other functional GI-disorder with similar symptoms (Barbara et al., 2006; Santos et al., 2006; for review Nassauw et al., 2007).

Frequent or excessive reduction of blood flow to the intestines, whether caused by distress or physical strain, may increase the permeability of the mucosa. The mucosa works as an adaptive barrier function and, modulated by stressors, might decrease its ability to resist bacterial translocation (Wallon and Söderholm, 2009). Interestingly, among patients with IBS, the incidence of colon ischemia is substantially more frequent than in the general population and may constitute a distinct part of the IBS natural history (Cole et al., 2004). If colon ischemia is due to long term emotional distress resulting in maladaptive functions of the ENS and vasculature we can only speculate.

Enteric nervous system

Since the ENS innervates all layers and regions of the GI-tract it, of course, plays an important role in GI-function as well as dysfunction. To be able to understand what happens with the GI-tract when exposed to stressors, a thorough knowledge of the ENS is desirable. Not surprisingly, the ENS is a very complex system considering its coordinating functions. I will as follow summarise of the ENS; its development, characterization and function. I will also highlight some of the most central neurotransmitter that I have included in this thesis.

Development

The development of ENS is an intriguing event. Nowhere in the embryo has migrations of cells been seen in such high speed. During the formation of the ENS, neural crest cells migrate long distances to colonize the entire length of the GI-tract. In the embryo ENS is derived from two specific regions. Neuronal crest cells migrating from neuronal tube adjacent to somites 1-7 (vagal region) are precursors for enteric neurons in the upper part of the GI-tract (foregut and midgut) and vagal ganglia, while caudal somites (sacral region) are precursors to the hindgut and the pelvic ganglia. A subpopulation of vagal neural crest cells also gives rise to major elements

in the cardiac outflow tracts, as well as to neurons and support cells in the intrinsic cardiac ganglia. After colonization of the gut, neural crest-derived cells differentiate to many different types of neurons or supporting glia cells forming a complex network participating in the peripheral nervous system (Young et al., 2000; Anderson et al., 2006).

Organisation and function

When the GI-tract is colonized it contains more than 10^8 neurons. The enteric neurons have been classified according to their content of neurotransmitters, their electrophysiological properties, their targets (circular muscle, longitudinal muscle, other neurons, blood vessels, epithelium etc) or their inputs and directions of their axons (ascending or descending projections) (Furness, 2006).

The neurons are situated in groups creating ganglia; each ganglion contains different types of neurons. The ganglia form two major networks of neurons, the myenteric ganglia situated in between the circular and longitudinal muscle layer and the submucous ganglia situated in the submucosa with close connections to the blood vessels and the mucosa. Nerve cell bodies outside the two major plexus, occur only infrequent in minor plexuses (the subserosal, deep muscle and mucosal plexus) which mainly contain nerve fiber bundles. The ENS plays a critical role in regulating various functions of the GI-tract (Furness, 2006). Due to its local position it is able to connect with the entire GI-tract, regulating motility, blood flow and water and electrolyte transport across the mucosal epithelium. Actually, ENS has been named “the second brain” since it can work autonomously without any input from the central nervous system (CNS). Although the majority of the intestinal nerve terminals are of intrinsic origin, projections from extrinsic (parasympathetic and sympathetic) neurons are also present. Afferent fibers (mainly originating from cranio-spinal sensory ganglia) connecting intrinsic neurons, also contribute to the communication between ENS and CNS (Wood J et al., 1999; Grundy et al., 2006; Furness, 2006).

Cellular morphology and electrophysiological properties

The kind of synaptic events that take place in ENS are basically categorized as fast and slow excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs). An enteric neuron may express mechanisms for both slow and fast synaptic neurotransmission. Fast synaptic transmissions have durations of milliseconds range while slow synaptic potentials lasts for several seconds or longer. Fast synaptic potentials are usually excitatory while slow synaptic potentials can be either excitatory or inhibitory (Grundy et al., 2006).

Enteric neurons are also characterized by their morphological appearance. The enteric neurons may have a non-polar or polar appearance and be adendritic or multidendtritic. They are uni-, pseudouni- or multiaxonal and their axons may have dendrites. Depending on their morphological characteristics the neurons are grouped into type I, II, III, IV, V, VI, VII or giant neurons (Stach et al., 2000).

Neurotransmitters

The ENS contains a plethora of neurotransmitters. Acetylcholine (Ach) is the main excitatory transmitter in the ENS. Ach acts on receptors situated on interstitial cells of Cajal (ICC), smooth muscle cells and other neurons which causes the muscle to contract (for review Bornstein et al., 2004). Ach is also known to cause intestinal vasodilatation and secretion (for review Vanner and Suprenant, 1996). Another excitatory transmitter used by motorneurons in the ENS is the tachykinin substance P (SP). SP is also an important neurotransmitter in sensory transduction and plays a role in neurogenic inflammation (for review Holzer et al., 2006). CRF is a neuropeptide recently identified in the ENS. CRF is suggested to have secretomotor and vasodilatation functions, is often co-localised with VIP, and induces contraction of muscle strips with adherent ganglia (Kimura et al., 2007). Functional studies where peripheral injections of CRF is given, inhibit gastric emptying but enhances propulsive motility and gives rise to watery diarrhoea in colon (for Review Taché et al., 2007).

Galanin is an example of a transmitter with both excitatory and inhibitory effects on GI motor function depending on the species studied. Galanin is found extensively in the ENS and is known to regulate transmitter release and secretory activity (Ekblad et al., 1985a and b; Anselmi et al., 2005).

The ENS also contains inhibitory motor neurons which receive input from primary afferent neurons. Adenosine triphosphate (ATP) is an example of a transmitter used by these neurons. ATP affects the intestine by binding to purinergic receptor present on afferent nerve terminals or secretomotor neurons containing VIP (Grundy et al., 2006). Enteric neurons use nitric oxide (NO) to induce inhibitory intestinal motor events but NO also modulates water and electrolyte transport (Ekblad and Sundler, 1997; for review Izzo et al., 1998). NO, which is produced by the neuronal enzyme nitric oxide synthase (nNOS), can be extensively found through out the GI-tract (Ekblad et al., 1994).

VIP is another important enteric neuropeptide with inhibitory properties. VIP containing neurons are numerous along the GI-tract acting directly or indirectly via ICC on smooth muscle cells (Furness, 2006). VIP is now looked upon also as a neuroprotective peptide since several studies with different aspects have pointed out VIP as anti-inflammatory and/or neuroprotective (Ekblad et al., 1996; Ekblad et al., 1998; Sandgren et al., 2003; Abad et al., 2003; Ekblad and Bauer, 2004; Arciszewski and Ekblad, 2005; Sigalet et al., 2007; Arciszewski et al., 2008; Sand et al., 2008; Sand et al., 2009).

A peptide considered to exert anti-secretory actions is neuropeptide Y (NPY). NPY, is found in extrinsic adrenergic fibers and intrinsic nerve cell bodies and is a potent vasoconstrictor thus inhibiting intestinal secretion (for review Cox, 2007). In addition ENS contains intramural calcitonin gene-related peptide (CGRP) (Ekblad et al., 1991; Kristensson et al., 2007). CGRP induces vasodilatation but is also an important peptide in sensory transmission from the GI-tract via spinal afferent fibers to the brain and is implicated in neurogenic inflammation (for review Holzer, 2006).

Somatostatin is a peptide synthesised and released from both enteric neurons and endocrine cells. It inhibits the release of many GI-hormones resulting in reduced muscle contraction and blood flow. Somatostatin also acts on sensory nerve endings to inhibit the release of proinflammatory neuropeptides (for review Bosch, 2009).

Enteric glia

Enteric glia is supportive cells, scaffolding enteric neurons in the ganglia and the nerve fibers connecting other intestinal cells. Enteric ganglia have been described a structural role, important for neuronal function (Grundy et al., 2006). Since glia cells are present in the lamina propria, scaffolding nerve fiber, they are associated with submucosal blood vessels and mucosal epithelium. Evidence point to glia cells being important for the maintenance of the mucosal epithelial barrier and also participating in inflammatory/immune responses (Grundy et al., 2006).

Mast cells

Mast cells have attracted much attention since they are found in many inflammatory conditions like asthma, dermatoses, IBD and IBS. Regarding the GI-tract we today know that it is colonized from birth by lymphoid and myeloid cells. The number of these cells fluctuates due to changes in luminal conditions and pathophysiological states. Cell types including lymphocytes, macrophages, dendrocytes and mast cells are present in varying numbers in the intestinal mucosa, submucosa and smooth muscle layers. This creates multiple opportunities for immune cells to interact with the ENS but also with the vascular system and other cells of significance to the intestine (for review Nassauw et al., 2007)

Mast cells derive from a distinct precursor in the bone marrow. Stem cell factor (SCF), nerve growth factor (NGF), RANTES and monocyte chemoattractor protein 1 (MPC-1) are chemoattractants which recruit mast cells to local tissues (Theoharides and Kalogeromitros, 2006). In place, mast cells mature under local mircoenvironmental factors which make them contain a diverse content of mediators. The mast cells can be subdivided into two predominant phenotypes based on their expression of distinct serine proteinases. In humans, mast cells are named MC_{TC} when they contain tryptase and chymase, these cells are located mainly in the connective tissues. MC_T are human mast cells only containing tryptase and these cells are primarily found in the mucosa (for review Nassauw et al., 2007).

In rodents connective tissue mast cells (CTMCs) are, as its name applies, preferably located in connective tissue, around blood vessels and in the

peritoneal cavity. Rodent MMCs are particularly found in intestinal and pulmonary mucosa (for review Nassauw et al., 2007). Mast cells can secrete a multitude of biologically potent mediators due to different triggers. When mast cells are triggered by allergens they degranulate which leads to a massive release of mediators. This creates increased vascular permeability and recruitment of different inflammatory mediators, in worst case scenario causing an anaphylactic shock. However, if mast cells are triggered by factors like cytokines, hormones, immunoglobulins, neuropeptides, serine proteinases and certain drugs they become activated and create a selective release of mediators from the mast cells without degranulation. Depending on their chemical properties mast cell mediators can be stored in granules or synthesised upon activation (Theoharides and Kalogeromitros, 2006).

The number of mature intestinal mast cells in mouse and rat are rather poor compared to other species, however, mast cells progenitors are abundant in the intestines. These progenitors ensure capacity for a rapid recruitment of mast cells if the intestinal wall is being exposed to pathophysiological or noxious stimuli's. Mast cells play an important role in protection of the host, but the defence becomes pathobiological when the trigger pathways lead to an effector response that creates more harm than good (for review Nassauw et al., 2007)

Aims of this thesis

The general aim of this thesis was to investigate how ischemia followed by reperfusion affects the large intestine and the ENS. We also wanted to investigate detailed processes of how the ENS copes with different stressors. Specific aims were in paper:

I. To quantify myenteric neuronal subpopulations expressing CGRP, galanin, NPY, somatostatin, VIP and nitric NOS in rat colon *in vivo* and after culturing. To investigate if culturing in the presence of CGRP, galanin, VIP, S-nitroso-N-acetyl-D,L-penicillamine (SNAP, a NO-donor) or N-nitro-L-arginine methyl ester (L-NAME, a NOS-inhibitor) affects neuronal survival.

II. To investigate if ischemia followed by reperfusion in rat large intestine affects the intestinal morphology, the presence and survival of enteric neurons, ICC and immune cells.

III. To study if rat mast cells, isolated by peritoneal lavage and mast cell mediators influence neuronal survival when co-cultured with myenteric neurons in primary culture from rat small intestine.

IV. To reveal the distribution of CRF immunoreactive (IR) neurons and possible co-localization of VIP in rat small and large intestine. To study a possible interplay between CRF, VIP and/or mast cells with myenteric neurons from rat small intestine in culture.

Methodology

As follows, aspects, advantages and disadvantages of the methods used in this thesis will be discussed. However, for details on various methods consult papers I-IV.

Primary cell culture

Cells in primary cell cultures are dissociated from animals or humans. These kinds of cells can not, in contrast to stem cell lines, grow and divide in infinity. Due to this new cultures must constantly be prepared. Primary cell cultures, however, provide us with the opportunity to perform detailed studies on how particular factors affect the cell culture system. Since its often possible to extract large quantities of cells, which can be cultured in several separate vials, many factors may be studied at the same occasion. There are, however, same drawbacks when using primary cell cultures. First, when extracting cells from its original habitat both mechanical and chemical treatments are used to separate the cells in question from each other and from the nearby tissue. This might not only harm the cells themselves but also stimulate them in different, maybe unwanted directions. Second, when the cells are grown they need well balanced cell culture media to survive. The basal medium provides the cells with a buffer system, salts, vitamins and nutrients. Most cells often also need various growth factors and substances providing support to help the cells to adhere to the culture vial. Several of these components are provided by the addition of foetal animal serum. The quality of the serum is depending on the health status of the animal donator and varies from one batch to another, which of course affects the conditions of the cells in culture. Third, cell cultures are sensitive to bacteria and fungus, which means that most cultures are continuously treated with antibiotics or other additives. Despite all the drawbacks with primary cultures, they do provide a good research tool. As long as the system is compared with it self and equipped with internal controls, the results can be considered reliable and indicate what happens *in vivo*.

Immunocyto- and histochemistry

Visualisation, of tissues or cultures, is essential in understanding functional aspects and differences between “normal” and pathological situations. Visualisation can be carried out by using different staining techniques or by the use of antibodies aiming at specific molecular targets. The result obtained, however, depends on the tissue used, how it is treated in terms of fixatives or washings and how long the tissue is exposed to the various staining compounds and chemicals. It is therefore important to thorough consider what to visualise before carrying out the experiments. Throughout this thesis we have used Stefanini’s solution or 4% paraformaldehyde as fixatives. Stefanini’s solution consists of picric acid which provides a quick penetrating fixation of the tissue, but also formaldehyde which has strong fixative properties. Tissues have been frozen, to preserve antigens to a high extent, or embedded in paraffin, to preserve the morphology. The staining techniques used are base on compounds that form strong stained complexes with certain metal ions (haematoxylin). Also used is metachromasia, a characteristic change in colour of staining due to aniline dyes which bind with chromotropes in the present tissue (toludine blue). Visualisation due to enzymatic peroxidase activity has been used to identify eosinophils. Most results in this thesis have been based on detection of proteins, enzymes or cells by antibodies. The advantage of using monoclonal compared to polyclonal antibodies is the specificity for a single antigen sequence. The polyclonal antibodies can, however, bind to several regions of the antigen molecule which provides enhanced detection capacity. The main requirement for a good antibody is that it displays high affinity for its antigen. A positively appearing immunoreaction can only be considered “specific” after performing absorption or at least, and second best, omission controls.

In order to amplify signals the ABC method, utilising the high affinity of avidin for biotin was used. Light or fluorescence microscope was used to verify and analyze signals. When analyzing antigens revealed by the fluorophores fluorescein isothiocyanate (FITC) or texas red selective excitation at 485 nm and 546 nm, respectively, was used. Disadvantages by using fluorophores are that they loose energy due to excitation, resulting in a constantly fading image.

Ischemia followed by reperfusion

Since the large intestine posses a rich network of blood vessels with arcades and collaterals supplying the intestine with oxygen rich blood it can be difficult to achieve ischemia. That is why we in our experimental approach, chose to clamp not only the arcades (microvascular clips) supplying the mid part of the intestine but also to clamp the intestine itself (hemostatic forceps) (Fig 1). This, of course, creates nondesirable crush damage, however, this is the only possible way to secure absence of blood supply. Sham operated rats served as controls; these rats were treated identical (incl. crushing) to the ischemia/reperfusion (I/R) rats except that the blood supply was not turned off. One should have in mind that surgery during approximately 1.5 h affects the animal. Due to this we also used naïve controls.

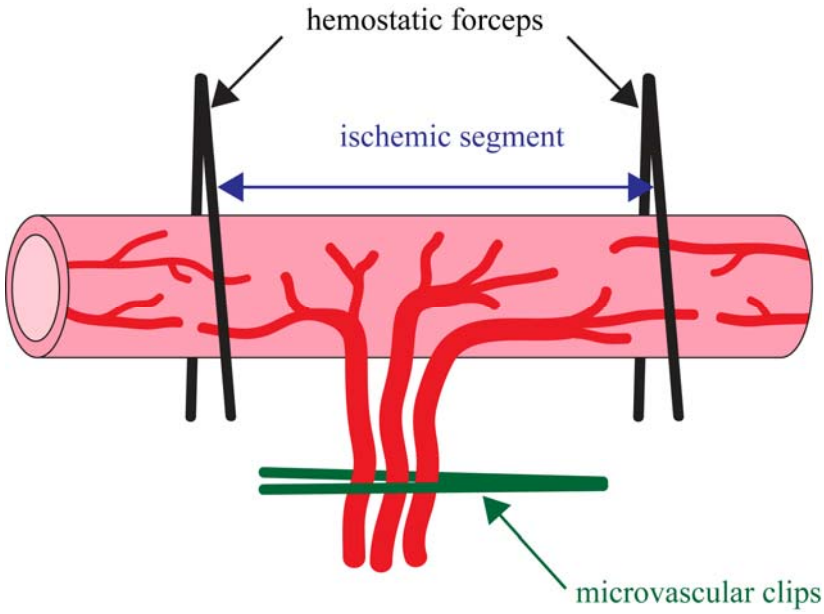

Fig 1. Schematic drawing indicating the sites of the segment clamped by hemostatic forceps on the rat large intestine.

Experimental design and results

Due to the findings that colon is the most prevalent location for intestinal ischemia and that IBS patients have an increased incidence thereof (Cole et al., 2004) we wanted to investigate the consequence of ischemia on rat colon. Our aim was to especially focus on the long term effects of ischemia followed by reperfusion since there is a lack of information in the literature on this. First, however, we wanted to investigate if the myenteric neurons of rat colon could be cultured since this would give us a clue of how well these adult neurons survive and adapt to injurious events (neuronal plasticity).

Paper I

With focus on myenteric neurons containing CGRP, galanin, NPY, NOS, somatostatin or VIP, which all play important roles in secretory and motor activity of the gut, we performed primary culturing of adult rat colonic myenteric neuron. Our aim was to elucidate the relative number of these various subpopulations of enteric neurons included in the cultures as compared to the *in vivo* situation (represented by cryo sections). The possible effect on overall survival as well as survival of selected neuronal subpopulation were investigated after addition of CGRP, galanin, VIP and the NO-donor SNAP and the NO-inhibitor L-NAME to the media when start of cell culture.

Methodologically we basically copied a previously used method for culturing myenteric neurons from rat ileum. Culturing was successful in that neurons, as well as, glia cell, attached to the culture dish, grew new terminals/sprouts and survived *in vitro*. A few smooth muscle cells and fibroblast were also present. A confluent cell layer with dispersed neurons were analysed after 4 days in culture. We found subpopulation of neurons containing CGRP, galanin, NPY, NOS (enzyme catalyzing production of NO from L-arginine), somatostatin and VIP. The relative numbers of neurons containing galanin and NOS were the same *in vitro* as *in vivo*. However the relative numbers of neurons containing CGRP, NPY,

somatostatin and VIP increased compared to the *in vivo* situation. Presence of CGRP, galanin, VIP, SNAP or L-NAME did not affect total neuronal survival. Neither did presence of CGRP affect the number of neurons containing CGRP, presence of galanin affect the number of neurons containing galanin or presence of VIP affect the number of neurons containing VIP. Presence of SNAP or L-NAME did not affect the number of neurons containing NOS.

Paper II

Rats were exposed to one hour of colonic ischemia, the blood supply was then restored and the rats were allowed to wake up. Sham operated and naïve rats were used as controls. The sham and I/R rats were sacrificed at day 1 and 3 and after 1, 2, 4, 10 and 20 weeks post ischemia. Morphometric measurements of the intestine showed no significant differences between sham and I/R operated rats. The neurons in the three groups of rats generally stained well and looked normal although in 4-, 10- and 20 weeks I/R rats some myenteric ganglionic formations lacked or displayed a low number of neurons. The relative numbers of neurons containing NOS or VIP were also studied. The relative number of myenteric neurons containing NOS increased 1 day after ischemia compared to sham operated rats. The relative number of myenteric neurons containing VIP was increased in both 1 and 3 days sham and I/R operated rats compared to naïve controls. The occurrence and topographic distribution of ICC did not differ between the sham and I/R operated groups.

The number and distribution of the immune cells eosinophils and mast cells were also studied. The number of eosinophils increased in 1 day sham and I/R operated groups compared to naïve controls. Eosinophila was particularly noted in the submucosa and serosa, but also to some extent in the mucosa, mainly basally mucosa. Colon from 3 days and 1 weeks sham and I/R rats also displayed an increased number of eosinophils in the mucosa, submucosa and serosa as compared with naïve controls. All other groups displayed no differences in number or distribution of eosinophils.

A few mast cells could occasionally be detected in naïve controls, sham or early I/R operated groups. However, 2 weeks post ischemia groups of mast cells could regularly be detected in submucosa, muscular layers and serosa. In 4-20 weeks post ischemia rats, mast cells were abundantly found in the

submucosa, muscular layers and the serosa. Mast cells were seen in close contact with myenteric neurons in the ganglia. The mast cells had not a confluent distribution through the intestinal segment exposed to ischemia but were found in clusters. Areas not containing mast cells had similar morphology as sham or naïve controls, however, in areas when mast cells were present the muscle fibers were tortuous and the muscle layers thickened. Our most intriguing finding was that in all segments containing muscular infiltrations of mast cells, the numbers of myenteric neurons were markedly reduced and short aganglionic segments could be detected. Consequently one must ask, do the mast cells kill the myenteric neurons or are the mast cells recruited due to failing myenteric functions?

In an attempt to answer the question if mast cells induce enteric neuronal cell death we set up a co-culture system of myenteric neurons and mast cells, which resulted in paper III. Although there may be regional differences we chose to culture mast cells in combination with myenteric neurons from rat ileum. This was due to a longer tradition and availability of more data from culturing ileal myenteric neurons. The use of small intestinal myenteric neurons in co-culture experiment with mast cells is also justified by the fact that Lindeström and Ekblad in 2004 showed that I/R on rat ileum resulted in mast cells infiltration and a loss of myenteric neurons. These results are well inline with the present results on rat colon I/R.

Paper III

To be able to study the influence of mast cells on cultured myenteric neurons from rat ileum we used an experimental approach similar to that used in paper I. In this study the myenteric neurons were allowed to establish in the culture for four days before being exposed to peritoneal mast cells or pharmacological agents or both. Co-culture with mast cells continued for 2 days before the cultures were fixed and stained for analysis. Mast cells adhered to the cell culture and survived well, their presence resulted in a disintegrated network of nerve fibers and a markedly enhanced loss of nerve cells. This effect was largely, but not wholly, reduced by simultaneous addition of a mast cell stabilizer, doxantrazole. Addition of the protease inhibitors chymostatin and nafamostat, as well as, the proteinase activated receptor 2 (PAR_2) antagonist FSLLRY-NH_2, the corticoid precortin or VIP in combination with mast cells, significantly and

concentration-dependently reduced neuronal cell loss. Presence of L-NAME in the co-cultures did not affect the number of surviving neurons.

In cultures without mast cells presence of precortin or VIP increased neuronal survival *per se*, while chymostatin, nafamostat or FSLLRY-NH_2 had no effect. Further efforts to explain through which mechanisms mast cells induce myenteric neuronal cell death were made by adding the mast cell mediators histamine, serotonin, heparin, tumor necrosis factor alfa (TNFα) and interleukin beta (IL1β) to the neuronal cultures. None of these affected neuronal survival. However, the mast cell mediators interleukin 6 (IL-6) and prostaglandin D_2 (PGD_2) concentration-dependently decreased neuronal survival. Also a PAR_2 agonist concentration-dependently decreased neuronal survival.
The relative number of neurons containing VIP increased after several days in co-culture with mast cells, while the relative number of neurons containing NOS was unaffected.

Paper IV

Mast cells contain a plethora of mediators, among them CRF, a peptide considered pivotal in acute stress. In addition, CRF-R_1 and CRF-R_2 has been identified on mast cells (Theoharides et al,. 2004). In paper IV we wanted to describe the possible presence and distribution of CRF containing neurons and mast cells in rat intestine and to quantify subpopulations of myenteric neurons in rat ileum containing CRF or VIP or both *in vivo* and *in vitro*. Our purpose was also to test if CRF, or its analogue Ucn1, affects myenteric neuronal survival in culture. Further we investigated if CRF interacts with mast cell-induced neuronal cell death.

The experimental design was in the latter case almost identical to that of Paper III. Cryostat sections from rat ileum and colon immunolabeled for CRF were shown to harbour nerve cell bodies in both submucous and myenteric ganglia and contained fine networks of fibers in the intestinal wall. The fibers were numerous in the mucosa and the majority of them contained also VIP. When quantifying ileum myenteric neurons *in vivo* it was found that approximately 67% of all CRF-IR neurons were also IR to VIP, while 90% of the VIP-IR neurons were also IR to CRF. However, after 6 days in culture numerous VIP-IR neurons were seen and these neurons possessed long and frequently varicose nerve terminals while CRF-

IR neurons were few and vaguely stained with CRF immunoreactivity concentrated to the cell bodies. All cultured neurons IR for CRF were also IR for VIP, whereas approximately only 5% of the VIP-IR neurons were IR also for CRF. Isolated and freshly fixed mast cells or mast cells cultured for 2 days displayed no immunoreactivity.

Cultured neurons were exposed to CRF, Ucn1, CRF antagonist, VIP, CRF + VIP, mast cells, mast cells + CRF or mast cells + CRF antagonist from day 4 to 6. CRF, Ucn1 or CRF antagonist did not affect neuronal survival, while VIP increased neuronal survival to approximately 160%. Notable was that simultaneous addition of CRF counteracted the neuroprotective effect of VIP.
The addition of peritoneal mast cells to the culture reduced neuronal survival to 46%. Addition of CRF or CRF antagonist in combination with mast cells did not interfere with the mast cells induced neuronal cell death.

Discussion

Looking at mammals, one easily notices the differences in size, appearance and behaviour within this group although there are many similarities as well. When doing research, which is aimed to help humans, the ultimate experimental model would of course be humans or a computerized human having all the human characteristics, without being a human. This model is at least not yet, available and let us hope it never will be. I have in my work used the rat as an experimental animal model, the rat is a good experimental model in many ways, its easy to house, its size is preferable and most importantly it has many biological similarities with man. Other experimental animals are also widely used like mouse, guinea-pig, rabbit and pig and the species differences are important to have in mind when interpreting research findings.

Another important point to consider is regional differences in the body, especially when studying the intestine which is a long hollow organ with several different specialisations along its way. In paper I we showed that myenteric neurons from rat colon could be cultured for at least 4 days *in vitro*. Earlier studies, performed in our group, have shown that submucous neurons from rat colon (Lin et al., 2004) and myenteric neurons from rat ileum (Lin et al., 2003; Sandgren et al., 2003) as well as myenteric neurons from pig small intestine (Arciszewski et al., 2005; 2008) can be successfully cultured for up to 21 days.

In paper I we also showed that the relative numbers of neurons containing CGRP, NPY, somatostatin and VIP increased *in vitro* compared to *in vivo*. These data may suggest that neuronal expression of CGRP, NPY, somatostatin and VIP is upregulated and that it selectively protects neurons subjected to injurious events e.g. isolation and culturing and promotes neuronal survival. NPY, somatostatin and VIP are all known to inhibit intestinal motility (Cox, 2007; Furness, 2006; Bosch et al., 2009) and this may be beneficial in this particular environment. An increased proportion of neurons expressing VIP have earlier been reported in primary cultures from both rat and pig small intestine, however, the relative number of

neurons expressing VIP was unchanged in submucous neurons from rat colon (Lin et al., 2004). Changes in enteric neuronal expression of CGRP, NPY and somatostatin in response to isolation and culturing have not previously been reported.

Addition of CGRP, galanin, VIP, NO-donor (SNAP)/inhibitor (L-NAME) did not affect total neuronal survival or the survival of the subpopulations of CGRP-, galanin-, VIP- or NOS-IR neurons. This indicates that the myenteric neurons from rat colon are relatively robust to environmental changes in neuropeptide concentrations. Neuronal survival of myenteric neurons from rat ileum has been shown to be enhanced by the addition of VIP and NO-donor while NOS-inhibitor reduced neuronal survival (Sandgren et al., 2003). We have by this report, implied that there are regional differences in injury-induced changes in expression of neurotransmitters and neurotransmitter evoked neuroprotection. We suggest that this is probably due to functional differences.

Paper II corroborates the above interpretation of different responses in different regions to the same stimuli. Previous I/R perfusion studies performed on rat small intestine showed intestinal hypertrophy, acidophilic neurons and eosinophilia (Lindeström et al., 2004) and necrotic mucosa (Takada et al., 1998). We demonstrated in paper II that rat colon subjected to ischemia followed by reperfusion showed no change in circumference or length of the affected segment, nor in thickness of the intestinal layers, nor in number and distribution of eosinophils as compared to sham operated rats. Thus regional structural differences following an ischemic insult points to colon being more robust to injurious events.

Notable similarities, like the mastocytosis and myenteric neuronal loss detected in the small intestine post ischemia, (Lindeström et al., 2004) also occur in the rat large intestine as shown in paper II and by Ravnefjord et al., 2008. We detected mast cells in and around the myenteric ganglia as well as in the circular and longitudinal muscle 4-20 weeks post ischemia. In regions with a high number of mast cells a markedly reduced number, or total lack, of myenteric neurons were found. If the lack of neurons is due to the presence of mast cells or if mast cells are recruited because of neuronal cell loss we can only speculate. Since mast cells contain a wide range of mediators, some with proinflammatory properties others with excitatory or inhibitory properties on the intestine, mast cells could be beneficial or/and

harmful to the intestine. This has been a topic of debate following the findings of an increased number of mast cells in both IBS and IBD patients. Most findings, however, indicate mast cells having a contributing role in the pathogenesis of IBS and IBD (Rijnierse et al., 2007)

Our results in paper III show that mast cells are capable of inducing death in myenteric neurons. Since the mast cell stabilizer doxantrazole were unable to fully rescue mast cell-induced neuronal death, mast cells are suggested to act on neurons through different mechanisms. The partial rescue could also be due to continuously dying mast cells releasing their injurious granules uncontrolled. Supporting the suggestion that mast cells execute neurons through different mechanisms is that addition of protease inhibitors or PAR_2 antagonist only partially rescue myenteric neurons from mast cell-induced cell death.

VIP and precortin also reduced mast cell-induced neuronal death, however, since both VIP and percortin *per se* increase neuronal survival in the absence of mast cells we suggest that this effect is mainly neuroprotective. It can, however, not be excluded that VIP and precortin also interfere with the release of mast cell mediators. These results are of high importance since mast cells today are considered as pharmaceuticals targets in IBS and IBD patients. These results indicate that the harmful effects performed by mast cells in the intestine most certainly target the neurons. Modulation of protease activity is today highly focused on since its receptor PAR_2 is expressed on both enteric and sensory neurons (for review Vergnolle, 2005). In this case mast cells are suggested to play a pivotal role since mast cells are one of the sources for protease release.

In Paper IV we showed that rat ileum and colon display CRF-containing nerve cell bodies in submucous and myenteric ganglia and that the intestinal wall possess a fine network of CRF-IR nerve fibers. A high degree of co-existence of CRF and VIP in submucous and myenteric neurons in both ileum and colon were detected. Our results are in line with previous immunocytochemical studies performed in guinea-pig small and large intestine (Liu et al., 2006). Functional studies of peripheral injections of CRF or Ucn1 in rats cause enhanced propulsive motility but also watery diarrhoea (Taché et al., 2004). Rats exposed to psychological distress display elevated CRF and decreased VIP plasma concentration (Huang et al., 2007). Taken together these findings indicate a possible interplay

between the two neuropeptides. The fact that VIP induces intestinal relaxation and secretion and that CRF induces intestinal contraction and secretion, indicates synergistic but also opposing effects.

Since the number of neurons expressing VIP is relatively much higher then those expressing CRF *in vitro* vs. *in vivo*, this is interpreted as CRF not being essential to express for neuroprotection, in contrast to VIP. Our results corroborate previous data supporting the idea that VIP is neuroprotective. In paper IV we present evidence for CRF counteracting VIP's neuroprotective effect, although CRF, Ucn1 or CRF antagonist did not affect survival of cultured neurons *per se*. This supports the idea of an interplay between CRF and VIP also in neuronal survival.

Earlier studies have shown that both human and rat mast cells synthesize and secrete CRF as well as its receptors and that psychological distress leads to mast cell activation and increased intestinal permeability (Santos et al., 1998; Santos et al., 2001; Theoharides et al., 2004; Cao et al., 2005; Santos et al., 2008; Keita et al., 2010). Neurons might therefore be affected by CRF secreted from mast cells. In paper IV we confirmed a mast cell-induced reduction of neuronal survival *in vitro* (Sand et al., 2009). Presence of CRF or CRF antagonist failed to affect mast cell-induced reduction in neuronal survival. This may be due to an already maximal reduction of neuronal survival. Our results indicate that mast cell-induced reduction in neuronal survival is not executed via CRF signaling pathways.

Conclusions

Paper I
Cultured rat colonic myenteric neurons increase their expression of CGRP, NPY, somatostatin and VIP, suggesting that these neuropeptides are of importance for neuronal survival.

Paper II
No structural remodelling of rat colon could be detected post ischemia. Local areas containing numerous mast cells appeared in the muscle layers, the serosa and in and around the myenteric ganglia 4-20 weeks post ischemia. Myenteric ganglionic formations within mast cell rich areas were virtually aganglionic.

Paper III
Mast cells induce a reduction of neuronal survival in cultures of myenteric neurons from rat small intestine via degranulation, PAR_2 activation or release of PGD_2 or IL-6. Corticosteroid and VIP are both neuroprotective and able to enhance neuronal survival when added to co-cultures with neurons and mast cells.

Paper IV
CRF and VIP co-exist in numerous neurons in rat small and large intestine. CRF signalling does not affect neuronal survival in cultures of myenteric neurons from rat small intestine, but attenuates the VIP mediated increase in neuronal survival. The mast cell-induced reduction in neuronal survival is not executed via CRF signalling pathways.

Populärvetenskaplig sammanfattning

Emotionell stress samt hårt fysiskt arbete reducerar blodflödet till tarmen. Om cirkulationen till tarmen är kraftigt reducerat under en längre period får tarmen syrebrist, den blir ischemisk. Vid ischemi bildas skadliga slaggprodukter vilka distribueras till andra organ när blodflödet ökar igen. Irritable bowel syndrome, IBS, är en vanlig mag-tarm sjukdom som man inte vet orsaken till. Patienterna är förstoppade eller har diarré, känner smärta eller obehag och är ofta svullna om magen. Det har noterats att IBS patienter har en ökad förekomst av tjocktarmsischemi. En annan patientgrupp som också drabbas av ischemi i tarm är de som har inflammatoriska tarmsjukdomar, tex Crohn's sjukdom eller ulcerös colit.

Tarmens funktion styrs till stor del av det enteriska nervsystemet som återfinns utmed hela tarmen. Nervcellerna ligger fördelade i framförallt två typer ganglier - ansamlingar av nervcells kroppar. Myenteriska ganglier ligger mellan tarmens två muskelskikt och submucosala ganglier ligger under sleminnan. Nervcellerna skickar signaler till tarmens övriga celler, men de kommunicerar också med varandra, samt med centrala nervsystemet. Genom studier som ingår i denna avhandling har vi sökt information om hur enteriska nervceller reagerar i utsatta situationer, samt hur dessa nervceller klarar av en tillfällig period av ischemi. Genom att isolera nervceller från råttans tarm och odla dom i primärkultur utsättes de för en stressande miljö. Vi har visat att de trots denna påfrestning kan överleva och tillverka signalsubstanser. Vi har också visat att vid odling av myenteriska nervceller från råttans tjocktarm är det en högre andel nervceller som uttrycker neuropeptiderna CGRP, NPY, somatostatin och VIP jämfört med när neuronen är intakta i tarmen. Dessa peptider är viktiga för tarmens funktion och slutsaten vi drar från dessa resultat är att de också är viktiga för nervcellerna överlevnad.

För att studera hur nervcellerna, samt övriga tarmstrukturer, klarar ischemi följt av reperfusion, stängdes blodflödet av under en timme till ett segment av råttans tjocktarm, för att därefter släppas på igen. Strukturellt klarade sig den behandlade tjocktarmen ganska bra, men 4-20 veckor efter ischemi

återfanns mastceller, som är inflammatoriska celler, i tarmens muskellager samt i dess ytterskikt. I områden där mastcellerna ackumulerades, vilket företrädesvis var nära de myenteriska ganglierna, saknades nervceller. För att utreda detta fenomen i detalj samodlades mast celler med myenteriska neuron. Det visade sig att närvaro av mastceller markant reducerade nervcellernas överlevnad. Mastceller innehåller många mediatorer som skulle kunna påverka nervcellsöverlevnad. Proteaser via sin mottagarmolekyl PAR_2, IL-6 och PGD_2 är de mediatorer vi visade kunde reducera nervcellernas överlevnad i kultur. Samtidigt fann vi att VIP och en corticosteriod hade förmåga att motverka den mastcellsinducerade nervcellsdöden. Dessa två visade sig också ha en gynnsam effekt på nervcellernas överlevnad i kulturer utan mastceller.

CRF är en peptid som frisätts vid emotionell stress. CRF och dess mottagarmolekyl produceras av mastceller samt av neuron i tarmen. I avhandlingens sista studie visade vi att råttan har många CRF innehållande nervceller i tunn- och tjocktarm och att dessa oftast också innehåller VIP. Vid odling av myenteriska nervceller återfanns relativt sätt få CRF- men många VIP-innehållande nervceller. CRF visade sig inte ha någon effekt på nervcellernas överlevnad i kultur men motverkade den neuroprotektiva effekten av VIP. Samtidigt kunde vi bekräfta att mastcellsinducerad nervcellsdöd, som noterats i samodlingarna, inte beror på närvaro av CRF.

Sammanfattningsvis har vi visat att myenteriska nervceller från råttans tjocktarm går att odla. Flera veckor efter en kraftig, men tillfällig, ischemi så dyker mastceller upp i tarmväggen och i dessa områden saknas myenteriska nervceller. Mastceller inducerar troligen död av nervceller mha sina mediatorer: proteas, PGD_2 och IL-6, dock inte via CRF signalering. VIP är neuroprotektiv och förhindrar nervcellsdöd men denna effekt motverkas av CRF.

Acknowledgements

I would especially like to thank:

Professor Eva Ekblad, for being a reliable, honest and great supervisor and for putting a lot of effort into the papers included in this thesis.

Erik Lindström, my former boss and source of scientific inspiration.

Anna Themner-Persson, for helping me on daily basis and being a supportive and good friend.

Anna Gunnarsdottir, Katarina Sandgren, Malin Josefson, Mikael Ekelund, Oskar Hammar and Bodil Ohlsson for putting clinical perspectives to my preclinical point of view and for being a bunch of really nice people.

Doris for having everything under control, Cecilia and Karin for making me laugh, Jonas, Britt-Marie, Ulrikke, Rama, Matteo, Nils and Frank for being cheerful, old or new members of the lab.

The people at B11 for providing interesting or less interesting conversations at fika or lunch!

Martin Nyström, Agneta Persson, Annette Jönsson, Adelé and LRI for making computers, education, technique and services work.

The most important people in my life Oscar and Fredrik for putting everything in perspective, mina solstrålar, jag älskar er!

My parents, Lena and Lennart, for always loving me and supporting me in all ways possible. Utan er hade jag inte varit så begåvad och vacker, moahhhhh! Ni är bäst!

My sister Anna for being a role model and taking care of me always. Stina, min goa lilla tjej, for making me go to Gothenburg very often and Christian

for being technical support and taking my family on very appreciated and needed holidays!

My extra family and best friends Britta, Kristin, Linda and Pernilla. Without you life would be dull!

Anna, Eva and Tomas for endless support in daily life, you mean a lot to me.

Ann-Katrin, Mattias, Amanda, Lovisa, Malin, Kim och Smilla, för mysiga stunder.

Åse, Therese, Åsa, Åsa and Dessiré with families for many nice moments and fikor.

My old colleges and food friends Anna, Terese, Marita, Dorota, Maria and Trädgårdsmästar'n, ska vi ta en middag snart?

Christer-redaktionen på P3, för att ni under alla år underhållit mig när jag suttit i ett mörkt rum och räknat celler, ni är fantastiskt roliga, Tack!!!

Sundsandvik with friends, for making me who I am today.

These studies were supported by:
The Swedish Medical Research Council
The Swedish Animal Welfare Agency
The Ihre Foundation
The 9-m-liv Foundation
The Crafoord Foundation
The Royal Physiographic Society

References

Abad C, Martinez C, Juarranz M, Arranz A, Leceta J, Delegado M, Gomariz R. Therapeutic effects of vasoactive intestinal peptide in the trinitrobenzene sulfonic acid mice model of Crohn's disease. Gastroenterology 2003;124(4):961-971

Almy T, Hinkle L, Berle B, Kern F. Alterations in colonic function in man under stress. Experimental production of sigmoid spasm in patients with spastic constipation. Gastroenterology 1949;12:437-449

Anderson R, Newgreen D, Young H. Neural crest and the development of the enteric nervous system. Neural crest induction and differentiation 2006;11:181-196

Anselmi L, Stella S, Lakhter A, Hirano A, Tonini M, Sternini C. Galanin receptors in the gastrointestinal tract. Neuropeptides 2005;39(3):349-352

Arciszewski M, Ekblad E. Lipopolysaccaride induces cell death in cultured porcine myenteric neurons. Dig Dis Sci 2005;50:1661-8

Arciszewski M, Sand E, Ekblad E. Vasoactive intestinal peptide rescues cultured rat myenteric neurons from lipopolysaccharide induced cell death. Regul Pept 2008;146(1-3):218-223

Barbara G, Stanghellini V, De Giorgio R, Corinaldes R. Functional gastrointestinal disorders and mast cells: implications for therapy. Neurogastroeterol Motil 2006;18:6-17

Bosch J, Adriaensen D, nassauw L, Timmermans J. The role(s) of somatostatin, structurally related peptides and somatostatin receptors in the gastrointestinal tract: a review. Regul Pept 2009;156(1-3):1-8

Bornstein J, Costa M, Grider. Enteric motor and interneuronal circuits controlling motility. Neurogastroenterol Motil 2004;1:34-38

Cannon, W. Bodily changes in pain, hunger, fear and rage. Charles T. Branford. Boston, Massachusetts, USA 1953:1–404

Cao J, Papadopoulou N, Kempuraj D, Boucher W, Sugimoto K, Cetrulo C, Theoharides T. Human mast cells express corticotropin-releasing hormone (CRH) and CRH leads to selective secretion of vascular endothelial growth factor. J Immuno 2005;174(12):7665-7675

Cole J, Cook S, Sands B, Ajenr A, Miller D, Walker A. Occurence of colon ischemia in relation to irritable bowel syndrome. Am J Gastroenterol 2004;99:486-491

Cox H. Neuropeptide Y receptors; antisecretory control of intestinal epithelial function. Autonomic Neuroscience: Basic and Clinical 2007;133:76-85

Ekblad E, Håkanssom R, Sundler F. Microanatomy and chemical coding of peptide-containing neurons in the digestive tract. In: Daniel EE (Ed) Neuropeptide function in the gastrointestinal tract. CRC Press, Boca Raton, Florida, pp. 1991:131-179

Ekblad E, Håkanson R, Sundler F, Wahlestedt C. Galanin: neuromodulatory and direct contractile effects on smooth muscle preparations. Br J Pharmacol 1985a;86(1):241-246

Ekblad E, Rökaeus A, Håkanson R, Sundler F. Galanin nerve fibers in the rat gut:distribution, origin and projections. Neuroscience 1985b;16(2):355-363

Ekblad E, Alm P, Sundler F. Distribution, origin and projections of nitric oxide synthase-containing neurons in gut and pancreas. Neuroscience 1994;63(1):233-248

Ekblad E, Mulder H, Sundler F. Vasoactive intestinal peptide expression in enteric neurons is upregulated by both colchicine and axotomy. Regul. Pept. 1996;63:113-21

Ekblad E, Sundler F. Motor responses in rat ileum evoked by nitric oxide donors vs. field stimulation: modulation by pituitary adenylate cyclase-activating peptide, forskolin and guanylate cyclase inhibitors. J Pharmacol Exp Ther 1997;283(1):23-28

Ekblad E, Sjuve R, Arner A, Sundler F. Enteric neuronal plasticity and a reduced number of interstitial cells of Cajal in hypertrophic rat ileum. Gut 1998;42:836-44

Ekblad E, Bauer AJ. Role of vasoactive intestinal peptide and inflammatory mediators in enteric neuronal plasticity. Neurogastroenterol Motil 2004;16:123-28

Furness J. Neuronal control of motility. In: Furness J, editor. The enteric nervous system. Massachusetts: Blackwell Publishing 2006:132-198

Grundy D, Al-Chaer E, Aziz Q, Collins S, Ke M, Taché Y, Wood J. Fundamentals of Neurogastroenterology: Basic Science. Gastroeterology 2006;130:1391-1411

Guyton A, Hall J. Textbook of Medical Physiology, ninth ed. 1996;60,77

Haglund U, Bergqvist D. Intestinal ischemia – the basic. Langenbeck's Arch Surg 1999;384:233-238

Hatoum O, Binion D, Gutterman D. Paradox of simultaneous intestinal ischemia and hyperaemia in inflammatory bowel disease. J Clin Invest 2005;35:599-609

Holzer P. Efferent-like roles of afferent neurons in the gut: Blood flow regulation and tissue protection. Autonomic Neuroscience: Basic and Clinical 2006;125:70-75

Huang Y, Yu J, Wang G, Chen Z, Wang Q, Xiao L. Effect of fluoxetine on depression-induced changes in the expression of vasoactive intestinal polypeptide and corticotrophin releasing factor in rat duodenum. World J Gastroenterol 2007;13(45):6060-6065

Izzo A, Mascolo N, Capasso F. Nitric oxide as a modulator of intestinal water and electrolyte transport. Dig Dis Sci 1998;(8):1605-1620

Keita A, Söderholm J, Ericson A. Stress-induced barrier disrution of rat follicle-associated epithelium involves corticotropin-releasing hormone, acteylcholine, substance P and mast cells. Neurogastroenterol Motil 2010; Epub ahead of print

Kimura T, Amano T, Uehara H, Ariga H, Ishida T, Torii A, Tajri H, Matsueda K, Yamato S. Urocortin 1 is present in the enteric nervous system and exerts an excitatory effect via cholinergic and serotonergic pathways in the rat colon. Am J Physiol Gastrointest Liver Physiol 2007;293(4):G903-G910

Kristensson, Themner-Persson A, Ekblad E. Survival and neurotransmitter plasticity in cultured rat colonic myenteric neurons. Regul Pept 2007;140:109-116

Lin Z., Sandgren K., Ekblad E. Increased expression of vasoactive intestinal peptide in cultured myenteric neurons from rat small intestine. Autonomic Neuroscience 2003;107:9-19

Lin Z, Sandgren K, Ekblad E. Increased expression of nitric oxide synthase in cultured neuron from adult colonic submucous ganglia. Autonomic Neuroscience: Basic and Clinical 2004;114:29-38

Lindeström LM, Ekblad E. Structural and neuronal changes in rat ileum after ischemia with reperfusion. Dig Dis Sci 2004;49:1212-22

Liu S, Gao N, Hu H, Wang X, Wang G, Fang X et al. Distribution and chemical coding of corticotropin-releasing factor-immunoreactive neurons in the guinea pig enteric nervous system. J Comp Neurol 2006;494(1):63-74

Mallick I, Yang W, Winslet M, Seifalian A. Ischemia-Reperfusion injury of the intestine and protective strategies against injury. Dig Dis Sci 2004;49:1359-1377

Nassauw L, Adriaensen D, Timmermans J. The bidirectional communication between neurons and mast cells within the gastrointestinal tract. Autonomic Neuroscience: Basic and Clinical 2007;133:91-103

Ravnefjord A, Pettersson M, Rehnström E, Martinez V. Acute colonic ischemia in rats results in long-term structural changes without alterations of colonic sensitivity. Int J Exp Pathol 2008;89(6):476-489

Rijnierse, Nijkamp A, Kraneveld A. Mast cells and nerve tickle in tummy implications for inflammatory bowel disease and irritable bowel syndrome. Pharmacol Ther 2007;116(2):207-235

Sand E, Themner-Persson A, Ekblad E. Infiltration of mast cells in rat colon is a consequence of ischemia/reperfusion. Dig Dis Sci 2008;53(12):3158-3169

Sand, Themner-Persson A, Ekblad E. Mast cells reduce survival of myenteric neruosn in culture. Neuropharmacol 2009;56:522-530

Sandgren K, Lin Z, Svenningsen Å, Ekblad E. Vasoactive intestinal peptide and nitric oxid promote survival of adult rat myenteric neurons in culture. J. Neurosci Res 2003;72:595-602

Santos J, Saperas E, Nogueiras C, Mourelle M, Antolín M, Cadahia A, Malagelada J. Release of mast cell mediators into the jejunum by cold pain stress in humans. Gastroenterology 1998;114(4):640-8

Santo J, Yang P, Söderholm J, Benjamin M, Perdue M. Role of mast cells in chronic stress induced colonic epithelial barrier dysfunction in the rat. Gut 2001;48(5):630-636

Santos J, Guilarte M, Alonso C, Malagelada J. Pathogenesis of irritable, bowel syndrome: the mast-cell connection. Gastroenterology 2005;40:1-12

Santos J, Alonso C, Guilarte M, Vicario M, Malagelada J. Targeting mast cells in the treatment of functional gastrointestinal disorders. Curr opin in Pharmaco 2006;6:541-546

Santos J, Yates D, Guilarte M, Vicario M, Alonso C, Perdue M. Stress neuropeptides evoke epithelial responses via mast cell activation in the rat colon. Psychoneuroendocrinology 2008;33(9):1248-1256

Sällstedt E-A, Hellström PM. Gastrointestinalkanalen och fysisk aktivitet, vad händer med mag-tarmkanalen under träning? Svensk idrottsmedicin. 2008;1:4-8

Sigalet D, Wallace L, Holst J, martin G, Kaji T, Tanaka H, Sharkey K. Enteric neuronal pathways mediate the anti-inflammatory actions of glucagons-like peptide 2. Am Physiol Gastrointest Liver Physiol 2007;293(1):G211-221

Stach W, Krammer H, Brehmer A. Structural organisation of enteric nerve cells in large animals including man. Neurogastroenterology from the basics to the clinics 2000;1:3-20

Taché Y, Martinez V, Wang L, Million M. CRF_1 receptor signalling pathways are involved in stress-related alterations of colonic function and viscerosensitivity: implications for irritable bowel syndrome. Brit J of Pharmacol 2004;141(8):1321-1330

Taché Y, Bonaz B. Corticotropin-releasing factor receptors and stress-related alterations of gut motor function. J Clin Invest 2007;117:33-40

Takada K, Yamashita K, Sakurai-Yamashita YShigematsu K, Hamada Y, Hioki K, Taniyama. Participation of nitric oxide in the mucosal injury of rat intestine induced by ischemia-reperfusion. J Pharmacol Exp Ther 1998;287(1):403-407

Theoharides T, Donelan J, Papadopoulou, Cao J, kempuraj D, Conti P. Mast cell as targets of corticotropin-releasing factor and related peptides. Trends Pharmacol Sci 2004;25(11):563-568

Theoharides T, Kalogeromitros D. The critical role of mast cells in allergy and inflammation. Ann N Y Acad Sci 2006;1088:78-99

Vanner S, Suprenant A. Neural reflexes controlling microcirculation. Am J Physiol 1996;(2Pt1):G223-G230

Veenestra R. Geelkerken R, Verhorst P, Huisman A, Kolkman J. Acute stress-related gastrointestinal ischemia. Digestion 2007;75:205-207

Wallon C, Söderholm J. Corticotropin-releasing hormone and mast cells in the regulation of mucosal barrier function in the human colon. Ann N Y Acad Sci 2009;1165:206-210

Wood J, Alpers P, Andrews P. Fundamentals of gastroenterology. Gut 1999;45(Suppl II):II6-II16

Young H, Hearn C, Newgreen D. Embryology and development of the enteric nervous system. Gut 2000;(Suppl IV)47:iv12-iv14