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Gut Feelings

From Brain to Gut and from Lumen to Gut

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FACULTY OF
MEDICINE

Gut Feelings

From Brain to Gut and from Lumen to Gut

Xiaowen Cheng

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Gut Feelings – From Brain to Gut and from Lumen to Gut

Abstract

Part 1: From Brain to Gut

The brain is an organ with a high energy requirement. To meet this demand, the brain needs a continuous and well-regulated blood supply. In situations where the brain, or parts of the brain, does not receive sufficient blood flow injury arise. This condition, commonly known as cerebral ischemia, is prevalent in today's society. In 2015, more than 6 million people died from stroke worldwide. In parallel to the central injury, more than half of the affected patients report gut dysfunctions such as constipation, dysmotility and incontinence after the ischemic event. In recent years an increased awareness and focus on the gut-brain axis has emerged. This bidirectional axis of communications has been showed to be involved in several conditions including anxiety, depression, Parkinson's disease and stroke.

A key player in gut regulation is the enteric nervous system (ENS), which sometimes is referred to as the "second brain". It is suggested that gut dysfunctions are rooted in an imbalanced ENS, since this system regulates motility, secretion, local blood flow and also interacts with the immune and endocrine systems.

In part 1 of this thesis the effects of different types of cerebral ischemia on the ENS in regards to survival and neuroplasticity are investigated in mouse. From this research, we are able to show that one type, focal ischemia, induces a significant of loss of enteric neurons as well as neurotransmitter plasticity. The underlying mechanisms are suggested to involve neuroimmune actions, including galectin-3 activation of toll like receptor 4. We further show that models of global cerebral ischemia and cerebral hypertension elicit a non-neurotoxic response on enteric neurons, suggesting that each type of ischemia triggers unique peripheral responses.

Part 2: From Lumen to Gut

In addition to taste thrills on the tongue, food also triggers sensations in the gastrointestinal (GI) tract. Similar to taste buds and taste receptors, specialized "intestinal sensor cells" in the GI epithelium are able to sense and mediate intestinal chemosensation. This includes sensing nutrients as well as harmful components in the luminal environment. One of the epithelial cells possessing chemosensory potential is the tuft cell, recognized by its fusiform shape and distinct apical "tuft" of microvilli extending into the lumen. Tuft cells are able to sense bitter, sweet and umami substances in lumen by expressing taste cell transduction proteins, α-gustducin, and activating taste related cation channel, transient receptor potential channel 5. They also contribute to the protection of the gut barrier and trigger type 2 immune reactions. Tuft cells are a rare cell type in the gut, its role in health and disease is just starting to be investigated.

In part 2 of this thesis we study the tuft cell, their distribution and proximity to endocrine cells and nerve fibers in the mouse intestine. From this research we are able to show that tuft cells have a specific distribution throughout the intestine, with a decreasing gradient from upper small intestine to distal small intestine and large intestine. We further describe a high degree of contacts with mucosal sensory nerve fibers and appetite associated endocrine cells. From this we suggest that tuft cells act as an interface between signals in the intestinal lumen and the host.

In part 2 of the thesis we also identify a novel subset of tuft cells which harbor serotonin (5HT) apically. This 5HT containing tuft cell, accounts for up to 80% of all tuft cells in mouse small intestine but are rare in the large intestine. We have not, yet, been able to discern the source of 5HT since neither the synthesizing enzyme tryptophan hydroxylase nor the 5HT transporter are found in tuft cells. However, we believe this subset cells have the possibility to play unique roles in gut regulation.

Key words
Enteric nervous system, cerebral ischemia, galectine-3, tuft cell, CGRP, PYY, CCK, GLP-1, 5HT

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Gut Feelings

From Brain to Gut and from Lumen to Gut

Xiaowen Cheng

LUND
UNIVERSITY

Cover painting by *Xin Yang*

No guts, no glory.

我有所念人，隔在远远乡。

我有所感事，结在深深肠。

乡远去不得，无日不瞻望。

肠深解不得，无夕不思量。

——白居易《夜雨》

Painting by Oscar Manouchehrian and Xiaowen Cheng

Thesis at a glance

	Main topics	Methods	Results	Conclusions
I	From Brain to Gut. Does cerebral ischemia cause enteric neuropathy?	- Focal and global ischemia (stroke) models - Gal-3 and TLR4 null mice - Primary myenteric neuronal cultures - Immunocytochemistry - Western blot	*In vivo* - Focal stroke in gal-3$^{+/+}$ mice → enteric neuronal loss - Focal stroke in gal-3$^{-/-}$ mice → no neuronal loss *In vitro* - Control neurons exposed to focal stroke serum or purified gal-3 → neuronal loss. TAK1/AMPK inhibitors counteracted neuronal loss - TLR4$^{-/-}$ neurons exposed to focal stroke serum → no neuronal loss	Focal stroke triggers gal-3 induced enteric neuronal loss, through a TLR4 mediated mechanism involving TAK1 and AMPK
II			- Focal stroke in mice induced enteric neuronal loss and up-regulation of VIP in submucous neurons - Global stroke did not induce enteric neuronal loss or change VIP or NOS expressions	Focal and global stroke influence enteric neuronal survival and plasticity differently, probably reflecting differences in peripheral neuroimmune effects
III	From Lumen to Gut. Tuft cells: Distribution and proximity to endocrine cells and nerve fibers	- "Swiss rolls" technique for mice intestinal preparations - Immunocytochemistry	- Tuft cells, identified by DCLK1-IR occurred throughout small and large intestine - Their numbers decreased in a proximal to distal manner - Tuft cells were in contact with nerve fibers and satiety associated peptide-IR endocrine cells	The topographic distribution of tuft cells suggests them being potential modulators of GI activities in response to luminal signaling
IV			- A large proportion of small intestinal tuft cells contained apical 5HT-IR - A small amount of tuft cells was in contact with enterochromaffin cells. - Tuft cell did not express 5HT synthesized enzyme TPH or reuptake transporter SERT	A novel subset of tuft cells, DCLK1/5HT-IR is described. These cells may have unique roles in the GI tract

5HT: 5-hydroxytryptanmine, serotonin, AMPK: AMP activated kinase, DCLK: doublecortin like kinase 1, Gal-3: galectin-3, IR: immunoreactivity, SERT: serotonin transporter, TAK1: transforming growth factor activated kinase 1, TLR4: toll like receptor 4, TPH: Tryptophan hydroxylase, VIP: vasoactive intestinal peptide

Table of Contents

List of Original Papers

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

I.   Cheng, X., Boza-Serrano, A., Turesson, M.F., Deierborg, T., Ekblad, E. and Voss, U., 2016. Galectin-3 causes enteric neuronal loss in mice after left sided permanent middle cerebral artery occlusion, a model of stroke. Scientific Reports, 6, p.32893, doi: 10.1038/srep32893, 2016.

II.   Cheng, X., Svensson, M., Yang, Y., Deierborg, T., Ekblad, E. and Voss, U., 2018. Focal, but not global, cerebral ischemia causes loss of myenteric neurons and upregulation of vasoactive intestinal peptide in mouse ileum. International Journal of Experimental Pathology, 99(1):38-45, doi:10.1111/iep.12263, 2018.

III. Cheng, X., Voss, U., and Ekblad, E. Tuft cells: Distribution and connections with nerves and endocrine cells in mouse intestine. Experimental Cell Research. In press, 2018

IV.   Cheng, X., Voss, U., and Ekblad, E. A novel serotonin-containing tuft cell subpopulation in mouse intestine. Manuscript.

Abbreviations

5HT	5-hydroxytryptamine, serotonin
AADC	Aromatic L-amino acid decarboxylase
AMPK	AMP-activated protein kinase
CC	Compound C
CCA	Common carotid artery
CCH	Chronic cerebral hypoperfusion
CCK	Cholecystokinin
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
DAB	Diaminobenzidine
DCLK1	Doublecortin-like kinase 1
EC	Enterochromaffin
EEC	Enteroendocrine cells
ENS	Enteric nervous system
FBS	Fetal bovine serum
HTX	Hematoxylin
IBD	Inflammatory bowel disease

IBS	Irritable bowel syndrome
ICA	Internal carotid artery
IL	Interleukin
IR	Immunoreactive
Gal-3	Galectin-3
GCIR	Global cerebral ischemia with reperfusion
GI	Gastrointestinal
GLP-1	Glucagon like peptide-1
HuC/D	Human neuronal protein
LI	Large intestine
LPS	Lipopolysacharide
MCA	Middle cerebral artery
nNOS	Neuronal nitric oxide synthase
PBS-T	PBS with 0.25% Triton X-100
pMCAO	Permanent middle cerebral artery occlusion
PGP	Protein gene product
PYY	Peptide YY
SERT	Serotonin transporter
SI	Small intestine
SP	Substance P
TAK-1	TGF-β-activated kinase

TLR-4	Toll-like receptor 4
TNF- α	Tumor necrosis factor α
TPH	Tryptophan hydroxylase
TRMP5	Transient receptor protein channel 5
VIP	Vasoactive intestinal peptide

Popular Summary

Part 1: From Brain to Gut

The brain is an organ with a high energy requirement. To meet this demand, the brain needs a continuous and well-regulated blood supply. In situations where the brain, or parts of the brain, does not receive sufficient blood flow injury arises. This condition, commonly known as cerebral ischemia, or stroke, is prevalent in today's society. In 2015, more than 6 million people died from stroke worldwide. In parallel to the central injury, more than half of affected patients report gut dysfunctions such as constipation, dysmotility and incontinence after the ischemic event. In recent years an increased awareness and focus on the gut-brain axis has emerged. This bidirectional axis of communications has been shown to be involved in several situations, such as anxiety, depression, Parkinson's disease and stroke.

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In addition to taste thrill by the tongue, food also triggers sensations in the gastrointestinal tract. Similar to taste buds and taste receptors, specialized "intestinal sensor cells" in the gastrointestinal epithelium are able to sense and mediate intestinal chemosensation. This includes sensing nutrients as well as harmfully components in the luminal environment. One of the epithelial cells possessing a chemosensory potential is the tuft cell. This cell is recognized by its fusiform shape and distinct apical "tuft" of microvilli extending into the lumen. Tuft cells are able to detect bitter, sweet and umami substances in the lumen through the expression of taste cell transduction proteins, α-gustducin, and activating taste related cation channel, transient receptor potential channel 5. Tuft cells also contribute to the maintenance and protection of the gastrointestinal barrier as well as triggering type 2 immune reactions. Tuft cells are a rare cell type in the gut, and its roles in health and disease are just starting to be investigated.

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Sammanfattning på svenska

Del 1: Från hjärna till tarm

Hjärnan är ett energikrävande organ. För att möta detta stora energibehov så behöver hjärnans blodflöde vara kontinuerligt och välreglerat. I situationer där hjärnan, eller delar av hjärnan, inte försörjs på ett tillfredställande sätt så uppstår skador, något som är relativt vanligt förekommande och kallas cerebral ischemi, eller stroke. Under 2015 dog mer än 6 miljoner mäniskor i världen av stroke. Efter stroke så beskriver mer än hälften av de drabbade patienterna att de, förutom hjärnskadan, upplever mag-tarm problem såsom förstoppning, motorikstörningar eller fekal inkontinens. Under senare år har en ökad medvetenhet och ett ökat fokus på den s.k. brain-gut-axis uppstått. Detta begrepp står för en kommunikation mellan de båda organen, i båda riktningarna, vid olika situationer såsom ångest, depression, Parkinsons sjukdom och stroke.

En nyckelspelare vad avser tarmens reglering är det enteriska nervsystemet, ibland kallat "second brain" eller "bukhjärnan". Tarmfunktionsrubbningar föreslås ha sitt ursprung i obalans i det enteriska nervsystemet, eftersom detta nervsystem reglerar tarmrörelser, sekretion, blodflöde och även interagerar med immunsystemet och det endokrina systemet.

I avhandlingens första del utreds hur olika modeller av cerebral ischemi, utförda i mus, påverkar det enteriska nervsystemet vad avser överlevnad

och neuroplasticitet. Utifrån dessa undersökningar kunde vi visa att en typ, fokal ischemi, orsakade en markant förlust av enteriska nervceller, och också förändringar i uttrycket av signalsubstanser. Bakomliggande mekanism förslås involvera nerv-immun interaktioner och inkludera galectin-3 aktivering av toll-like receptor 4. Vi kunde också visa att global cerebral ischemi och cerebral hypotension inte negativt påverkade enteriska nerver vilket tyder på att olika typer av ischemi triggar igång olika typer av nerv-immuninteraktioner.

Del 2: Från tarmlumen till tarm

Förutom smakupplevelsen på tungan så sätter mat också igång sensoriken i mag-tarmkanalen. I likhet med smaklökar och smakreceptorer så kan specialiserade "intestinala känselceller" känna av och vidarebefordra kemosensorik i tarmen. Detta innefattar att känna av näringsämnen såväl som skadliga komponenter i tarmlumen. En av de epitelceller som tillskrivs kemosensorik är tuftcellen. Denna cell känns igen på att den är spolformig och har en "tofs" (eng. "tuft") av långa utskott (mikrovilli) in mot lumen. Tuftceller kan detektera smakerna bitter, sött och umami eftersom de, i likhet med smakceller, uttrycker transduktionsproteinerna α-gustducin och transient receptor potential channel 5. Tuftceller har även förmåga att upprätthålla och skydda tarmmukosabarriären och att igångsätta typ 2 immunreaktioner. Tuftceller är sparsamt förekommande i tarmen och deras betydelse för hälsa och sjukdom har nyligen börjat undersökas.

课题简介

第一部分：脑-肠

大脑是一个高能耗、低存储的特殊器官，其正常运转依赖于源源不断的血液供应。一旦发生血供不足，部分脑组织将会遭受严重损伤，临床称之为中风。近年来，中风发病率高，仅在 2015 年全球就有超过 600 万人死于中风。奇怪的是，半数以上的中风患者不仅有脑损伤，还伴发一系列胃肠功能障碍的症状，如便秘、动力障碍、失禁等。脑肠轴（brain-gut axis）引起了广泛关注。脑肠轴双向调节，在焦虑症、抑郁症、帕金森综合征、中风等疾病中都有表现。

肠神经系统（enteric nervous system）是胃肠功能的主要调控系统，也被称为"第二大脑"。胃肠道的动力，分泌，血流，免疫等重要功能都受肠神经系统支配，故而胃肠功能障碍很可能根源于肠神经系统紊乱。

在第一部分，我们重点观察了不同类型的脑缺血对肠神经元的影响。我们发现只有局部脑缺血造成了肠神经元损伤与重塑。这可能是由于中枢系统损伤后释放 galectin-3，激活 TLR4 而引起的神经免疫反应，牵连到肠道神经系统。而全脑缺血再灌注与慢性低血容量灌注型脑缺血却并没有引起肠神经元损伤或重塑，可能是不同类型的脑缺血激活炎症反应机制不同。

第二部分：腔-肠

味觉刺激不仅存在于口腔，也存在于胃肠道。胃肠道上皮细胞中也有自己的味蕾 ——一种"感受器细胞"，用来感知和调节肠腔内的化学变化，分辨营养物质与有害成分。Tuft 细胞便是一种重要的肠上皮感受器细胞。这类细胞形似纺锤，顶生微绒毛，能表达味觉细胞转导蛋白 α-促胃液素，激活味觉相关阳离子通道 TRMP5。Tuft 细胞能感受到肠腔内苦、甜、鲜等物质。同时，tuft 细胞还能触发 2 型免疫反应，保护胃肠屏障。但由于 tuft 细胞是一种罕见的细胞型，其细胞功能及其在疾病中作用等研究刚刚起步。

在本文第二部分，我们研究了在小鼠肠道中 tuft 细胞的分布情况，及其与神经纤维、内分泌细胞的关联情况。我们发现 tuft 细胞大量聚集于近端小肠，并呈现出近端—远端梯度逐减的规律。我们进一步发现大量 tuft 细胞与肠粘膜感受神经纤维、食欲相关性内分泌细胞接触紧密，由此推断 tuft 细胞可能是肠腔信号与宿主之间的重要界面。

在这一部分，我们首次观察到了 tuft 细胞的新亚型，这种细胞亚型顶端含五羟色胺（5HT）。此亚型在小鼠近端小肠高占 80%，但在大肠中却极为罕见。目前我们尚不能确定其五羟色胺来源，因为 tuft 细胞中既未发现五羟色胺合成酶，也未发现五羟色胺转运蛋白。然而，我们认为这种亚型细胞在肠道中有其独特的作用。

Introduction

The gastrointestinal tract

The inner lining of the gastrointestinal (GI) tract makes up a large and vulnerable surface facing the luminal environment. It fulfils two important roles: to absorb nutrients and, simultaneously, to defend against pathogens and harmful elements contained in the GI lumen. Such elaborate and complex work requires diverse cellular cooperation.

The structure of the GI tract

While the different regions of the GI tract perform different and diverse roles along the, in man 5-6 m long, canal their anatomical structure is similar. It consists of four layers, from inside to out; these are mucosa, submucosa, muscularis propria and serosa. The mucosa consists of columnar epithelium, lamina propria and muscularis mucosa. This layer is the prime barrier separating the host and the outside environment. The submucosa is made up of dense connective tissue and is situated underneath the mucosa. A ganglionated plexus, the submucous ganglia, and glands are present in this layer. The muscularis propria is composed of two sublayers of smooth muscle cells, an outer longitudinally and an inner circularly orientated, and in between them are the myenteric ganglia positioned. Outmost the GI tract is covered by a serosa.
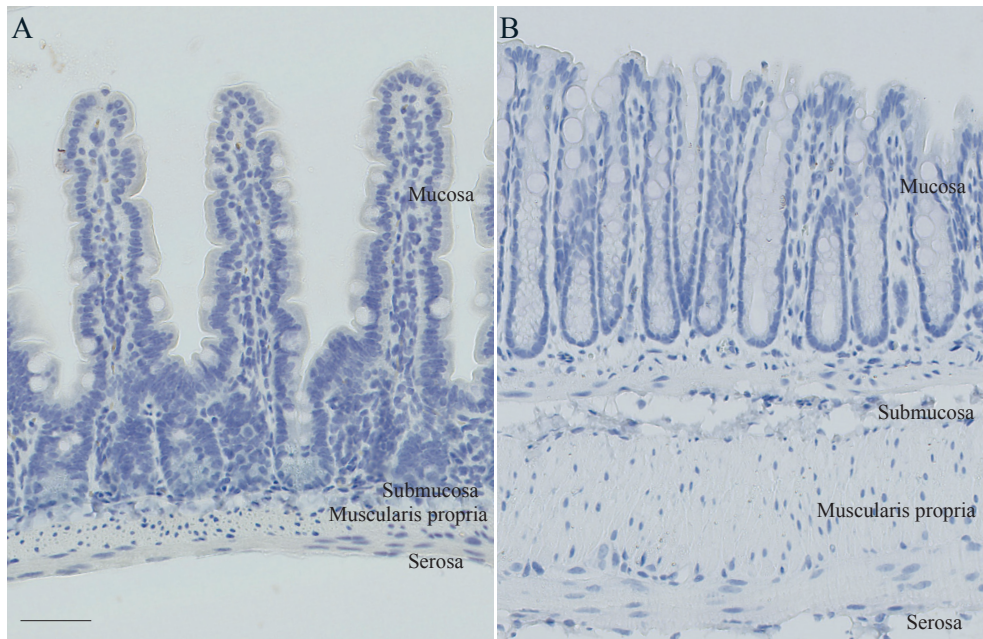
Figure 1 The structure of the GI tract. Paraffin sections stained with hematoxylin of small (A) and large (B) intestine from naive mouse showing the four principle layers of the intestinal wall. Bar = 50 μm

Enteric nervous system

The myenteric and submucous ganglia comprise the enteric nervous system (ENS). This extensive, intrinsic system innervates the GI tract and plays important roles in regulating GI motility, secretion, local blood flow and it also interacts with the immune and endocrine systems [1]. The ENS is an independent and integrative nervous system influencing, and being influenced by, the central nervous system (CNS, reviewed by [1,2]). The myenteric ganglia and submucous ganglia are highly interconnected and nerve fibers from both innervate the smooth muscle layers, the submucosa, the mucosa, endocrine cells and blood vessels.

Classification of enteric neurons

Neurons in the ENS can be classified based on e.g. their morphology, function, electrochemical properties or neurotransmitter content.

1 Morphology: based on neuronal shape and numbers of dendrites and axon, enteric neurons can be roughly divided into Dogiel I-VII types. Most neurons are type I-III [3,4]. In addition, neurons can be classified based on size, shape and electron density of their terminal vesicles.

2 Function: enteric neurons can be divided into intrinsic primary afferent neurons (IPANs, sensory neurons), interneurons, motor neurons, and intrinsic intestinofugal afferent neurons (IFANs). Stimulus in GI tract is detected by IPANs, an impulse is generated and transferred to motor neurons via interneurons. IFANs send neuronal projections to pancreas, bile ducts and extrinsic, in particular sympathetic, ganglia [5].

3 Electrophysiological properties: in principle two types of neurons, synaptic type (S neurons) and after-hyperpolarization type (AH) neurons are identified. Fast, large amplitude potentials are transmitted by S neurons, and slow postsynaptic potentials by AH neurons [3,6,7].

4 Neurotransmitters: to identify subsets of enteric neurons on basis of their transmitter content is a widely used procedure [8,9].

Neurotransmitters

A large number of neurotransmitters are expressed and released by the ENS. Its neurotransmitter diversity resembles that of the central nervous system (CNS). In general, excitatory transmitters, such as acetylcholine and substance P, stimulate motility, increase intestinal secretion, release enteric

hormones and dilate blood vessels. Inhibitory peptides, such as vasoactive intestinal peptide (VIP) and nitric oxide (NO), play important roles in the peristaltic reflex of GI tract [6,9,10]. Enteric neurons often co-express and co-release two or more different neurotransmitters. Neurotransmitters examined in this thesis are described below.

Vasoactive intestinal peptide (VIP)

VIP, is a 28 amino acid peptide and a member of the glucagon/VIP peptide family [11-13]. It is abundant in both nerve cell bodies and nerve fibers in the GI tract and is to a large extent colocalized with neuropeptide Y [14-16]. VIP expressing neurons are particularly numerous in submucous ganglia, while VIP positive fibers are found in all layers of the gut wall [17,18]. VIP is an important non-adrenergic non-cholinergic (NANC) inhibitory neurotransmitter in the GI tract. The major functions of VIP include vasodilation, smooth muscle relaxation, modulation of inflammation and neuroprotection [19-23].

Nitric oxide (NO)

NO, produced by NO synthase (NOS), is also a NANC inhibitory neurotransmitter in the GI tract [19]. Three NOS isoforms, neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) exist [24]. The vast majority of nNOS expressing neurons is located in the myenteric ganglia and nNOS positive nerve fibers are frequently observed within the smooth muscle layers but sparse in the mucosa/submucosa [18,25]. As an inhibitory neurotransmitter, NO causes relaxation of intestinal smooth muscle, but has also been ascribed important roles in protection and maintenance of enteric

neurons [20,26]. Loss of NO secreting neurons is reported to result in intestinal functional disorders [27-29].

Calcitonin gene-related peptide (CGRP)

CGRP is a 37 amino acid neuropeptide belonging to the calcitonin/CGRP family of peptides and primarily located to sensory fibers centrally and peripherally [30]. Two major CGRP isoforms are found: αCGRP widely distributed in both central and peripheral nerves, and βCGRP mainly expressed in the ENS. They have similar structure and function but are synthesized by different genes. CGRP-IR fibers, either of intrinsic or extrinsic origin, innervate all layers within the intestine and are particularly abundant in myenteric ganglia [31-33]. Nerve fibers co-expressing CGRP and substance P are considered as primary afferents of extrinsic origin [31,34,35]. CGRP is a highly potent vasodilator and improves mesenteric blood flow [36].

The thesis consists of two parts which in the following will be described separately.

Part 1: From Brain to Gut

Brain-gut axis

The brain-gut axis is a bidirectional pathway, through which various signals are exchanged. This axis has attracted much attention since diseases originating in one of the involved organs often affect the function of the other. It has for example been shown that anxiety [37] and depression [38], as well as neurodegenerative disorders such as stroke [39] are associated

with alterations in gut microbiota composition. GI complications are also associated with CNS damages and e.g. Parkinson's disease [40,41] and stroke [42] patients often suffer GI complications. Several homeostatic systems besides the CNS and the ENS have been found to be involved in gut and brain interactions, e.g. the autonomic nervous system (ANS), the immune system and the gut microbiota (reviewed by [43,44]).

Cerebral ischemia

Cerebral ischemia, also called stroke, is the second leading cause of death and the third leading cause of disability worldwide. In 2015 more than 6 million people died from stroke [45]. Complications, such as paralysis, cardiac disease, pneumonia, constipation and incontinence, as well as infections and sepsis, all reducing quality of life are common among the survivors [42,46,47].

There are two main types of cerebral ischemia, focal, caused by thrombosis or embolism [48,49], and global, caused by hypoperfusion or heart arrest [50]. Where focal ischemia results in insufficient blood supply to restricted parts of the brain, global ischemia affects widespread brain areas. Reperfusion following the ischemic insult, may further exacerbate the brain injury through increased levels of oxidative stress, inflammation and homeostatic imbalance [51].

In addition to the ischemic brain injury, studies on cerebral ischemia in mice have shown altered GI balance and dysfunction. These include mucosa barrier disruption, increased levels of circulating ghrelin [52],

reduced T and B cell counts in Peyer's Patches, and bacterial translocation [53], implying a complex brain-gut interaction in the post stroke situation.

Neuronal energy metabolism

Cerebral ischemia is known to lead to neuronal stress including imbalanced energy supply [51,54,55]. Glucose and oxidative phosphorylation were previously believed to be the main driver for neuronal ATP production, but neurons also utilize lactate and even lipids as energy [56-60].

Utilization of energy is by break down of ATP into ADP, which can be further converted to adenosine monophosphate (AMP). When cellular energy is depleted (increased AMP:ATP ratio), AMP protein kinase (AMPK) is rapidly activated. AMPK is a heterotrimeric protein complex that regulates cellular energy status by monitoring the concentrations of ATP, ADP and AMP. AMPK is able to switch on and off catabolic and anabolic pathways, thus adjusting energy imbalances [61-64].

Neuroplasticity and neuropathy

Neuroplasticity is the ability of neurons to adapt both to intrinsic and environmental stimuli by changing its structure, numbers, or transmitter expressions during development and in pathophysiological situations [65-68]. The ENS displays a high degree of neuroplasticity, for example, up-regulated neuronal expressions of VIP and/or nNOS have been shown in neuronal stress situation and are believed to be part of a cellular protection mechanism [20,21,64,69,70].

Enteric neuroplasticity may alter GI functions, and be linked to digestive disorders [22,71], including chronic pain, nausea, bloating, abnormal distention, constipation and incontinence. Mal-adaptive changes lead to neuropathies like neuro-degeneration and /or inflammation [68]. Reduction in neuronal numbers, swollen nerve cell bodies, aberrant mitochondria, cytoplasmic vacuolization and neuronal structure loss are signs of a degenerative ENS [72]. Inflammatory enteric neuropathy is characterized by lymphocytes infiltrating the enteric ganglionated plexuses [73,74]. Experimental evidence suggests that myenteric ganglia are more vulnerable to neuropathies, compared to the submucous ones, but the reason behind is unknown [73,75].

Innate immune response

The innate immune system within the GI tract provides short-term defense against invading pathogens, and tolerance to dietary and commensal microbiota [76]. Several cell types, including macrophages, mast cells, and lymphoid cells, take part in the innate immunity response [76]. It is noteworthy that the GI tract comprises the largest mass of lymphoid tissue in the body. The innate immune system identifies and responds through pattern recognition receptors (PRR), which includes the membrane associated toll-like receptors (TLR) [77-79].

Galectin-3 (gal-3)

Gal-3 is a member of the β-galactoside-binding protein family, containing a C-terminal carbohydrate-recognition domain (CRD) [80]. Gal-3 was recently found to be secreted from IBA-1 (a marker of microglia and

peripheral macrophages) positive cells [81]. Gal-3 shows ubiquitous subcellular distribution with multiple intra- and extracellular effects. Its expression significantly elevates upon stimuli, e.g. lipopolysaccharide (LPS) exposure, and it plays both pro- and anti-inflammation roles [81]. In the GI tract, gal-3 is suggested to stabilize cell-cell junctions and enabling polarization of intestinal epithelial cells. Furthermore, it has been associated with GI cancers, as well as with Crohn's disease and ulcerative colitis [82,83]. Gal-3 was recently shown to be a ligand for TLR4 [81].

Part 2: From Lumen to Gut

The intestinal epithelium

The barrier between the luminal environment and the host is made up of a single cell layer, the intestinal epithelium. This layer comprises different cell types, including enterocytes, goblet cells, Paneth cells, enteroendocrine cells (EECs), and tuft cells, each optimized to adopt its engagement in the ever-changing luminal environment. The intestinal epithelium is an important interface in the body. It is essential for chemosensation, absorption, water and electrolyte secretion, as well as constituting the key barrier against uncontrolled entry of microbes and harmful substances into the body. Chemosensation in the gut is mediated by specialized epithelial cells, the tuft cells and EECs [84,85].

Tuft cells

Tuft cells in the GI tract were initially described in stomach and duodenum of mice; recognized by their characteristic fusiform shape and distinct tuft of microvilli [86]. Tuft cells contain an intracellular tubular network spanning between microvilli and endoplasmic reticulum, enabling molecular exchange between lumen and cell [87]. They also issue lateral projections reaching into the nuclei of neighboring cells, possibly enabling them to cross talk [87]. Close contacts between tuft cells and enteric nerve fibers are also reported [88-90]. Tuft cells make up 0.4-2% of the cells in the intestinal epithelial lining and they are preferentially located in villi and crypt-villus junctions [91,92]. Due to their expression of taste cell specific GTP-binding protein, α-gustducin, and taste-related TRMP5 [93], tuft cells are considered as luminal sensors. Recent studies also revealed their role in triggering interleukin (IL) -25 associated immune reactions leading to expulsion of pathogens and to be involved in epithelial protection and regeneration [94-96].
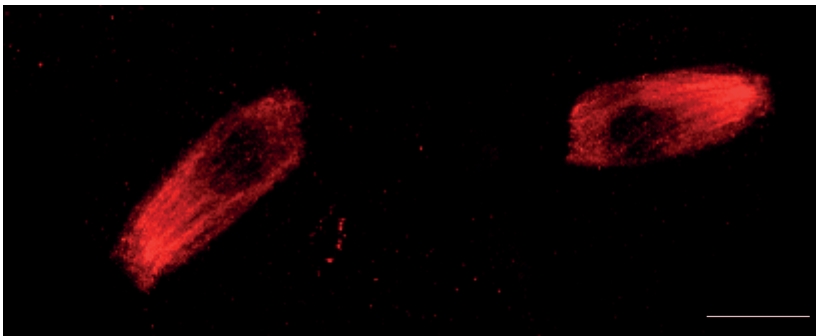

Figure 2 morphology of tuft cells. Cryostat section of small intestine from naive mouse immunostained for DCLK1, a tuft cell marker. Bar = 10 μm

Enteroendocrine cells

The GI tract comprises the body's largest population of endocrine cells, often identified and described by their hormone signature. In response to nutrient stimulation several hormones, e.g. cholecystokinin (CCK), peptide YY (PYY) and glucagon-like peptide-1 (GLP-1), are secreted from EECs (reviewed by [97,98]). These hormonal signals can be transmitted to nerves or into the circulation.

Cholecystokinin

The CCK peptide occurs in different forms, identified by the number of amino acids it contains, such as CCK58, CCK33, CCK22 and CCK8 [99]. The length of CCK depends on post-translational modification of its precursor, prepro-CCK [100]. All members of the gastrin/CCK family, have a common C-terminal amino acid sequence [101,102]. CCK is mainly secreted by EECs in the upper small intestine [103], and its release is strongly stimulated by infusion of fatty acids. CCK facilitates digestion by increasing secretion of pancreatic and bile juices but also by short-term inhibition of gastric emptying and acid secretion [104-106]. In addition, it induces satiety [107].

Peptide YY

PYY is an important appetite suppression hormone, often co-expressed with GLP-1 in a subpopulation of EECs. It is a 36 amino acid peptide and belongs to the neuropeptide Y family [108]. Most PYY is produced in ileum and colon, and its release is stimulated by intestinal infusion of fat [108,109]. It suppresses food intake by activating Y2 receptors in the

hypothalamus and vagal afferent neurons, as well as by inhibiting gastric acid secretion and motility [110].

Glucagon-like peptide-1

GLP-1, a gut-derived incretin hormone, is produced by EECs. GLP-1 containing EECs are abundant in ileum and colon [111]. GLP-1 release is strongly stimulated by luminal glucose and fat and improves glucose tolerance by elevating insulin secretion from pancreatic β cells and inhibits food intake by reducing glucagon secretion and by delaying gastric emptying [112]. Based on these functions, GLP-1 mimetics are used in patients with type II diabetes and/or obesity in clinics [113,114].

Serotonin

Serotonin (5-hydroxytryptamine, 5HT) is a bioactive monoamine synthetized from the amino acid L-tryptophan. It plays important roles both centrally and peripherally. The GI tract harbors the largest store of 5HT in the body, and the vast majority of 5HT is synthesized, stored and secreted by enterochromaffin cells, a subset of EECs [115,116]. Enteric neurons and mast cells are also found to generate small amounts of 5HT in the GI tract [117]. 5HT secreting enterochromaffin cells are distributed in both small and large intestine, with the highest density in upper small intestine. Tryptophan hydroxylase (TPH) is the rate-limiting enzyme in 5HT synthesis. Two isoforms of TPH exist, TPH1 used by enterochromaffin cells and TPH2 by neurons [118]. In the GI tract, 5HT from enterochromaffin cells can either be released into the lumen from the apical part or to the circulation and local environment from the basal part [119]. Extracellular 5HT are recycled by reuptake into nearby cells by a selective

serotonin transporter (SERT) [116]. In the GI tract, 5HT is involved in many physiological functions, including motility, secretion, blood flow and sensation [115].

$$\text{Tryptophan} \xrightarrow{\text{TPH}} \text{5HTP} \xrightarrow{\text{AADC}} \text{5HT} \xrightarrow{\text{SERT}} \text{Reuptake}$$

Figure 3 5HT synthesis and reuptake. 5HT: 5-hydroxytryptamine or serotonin, 5HTP: 5HT precursor, AADC: Aromatic L-amino acid decarboxylase, SERT: serotonin transporter, TPH: Tryptophan hydroxylase.

Methods

Part 1 From Brain to Gut (Papers I and II)

Experimental design

In vivo
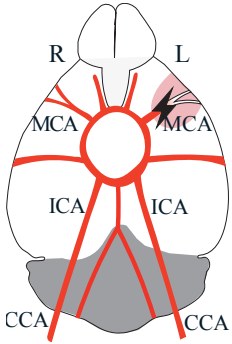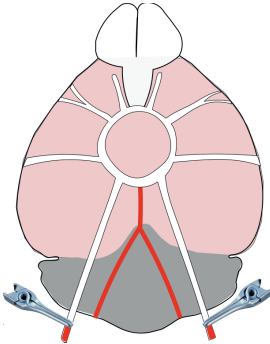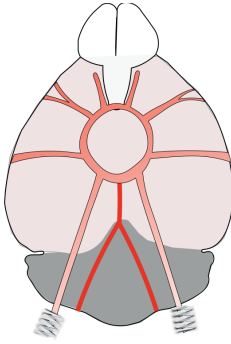
	pMCAO	GCIR	CCH
Illustration			
Occluded arteries	Middle cerebral artery, left side	Common carotid arteries, bilateral and transient	Common carotid arteries, 30% bilateral reduction of blood flow
Duration ischemia	Permanent (6 h, 3 d and 7 d)	13 min + 2 w reperfusion	17 w
Animals	Gal-3$^{+/+}$ and gal-3$^{-/-}$	Naive	Naive
Sampling	Ileum, colon, blood serum	Ileum	Ileum
Examination	Neuronal numbers, neuroplasticity, morphology		

Table 1 Experimental design of *in vivo* experiment. Gal-3$^{+/+}$ and gal-3$^{-/-}$ mice were subjected to either cerebral ischemia or sham surgery. The enteric neuronal numbers, neuroplasticity and intestinal morphology were estimated at selected time points. The affected brain area was labeled by pink color. CCA: common carotid artery, CCH: chronic cerebral hypoperfusion, Gal-3: galectin-3, GCIR: global cerebral ischemia with reperfusion, MCA: middle cerebral artery, ICA: internal carotid artery, pMCAO: permanent middle cerebral artery occlusion.

In vitro

Cultured myenteric neurons from C57BL/6 mice were exposed to:

1   Serum from gal-3$^{+/+}$ mice, sham or pMCAO operated

2   Serum from gal-3$^{-/-}$ mice, sham or pMCAO operated

3   Purified gal-3 with or without inhibitors of TAK1 or AMPK

4   Serum from gal-3$^{+/+}$ mice with or without inhibitors of TAK1 or AMPK

Cultured myenteric neurons from TLR4$^{-/-}$ mice were exposed to

1   Serum from gal-3$^{+/+}$ mice, sham or pMCAO operated

2   Serum from gal-3$^{-/-}$ mice, sham or pMCAO operated

3   LPS

In vivo experimentation

Cerebral ischemia models

Three different stroke models were used (see Table 1): focal cerebral ischemia (mimicking thrombus and embolism in the clinics), and two models of global cerebral ischemia (mimicking heart arrest or chronic low blood pressure). Focal cerebral ischemia was modeled by pMCAO, which permanently occludes the left sided middle cerebral artery. Global cerebral ischemia was performed by either transient occlusion of both common carotid arteries following reperfusion (global cerebral ischemia with reperfusion, GCIR), or 30% reduction of blood flow from the common carotid arteries to the brain (chronic cerebral hypoperfusion, CCH). Animals were all of C57BL/6 background (papers I and II), gal-3$^{+/+}$ and

gal-3$^{-/-}$ mice were used (Paper I). Surgical procedures are described in detail in papers I and II.

Tissue and blood sampling

On the day of sacrifice animals were deeply anesthetized and guillotined after which intestines and blood were sampled. An incision was made along the abdominal midline and visceral organs exposed. The intestines were removed and fixed in 4% buffered paraformaldehyde (PFA) or Stefanini's fixative, rinsed in tyrode solution containing 10% sucrose three times before segments of ileum and colon were cut out, orientated and mounted for longitudinal and cross section in FSC 22 Clear, frozen on dry ice and sectioned. Sections were processed for immunocytochemistry.

Collected blood was centrifuged 5 min at 1000 rcf, serum collected and pooled in respective treatment groups. Aliquots (20 μl) were stored at -80 °C until use (paper I only).

In vitro experimentation

Primary myenteric neuronal cultures

Myenteric neurons were dissociated from mouse small intestine (see paper I for details). Cell cultures were prepared by adding 50 μl of a constantly mixed cell suspension into 8-well chambers prefilled with 450 μl cell culturing medium. From each animal two 8-well chambers were prepared. Fresh medium containing applicable experimental test agents was added to cultures after a four-day equilibration culture period. Control wells were cultured in parallel. After 4 days, cells were fixed in Stefanini's fixative, rinsed in tyrode solution containing 10% sucrose, frozen, thawed and

processed for immunocytochemistry. Cultures were obtained from C57BL/6 mice as well as from TLR4$^{-/-}$ mice (Paper I).

Part 2 From Lumen to Gut (Papers III and IV)

Experimental design

Distribution (Paper III)

CCK

Satiety associated EECs — GLP-1

PYY

Tuft cells — Contacts (Paper III) — PGP 9.5

Nerves

CGRP

Distribution

TPH

New subset: DCLK1/5HT-IR cells (Paper IV) — 5HT source

SERT

DCLK1 in contact with 5HT-IR EECs
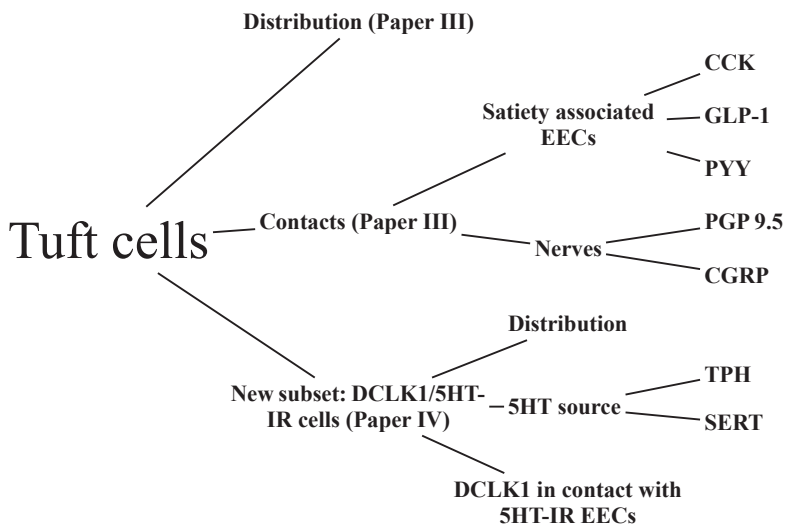
Figure 4 Experimental design of part 2. The regional distribution of tuft cells and their spatial connections with EECs and mucosal nerve fibers were investigated in paper III. A novel subset of tuft cells, DCLK1-5HT-IR cells, were described in paper IV, in terms of number, distribution, possible synthesis 5HT and contact with 5HT-containing ECs. 5HT: 5-hydroxytryptamine, CCK: cholecystokinin, CGRP: calcitonin gene-related peptide, DCLK1: doublecortin like kinase 1, EC: enterochromaffin cell, EEC: enteroendocrine cells, GLP-1: glucagon-like peptide-1, PYY: peptide YY, SERT: serotonin transporter, TPH: tryptophan hydroxylase

Swiss roll technique for intestinal tissue preparation (Papers III and IV)

The Swiss roll technique is an ideal method when preparing long intestinal segments for histology (Figure 5). In brief, mouse intestine was removed and opened along the mesenteric line, and its content washed out. The small intestine was divided in five equally long segments while the large intestine was kept intact (Figure 5, A and B). The segments were coiled on wood sticks to form Swiss rolls and carefully placed into cassettes (Figure 5, C). For paraffin sectioning, the cassettes were immersion fixed in PFA overnight at 4 ^{o}C, rinsed in 70% ethanol three times, dehydrated, cleared and embedded in paraffin (paper III only). For cryo sectioning (Papers III and IV), the cassettes were fixed in Stefanini's fixative, rinsed in tyrode solution (containing 10% sucrose) and processed for immunocytochemistry.
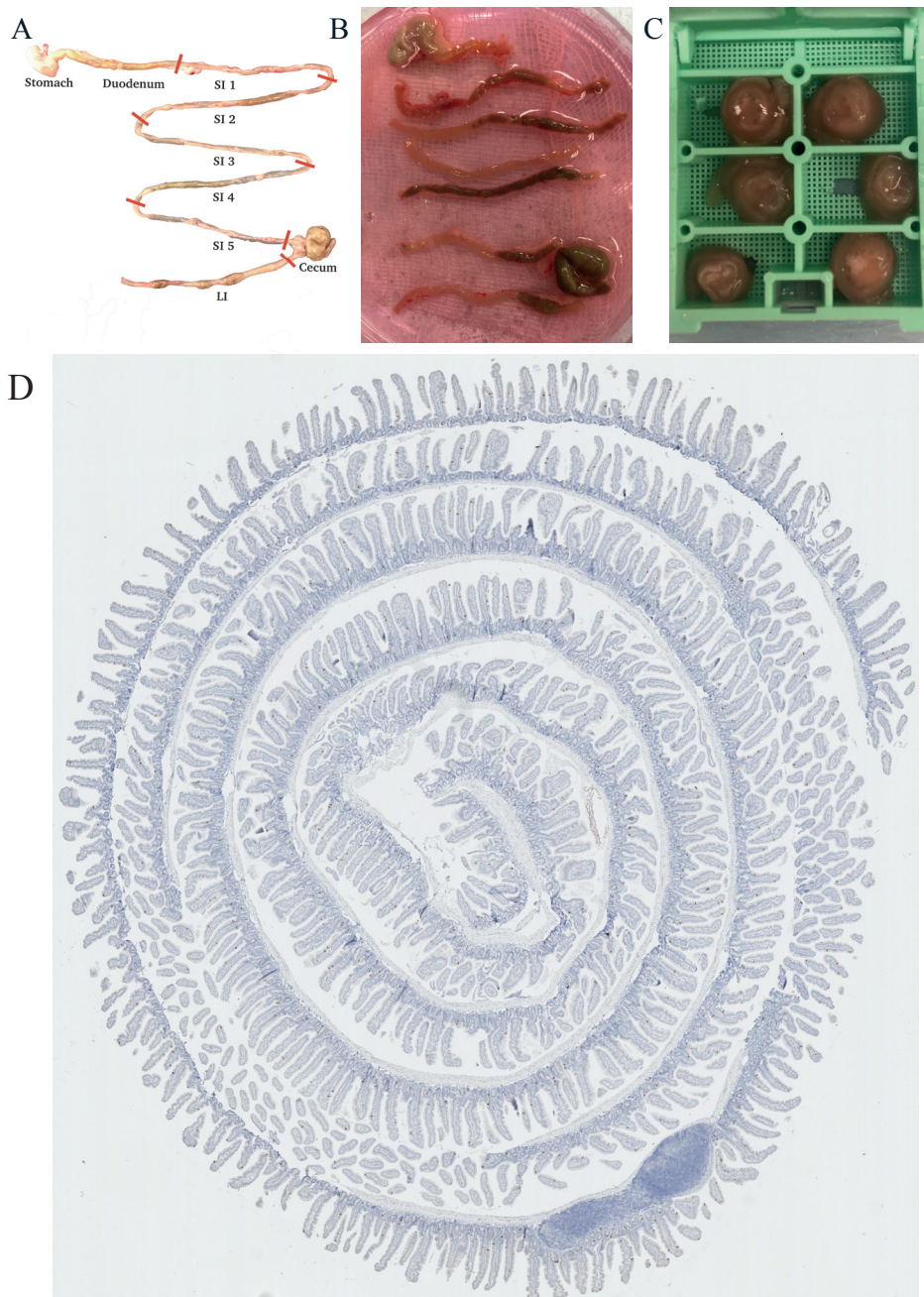
Figure 5 the procedure of Swiss roll technique. A) and B) Intestines were harvested. Small intestine was equally divided into 5 segments, labeled SI 1-5 and the large intestine was undivided. C) Intestines were cleaned, coiled into "Swiss roll" and placed in cassettes. D) Histological image of intestinal segment with hematoxylin staining (SI 2).

Part 1 and 2: Immunocytochemistry

Immunocytochemistry is a powerful tool for cellular identification of peptides or cellular markers. The majority of results, both from *in vivo* and *in vitro* experiments, in this thesis are obtained by using immunocytochemistry.

Primary antibodies directed against particular molecular or protein targets were visualized using indirect detection techniques with fluorophore (Daylight or Alexa Fluor) conjugated secondary antibodies against the primary IgG. In papers I and III, signal stain Boost IHC detection reagent (paper I) or IgG ImmPRESS reagent (paper III) were also used together with 3,3'-diaminobenzidine (DAB) substrate reaction to detect primary antibodies in tissues. Toluidine blue (papers I and II) or hematoxylin (HTX, paper III) was used to visualized general tissue morphology.

Reagent	Content
Cell culture medium	NBA with 10%v/v fetal bovine serum, 0.5 mM L-glutamine and 50 U/mL penicillin, 50 µg/mL streptomycin
Citrate acid buffer	0.01 M sodium citrate in distilled water, adjust to pH 6
PBS-T	Phosphate buffer saline (PBS) with 0.25% Triton X-100
PFA	4% paraformaldehyde in 0.1 M phosphate buffer
Stefannini's fixative	0.2% picric acid, 2% formaldehyde in 0.1 M phosphate buffer, pH 7.2
Tyrode's solution	Isotonic solution of NaCl, KCl, $CaCl_2$, $MgCl_2$, NaH_2PO_4, $NaHCO_3$, with 10% sucrose added

Table 2 A list of buffers and solutions used in this thesis

Antigen	Paper#	Host	Working Dilution	Source and Code #
5HT	IV	Goat	1:1000	Abcam; 66047
5HT	IV	Rabbit	1:1000	Produced and donated by Professor Steinbush; Ser1-16
CCK	III	Guinea pig	1:2000	Produced and donated by Professor J Rehfeld, Rigshospitalet, Copenhagen University, Denmark
CGRP	III	Rabbit	1:4800	Euro-Diagnostica; 8724
DCLK1	III and IV	Rabbit	1:250	Abcam; 37994
GLP-1	III	Goat	1:250	Santa Cruz; sc7782
HuC/D	I and II	Mouse	1:400	Thermo Fisher Scientific; A21271
nNOS	II	Rabbit	1:1200	Euro-Diagnostica; 9223
PGP 9.5	I and III	Rabbit	1:1600	Ultraclone; RA95101
PYY	III	Guinea pig	1:2400	Euro-Diagnostica AB; 8704
SERT	IV	Goat	1:100	Abcam; 130130
TPH	IV	Sheep	1:800	Abcam; 32821
VIP	II	Rabbit	1:1200	Euro-Diagnostica; 7852

Table 3 A list of primary antibodies used in this thesis. 5HT: 5-hydroxytryptamine, CCK: cholecystokinin, CGRP: calcitonin gene-related peptide, DCLK1: doublecortin like kinase 1, GLP-1: glucagon-like peptide-1, Hu: human neuronal protein, nNOS: neuronal nitric oxidase synthase, PGP: human gene product, PYY: peptide YY, SERT: serotonin transporter, TPH: tryptophan hydroxylase, VIP: vasoactive intestinal peptide.

Statistics

Data is presented as mean ± standard error of mean (SEM) and analyzed by GraphPad Prism (GraphPad software Inc, USA). Significant differences were determined using one-way ANOVA (papers I-IV) or two-way ANOVA (paper I), with Turkey's multiple comparison test. P-value < 0.05 were considered significant.

Results and Discussion

Part 1 From Brain to Gut

Intestinal morphometrics, neuronal density and relative numbers of VIP-IR and nNOS-IR enteric neurons were assessed in all models of cerebral ischemia, these findings are presented in paper II. One model, pMCAO, displayed a remarkable loss of myenteric neurons in both ileum and colon at 3 and 7 days, but not 6 h after the occlusion. Mechanisms underlying this loss were investigated in paper I.

Focal, but not global, cerebral ischemia causes loss of myenteric neurons and upregulation of vasoactive intestinal peptide in mouse ileum (paper II)

In this study C57BL/6 animals were used, the time-point after which animals in each of the cerebral ischemia models were sacrificed were chosen based on manifestations in the brain. Sham operated animals sacrificed at the same time point served as controls. This led to time-points ranging from 1 week in pMCAO animals to 17 weeks in the CCH animals. Results showed that all models maintained a normal intestinal morphometry. Assessment of neuronal density in the ileum showed that one model, pMCAO, induced loss of myenteric neurons without loss of submucous neurons. No loss was present in the either of the other models. Analyses of the relative numbers of VIP-IR neurons showed pMCAO to

induce significantly increased numbers of VIP-IR submucous neurons, no alteration in relative numbers of VIP-IR neurons were seen in the other models. The relative numbers of nNOS-IR neurons were also assessed but was not found to be affected.

Gal-3 causes enteric neuronal loss in mice after left sided permanent middle cerebral artery occlusion, a model of stroke (paper I)

In this study the effects of pMCAO on enteric neuronal survival was investigated using gal-3^{+/+} and gal-3^{-/-} mice on a C57BL/6 genetic background. Time points ranged from acute (6 h) effects to more chronic effects (3 and 7 days) after either sham or pMCAO operation. In mice lacking gal-3 no losses of myenteric neurons in ileum and colon were observed after pMCAO. This was further investigated in vitro, using cultures of primary myenteric neurons isolated from C57BL/6 mice or TLR4^{-/-} mice.

In vitro **studies**

To investigate the mechanisms underlying the pMCAO induced neuronal loss and the apparent role of gal-3, primary cultures of myenteric neurons from C57BL/6 mice were exposed to serum from sham or pMCAO operated gal-3^{+/+} or gal-3^{-/-} mice. Compared to control neurons grown in parallel, exposure of serum from pMCAO operated gal-3^{+/+}, but not gal-3^{-/-}, mice caused a significant neuronal loss. A similar loss was observed in cultures exposed to purified gal-3.

Gal-3 was recently shown to be a TLR4 ligand. To analyze if TLR4 was involved in the pMCAO serum induced loss in vitro, primary cultures of myenteric neurons from TL4^{-/-} mice were exposed to serum from sham or pMCAO operated gal-3^{+/+} mice. We found that TLR4^{-/-} cultures were protected against the gal-3^{+/+} pMCAO serum-induced loss.

Lipopolysaccharide (LPS) is a TLR4 ligand. LPS has been shown to induce enteric neuronal loss through activation of a pathway involving TAK1 and AMPK [64]. To evaluate if gal-3 elicited the same pathway, primary cultures of C57BL/6 mice were exposed to purified gal-3 or gal-3^{+/+} pMCAO serum in the presence of inhibitors of either TAK1 or AMPK. Presence of inhibitors counteracted both the gal-3 and the gal-3^{+/+} pMCAO serum induced neuronal loss.

Since cerebral ischemia has been suggested to cause elevated levels of circulating LPS, sera from sham and pMCAO operated gal-3^{+/+} and gal-3^{-/-} mice were analyzed for LPS content. Low concentrations were found in all samples regardless of treatment or genotype.

Discussion part 1

In cerebral ischemia, reactive oxygen, nitrogen species and damage associated molecular pattern signals are released from the affected area activating both innate and adaptive inflammation, causing injury of central neurons. In the three models of cerebral ischemia tested we were able to show that one model, pMCAO, displayed significant peripheral effects on enteric neurons and their VIP expression. These findings were surprising since all models are shown to cause loss of central neurons (pMCAO and

CCH (Deierborg, unpublished); GCIR [120]). However, each model displays different time-resolutions in regards to occlusion duration, barrier integrity as well as immunological response [120-122] It is our belief that the reason underlying the different effects on enteric neurons are based on these parameters and the ability of the body to eliminate or stop the damaging signal cascade.

Through our investigation into the underlying mechanism of the enteric neuronal loss and VIP upregulation in the pMCAO model, we were able to pin point gal-3 as a key mediator of the neuronal loss. Gal-3 is an endogenous ligand of TLR4 [81], and is released from microglia post stroke [81]. In a previous in vitro study LPS, a TLR4 ligand [123], was shown to cause enteric neuronal loss through a pathway involving TAK1/AMPK [64]. We have been able to show that gal-3 mediate enteric neuronal loss through the same pathway as LPS. Since absence of gal-3, TLR4, as well as inhibitors of TAK1 and AMPK prevented the neuronal loss (Figure 6).
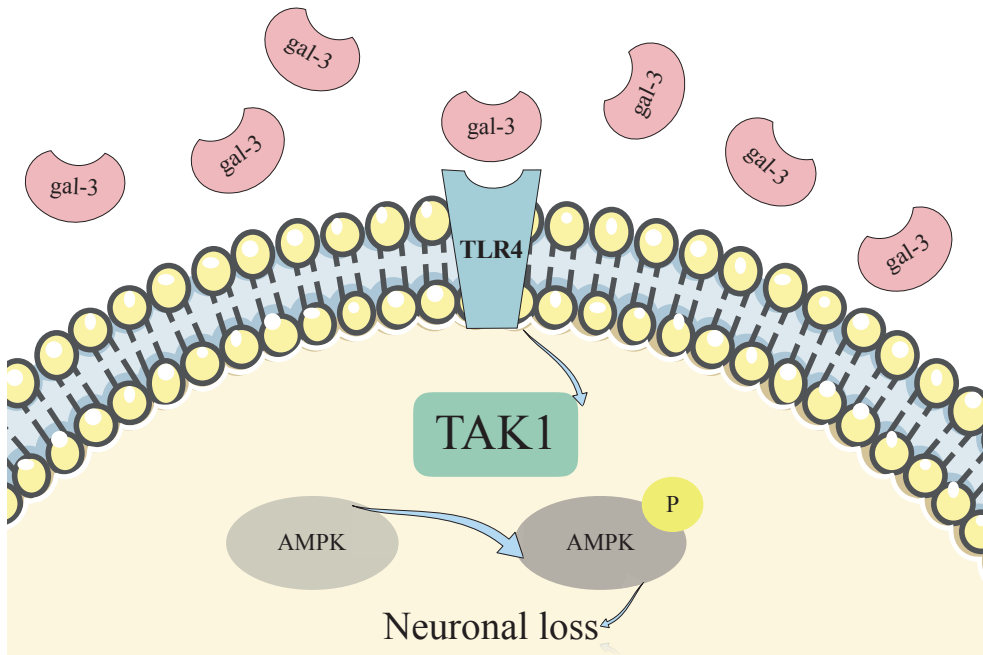
Figure 6 Overview of gal-3 activated TLR4 induced enteric neuronal loss, via TAK1/AMPK pathway. AMPK: AMP-activated protein kinase, Gal-3: Galectin-3, TAK-1: TGF-β- activated kinase 1, TLR-4: toll-like receptor 4

An elevated proportion of VIP-IR neurons in submucous ganglia were observed 7 days post pMCAO. It is our belief this is a neuronal survival response due to the pathophysiological situation. Neuroprotective properties of VIP in response to injurious factors have been found both in vitro and in vivo. One of the mechanisms by which VIP executes its neuroprotective effects on myenteric neurons is through modulation of immunological pathways including downregulation of TLR4 expression [23,124,125]. In addition, to the TLR4 downregulation VIP is also able to mediate neuroprotection through other pathways including improving the intestinal barrier. Thereby suggesting that the increase in VIP-IR neurons is a self-protection mechanism against gal-3 induced TLR4 activation post pMCAO.

Combined our findings show the existence of parallel manifestations in central and enteric nervous system, after a stroke.

Part 2 From Lumen to Gut

The signals and underlying mechanisms mediating the interaction between the luminal environment and host is still not fully understood. Chemosensory cells situated in the epithelial barrier is in this regards an interesting population to investigate and understand. The tuft cell is a relatively poorly described and understood chemosensory cell. To further our knowledge of the tuft cell a detailed and systematic evaluation and description of its distribution across the GI tract as well as its proximity to other chemosensory cells and neurons were undertaken.

In these studies, C57BL6 mice were used. Their intestines were systematically divided into equal segments with the small intestine divided in 5 (SI 1-5) and the large intestine left undivided (LI). Tuft cells were found throughout the intestines in a proximo-distal gradient; the highest number was in SI 1, corresponding to proximal small intestine. Their number decreased distally and was at their lowest in SI 4, proximal ileum. Tuft cells numbers remained low in segment SI 5, distal ileum, and in large intestine.

Proximity to EECs and nerve fibers

To further describe the tuft cell and their cellular environment, their possible connection with another population of chemosensory cell, namely the satiety associated EECs as well as nerve fibers were estimated.

Evaluation tuft cells in contact with EECs containing the satiety associated peptides CCK, PYY or GLP-1 were estimated. Results showed that 5% of all tuft cells were found in close proximity to CCK-IR EECs, and up to 10% were in contact with PYY and GLP-1-IR EECs.

To evaluate possible connections between tuft cells and nerve fibers the numbers of tuft cells in close proximity to PGP 9.5- and CGRP-IR nerve fibers were determined. PGP 9.5-IR fibers represent the bulk of enteric nerve fibers while those IR to CGRP represent a subpopulation thereof. Results showed that 60% of tuft cells in the small intestine and 40% in the large intestine were found to be in close contact with PGP 9.5-IR nerve fibers. Further, CGRP-IR fibers constituted one-third of these fibers in the small intestine and two-thirds in the large intestine.

A novel subset of tuft cells

During initial tuft cell studies, a novel subset of tuft cells was observed which contained 5HT apically. These DCLK1/5HT-IR cells accounted for up to 80% in villi and 30% in crypts of all tuft cells in the small intestine but were rare in the large intestine. Focusing on the small intestine we found that a 3-12% of all tuft cells were in close proximity to 5HT-IR enterochromaffin cells and 2-10% of all enterochromaffin cells in close proximity to tuft cells. To evaluate the source of 5HT in tuft cells the presence of TPH, the enzyme responsible for 5HT synthesis, or SERT, the selective transporter in 5HT reuptake was investigated. Investigations showed that neither TPH nor SERT were present in tuft cells, and thus the source of 5HT in tuft cells is currently unknown.

Discussion part 2

Through a systematic and detailed investigation and description of tuft cell number, topographic distribution and proximity to satiety associated EECs and nerve fibers in mouse small and large intestine we have laid the baseline for future studies of the role tuft cells in health and disease. Through these studies we have further been able to identify a novel subset of tuft cells containing 5HT.

We observed that tuft cells were preferentially located in the upper small intestine, and that their numbers gradually decreased distally. Both tuft cells and enterochromaffin cells are stimulated by nutrients and luminal compounds (e.g. short chain fatty acids, glucose, acids and bases), which are present in higher concentrations in the upper small intestine [126,127]. Tuft cells express α-gustducin and TRMP5, which are important for transduction of bitter, sweet and umami sensations [85]. We believe the proximo-distal distribution of tuft cells reflects higher concentrations of nutrients in upper small intestine. The gradient would in such case enable a fine tuning of homeostasis responses in response to luminal content. An intracellular tubular network from the microvilli to the endoplasmic reticulum enables information exchange between the cell and lumen [87]. Additionally, the tuft cell gradient may serve to prevent intestinal mucosal damage by gastric acid injury [128,129].

We found a fraction of tuft cells to be in close proximity to satiety associated EECs (containing either CCK, PYY or GLP-1) and nerve fibers, in particular CGRP-IR fibers. These observations strengthen our hypotheses that tuft cells act as an interface, modulating signals between intestinal lumen, EECs and sensory nerves. It should be noted that ultrastructural

analyses show tuft cells in physical contact with neighboring cells by extending cytospinules laterally into their nuclei [87]. If these are present between tuft cells and EEC were not investigated.

A new subset of tuft cells harboring 5HT apically was identified. This DCLK1/5HT-containing cell type accounts for up to 80% of all DCLK1-IR tuft cells in mice small intestine, but it was absent in large intestine.

We further explored the possible source of apical 5HT in this large subset of tuft cells. The synthesis of 5HT requires TPH, the rate-limiting enzyme, both in endocrine cells and neurons. Two forms of TPH exists, TPH1 is expressed in enterochromaffin cells and TPH2 in neurons [130,131]. In the present study, we used a TPH antiserum that cross reacts with both isoforms. No TPH-IR was found in tuft cells, suggesting that the apically located 5HT is not synthesized in these cells. According to previous studies, luminal 5HT is able to be taken up by epithelial cells, via the selective 5HT transporter SERT [116]. However, we failed to identify SERT-IR in tuft cells throughout the intestine. Thus, we are so far, unable to identify the origin of 5HT in tuft cell apex. Since tuft cells are able to communicate with neighboring cells through cytospinules, it may be suggested that 5HT are transported into the tuft cells by this route. If this route is utilized has not been investigated. However, it should be noted that while 80% of tuft cells contain 5HT only between 3-12% of all tuft cells was in contact with 5HT-IR enterochromaffin cells.

Combined our findings underline the unique possibilities tuft cell may have in the fine-tuned homeostatic responses in response to luminal content.

Conclusions

Collectively results presented in this thesis suggest that

1   Loss of enteric neurons and a change in VIP expression after focal cerebral ischemia, but not after global, suggests differences in peripheral neuroimmune responses induced by cerebral ischemia.

2   Enteric neuronal cell death after focal cerebral ischemia, involves neuroimmune interactions; gal-3 activation of TLR4 and downstream TAK1 and AMPK pathways.

3   Tuft cells have an optimal strategic position being in contact with enterochromaffin cells and mucosal nerve fibers. Thus, they can act as an interface between the intestinal lumen and the enteric neurons. This renders them a unique possibility to orchestrate gut homeostasis by modulating intestinal activities.

4   A novel subset of tuft cells, DCLK1/5HT-IR cells, was observed in small intestine. They may have a unique role in mediating GI functions.

Future Perspectives

Research is traditionally divided into basic and applied research. Over the last decades, studies on both have developed dramatically. Integrating experimental data with clinical observation is challenging. During my four years of research, I have studied stroke induced enteric neuronal loss, and tuft cells. This have generated some aspects to consider in upcoming years.

From Bench…

In part one of this project, we found focal cerebral ischemia to induce enteric neuronal loss and upregulation of VIP-IR neurons. The underlying mechanism involves neuroimmune reactions, including gal-3 activated TLR4. Many questions remain to be answered.

1   Which properties make the enteric neurons susceptible in the inflammatory environment? Which subsets of enteric neurons are most vulnerable?

2   Do the neuronal "survivors" function normally and adequately?

3   Does the neuronal up-regulation of VIP rescue some enteric neurons and does it influence GI motility or other GI activities?

4   What else is influenced in the intestinal neuroinflammatory processes, for example, microbiota?

5   The finding that neuropathy was induced by focal, but not global, cerebral ischemia raises questions. Does the global cerebral ischemia induce peripheral neuronal functional disorder? In what way does global cerebral ischemia trigger peripheral inflammation differently? To what extent do central triggers of neuroinflammation cause peripheral inflammation that leads to enteric neuropathy?

In part two of the project, a novel subset of tuft cells was found. There are some unanswered questions related to this part.

1   What is the source of 5HT in the here described novel subset of tuft cells?

2   Tuft cells were found in both small and large intestine, but 5HT/DCLK1-IR tuft cells were only observed in the small intestine. Have this subpopulation unique properties or functions? Do tuft cells have identical functions in both small and large intestine, or do they differ?

3   What is the apically located 5HT used for in the tuft cells?

… to Bedside

TAK1/AMPK inhibitors are able to prevent enteric neuronal death in vitro when exposed simultaneously with serum collected from mice subjected to cerebral ischemia.

1   Can these inhibitors in future be used in the clinical settings? If so, what dose and treatment regime should be used?

2   Can these inhibitors be used in other diseases causing enteric
     neuropathy?

3   Can these inhibitors play roles in the protection of central neurons?

In part two, the regional distribution of tuft cells and their spatial connections with EECs and mucosal nerve fibers were investigated. Further a specific population of the tuft cells containing 5HT were identified. Intestinal 5HT plays diverse roles e.g. they regulate intestinal peristalsis, maintenance and inflammation, blood glucose concentration, cell regeneration and bone metabolism [132]. Dysregulation of 5HT may lead to diarrhea or constipations, and may thus be involved in irritable bowel syndrome (IBS) and inflammatory bowel diseases (IBD) [133,134]. Tuft cells may be a new target to understand the pathologies of IBS and IBD, and a potential target to treat these diseases.

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