Peripheral and Central Mechanisms in Irritable Bowel Syndrome

- in search of links

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Cover: schematic diagram of brain gut axis in IBS

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To my Dad

Have no fear of perfection; you'll never reach it.

Maria Skłodowska-Curie

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ABSTRACT

Irritable bowel syndrome (IBS) is a chronic visceral pain disorder with female predominance, characterized by recurrent abdominal pain and disturbed bowel habits in the absence of an identifiable organic cause. This prevalent and debilitating disease, which accounts for a substantial economic and individual burden, lacks exact diagnostic tools and effective treatment, since its pathophysiology remains uncertain. The bidirectional and multilayered brain-gut axis is a well-established disease model, however, the interactions between central and peripheral mechanisms along the brain-gut axis remain incompletely understood. One of the well-described triggering factors, yet accounting for only a fraction of IBS prevalence, is bacterial gastroenteritis that affects mucosal barrier function. Altered gut microbiota composition as well as disturbed intestinal mucosal barrier function and its neuroimmune regulation have been reported in IBS, however, the impact of live bacteria, neither commensal nor pathogenic, on intestinal barrier has not been studied yet. Furthermore, abnormal central processing of visceral sensations and psychological factors such as maladaptive coping have previously been suggested as centrally-mediated pathophysiological mechanisms of importance in IBS. Brain imaging studies have demonstrated an imbalance in descending pain modulatory networks and alterations in brain regions associated with interoceptive awareness and pain processing and modulation, particularly in anterior insula (aINS), although biochemical changes putatively underlying these central alterations remain poorly understood. Most importantly, however, possible associations between these documented changes on central and peripheral levels, which may as complex interactions contribute to disease onset and chronification of symptoms, are widely unknown.

This thesis aimed to investigate the peripheral and central mechanisms in women with IBS compared to female healthy controls (HC) and to explore possible mutual associations between these mechanisms.

In Paper I, we studied paracellular permeability and passage of live bacteria, both commensal and pathogenic through colonic biopsies mounted in Ussing chambers. We explored the regulation of the mucosal barrier function by mast cells and the neuropeptide vasoactive intestinal polypeptide (VIP) as well as a correlation between mucosal permeability and gastrointestinal and psychological symptoms. We observed increased

paracellular permeability and the passage of commensal and pathogenic live bacteria in patients with IBS compared with HC, which was diminished by blocking the VIP receptors as well as after stabilizing mast cells in both groups. Moreover, higher paracellular permeability was associated with less somatic and psychological symptoms in patients.

In Paper II, we aimed to determine the association between colonic mucosa paracellular permeability and structural and resting state functional brain connectivity. We demonstrated different patterns of associations between mucosa permeability and functional and structural brain connectivity in IBS patients compared to HC. Specifically, lower paracellular permeability in IBS, similar to the levels detected in HC, was associated with more severe IBS symptoms and increased functional and structural connectivity between intrinsic brain resting state network and descending pain modulation brain regions. Our findings further suggested that this association between mucosa permeability and functional brain connectivity was mainly mediated by coping strategies.

In Paper III, we investigated putative alterations in excitatory and inhibitory neurotransmission of aINS, as the brain's key node of the salience network crucially involved in cognitive control, in IBS patients relative to HC and addressed possible connections with both symptoms and psychological factors. We found decreased concentrations of the excitatory neurotransmitter Glx in bilateral aINS in IBS patients compared to HC, while inhibitory neurotransmitter GABA+ levels were comparable. Further, we demonstrated hemisphere-specific associations between abdominal pain, coping and aINS excitatory neurotransmitter concentration.

In conclusion, this thesis broadens the knowledge on peripheral and central mechanisms in IBS and presents novel findings that bring together the ends of brain-gut axis. Our results depict association between mucosal permeability, IBS symptoms and functional and structural connectivity engaging brain regions involved in emotion and pain modulation as well as underlying neurotransmitter alterations.

SVENSK SAMMANFATTNING

IBS (irritable bowel syndrome) är en kronisk tarmsjukdom som kännetecknas av buksmärtor i kombination med förändrade tarmvanor. Sjukdomen drabbar ungefär 11 % av befolkningen, övervägande kvinnor och kan vara förenat med påtaglig försämrad livskvalitet för den drabbade individen och ökade hälso- och sjukvårdskostnader. Förutom magtarmsymptom lider patienter med IBS ofta av andra kroniska smärttillstånd och olika psykologiska symptom, såsom nedstämdhet, ångest och bristande förmåga att hantera kronisk smärta, så kallad coping. Orsaken till IBS är ännu inte helt klarlagd, men störd kommunikation mellan tarmen och hjärnan, via den så kallade "hjärna-tarmaxeln", anses vara väsentligt. Personer med IBS kan skilja sig ganska mycket från varandra avseende symptomens karaktär, vilket skulle kunna tyda på skillnader i de bakomliggande patofysiologiska faktorerna. I hjärna-tarmaxeln ingår bland annat det centrala nervsystemet som även påverkas av psykologiska faktorer, delar av immun- och neuroendokrina systemet, tarmens slemhinna samt tarmfloran. Störningar i dessa system har påvisats vid IBS. Däremot är sambandet mellan störd hjärnfunktion, tarmbarriärfunktion och patientens symptom fortfarande oklart. I denna avhandling undersöktes både hjärnan och tarmbarriären hos kvinnor med IBS och friska kvinnor, samt eventuella samband med magtarmsymptom och psykologiska symptom.

Kvinnor med IBS och friska kvinnor genomgick tjocktarmundersökning med vävnadsprovtagning för att undersöka tarmbarriärfunktion samt magnetkameraundersökning (MR) för att mäta struktur och aktivitetsmönster i hjärnan vilka korrelerades till tarmbarriärfynd. En speciell del av MR-undersökning, kallad MR-spektroskopi, genomfördes för att mäta signalsubstanskoncentration i ett område i hjärnan, inblandad i den känslomässiga upplevelsen och bearbetningen av smärtsignaler från tarmen, kallat insula. Aktivitetsändringar i detta hjärnområde har tidigare påvisats vid IBS. Alla deltagare fyllde också i frågeformulär om magtarm- och psykologiska symptom.

I delstudie I undersökte vi tarmslemhinnans genomsläpplighet genom att ta vävnadsbitar från tjocktarmen och undersöka dem i så kallade Usingkammersystem. Vi undersökte passagen genom tarmslemhinnan för små molekyler och för levande bakterier samt studerade regleringen av denna passage. Resultaten visade att patienter med IBS hade tydligt ökad genomsläpplighet både för små molekyler men också för bakterier. Vi

fann att genomsläppligheten regleras av tarmslemhinns immunceller, så kallade mastceller och ett neuroendokrint hormon vasoaktivt intestinalt polypeptid. Vi fann också att passagegraden för små molekyler var kopplad till vissa kroppsliga och psykologiska symptom hos IBS patienterna.

I delstudie II studerade vi sambanden mellan hjärnans aktivitetsmönster och struktur, tarmbarriärfunktion och symptom. Våra data visade att IBS patienterna med normal tarmbarriärfunktion hade liknande avvikelser i hjärnan som man också kan se vid störda centrala smärtregleringsmekanismer. De patienter med IBS som uppvisade ökad tarmgenomsläpplighet hade mindre av dessa centrala störningar. Statistisk analys visade att sambandet mellan tarmbarriärfunktionen och hjärnfunktionen till viss del var förmedlad av en sämre copingförmåga.

I delstudie III undersökte vi sambanden mellan koncentrationen av signalsubstanter i insula och symptom. Resultaten visade en minskad koncentration av den stimulerande signalsubstansen Glx i insula hos patienter med IBS jämfört med friska. Dessutom indikerade våra data att den vänstra och högra hjärnhalvan kan ha olika funktioner avseende smärthantering, eftersom smärta var mer associerad till Glx koncentrationen i höger insula och copingförmåga mer till Glx koncentrationen i vänster insula.

Sammanfattningsvis bidrar denna avhandling till ökad kunskap om tarmens perifera och hjärnans centrala mekanismer vid IBS samt presenterar nya resultat om en avvikande tarmbarriär och hjärnfunktion vid IBS. Avhandlingen belyser vidare sambanden mellan förändringar i tarm och hjärna och psykologiska symptom.

LIST OF PAPERS

I. Vasoactive Intestinal Polypeptide and Mast Cells Regulate Increased Passage of Colonic Bacteria in Patients with Irritable Bowel Syndrome

Olga Bednarska, Susanna Walter, Maite Casado-Bedmar, Magnus Ström, Eloisa Salvo-Romero, Maria Vicario, Emeran A. Mayer, Åsa V Keita

Gastroenterology, 2017 Oct;153(4):948-960.e3.

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II. Interactions between Gut Permeability and Brain Structure and Function in Health and Irritable Bowel Syndrome

Suzanne T Witt, Olga Bednarska, Åsa V Keita, Adriane Icenhour, Michael P Jones, Sigrid Elsenbruch, Johan D Söderholm, Maria Engström, Emeran A Mayer, Susanna Walter

Neuroimage Clinical, 2019; 21: 101602.

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III. Reduced Excitatory Neurotransmitter Levels in Anterior Insulae are Associated with Abdominal Pain in Irritable Bowel Syndrome.

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Accepted for publication in *Pain*

ABBREVIATIONS

ACC	anterior cingulate cortex
aINS	anterior insula
ANS	autonomic nervous system
BOLD	blood-oxygen-level dependent
BPI	Brief Pain Inventory
CAN	central autonomic network
CEN	central executive network
CNS	central nervous system
⁵¹ Cr-EDTA	chromium labeled ethylenediaminetetraacetic acid
CRF	corticotropin releasing factor
CRFR	corticotropin releasing factor receptor
CSQ	Coping Strategies Questionnaire
DMN	default mode network
DTI	diffusion tensor imaging
EAN	emotional arousal network
ENS	enteric nervous system
FA	fractional anisotropy
FC	functional connectivity
fMRI	functional magnetic resonance imaging
GABA	γ-aminobutyric acid
GABA+	γ-aminobutyric acid + co-edited macromolecular signals
GALT	gut-associated lymphoid tissue
Gln	glutamine
Glu	glutamate
Glx	glutamate + glutamine
GSD	Gastrointestinal Symptom Diary
HADS	Hospital Anxiety and Depression Scale
HC	healthy controls
HPA	hypothalamic-pituitary-adrenal axis
IBS	irritable bowel syndrome
IBS-SSS	IBS Severity Scoring System
ICA	independent component analysis

INS	insula
Isc	short circuit current
MC	mast cells
MEGA-PRESS	Mescher-Garwood point resolved spectroscopy
mM	millimolar
MRI	magnetic resonance imaging
NSAID	non-steroidal anti-inflammatory drugs
NTS	nucleus tractus solitarius
PACAP	pituitary adenylate cyclase polypeptide
PAG	periaqueductal grey matter
PD	potential difference
PHQ-15	Patient Health Questionnaire
PI-IBS	postinfectious irritable bowel syndrome
pINS	posterior insula
ppm	parts per million
qMRS	quantitative magnetic resonance spectroscopy
ROI	region of interest
rs-fMRI	resting-state functional magnetic resonance imaging
RVM	rostroventral medulla
SMN	sensorimotor network
SN	salience network
TE	echo time
TER	transepithelial resistance
TR	repetition time
VIP	vasoactive intestinal polypeptide
vlPFC	ventrolateral prefrontal cortex
VMb	ventromedial thalamic nucleus basal portion
VMpo	ventromedial thalamic nucleus posterior portion
VPAC	vasoactive intestinal polypeptide receptor
VSI	Visceral Sensitivity Index

INTRODUCTION

Irritable bowel syndrome

Irritable bowel syndrome (IBS) is a widespread gastroenterological disorder with a pooled prevalence of around 11% [141] that ranges from 1.1% in France and Iran to 35.5% in Mexico [225]. Although the term “IBS” first appeared in 1950 [27], the disease was known long before, and had been described by Jacob Mendes da Costa as early as in 1871 using the term – “mucous colitis” [51]. Recurrent abdominal pain and disturbed bowel habits are the hallmarks of the disease, which still lacks established disease-specific biomarkers. IBS is diagnosed based on diagnostic criteria in the absence of alarm symptoms after the exclusion of some differential diagnoses such as coeliac disease, inflammatory bowel diseases and colorectal cancer, which can be arrived at after minimal laboratory tests and colonoscopy when appropriate [159]. Over the last 40 years, since the first release of the Manning criteria in 1978 [145], IBS diagnostic criteria have been systematically changed and improved, with the publication of the Rome criteria in 1989 [239], Rome II in 1999 [240], Rome III in 2006 [139] (see Box 1, these are the criteria used in this thesis) and Rome IV in 2016 [159]. The most recent Rome IV criteria sharpened the description of pain as an IBS symptom, by removing the word “discomfort” since it was considered to be imprecise in certain languages, and introducing the overall relationship between abdominal pain and bowel movements, not necessarily improvement of pain following bowel movements, as it was defined in Rome III [204].

Although IBS diagnostic criteria may be fulfilled in individuals with some organic diseases [157], the robustness of these criteria has been confirmed in studies showing that the prevalence of missed organic disease is about 1.3% [60] when the IBS diagnosis is based on the criteria, which is to be compared to approximately 44% when the diagnosis is based on clinical impression only [59,234]. In the scarcity of effective IBS medication, the current treatment for IBS targeted for stool consistency alterations is well established, well tolerated by the patients and easy to evaluate, hence, it is widely used. To aid the clinician, IBS patients are subgrouped based on the prevalent stool consistency pattern, with groups that include IBS-C with predominant constipation, IBS-D with predominant diarrhea, IBS-M with mixed type of stools and IBS-U for unsubtyped

Box 1 ROME III Diagnostic Criteria* for Irritable Bowel Syndrome

Recurrent abdominal pain or discomfort** at least 3 days per month in the last 3 months associated with 2 or more of the following:

1. Improvement with defecation
2. Onset associated with a change in frequency of stool
3. Onset associated with a change in form (appearance) of stool

*Criteria fulfilled for the last 3 months with symptom onset at least 6 months prior to diagnosis.

**Discomfort means an uncomfortable sensation not described as pain. In pathophysiology research and clinical trials, a pain/discomfort frequency of at least 2 days a week during screening evaluation for subject eligibility.

(Longstreth *et al*, 2006)

IBS [159]. This subgrouping, however, has substantial flaws, as the stool consistency fluctuates in the same individual in at least 80% of cases [178]. Likewise, stool consistency itself poses negligible burden for some of the patients in comparison to prevalent and often disabling extraintestinal or psychological symptoms associated with the disease. In fact, the plethora of extraintestinal symptoms such as fatigue, headache, insomnia, musculoskeletal and urinary symptoms, along with psychological comorbidities, such as anxiety and depression [19] constitute the prevailing reason that IBS patients seek care [55].

IBS, although not lethal, has a considerable detrimental effect on the patients' quality of life [217] and is a significant economic burden [33], due not only to frequent work absenteeism [11], but also substantial healthcare utilization [33]. This debilitating disease that constitutes a major burden to individuals, the healthcare system and society, lacks exact diagnostic tools and adequate treatment, since its pathophysiology remains obscure. A fundamental comprehension of the heterogeneity of IBS appears to be the only way to tackle it.

The brain-gut axis

Probably due to its heterogeneity, the pathophysiology of IBS is still obscure, but several predisposing factors (such as genetics, environmental and psycho-social factors) have been identified, as have several triggering factors (such as gastroenteritis, trauma, stress, and food intolerances). These factors influence the physiological mechanisms that lead to altered gut microbial flora, enhanced intestinal permeability, immunesystem acti-

Figure 1. The brain-gut axis and its role in the pathophysiology of IBS. Abbreviations: GI, gastrointestinal; HPA, hypothalamic-pituitary-adrenal axis.

vation, gastrointestinal dysmotility, autonomic dysfunction and visceral hypersensitivity. This complex multifactorial pathophysiology has been summarized as a dysregulation of the brain-gut axis comprising all of the abovementioned pathophysiological mechanisms (Figure 1) [153,165]. Explaining all the hallmark symptoms of IBS as disturbances in different levels of this bi-directional system has gained increasing attention in recent decades, resulting in a principal change of the view of all the functional gastrointestinal disorders, that according to the latest Rome IV terminology are now known as "Disorders of gut-brain interaction" [159]. Particular components of the bottom-up and top-down model will be discussed in detail below.

Mucosal barrier function

The mucosa of the gastrointestinal tract is directly exposed to hazards from the environment, while being responsible for the absorption of all essential nutrients, electrolytes and water. To keep the balance between protection and absorption, the mucosa in different parts of the digestive tract has different properties, and these parts interact with each other in a coordinated manner. The anatomy of the intestinal barrier in general consists of two components: extracellular and cellular (Figure 2). The first element is the lumen itself, where harmful substances and microorganisms are destroyed in an unspecific manner by the acidic environment and by digestive enzymes from the gastric, pancreatic and biliary fluids [203]. The host luminal bacterial flora is also a part of the defence, competing for space and nutrients with ingested pathogens [32]. The next extracellular component is the microclimate, i.e. unstirred water and the mucus layer. In the colon, in order to oppose microbial invasion, the outer, stirred, part of the mucus layer contains immunoglobulins, phospholipids, mucins and peptides with antimicrobial properties. This part of the layer also prevents bacterial adhesion to the epithelium due to its hydrophobic properties. The inner non-stirred mucus layer is known as the “glycocalyx”, and protects the epithelium from digestive enzymes, and dehydration, and facilitates nutrient absorption, epithelial renewal and oral antigen tolerance. This part of mucus is mainly free from bacteria [86,106]. Finally, intestinal motility and water excretion form the final elements of the extracellular components of the intestinal barrier.

Figure 2. Intestinal barrier. Abbreviation: VIP, vasoactive intestinal peptide. Adapted from Keita *et al.* The intestinal barrier and its regulation by neuroimmune factors. *Neurogastroenterology & Motility*, 2010 with permission.

The most important cellular component of the intestinal barrier is the epithelium itself. It is a monolayer of enterocytes and several types of specialized cells, such as Goblet cells, producing mucus [117], Paneth cells, secreting defensins and enterochromaffin cells, secreting hormones and neuropeptides, located both in the small and the large intestine [201]. Membranous (M) cells, responsible for antigen uptake and presentation, are located only in the small intestine [201]. All these specialized cells, scattered among enterocytes, are the key cells of the epithelial lining. They present digestive, metabolic and immune functions, and are involved in the innate immune response, and the activation and recruitment of leukocytes. The lamina propria, which lies underneath the epithelium, contains immune cells that can be classified as organized GALT (gut-associated lymphoid tissue), which comprises lymphoid follicles (in the colon), Peyer's patches (in the ileum), and mesenteric nodes, which are responsible for inducing immune response - and diffuse GALT, which comprises lymphocytes, eosinophils, dendritic cells, macrophages and mast cells, which are general effectors of the immune response [201]. The lamina propria harbors also connective tissue and cells of the enteric nervous system (ENS). Neurons and enteric glial cells of the ENS, together with the central nervous system (CNS), maintain the homeostasis in the gastrointestinal tract, for example via secretion of neurotransmitters [111], as described in the following section.

Transcellular and paracellular permeability

Epithelial cells, that are lining the digestive tract, create a monolayer of semi-permeable, polarized cells connected to each other (and to basal membrane) by protein complexes known as tight junctions (TJ), anchoring junctions (adherens junctions and desmosomes connected to the cells cytoskeleton) and communication junctions (building communication channels between neighboring cells) [201]. TJ are localized most apically between the cells and control the passage of small water-soluble molecules. They are built of four different protein complexes: occludin, claudins, junctional adhesion molecules and tricellulin, that are connected to the cells cytoskeleton via zonula occludens proteins [250]. These proteins together regulate the *paracellular route* of the intestinal permeability, accounting for 85% of the passive transepithelial transport. The *transcellular route* across the epithelial membrane may occur both as diffusion (passive transport of smaller lipophilic and hydrophilic molecules) and as active transport by endocytosis: micropinocytosis, phagocytosis, receptor

mediated (clathrin-mediated) and clathrin-independent caveolar endocytosis [32,110]. Micropinocytosis enables the fluid to enter the cells through the formation of an invagination in the cell membrane. Phagocytosis is a process, which internalizes larger particles that engage specific membrane receptors. One of the most well-described endocytic routes playing important role in cellular homeostasis is clathrin-mediated endocytosis. In this process small vesicles are coated by cytosolic protein, clathrin, and trap molecules that are ligands for clathrin receptors. Clathrin-independent endocytosis is still not fully understood, where small flask-shaped lipid rafts termed caveolae form vesicles to internalize the cargo. Interestingly, this pathway seems to be used by some pathogens, such as *Escherichia coli* [40,110].

One of the strongest known trigger factors in IBS is gastroenteritis, accounting for ca 9% of all IBS cases [14,210], however purely mathematical modelling estimates that postinfectious IBS (PI-IBS) may constitute the majority of IBS prevalence [210]. Bacterial infection (such as *Campylobacter jejuni*, *Salmonella enteridis* [112,206], *Salmonella thyphimurium* [114,158] and *Escherichia coli* [148]) poses higher risk for development of PI-IBS than viral [186], or parasitic gastroenteritis [254]. This is probably due to more severe mucosal damage, as a result of bacterial invasion and cytotoxins [237]. This evident causality between gastroenteritis, impaired mucosal barrier and development of PI-IBS resulted in studies on intestinal permeability, initially done by using oral probe multi-sugar excretion tests [147,227], that measured small bowel intestinal permeability. Subsequently mucosal barrier studies expanded to different types of IBS [58,116,272,271] and both small and large bowel assessments [131,185], however showing conflicting results [58,116,272].

The detrimental effect of non-steroidal anti-inflammatory drugs (NSAID) on intestinal permeability is well investigated, and seems to be enhanced in IBS in comparison to HC, supporting the notion of impaired mucosal barrier in IBS [116]. The impact of NSAID on gut mucosa is diverse, e.g. topical toxic, by damaging the mucus layer and inducing mucosal damage by inhibiting the prostaglandins; intracellular, by influencing mitochondrial phosphorylation resulting in disruption of the TJ [116,121]. Thus, the exclusion of NSAID use is mandatory for the participants that contribute to mucosal barrier studies.

Intestinal permeability can be measured using several techniques. The most widely used assays are urine excretion of oral probe tests, which use different (mostly indigestible) molecules of known absorption site, like saccharides, polyethylene glycol (PEG) or radioactive chromium la-

beled ethylenediaminetetraacetic acid (^{51}Cr -EDTA). There are several studies that employ these techniques exploring mainly PI-IBS and IBS-D and show increased intestinal permeability [58,76,147,195,214,227,271]. Some of the pitfalls of those techniques, such as gastric emptying time and dilution, bacterial degradation and intestinal motility, have been circumvented by using multi-sugar permeability test. Other confounders are there to remain, such as renal dysfunction, saccharides-derived intestinal motility changes or PEG high inter- and intra-individual variations [82,93,172,175]. Oral probe tests, although non-invasive, are unable to provide more detailed information about the mechanisms of the observed disturbance in intestinal permeability.

The method that enables permeability studies *ex vivo* in real time on viable human tissue in a diffusion chamber called Ussing chamber was established in 1951 by zoologist Hans Henriksen Ussing and his colleague chemist Karl Zehran [245]. Their work concerning active transport of sodium through isolated frog skin was a cornerstone of epithelial permeability research, being nowadays, with some modifications, a golden standard technique [79]. Ussing's "little chamber" consist of two half-chambers filled with physiological buffered Krebs solution separated by the examined tissue. In this way the mucosal and serosal side of the tissue may be exposed to different milieu. The solution in both half-chambers is stirred by the gas flux of oxygen and carbon dioxide and kept at 37°C, i.e. the temperature of human body. Active transport of ions produces a potential difference (PD) measured by agar bridges. To determine this voltage, two platinum electrodes, separated from the epithelium, transduce external current nullifying PD. This current, termed short circuit current (I_{sc}), together with PD, enables the calculatation of transepithelial resistance (TER) in accordance with Ohm's law (voltage= curent x resitance). TER indicates electrical resistance mainly of the paracellular permeability route via TJ.

To determine transcellular and paracellular permeability, different substances of known transport route are used, such as ^{51}Cr -EDTA for paracellular permeability, and horseradish peroxidase for transcellular permeability. Although this method is a hallmark of all epithelial permeability studies, it carries several limitations, such as *ex vivo* environment, where the biopsy taking *per se* constitutes tissue injury, following ischemia and denervation. However, meticulous monitoring of the viability of the tissue diminishes this problem. Another limitation is the timing of the experiment, as the viability of the tissue lasts no more than about 5-6 hours after the biopsy taking. Yet the possibility of subsequent microscop-

ic examination of the challenged tissue in order to explore, for instance, a transport route of a so far unknown permeation marker, is unique for this method.

Neuroimmune regulation of mucosal barrier function

Mast cells

Disruption in mucosal barrier function is known to go hand in hand with low grade immune activation [252], which is also true in a proportion of IBS patients [97,136]. The lamina propria, the final layer of intestinal barrier, harbors an abundance of both innate and adaptive immune system cells, whereof one of the most widely investigated innate immune cells are the mast cells (MC). MC are of great importance in different aspects of the function of intestinal tract, aside from host defense against microbes, such as epithelial water and electrolyte secretion and mucosal barrier function, blood circulation, neuroimmune interactions, pain mediation and motility (Figure 3) [21]. MC distributed in the main host-barrier tissues such as skin, respiratory, urinary and intestinal tract, show significant heterogeneity and adaptability to different tissue conditions. Most MC of the intestinal tract are located in lamina propria (ca 2-3% of all cells [28]) and are of phenotype MC_T (secreting only tryptase), while those located in submucosa (about 1% of all cells) are termed MC_{TC} phenotype (secreting both tryptase and chymase) [202,265,269]. The most effective way to activate MC is via an IgE dependent high affinity receptor, which plays a crucial role in allergic reactions. Other immune-dependent (such as IgA, IgG, complements and Ig-free-light chains) as well as immune-independent stimuli (such as neurotransmitters, neuropeptides and hormones) may also trigger MC activation. Upon activation MC release an overabundance of mediators, that can be mainly divided into the ones that are preformed in granules (such as histamine, serotonin, tryptases, chymase, and to some degree TNF α and IL-6) and are secreted within seconds from activation, as well as those synthesized de novo (such as cytokines, chemokines, growth factors and some neuropeptides) and are responsible for late phase reaction. Increased MC infiltration in both the small bowel [83,149,150,249] and the colon [3,15,16,42,50,49,180,263] in different IBS subtypes as well as MC activation [15,16,29,35,42,50,83,120,149,150,263] has been reported in many studies, however conflicting data exist, showing comparable MC counts in IBS and HC [17,25,35,36]. Positive correlation between MC infiltration

and increased intestinal permeability in IBS has been implicated [235] and MC mediators such as tryptase, TNF α or interleukins are known to disrupt both paracellular [101,259] as well as transcellular permeability [111].

Figure 3. The role of mast cells in IBS. Abbreviations: HPA, hypothalamic-pituitary-adrenal axis; SAM, sympathetic adrenal-medullary axis; DCs, dendritic cells; ENS, enteric nervous system; PNS, peripheral nervous system; CNS, central nervous system. Reprinted from Zhang *et al* Mast Cells and Irritable Bowel Syndrome: From the Bench to the Bedside. J Neurogastroenterology and Motility, 2016 with permission.

Vasoactive intestinal polypeptide

One of the neuroimmune mediators released by MC is vasoactive intestinal polypeptide (VIP) that consists of 28 aminoacids. It is produced in various tissues, such as intestinal tract, pancreas and respiratory airways. Nevertheless, VIP is primarily localized in neurons, both centrally- in suprachiasmatic nuclei of the hypothalamus, and peripherally- in the neurons and other cells of peripheral nervous system and of ENS. VIP concentrations measured in serum are mainly of neural origin and are com-

monly low, but may rise substantially in rare VIP producing tumors (VIPomas). They can occur in adults mainly in pancreas, although in children VIPomas appear as tumors of the nervous system, ganglioneuromas or ganglioneuroblastomas [73].

VIP and pituitary adenylate cyclase polypeptide (PACAP) belong to a superfamily, together with other structurally related hormones (such as glucagon, glucagon-like peptide, and secretin) and share some receptors, termed PACAP type I and II receptors [87,127]. PACAP type I receptors show mainly affinity for PACAP, whereas PACAP type II receptors are sensitive to both PACAP and VIP. They are known as VIP receptors, and are subdivided in VPAC₁ and VPAC₂. Both receptors are expressed in MC [87,127,224]. VPAC₁ is mainly distributed in CNS (cortex and hippocampus), liver, lung, intestine and T lymphocytes. VPAC₂ receptor is also abundant in CNS (thalamus, brainstem, spinal cord and dorsal root ganglia), and is found in smooth muscles, intestines and in cardiovascular system [87]. Apart from smooth muscle relaxation in the cardiovascular (vasodilatation), gastrointestinal, urogenital and respiratory tract, VIP exerts a plethora of different functions in various organs, such as: water and ion secretion in the intestines and pancreas, stimulation of biliary secretion, endocrine stimulation in adrenal glands, kidneys, pancreas, pituitary/hypothalamus and thyroid gland as well as in CNS, regulation of cardiac rhythm, neuroprotection and neurodevelopment [87].

As far as inflammation and MC are concerned, VIP shows anti-inflammatory properties by modulating T cell differentiation as well as trafficking and stabilizing MC degranulation [87]. Nevertheless, evidence on the impact of VIP on intestinal barrier is divergent, demonstrating both stabilizing and disrupting effect on gut mucosa. The effect of VIP may plausibly vary depending on different ongoing pathological processes [34]. In fact, both VIP agonists and antagonists have been suggested as promising anti-inflammatory agents in different inflammatory diseases, such as rheumatoid arthritis (VIP agonists) or inflammatory bowel diseases (both VIP agonists and antagonists) [34]. In IBS, increased amount of VIP was detected in colonic biopsies in smaller studies [179,216], however data seem to be inconsistent [196].

Mast cell-nerve axis

The ENS is a part of the autonomic nervous system (ANS) and consists of about 100 million nerve cells (intrinsic primary afferents [153]) organized in three major plexuses: the myenteric (Auerbach plexus, located between the longitudinal and circular muscle layers and regulating motility), the

submucous (three ganglionated layers situated between the circular muscle layer and mucosa) and the mucous plexus. The two latter ones, known as Meissner plexus, are mainly engaged in interplexal communication and secretion of about 30 different neurotransmitters [28]. ENS neurons communicate with CNS through both afferent and efferent sympathetic (via prevertebral ganglia) and parasympathetic neurons (via nervus vagus and plexus pelvici) that innervate the gut and, hence, contact ENS and gut wall immune cells, such as MC [71]. In fact, MC have been shown to be located in close proximity to extrinsic afferent nerves and enteric neurons [12,91,202,230,231]. MC-nerve interaction is reciprocal [28,196], as MC respond to neurotransmitters released by the neurons, while the bioactive substances released by MC affect relevant neural receptors, resulting in visceral pain. However, this crosstalk requires activated MC, as only then MC response to neuropeptides [202,246]. There is accumulating evidence that MC activation and MC-nerve signaling may play crucial role in peripheral sensitization and visceral hypersensitivity in IBS [183,238,269]. However, data show both positive correlation between MC activation/density and pain in IBS [15,185,214,263] as well as doubtful or no correlation at all [15,150]. Nerve fiber overgrowth due to release of nerve growth factor produced by MC [54] was observed in colonic biopsies of IBS patients. MC stabilizers (ketotifen [120], sodium cromoglycate [232]) as well as anti-inflammatory aminosalicylates (mesalazin [44]) demonstrated positive effect on visceral hypersensitivity and MC activation in several studies, although vague and doubtful effect on IBS symptoms [13].

Brain function

Brain is undoubtedly the ultimate instance for all the peripheral bodily impulses, deciding which of them becomes conscious and creating the feeling of pain. It is, however, not before the advancement of neuroimaging techniques that the breakthrough in the appreciation of central aspects of pain modulation took place, even though some other techniques (such as electroencephalography [109], computed tomography [26], positron emission tomography [132] or magnetoencephalography [43]) were available long time before the dawning of magnetic resonance imaging [129,146].

MRI utilizes the spin property of all the unbound protons or electrons, in particular hydrogen protons abundant in the human body, creating small magnetic charge that aligns with a strong magnetic field intro-

duced by the MRI scanner. Thereafter high radiofrequency pulse is introduced on the aligned protons that disrupts them and forces them to re-align in the magnetic field. When the pulse is turned off, the protons return to the primary alignment along the magnetic field, releasing electromagnetic energy that is detected and differentiated by the scanner based on how quickly the energy is released by different tissues. Grey and white matter density as well as cortical thickness can be visualized, measured and statistically compared by the means of high resolution structural MRI of the whole brain or specific regions of interest (ROI). Regional grey matter density changes in IBS have been indicated, even though differences between IBS and HC were less obvious and limited after controlling for anxiety and depression [23,208,241].

Another type of structural MRI, diffusion tensor imaging (DTI), measures movement of water molecules along white matter tracts. In case of tissues with intrinsic fibrous structure such as brain white matter (neural axons) or muscles (muscle fibers), water molecules move easily along those structures, which can be detected with DTI. The degree of anisotropy (direction-dependency) of the diffusion of water molecules is delineated using fractional anisotropy (FA). FA is a scalar value between zero (for isotropic diffusion, similar in all directions as in a sphere, which depicts no movement) and one (rarely in reality, ellipsoid reduced to a line when diffusion is restricted to one direction). It depicts the microstructure of the white matter, such as the white matter integrity, the diameter of the axons, their density and myelination. Changes in white matter microstructure of regions associated with nociception or innervating sensorimotor cortex have been implicated in IBS [39,61,100].

In order to further investigate the function of particular brain areas functional MRI (fMRI) utilizes changes in magnetic properties of oxygenated and deoxygenated blood measured in real time. Almost a hundred years ago Pauling determined that oxygenated hemoglobin was nonmagnetic (diamagnetic) while deoxygenated hemoglobin had 4 unpaired electrons not bound to oxygen, which made it paramagnetic [181]. Blood-oxygen-level dependent (BOLD) contrast arises when neural activity causes increase in cerebral blood flow and volume to optimize oxygen supply with following higher oxygen consumption rate [138]. Due to high spatial resolution (1-2mm) and satisfactory temporal resolution (of a few seconds), this method has been widely adopted in a variety of task-based studies, such as brain responses to visceral stimulation in rectal distension protocols in IBS. Although inconsistent and difficult to compare due to technological and procedural differences, those fMRI studies im-

plicate activation changes in brain regions involved in salience and emotional arousal networks [242]. That can be determined by examining functional connectivity (FC) between different brain regions that express neural activity simultaneously and build specific functional networks. Even during rest, fMRI, called resting-state fMRI (rs-fMRI), can detect slow fluctuations in the BOLD signal in functionally connected brain regions, which provides evidence for task-negative default mode network (DMN) as well as other task-positive intrinsic brain networks described below.

Brain imaging studies provide knowledge about FC of different brain regions. However, these data are based on detection of changes in oxygen utilization and blood flow and do not reflect underlying chemical processes taking place in the synapses in specific brain regions. The most abundant excitatory neurotransmitter in the CNS, the glutamate (Glu), accounts for more than 90% of the synaptic brain connections [160]. There is growing body of evidence implicating dysregulation of both central and enteric glutamergic neurotransmission in gastrointestinal diseases such as IBS [69]. Glu is transformed to glutamine (Gln) in the glial cells and taken up by the neurons, where it is recycled again to Glu, which is the precursor of γ -aminobutyric acid (GABA), the main inhibitory neurotransmitter produced in the neurons and released to the synaptic space. From there, GABA is taken up by the glial cells and transformed again to Glu, and that closes the cycle [182]. Specific technique, existing in vitro even before MRI development, that explores chemistry and cellular features in vivo, is known as quantitative magnetic resonance spectroscopy (qMRS). It enables to detect and quantify Glu (reported with its precursor Gln as a combined measure Glx) and GABA (reported with coedited macromolecular signals as GABA+) in the human brain [160,182]. As for MRI, qMRS is based on magnetic field properties of ^1H hydrogen that depends not only on external magnetic field but also on local magnetic field build by nearby electrons (so called shielding). It causes a variation of resonance frequencies of ^1H , called chemical shift (expressed in parts per million, ppm), that is specific for the structure of a molecule and can be depicted as a spectral peak approximately proportional to the number of nuclei that create this peak [182].

The most prevalent molecule in human tissue is water, with a concentration about 1000x higher than other metabolites, which makes it impossible to detect other chemicals in qMRS, unless water signal is suppressed. Water suppression is done by applying a frequency selective pulse at 4.7 ppm before the excitation phase and then dephasing water

spins with a crusher gradient. Lipids from the scalp resonate at about 1.3 ppm and may distort the spectra of other metabolites. The fat signal in MRI and MRS is suppressed by using outer volume suppression techniques, restraining the detected area to brain tissue only. GABA+ and Glx are determined using MEGA-PRESS technique described in the Method section [163,167].

Despite accumulating evidence of aberrant brain neurochemistry in other chronic pain conditions [8,68,88], little is known about neurotransmitter levels in IBS [170].

Visceral pain pathways

In order to understand the brain's role in visceral pain recognition and modulation, basic knowledge about visceral pain circuitry is mandatory. CNS and ENS interact in order to maintain homeostasis, for instance by regulating MC and sharing the same neuropeptides and receptors, such as substance P, vasopressin, somatostatin, and others [165,264]. Moreover, ENS communicates with extrinsic vagal and spinal splanchnic and pelvic afferents that project to the nucleus tractus solitarius (NTS) in the medulla and to lamina I of the dorsal horn, respectively. Both NTS and lamina I project to parabrachial nucleus in the brainstem. Signals from the parabrachial nucleus are routed to the thalamus (VMpo and VMb, ventromedial thalamic nucleus posterior and basal portion) and thereafter both to insula (INS) and anterior cingulate cortex (ACC), as well as to the hypothalamus, amygdala and periaqueductal grey matter (PAG) [46,152,155]. Visceral afferent signals are processed in the brain in regions related to brain networks, such as salience network (anterior INS - aINS, anterior midcingulate cortex, amygdala), emotional arousal network (locus coeruleus, amygdala, subgenual and pregenual ACC, hippocampus), as well as regions related to perception of gut homeostasis (brainstem nuclei, thalamus, posterior INS - pINS) and descending pain modulation pathway (PAG, rostroventral medulla.- RVM) [108].

The amygdala, attributable to both salience and emotional arousal networks, is essential in decoding the emotional significance of afferent stimuli, implicated in fear conditioning and processing of anxiety [10,200]. It is an important center of pain modulation with a proven functional lateralization, i.e. prevailing pro-nociceptive function of the right central nucleus of amygdala, while excitation of the left central nucleus of amygdala leads to decrease of pain perception [10,200]. Amygdalar neu-

rons are able to switch off and on nociceptive pain perception by two opposite corticotropin releasing factor (CRF) receptors: CRFR1 mediating hyperalgesia, and CRFR2 mediating analgesia [198]. Almost all amygdalar subnuclei heavily project to aINS [92]. The parabrachial nucleus in the pons contains the same CRF receptors and connects amygdala to spino-parabrachial pain pathway, i.e. PAG and RVM [198].

PAG is a hub of descending opioid-mediated inhibition of nociceptive stimuli, receiving input from cortical sites, amygdala, as well as ascending spino-parabrachial nuclei [176]. PAG-driven descending pain modulation occurs via antinociceptive, opioid-mediated effect on RVM's on-cells and activation of off-cells, resulting in analgesia. RVM modulates pain bidirectionally, both inhibits pain through the off-cells and facilitates pain through the on-cells. RVM is considered to be the final relay in descending modulation of noxious stimuli. Its activation is pivotal in development and maintenance of central sensitization [176].

Output signals from the brain to the gut are led through ANS and the hypothalamic-pituitary-adrenal (HPA) axis, both of them are simultaneously responsible for stress response regulation. Pain, as physiological stressor, as well as psychological stress may increase ANS sympathetic and decrease parasympathetic tones, upregulate the HPA axis and increase the release of CRF. This results in amplifying peripheral inflammation, which in turn increases visceral afferent signaling and closes the vicious circle [108].

The CNS has the ability to modulate the intensity and perception of afferent nociceptive signals, via endogenous pain inhibitory pathways (known as "conditioned pain modulation") or excitatory pathways (known as "central sensitization") both on the spinal and supraspinal level [165]. The latest meta-analysis of 12 studies on conditioned pain modulation in IBS populations showed diminished conditioned pain modulation in IBS in comparison to HC with odds ratio of 4.84 (95%CI, 2.18-10.71, $p < .0001$) [4]. However 88% of all the study participants were female, whereas previous meta-analysis performed on males reported less deficiency in conditioned pain modulation [140]. Regulation of the fragile balance between pain facilitation and inhibition takes place within RVM-PAG axis and cortical and supraspinal centers, such as medial prefrontal cortex, ACC, midcingulate cortex, INS, amygdala, hypothalamus and brainstem nuclei [260]. Heterotopic stimulation, i.e. the phenomenon of diminished pain perception due to application of another painful stimulus, has been widely applied in the studies of visceral pain modulation [4]. Graham *et al* presented that in IBS, pain modulation by heterotopic stimulation acti-

vated brain areas connected to bottom-up control such as amygdala or hippocampus, whereas in HC it activated areas of top-down control such as ACC, INS, PAG and RVM [78]. Evidence of conditioned pain modulation and central sensitization in visceral pain entities, such as IBS, is accumulating [4,155,260].

Brain networks

Already in the late 19th century, based mainly on studies on non-human primates, scientists begun to understand that brain function, cognition in particular, relies on complicated architectural network of different brain regions (Figure 4). The breakthrough in brain function studies came with the development of fMRI, especially BOLD signal technique, which gave rise to exploration of functional connections of specific brain regions during cognitive tasks, based on observed spatio-temporal patterns of slow BOLD fluctuations. Those patterns corresponded to task-positive functional networks described below. Up to the 1990-ties it was believed that in health, brain activity is switched off when the person is awake and resting, not performing any mental or physical task [30]. However, other techniques than fMRI (such as positron emission tomograph [193] or electroencephalography [77]) provided evidence of coordinated spontaneous brain activity.

The only task-negative resting state network detected and widely researched using FC fMRI, is known as *default mode network* (DMN), with key nodes in ventrolateral prefrontal cortex (vlPFC) and posterior cingulate cortex. DMN is active at wakeful rest, such as remembering the past, planning the future, daydreaming or mind-wandering and is usually deactivated in goal-oriented tasks, which makes it negatively correlated to task-positive networks. Decreased FC among DMN brain regions are widely reported in chronic pain conditions [66,168,190] and implicated in scarce IBS studies [84,98,133].

Salience network (SN) has been associated with the detection of salient stimuli (not limited to painful stimuli) and integration of sensory and emotional stimuli in order to recruit relevant functional brain network. The core brain regions of SN are aINS, vlPFC and ACC, particularly anterior midcingulate cortex. Alteration in SN in IBS have been consistently reported [96,95,156], confirming the key role of this network in IBS pathophysiology.

Figure 4. Brain networks. Abbreviations: Amyg, amygdala; aINS, anterior insula; aMCC, anterior midcingulate cortex; BG, basal ganglia; dlPFC, dorsolateral prefrontal cortex; Hipp, hippocampus; Hypo, hypothalamus; LCC, locus coeruleus complex; M1, primary motor cortex; M2, supplementary motor cortex; mPFC, medial prefrontal cortex; NTS, nucleus tractus solitarius; OFC, orbitofrontal cortex; PAG, periaqueductal grey; pgACC, pregenual anterior cingulate cortex; pINS, posterior insula; PCC, posterior cingulate cortex; PPC, posterior parietal cortex; sgACC, subgenual anterior cingulate cortex; Thal, thalamus; vIPFC, ventrolateral prefrontal cortex. Adapted from Mayer *et al*, Towards a systems view of IBS. Nat Rev Gastroenterol Hepatol. 2015, with permission.

Central executive network (CEN) is implicated in cognitive operating on identified salience, i.e. direction of attention, planning, selection of response against changing conditions and homeostatic contexts. Key nodes include the posterior parietal cortex and dorsolateral prefrontal cortex. Evidence suggests functional alterations in cognitive processes connected with CEN in patients with IBS [2,115].

Sensorimotor network (SMN), that receives afferent input, is involved in processing, integration and modulation of this sensory input. Key nodes of SMN are basal ganglia, pINS, thalamus, primary and sup-

plementary motor cortices (located in precentral gyrus: M1 and M2 respectively), primary and secondary somatosensory cortices (located in postcentral gyrus: S1 and S2 respectively). Current evidence indicates increased activation, along with neuroplastic alterations, in brain regions associated with SMN in IBS patients [96,105,184].

Emotional arousal network (EAN) plays an important role in the assessment of salient stimulus (both delivered and expected) and mediation of ANS output via central autonomic network. EAN includes medial prefrontal cortex, vlPFC, pregenual (known also as rostral) ACC, subgenual ACC, amygdala, hippocampus, locus coeruleus complex. Studies support increased reactivity of EAN in response to both actual and perceived visceral stimuli in IBS [20,242,255].

Central autonomic network (CAN) plays essential role in visceromotor, neuroendocrine, and pain control as well as behavioral responses necessary to sustain homeostasis. It includes aINS, amygdala, hypothalamus, PAG, NTS, and ventrolateral medulla. Upregulation in CAN, in response to chronic stress, has been presented in animal studies [52,94] and in human brain imaging studies both in HC [199] and IBS patients [125].

The DMN, CEN and SN are the three canonical brain networks. Alterations in salience detection in IBS, i.e. deciding about the importance of the detected stimulus, are closely connected to increased cognition and aberrant activity in DMN [85]. Proper evaluation of both internal and external stimuli is vital for maintaining homeostasis and survival. Aberrations in salience circuits are known to contribute to chronic pain [24].

Insula

Growing body of evidence also supports the pivotal role of SN aberrations in response and connectivity in IBS [96,95,156]. The key node of SN, insula, is a portion of cerebral cortex hidden deep within the lateral sulcus, the fissure between the frontal, temporal and parietal lobes. Anatomically, insula is divided by the central sulcus into two sections, the bigger, frontal, ventral and dorsal part- aINS and the smaller, posterior, dorsal and ventral part- pINS. Regarding insular cytoarchitecture, the dysgranular medial part (mid-insula, mid-INS) is distinguished from the agranular aINS and the granular pINS [215]. The current model of insular function suggests that all the interoceptive impulses, including visceral pain are collected in the pINS, integrated and processed in mid-INS. They are then transferred to the aINS, which is the center of interoceptive awareness as

Figure 5. Ascending pathways of the sympathetic and parasympathetic autonomic nervous system and the lateralization in the forebrain. Abbreviations: VMpo, ventromedial thalamic nucleus posterior portion; VMb, ventromedial thalamic nucleus basal portion; NTS, nucleus tractus solitarius; ACC, anterior cingulate cortex; dIPFC, dorsolateral prefrontal cortex; OFC, orbitofrontal cortex. Adapted from Craig, *Forebrain emotional asymmetry: a neuroanatomical basis?* Trends in Cognitive Sciences 2005, with permission.

well as pain perception and cognitive and emotional pain modulation, with heavy projections from amygdala, as mentioned before. It is aINS that harbors large spindle shaped projection neurons, termed von Economo neurons [164,171], that form the fast relay between limbic sensory (aINS) and motor (ACC) cortices and are implied in self-awareness [47]. Increasing evidence supports crucial role of aINS in switching between CEN and DMN in order to focus attention on salient stimuli [161,228,257]. aINS is implicated in almost all kind of interoception, emotional and bodily awareness, and displays strong functional lateralization in line with well-recognized forebrain asymmetry of autonomic afferents (Figure 5) [46]. In general, the right aINS is linked to energy spending, survival processes and emotions, arousal and sympathetic activity, whereas, on the contrary, the left aINS, is associated with energy saving activities, nourishment, affiliative emotions and parasympathetic activity [46-48]. However, studies report sex differences in lateralization of emotional processing involving aINS, with the prevalence of left aINS activation by emotional stimuli in men, while similar activation of both aINS in women [107].

Psychological comorbidities

Stress and psychological factors are well-established and influential underpinnings in the pathophysiology of IBS and the bio-psycho-social disease model. Numerous studies demonstrate that psychiatric comorbidities, such as anxiety and depression, chronic emotional stress, history of abuse, specific personality traits and impaired coping play a prominent role in peripheral and central responses to visceral stimuli [62]. The prevalence of psychiatric comorbidities may be as high as 60-90% in selected IBS populations [126,233,256] in particular with overrepresentation of anxiety and depression [130]. History of abuse, reported by over 40% of patients seeking a gastroenterologist due to functional gastrointestinal diseases including IBS [57], contributes to increased vulnerability and exacerbation of IBS symptoms [151,258]. In fact, apart from type and severity of gastrointestinal infection, psychological factors, such as adverse life events, increased stress susceptibility, anxiety, depression, and personality traits, such as neuroticism, are independent risk factors in the development of postinfectious IBS [14,119,226].

Visceral hypersensitivity (painful perception of non-noxious stimuli-allodynia or increased sensitivity to noxious stimuli- hyperalgesia) that 20 years ago was supposed to be “a reliable biological marker of IBS” [162], now is proven to exist in only about half of the IBS patients [188]. Although there is conflicting evidence on eventual correlation between hypersensitivity and symptom severity [63,128,218], positive association of hypersensitivity and anxiety [81] and depression [63] is widely recognized. Visceral hypersensitivity has been associated with hypervigilance (increased attention to visceral stimuli) and cognitive bias due to an inclination of negative categorization of visceral sensations commonly observed in IBS [53,187].

Aforementioned dysregulation of ANS and HPA axis, known component of IBS pathophysiology, is the well-documented way of the influence of stress on living organisms, which substantiates the role of stress in IBS symptomatology [151,177]. Evidence from clinical and experimental studies implicate prevailing impact of psychological stress on all the aspects of IBS pathophysiology, such as mucosal immune activation, secretion and permeability, intestinal sensitivity, alterations in peripheral and central nervous system as well as gastrointestinal microbiota [191].

Individual susceptibility to stress is tightly coupled with the ability to engage adaptive (in general: approaching the threat) or maladaptive (in general: avoiding the threat) cognitive and behavioral strategies to deal

with stressors, called coping. Maladaptive coping is widely associated with both intestinal and extraintestinal symptom severity in IBS [261]. Particularly catastrophizing, a prime maladaptive coping strategy, is overrepresented in IBS and related to diminished quality of life [212,213] and increased IBS symptom severity [56,247]. On the other hand one of the most effective IBS treatments, cognitive behavioral therapy is mainly based on implementing adaptive coping, in order to face and handle the pain, and demonstrates long-term positive effects on both IBS symptoms as well as quality of life [118,209].

Sex differences in IBS

IBS is a disease with female predominance of varying reported female-to-male ratios of 2-3:1, for different populations. The recent overall prevalence, however, is 67% higher in women (odds ratio 1.67 CI 1.53/1.82) [37,140], which is probably biased by the selection of health care seeking samples [37]. Sex differences in IBS have to be viewed from the perspective of known sex differences between healthy men and women, such as slower gastrointestinal transit and lower pain thresholds to nociceptive stimuli in women, increased cardiosympathetic tone in men and gender differences in CNS activation (increased activation of sensory brain regions in men while in ACC and INS in women) [37]. Although the role of sex hormones in mediating sex differences in IBS is still incompletely understood, evidence indicates pronociceptive effect of estrogen in the CNS, as well as estrogen-derived increase in MC activation, decrease in intestinal permeability and motility and even modulation of gut microbiota [166]. This is because estrogen receptors are widespread in the gastrointestinal tract (in the mucosa, smooth muscles, afferent sensory fibres) as well as along the brain-gut axis (at spinal and supraspinal regions) [166]. Menstrual cycle increases IBS symptoms when estrogen and progesterone concentrations increase, i.e. during the luteal phase and at the onset of menses [90]. Women with IBS seem to report greater IBS symptom burden such as nausea, bloating and constipation, increased extraintestinal manifestations and worsened quality of life in comparison to male IBS patients, however the differences in IBS symptoms between pre- and postmenopausal women are still unclear [37,236]. Furthermore, women with IBS exhibit reduced conditioned pain modulation (used as a proxy of central sensitization), shown in some studies to vary during different phases of menstrual cycle [102]. There is also a growing body of evidence that indicates sex differences in central processing of visceral sensation in IBS. Specifically, studies demonstrate increased activation in affective and

autonomic brain regions in women (amygdala, infraglenal cortex) and in corticolimbic pain inhibition regions in men (INS and midcingulate cortex) during both cued and delivered aversive visceral stimuli [37,124,166]. There is also evidence on higher sensitivity to salient somatosensory and interoceptive stimuli in women than in men [107].

Summary

IBS is clearly a multifactorial disease of incompletely understood and definitely complex pathophysiology. Therefore, in line with the bio-psycho-social model of IBS, the way to understand the disease is to deepen the knowledge of the mechanisms behind the interplay between the peripheral and central processes in IBS.

AIMS

The overall aim of the thesis was to study peripheral and central mechanisms in IBS and explore relationship between them.

Specific aims:

- to determine colonic mucosa paracellular permeability, permeability to live bacteria, both commensal and pathogenic, and the influence of MC and VIP, as well as associations between mucosal permeability and symptoms
- to determine the association between colonic mucosa permeability and structural and functional brain connectivity
- to investigate putative alterations in excitatory and inhibitory neurotransmission of aINS, as the brain's key node of the salience network crucially involved in cognitive control, and determine possible connections with both IBS symptoms and psychological factors

METHOD

Subjects

Patients aged 18-60 years, diagnosed with IBS, attending Gastroenterology Department, University Hospital in Linköping, were screened for fulfilling of Rome III criteria. HC aged 18-60 years, without any history of gastrointestinal disease or complaints, were recruited by an advertisement and offered monetary compensation (Figure 6). Exclusions criteria for both groups were organic gastrointestinal disease, metabolic, neurological or psychiatric disorders and nicotine intake within 2 months before the study, as well as separate sigmoidoscopy exclusion criteria (self-reported allergy, NSAID intake) and MR exclusion criteria (ferromagnetic implants, claustrophobia or large tattoos). All participants were right-handed.

83 patients were initially included in the study, whereof there were only 12 men (13.6%). Because of the substantial underrepresentation of the male patients, only female individuals were finally enrolled in the study. 39 female healthy controls were initially recruited by an advertisement. As neurotransmitter concentrations alter with age [207], meticulous age-matching was performed with an age range of ± 3 years. From the patients' cohort, 32 women accomplished both sigmoidoscopy and fMRI, while 34 women, who declined sigmoidoscopy, underwent only

Figure 6. Overview of inclusion process for both IBS patients (A) and healthy controls, HC (B). INS, insula.

fMRI examination. As for the HC, 15 individuals completed both fMRI and sigmoidoscopy, while 10 participants did only fMRI. Moreover, for the assessment of bacterial passage routes in electron microscopy and immunofluorescence of TJ, five more patients and five more HC were enrolled, who underwent both sigmoidoscopy and fMRI.

Because the insula voxel in fMRI examination was added subsequently during the enrollment, INS qMRS data was finally available for 37 patients (drop out: 1- water data missing, 1- no left insula data) and 21 HC (drop out 1-no left insula data).

Questionnaires

An overview of the type of questionnaires used in each paper is depicted in Table 1.

IBS Severity Scoring System (IBS-SSS) evaluated overall IBS symptom severity by assessing 5 items: frequency and intensity of abdominal pain, intensity of bowel distension, satisfaction with bowel habits and interference with daily life [72]. Each item scores between 0 and 100 and the sum score indicates mild (75-175), moderate (175-300) or severe (>300) symptoms.

Table 1: Overview of the questionnaires used in each study of the thesis

Questionnaire	Paper I	Paper II	Paper III
IBS-SSS	X	X	X
VSI		X	X
HADS		X	X
CSQ		X	X
PHQ-15		X	X
BPI			X
GSD			X
Mac Arthur			X

IBS-SSS, IBS Severity Scoring System; VSI, Visceral Sensitivity index; HADS Hospital Anxiety and Depression Scale; CSQ, Coping Strategies Questionnaire; PHQ-15, Patient Health Questionnaire; BPI, Brief Pain Inventory; GSD, Gastrointestinal Symptom Diary; Mac Arthur, MacArthur Scale of Subjective Social Status.

Visceral Sensitivity Index (VSI) was implemented to assess gastrointestinal symptom-specific anxiety [123]. The 15-item self-report questionnaire evaluates cognitive, emotional and behavioral responses to fear of gastrointestinal sensations, symptoms and the context in which the symptoms are experienced. Items are scored on a reversed 6-point scale ranging from 0 to 5, with sum scores between 0 and 75. Higher scores indicate more severe symptom-specific anxiety.

Hospital Anxiety and Depression Scale (HADS) was applied to estimate states of depression and anxiety [274]. The scale consists of seven items for depression (HADS-D) and anxiety subscales (HADS-A), with scores on each subscale ranging from 0 to 21. Cut-off values are indicated as ≥ 8 for subclinical (suspicious) anxiety or depression and ≥ 11 as definite cases on both the HADS-D and HADS-A, respectively [22].

Coping Strategies Questionnaire (CSQ) was used to evaluate cognitive and behavioral strategies to cope with pain [197], involving six subscales for cognitive strategies (ignoring pain, reinterpreting pain, diverting attention, calming self-statements, catastrophizing, praying/hoping) and two subscales for behavioral strategies (increasing activity, increasing pain behavior). Sum scores between 0 and 36 for each subscale indicate how frequently a coping strategy is used.

Patient Health Questionnaire (PHQ-15) was applied to assess the degree of somatic symptom severity across 15 different somatic symptoms [122]. Sum scores ranging from 0 to 30 indicate levels of somatic symptom severity.

Brief Pain Inventory (BPI) is a validated pain assessment tool, that measures both the intensity of pain (4 items, sensory dimension) and interference of pain with the patient's life (7 items, reactive dimension) [41]. Each item is scored on a 0-10 scale with sum scores ranging from 0-40 for pain severity and from 0-70 for interference and higher scores indicating higher levels of pain severity and interferences, respectively.

Gastrointestinal Symptom Diary (GSD) was used by IBS patients to record their gastrointestinal symptoms on 14 consecutive days [192] (Figure 7). The symptoms (abdominal pain, nausea, bloating) as well as every single bowel movement and stool consistency (defined by Bristol Stool Chart [134]) were reported along a 24-hour time axis. The values were manually scored, and the mean frequency of symptom episodes per week and symptom duration per day was extracted from the diary data.

Mucosal barrier measurements

Using chamber experiments

Mucosal barrier studies were performed in modified Ussing chambers (Harvard apparatus Inc., Holliston, MA, USA; Figure 8) [79]. Mucosal biopsies were carefully mounted to cover the 1.8mm² of exposed tissue surface area. Thereafter 1.5 ml 10 mM mannitol in Krebs buffer was added to the mucosal side of the chamber, while the serosal side was filled with 1.5ml 10 mM glucose in Krebs buffer. The system was continuously circulated by gas flow, 95% O₂/5% CO₂, while simultaneously oxygenated and kept warm at 37°C. Totally 40 min equilibration prior to the experiment start was completed to obtain steady-state in transepithelial PD. The electrophysiology measurements, *I*_{sc}, TER and PD were monitored by the means of two platinum electrodes for current passage and two silver chloride Ag/AgCl-electrodes for PD measurements, all in open circuit conditions. Every second minute of the whole experiment period, sampling of electrophysiology data were performed via LabVIEW software (LabVIEW, National Instruments, Solna Sweden) by sending direct alternating current pulses through the specimens and measuring the voltage response. A current (*I*) – voltage (*U*) pair relationship linear-squares fit was done to calculate the TER from the slope of the line and the PD from the intersection of the voltage ($U = PD + TER \times I$). All parameters were calculated during the 30-60-90-120 min period.

Figure 8: The drawing (A) and the photograph (B) of the Ussing chamber used in the thesis. Ag/AgCl, silver chloride electrodes. Reprinted from Medical Dissertation No 1006, Linköping University, with permission from Åsa Keita.

Bacterial permeability

The aim of the study was to determine mucosal permeability to live bacteria, both commensal and pathogenic. As mentioned before, the commensal bacteria *Escherichia coli* seems to play a role in dysbiosis, lately associated with IBS pathophysiology [174], and *Salmonella typhimurium* infection of the gastrointestinal tract is widely recognized as a risk factor for the subsequent development of IBS [114,158]. Therefore the choice of these two bacterial strains seemed to be warranted. *Escherichia coli* HS and *Salmonella typhimurium* were prepared according to routines described previously [113]. Specifically, 5 ml of plasmid enhanced green fluorescent protein 1:100 was added to 50 ml of bacterial competent, carefully shaken and incubated on ice for 30 min before heat shock at 40°C for 90 s. Subsequently, the bacteria were incubated added 450 ml SOC medium (Super Optimal broth with Catabolite repression), at 37°C, 200 r.p.m.(revolutions per minute), for 1 hour, followed by 12 hours incubation at 37°C plated on LB (Lysogeny broth)+ ampicillin agar plate. After 20 min of Ussing chamber equilibration final concentrations of 10⁸ CFU/ml of live green fluorescent protein (GFP) -labelled *Escherichia coli* HS and *Salmonella typhimurium* were added to the mucosal side of separate chambers. Bacterial passage was designated in a multileader plate reader.

Paracellular permeability

⁵¹Cr-EDTA is a widely known marker for the paracellular permeability used in Ussing chamber experiments that is proven to pass through the mucosa between the cells. The firm binding of the radioactive Cr to the EDTA molecule ensures equal Cr and EDTA passage and minimizes reaction between the tissue Ca²⁺ and EDTA. After 20 min of Ussing chamber equilibration mucosal side of each chamber was added 34μCi/ml ⁵¹Cr-EDTA. At 0, 60 and 120 min of experiment, serosal samples were collected and replaced with an equal volume of Krebs solution. The paracellular passage was calculated by gamma-counting (1282 Compugamma; LKB, Bromma, Sweden).

Blocking agents

In order to explore the role of MC and VIP in intestinal barrier function specific blocking agents were used. After initial 20 min equilibration, the biopsies were treated on the serosal side with either 1μM of VIP receptor blocker anti-VPAC, 1μM of MC stabilizer ketotifen in duplicate systems, or

vehicle, and kept in equilibrium in additional 20 min before the bacteria or ^{51}Cr -EDTA were added. VIP receptor blocker is a fragment of VIP signal peptide containing only 23 amino acids (6-28) out of 30, thus it maintains binding affinity to both VIP receptors, while exerting no activation (V4508 Sigma-Aldrich). Ketotifen is a MC degranulation stabilizer that demonstrated some positive effect on IBS patients as described in the introduction [120].

Immunofluorescence of MC tryptase, VIP, VPACs

Different processes of detecting particular antigens in tissue sections based on the affiliation of these cellular antigens to specific antibodies are termed immunohistochemistry. Immunofluorescence is an immunohistochemical staining method of visualizing antigens after conjugating and binding reaction with fluorescent dyed antibodies. Fluorescence is activated when an exciting light of a specific wavelength is applied on the pre-treated specimen under a fluorescent microscope.

Colonic biopsies were fixed with 4% formaldehyde, dehydrated according to standard procedures before embedded in paraffin blocks, microtome sliced and mounted on a microscope slide. After heat fixation an inverse process of deparaffinisation and hydration took place, prior to treating with blocking reagent, in order to reduce nonspecific background staining. Then, the samples were stained with primary antibodies and incubated in a dark humidified chamber over night, at 4°C. After washing with phosphate buffered saline (a water-based isotonic salt solution, PBS) and incubating with secondary antibody, the samples were cover slipped with anti-fade reagent and were ready for immunofluorescence examination the next day.

Intestinal MC contain huge amounts of tryptase in their granules, and therefore anti-MC tryptase antibody was used to detect MC. As VIP is a neuropeptide widely recognized to influence human digestive system in various ways [169], for instance regulating intestinal permeability [112], we stained the biopsies for both VIP and its receptors, VPAC1 and VPAC2 by using anti-VIP, anti-VPAC1 and anti-VPAC2 antibodies in immunofluorescence and examined co-localization of VIP/VPAC1/VPAC2 and MC. Expressions of MC, VIP/VPAC1/VPAC2 were evaluated in fluorescent confocal microscopy.

Magnetic Resonance Imaging data acquisition and analysis

Functional Magnetic Resonance Imaging protocol

In order not to influence the fMRI examination output following a potentially unpleasant examination, such as sigmoidoscopy, according to the study protocol, the participants underwent MR scan firstly, whereas sigmoidoscopy was performed within 2 weeks' time afterwards. This time-period was set as a general recommendation and its protraction was not an exclusion criterium, as individual intestinal permeability is supposed to be quite stable over a period of at least 8 weeks, when repeating measurements [144,248,273].

Participants were requested to fast for at least four hours (water was acceptable) and refrain from consuming alcohol and taking any IBS-related, pain, or sleep medication for at least 24 hours before the MRI scan. Security protocol was collected from the participants to ensure no contraindications to MR scanning. Images were collected on a 3 T Philips Ingenia (Philips Healthcare, Best, The Netherlands) equipped with a 32-channel head coil at the Center for Medical Image Science and Visualization (CMIV) at Linköping University, Sweden. Prior to fMRI and qMRS, a standard MR screening with T1-weight images was performed in all participants to exclude substantial brain abnormalities, followed by qMRS and thereafter fMRI and DTI examination.

Resting state functional MRI

Functional MRI data were obtained using the following parameters: repetition time (TR) 2 seconds, echo time (TE) 37 milliseconds and voxel size $3.59 \times 3.59 \times 4\text{mm}^3$ in 28 slices. As magnetic resonance imaging is extremely sensitive to motion, the rs-fMRI images were preprocessed following the guidelines of the GIFT group independent component analysis (ICA) toolbox, as described below [5].

fMRI uses BOLD as an indirect measure of neural activity encoded for each voxel (volume element) of the brain, resulting in an abundance of relevant and irrelevant signals. To organize and analyze the data mathematical models are used to separate the signals of interest and these not of interest. ICA is a blind source signal separation, which means that it

isolates unknown signals in a single subject, grading them according to most explained variance. It extracts independent patterns of comprehensible fMRI activity that varies together over time, both in positive and negative direction, and is significantly distinguishable from activities in other sets of voxels. GIFT is an application allowing group assumption from fMRI data using ICA, which is conducted in three main stages: data compression, ICA and finally back reconstruction to recreate individual subject and group components of the signals of interest.

Statistical parametric mapping SPM8 (<http://www.fil.ion.ucl.ac.uk/spm>) was used to realign the images that were subsequently spatially normalized using a standard brain atlas of the Montreal Neurologic Institute (MNI). They were additionally smoothed with an 8 mm full width half maximum (FWHM) Gaussian kernel to diminish image noise and further reduce the effects of individual anatomical variability. Head motion less than one voxel in any direction was set as a limit that has not been exceeded in any of the examinations. Group ICA toolbox GIFT v4.0a (<http://icatb.sourceforge.net>; [31]) was used to calculate functional connectivity. After performing single ICA on all cases [18] with back recon-

Figure 9. Group independent DMN component. The one-sample t-test statistical map over all 47 subjects is thresholded at $p < 0.05$, Family Wise Error corrected for comparing across the whole brain. Color bar given in terms of t-statistic.

struction of every subject's spatial maps from the raw data [65], 45 independent components were calculated [135] and assured for stability using ICASSO [135] with 500 iterations. ICASSO deals with the major ICA problem of reliability of the estimated components due to statistical error of limited sample size as well as practical issues with ICA algorithm and the real data that not always follow ICA model. ICASSO investigates ICA statistical reliability and stability by running the algorithm multiple times with differently bootstrapped data sets in order to assess ICA estimates.

To identify the DMN components spatial regression with a template of the DMN [222] was employed (Figure 9). The color brain maps use t-statistics of the brain areas, presenting positive synchronous activation marked in warm colors (red- low significance, yellow- high significance) and negative simultaneous activation marked in cold colors (blue- low significance, green- high significance), as shown in Figure 9.

Diffusion tensor imaging

DTI data were obtained using a 64-direction sequence with TR 9,339 seconds, TE 82 milliseconds and voxel size 2 mm³ and diffusion weighting of b=1000 seconds/mm². The data were processed using standard procedures (<https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSL>; [104,223]) after being reconstructed on the scanner. FLIRT toolbox [80,103] was employed to correct for distortion and subjects movement due to eddy-current. Individual FA images were spatially normalized to MNI template using tract-based spatial statistics (TBSS; [221]) according to FNIRT toolbox [6] to obtain group-level maps of FA.

Quantitative Magnetic Resonance Spectroscopy

qMRS measures were accomplished using MEGA-PRESS sequence (kindly provided by R. Edden, Johns Hopkins University; [163,167]) with TR 2 seconds and TE 68 msec, edited pulses "ON" at 1.90 ppm and "OFF" at 7.46 ppm and water suppression MOIST (multiple optimizations insensitive suppression train), in a voxel of 4.5x2.0x3.0 cm³ located in the right and left aINS (Figure 10A, B). MEGA-PRESS sequence employs splitting of the spectrum peak that varies in phase and amplitude depending on TE due to J coupling (spin-spin coupling), occurring when protons in a molecule are affected by other protons due to sharing of the electrons. MEGA-PRESS is primarily optimized for evaluation of the weak GABA molecules

spectrum, brain neurotransmitter with editing coupled signals at 1.9 and 3 ppm due to J coupling [173,205]. MEGA-PRESS exploits J-difference editing by subtracting two data sets: “ON” and “OFF”. During “ON” acquisition pulses at 1.9 ppm are applied, that selectively resonate some of the GABA protons and simultaneously refocus the coupled protons at 3 ppm. During “OFF” acquisition impulses applied have no influence on GABA signals. Subtraction of “OFF” from “ON” spectra results in revealing only those 1.9 ppm signals that are coupled with 3 ppm signals, which is specif-

Figure 10: Typical qMRS volume of interest (voxel size 4.5x2.0x3.0 cm³) placement in the (A) right and (B) left aINS. The yellow box illustrates the voxel targeting the GABA signal at 3.0 ppm, and the white box illustrates the voxel the water residual at 4.7 ppm originates from, which shows the chemical shift displacement. Representative spectra with LCModel fitting depict (C) an averaged difference spectrum for the extraction of GABA+ and (D) an averaged MEGA-PRESS OFF spectrum for Glx extraction (labeled as 2 and 7, extracted solely from OFF-spectra) from a healthy volunteer (black line: post-processed spectra prior to fitting; red line: LCModel fit). Residuals are shown at the top of each panel. (Assignments: 1, Creatine (-2CH₂-); 2, Glx (-2CH-); 3, Choline (-N(CH₃)₃); 4, Creatine (-N(CH₃)); 5, GABA+ (-4CH₂-); 6, tNA (-3CH₂-); 7, Glx (-4CH₂-); 8, GABA+ (-2CH₂-); 9, tNA (-2CH₃); 10, GABA+ (-3CH₂-); 11-13, Macromolecules and lipids, (-CH₂-). Abbreviations: Glx, glutamate+glutamine; GABA+, γ-Aminobutyric acid + coedited macromolecular signals; tNA, total N-Acetyl compounds (NAA + NAAG).

ic for GABA. Linear combination of model spectra LCMModel (Version 6.3-1L; [189]) was applied to calculate GABA+ from difference spectrum after subtracting “OFF” from “ON” spectrum (Figure 10C). Glx was computed from “OFF” spectrum (Figure 10D).

Statistics

Paper I

All parametric data, according to Shapiro-Wilk normality test, such as VIP concentration in blood samples and biopsy lysates, MC density and expression of VPAC1 and VPAC2 receptors were shown as mean $\pm$ standard error of mean (SEM) and comparisons between groups were performed with Student t test (for comparing two groups) and ANOVA test (for more than two groups).

Non-parametric data such as bacterial and paracellular permeability were given as median (25th–75th interquartile range). Comparisons between groups were done with Mann–Whitney U tests for comparing of two groups and Kruskal–Wallis test was used for comparing three or more groups. Wilcoxon matched-pairs signed rank test was used for paired comparisons of immunofluorescence measures of TJ with and without Salmonella exposure. Differences with $p < .05$ were considered significant. Correlation of non-parametric data were performed using Spearman Correlation test. Analyses were calculated on GraphPad Prism 7 (<https://www.graphpad.com/scientific-software/prism>).

Paper II

Although not all questionnaire data passed the Shapiro-Wilk test, the results were presented as mean $\pm$ standard deviation (SD) in order to facilitate possible future interpretation and comparisons. Mann-Whitney U test was used to calculate between group differences and Spearman’s rho for the correlation between permeability and questionnaire data.

Analyses on the behavioral data were calculated using SPSS v24 (<https://www.ibm.com/products/spss-statistics>). GraphPad Prism 7 (<https://www.graphpad.com/scientific-software/prism>) was used to create the graphs.

For correlations of permeability, DMN functional connectivity and DTI data both between group (IBS vs HC) and within group (IBS and HC)

permutation analyses using PALM software tool (<https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/PALM>; [262]) with 10,000 iterations were performed. In general, a permutation is an alignment of all or part of objects in a set, with regard to the sequence of the alignment. The within-group analyses were restricted to the brain regions significant in the initial between group analyses (masked at $p < 0.05$, Family Wise Error (FWE) correction for the whole brain comparisons). FWE is defined as the probability of making at least one false conclusion in the series of hypothesis tests. As depression and anxiety are known to influence functional connectivity, both subscores from HAD questionnaire (HAD-A and HAD-D) were included in all analyses as covariates of no interest, likewise the time slot between the MR scan and sigmoidoscopy examination. Moreover, Threshold Free Cluster Enhancement (TFCE; [220]) was used to define the clusters with significance set at $p < 0.05$ and FWE correction. In a fMRI analysis numerous statistical tests were used due to >80,000 voxels included in the analysis, which makes it necessary for appropriate correction for multiple comparisons. TFCE is a well-established method for multiple comparisons correction of statistical maps in fMRI studies. The calculations were performed using the software Mango (<http://ric.uthscsa.edu/mango/>; J.L. Lancaster and M.J. Martinez).

Path analysis

Path analysis is a type of multiple regression analysis, which is in general a statistical analysis designated for normally distributed data sets. It is a confirmatory statistical method that is based on a created by the researcher theory about some associated regression relationships. Path analysis tests the goodness of fit of this theory against a perfect model. In our study the path analysis was done to test if the detected associations between permeability and functional brain connectivity were related directly or they were mediated by some clinical characteristics from the questionnaires. Prior to creating the model, significant correlations between the components had to be detected. As Spearman's rho from barrier function data showed, only the paracellular permeability was significantly correlated to some of the IBS questionnaire data, namely symptom severity (IBS-SSS), gastrointestinal related anxiety (VSI) and coping (CSQ-pain behavior). In order to define the association between ^{51}Cr -EDTA permeability and RVM functional connectivity permutation analyses were used. These findings were confirmed by a simple calculation with Pearson's correlation coefficient. The path model was constructed

delineating both a direct relation between the gut and brain data as well as indirect relations through the three clinical components mentioned above. The path analysis was calculated using Mplus (<https://www.statmodel.com/>) with all path coefficients presented in a standardized form. As mentioned before, not all of the data were normally distributed; therefore nonparametric bootstrapping with 2000 replications was also used. It is a method of inferential statistics based on the principle of multiple sampling of a set of data while allowing for replacement, and measuring a statistic of this sample. By replicating the procedure numerous times, one can infer that the statistic measured on the multiple samples corresponds with the statistic of the population.

Paper III

As not all questionnaire data passed the Shapiro-Wilk test, results were presented as median (25th–75th interquartile range). Mann-Whitney U test was used to calculate between group differences in Glx and GABA+ concentrations and questionnaire data. Chi Square test was applied for categorical data group comparisons of medical journal data on previously diagnosed anxiety and / or depression and medication use. Effect sizes were indicated using Cohen's d for results from U-test and as Cramér's V for Chi Square test.

Stepwise multiple linear regression analyses (criteria: probability to enter $\leq .05$, probability to remove $\geq .10$) were carried out in IBS patients to determine the significant predicting factors of aINS Glx and GABA+ at $p < .05$. Statistical analyses were calculated using SPSS version 25 (IBM Corporation, Armonk, NY) and GraphPad Prism 7 (<https://www.graphpad.com/scientific-software/prism>).

Ethical approval

Ethical approval for the study was obtained from the Regional Ethical Review Board in Linköping, Sweden (Dnrs. 2013/506-32; 2014/264-32). All the participants gave written, informed consent according to the Helsinki Declaration.

RESULTS

Clinical characterization of the patients

Demographic data for the female patients' and the female HC' cohorts in all three Papers are depicted in Table 2. The cohort from Paper I and II was identical except for the 5 additional patients and HC in Paper I, who were included for the purpose of supplementary electron microscopy studies. The patients and some of the HC from the Paper III cohort were included subsequently, and thereby are not the same individuals as in Paper I and II. There was no statistical difference between age of IBS and HC in any of the cohorts. The vast majority of IBS patients had moderate to severe disease intensity according to IBS-SSS questionnaire. The percentage of different types of IBS subgroups according to the Rome III criteria is depicted in Table 2. This IBS subgrouping however, turned out not to be significant in any of the main correlations explored in the thesis.

Table 2. Clinical characteristics of the cohorts included in Papers I, II and III

		Paper I*	Paper II*	Paper III**
IBS	Total	37	32	39
	Age	32.2 (19-55)	32.6 (19-55)	32.1 (18-57)
	IBS-SSS	347 (167-480)	343 (167-480)	336 (203-468)
	IBS-D	8 (21.5%)	7 (22%)	10 (26%)
	IBS-C	8 (21.5%)	8 (25%)	7(18%)
	IBS-M	21 (57%)	17 (53%)	22(56%)
HC	Total	20	15	21
	Age	29.9 (20-48)	29.8 (21-48)	32.1 (20-55)

Age and IBS-SSS given as mean (range). IBS-SSS, IBS Severity Scoring System; IBS-D, IBS with predominant diarrhea; IBS-C, IBS with predominant constipation; IBS-M, IBS with mixed type of stools

*overlapping cohorts in Paper I and II; **separate cohort in Paper III

Severe psychiatric diseases such as schizophrenia, bi-polar disorder, psychosis, severe depression etc, were exclusion criteria, as well as contraindications for MRI and structural neurological disorders. Roughly one third of the patients as well as two HC (one HC in Paper I & II; two HC in Paper III) had elevated HADS anxiety scores, and only a fraction of IBS

patients had elevated HADS depression subscores (Table 3). The antidepressants used by the patients, depicted in Table 3, especially low dose tricyclic antidepressants, were prescribed due to IBS pain treatment.

Table 3. IBS patients with minor psychiatric disorders and their medication (n (%))

		Paper I	Paper II	Paper III
HADS	HADS-A>11	14 (38%)	12 (38%)	11 (28%)
	HADS-D>11	6 (16%)	5 (16%)	3 (8%)
Antidepressants	Total	17 (46%)	14 (44%)	16 (41%)
	TCA	8 (22%)	6 (19%)	6 (15%)
	SSRI	6 (16%)	5 (16%)	7 (20%)
	SNRI	3 (8%)	3 (9%)	2 (5%)
	NDRI	0	0	1 (3%)

All data are given as number and percent from the IBS cohort respectively. HADS, Hospital Anxiety and Depression Scale; TCA, low dose tricyclic antidepressants; SSRI, selective serotonin reuptake inhibitors; SNRI, serotonin and norepinephrine reuptake inhibitors; NDRI, norepinephrine-dopamine reuptake inhibitors.

Questionnaire data

Comparisons between IBS and HC groups in the cohort from Paper II and III, with regard to significant IBS-related and psychological symptoms are depicted in Table 4 and 5, respectively. Women with IBS displayed significantly lower socioeconomic status assessed with MacArthur SES ladder in comparison with HC in all study populations, with a pooled median of 6 (IQR 5-7) for IBS and 7 (IQR 7-8) for HC; Mann Whitney U 574.5; p=.00078.

In all cohorts IBS patients showed significantly higher somatic symptom severity, anxiety and depression scores, as well as symptom-specific anxiety compared to HC. In Paper II, three subscales from CSQ questionnaire were used for further analyses, namely CSQ-catastrophizing, CSQ-pain behavior and CSQ-diverting attention, as they were significantly correlated with disease specific symptoms measured with IBS-SSS (Spearman's rho> ±0.3). In Paper III, only one coping strategy was significantly different between IBS and HC group, namely CSQ-catastrophizing, however regression analysis revealed instead CSQ-pain behavior, diverting attention and calming self statements (self-talk) coping strategies to predict neurotransmitter concentrations in aINS.

Table 4: Characteristics of patients with IBS and healthy controls (HC) from Paper II

Questionnaire	IBS (N = 32)	HC (N = 15)	<i>U</i>	<i>p</i>	<i>d</i>
Anxiety (HADS)	11.0 (8.0-14.0)	4.0 (2.0-5.0)	22.00	<.0001	2.51
Depression (HADS)	5.5 (4.0-9.7)	1.0 (0-3.0)	52.50	<.0001	2.53
Somatic symptom severity (PHQ-15)	17.0 (13.0-18.0)	3.0 (2.0-7.0)	6.50	<.0001	2.60
Symptom-specific anxiety (VSI)	44.0 (32.0-54.0)	0 (0-3.0)	3.50	<.0001	2.56
Catastrophizing (CSQ)	14.0 (7.0-21.0)	2.5 (0-8.0)	69.50	<.0001	2.50

Significant results of Mann-Whitney U-test and effect size (Cohen's *d*) comparing clinical characteristics in IBS patients and HC. All data are given as median and interquartile range (IQR). HADS, Hospital Anxiety and Depression Scale; PHQ-15, Patient Health Questionnaire; VSI, Visceral Sensitivity Index; CSQ, Coping Strategies Questionnaire. For questionnaire reference, see Method section.

Table 5: Characteristics of patients with IBS and healthy controls (HC) from Paper III

Questionnaire	IBS (N = 39)	HC (N = 21)	<i>U</i>	<i>p</i>	<i>d</i>
Symptom severity (IBS-SSS)	355.0 (291-376)	13.0 (0-49)	.00	<.0001	2.94
Anxiety (HADS)	9.0 (5.75-14.0)	4.0 (2.0-6.5)	176.00	<.001	1.06
Depression (HADS)	5.0 (2.0-8.0)	1.0 (0-3.0)	131.50	<.0001	1.34
Pain intensity (BPI)	15.5 (10.5-22.25)	0.0 (0.0-1.5)	51.50	<.0001	2.05
Pain interference (BPI)	32.0 (14.0-44.0)	0.0 (0.0-0.0)	44.00	<.0001	2.14
Somatic symptom severity (PHQ-15)	15.5 (12.0-18.25)	4.0 (3.0-5.5)	11.50	<.0001	2.63
Symptom-specific anxiety (VSI)	45.0 (30.0-58.75)	2.0 (0-6.0)	14.00	<.0001	2.59
Catastrophizing (CSQ)	14.5 (9.0-18.5)	2.5 (0.0-7.0)	97.50	<.0001	1.60

Significant results of Mann-Whitney U-test and effect size (Cohen's *d*) comparing disease-related and psychological characteristics in IBS patients and HC. All data are given as median and interquartile range (IQR). IBS-SSS, IBS Severity Scoring System; HADS, Hospital Anxiety and Depression Scale; BPI, Brief Pain Inventory; PHQ-15, Patient Health Questionnaire; VSI, Visceral Sensitivity Index; CSQ, Coping Strategies Questionnaire. For questionnaire reference, see Method section.

In order to diminish recall bias, prospective daily pain data reports were used in Paper III. Data from six patients were missing. In general, IBS patients reported about three hours of daily abdominal pain with a

frequency of about seven episodes per week (for details see Table 3 Paper III).

Mucosal barrier data

Electrophysiology, measured by TER after equilibration, was stable, as well as Isc measured throughout the experiment.

There was a significantly decreased TER in biopsies from IBS compared to HC in the beginning of the experiments, after 30 minutes, as well as after 60 minutes from the start. At 90 minutes TER was still lower in IBS, however it did not reach statistical significance.

Interestingly, only in biopsies from IBS added *Salmonella typhimurium* compared to vehicle, TER was significantly decreased, both after 30, 60 and 90 min and this decrease was hampered by pretreating with Keto-tifen but not with anti-VPACs.

Bacterial and paracellular permeability

Both bacterial and paracellular mucosal passage was significantly higher in biopsies of IBS group compared to HC. Bacterial permeability was 2.79 times higher for *Salmonella* in biopsies from IBS than from HC and 1.88 times higher for *Escherichia coli* in biopsies from IBS than from HC (Figure 11). Bacterial passages were not intercorrelated, possibly implicating different uptake mechanisms of the commensal and pathogenic bacteria strains.

Figure 11. Bacterial passage through colonic biopsies mounted in Ussing chambers for 120 min. Comparisons were performed with Mann-Whitney U test, *** $p < .0005$.

The differences were not that robust for paracellular permeability, but still significantly increased in biopsies from IBS compared to HC (Figure 12). Despite substantial overlap between IBS and HC results, a small subgroup of IBS patients showing elevated paracellular permeability could be defined (Figure 12, red dots). Further analysis revealed that this small subgroup also reported significantly less severe IBS symptoms and less symptom-specific anxiety and somatization than the subgroup with more "normal" paracellular permeability. ^{51}Cr -EDTA permeability was not affected by adding bacterial strains into chambers. There was no correlation between ^{51}Cr -EDTA and bacterial passages.

Figure 12. ^{51}Cr -EDTA passage through colonic biopsies of IBS and HC mounted in Ussing chambers at 120 min. Comparisons were performed with Mann–Whitney U test, * $p < .05$ vs vehicle. Red dots, a subgroup of IBS patients with elevated permeability (1.35-2.15cm/sx 10^{-6}).

Regulation by VIP and MC

In chambers pre-treated with ketotifen or anti-VPACs bacterial permeability was significantly decreased not only in IBS but also in HC (Figure 13 A, B).

There was higher plasma VIP concentration in IBS compared to HC, although it did not correlate with any of the Ussing chamber measurements.

Immunofluorescence examinations showed increased amount of MC in colonic biopsies from IBS compared to HC as well as the percentage of MC expressing VPAC1, but no significant difference neither for MC expressing VPAC2, nor for MC containing VIP.

MC density correlated with the percentage of MC expressing VPAC1 as well as the percentage of MC expressing VPAC1 was associated with the percentage of MC expressing VPAC2.

Figure 13. Influence of VIP and MC blockers on passage of *E coli* HS (A) and *S typhimurium* (B) through colonic biopsies of IBS and HC mounted in Ussing chambers at 120 min. Comparisons were performed with Mann–Whitney U test, ** $p < .005$, *** $p < .0005$ vs vehicle.

Questionnaire data and intestinal permeability

Bacterial permeability revealed no significant correlation with any of the symptoms assessed using the questionnaires.

Quite surprisingly, we found a negative correlation between paracellular permeability and some of the IBS-specific symptoms, such as intestinal symptom severity (measured with IBS-SSS, Spearman's $\rho = -0.41$, $p < 0.025$), symptom-specific anxiety (measured with VSI, Spearman's $\rho = -0.42$, $p < 0.024$) as well as coping strategy, pain behavior (assessed with CSQ, Spearman's $\rho = -0.502$, $p < 0.006$) showed in Figure 14.

Figure 14. Graphs of questionnaires exhibiting significant correlations with levels of ^{51}Cr -EDTA passage in the IBS participants only. From left to right, these questionnaires were IBS-SSS, IBS Severity Scoring System; VSI, Visceral Sensitivity Index; and CSQ, Coping Strategies Questionnaire, pain behavior subscale.

Resting state functional connectivity data

Paracellular permeability and DMN connectivity

All the within-group results both for paracellular permeability and bacterial permeability (see below) were masked with the between-group results thresholded at $p < 0.05$ with FWE correction.

Within-group and between-group results of correlation between paracellular permeability and DMN connectivity are depicted in Table 6.

Overall, brain features that correlated with paracellular permeability in the two study groups were hardly overlapping, which would suggest IBS-specific character of the alterations found in this group. In the main, the correlations between paracellular permeability and FC in HC were more various and more widely distributed than in IBS group.

Most interestingly, right amygdala exhibited negative correlation between FC with DMN and paracellular permeability in IBS group, whereas in HC group changes in connectivity in this brain area were not detected at all.

In HC, on the other hand, decreasing paracellular permeability was associated with increased FC between DMN and PAG, which was not observed in IBS group.

Correlation between paracellular permeability and RVM connectivity with DMN was detected in both groups, however in HC the correlation was positive (increased paracellular permeability - increased connectivity RVM-DMN), while in IBS negative (increased paracellular permeability -

Table 6. Within-group and between-group results in correlation between paracellular permeability and DMN connectivity

DMN connectivity with areas belonging to brain networks

	PP	Pain	CEN	SN	EAN	SMN
HC	pos	RVM	right middle frontal gyrus			bilateral pre-/postcentral gyri, thalamus, SMA paracentral lobule
	neg	PAG		ACC, right INS		bilateral precentral gyri
IBS	pos		left inferior frontal gyrus, right superior and medial frontal gyrus, caudate	ACC		SMA and medial paracentral lobule/post-central gyrus
	neg	RVM	left middle and superior frontal gyrus, right inferior frontal gyrus	ACC	right amygdala/parahippocampus	left precentral gyrus right post-central gyrus
IBS vs HC		PAG RVM	bilateral middle and inferior frontal gyri		right amygdala, left orbital frontal cortex	bilateral pre-/postcentral gyri, thalamus, SMA, paracentral lobule

DMN, default mode network; PP, paracellular permeability; Pain, pain processing brain regions; CEN, central executive network; SN, salience network; EAN, emotional arousal network; SMN, sensorimotor network; IBSvsHC, between-group differences; RVM, rostroventral medulla; PAG, periaqueductal grey matter; ACC anterior cingulate cortex; INS, insula; SMA, supplementary motor area

- decreased connectivity RVM-DMN).

As far as DTI results are concerned, the association between paracellular permeability and white matter microstructure would suggest that the findings from rs-fMRI on FC between diverse brain regions and DMN in relation to paracellular permeability correspond to increased white matter fiber organization in the tracts subserving those areas.

Bacterial permeability and DMN connectivity

Mucosal permeability for *Escherichia coli* showed no specific correlation with functional rs-fMRI connectivity of DMN, hence bacterial permeability described below refers to mucosal permeability for *Salmonella typhimurium*.

Within-group and between-group results of correlation between bacterial permeability for *Salmonella typhimurium* and DMN connectivity are depicted in Table 7. Correlations between bacterial permeability for *Salmonella typhimurium* and DMN connectivity in HC and IBS showed more overlap than the findings for paracellular permeability did. Bacterial permeability showed no correlation to RVM-DMN connectivity, as it was the case for paracellular permeability, suggesting that probably paracellular permeability may represent more important source of central sensitization than the passage of live bacteria.

From DTI measurements, white matter fiber organization between some of these brain regions was also increased, suggesting structural underpinnings of observed functional connectivity.

Table 7. Within-group and between-group results in correlation between bacterial permeability and DMN connectivity

DMN connectivity with areas belonging to brain networks

	BP	Pain	CEN	SN	EAN	SMN
HC	pos				bilateral orbital frontal cortices	
	neg	RVM	middle frontal gyri	ACC		pre- and postcentral gyri
IBS	pos	pons	medial frontal gyrus			SMA and medial paracentral lobule/post-central gyrus
	neg			left INS		left precentral gyrus right post-central gyrus
IBS vs HC		RVM pons	middle and inferior frontal gyri	ACC INS	orbital frontal cortex	bilateral pre-/postcentral gyri, SMA, paracentral lobule

DMN, default mode network; BP, bacterial permeability; Pain, pain processing brain regions; CEN, central executive network; SN, salience network; EAN, emotional arousal network; SMN, sensorimotor network; IBSvsHC, between-group differences; RVM, rostroventral medulla; ACC anterior cingulate cortex; INS, insula; SMA, supplementary motor area

Paracellular permeability, DMN-RVM functional connectivity and questionnaire data

Given on one hand, that in IBS only paracellular permeability showed correlation with clinical and behavioral parameters, and on the other hand that in this group the correlation between paracellular permeability and DMN-RVM functional connectivity was reversed compared to HC, the question arised if and how the observed FC within RVM was related to IBS and psychological symptoms. To explore this, a path analysis was performed (Figure 15). Because RVM is known to be a key brain region involved in descending pain modulation, we assumed in our path analysis the unidirectional effect of paracellular permeability on RVM functional connectivity and chose the questionnaires correlated with paracellular permeability (IBS-SSS, VSI and CSQ-pain behavior) to model indirect effects on RVM (Figure 15). Path analysis indicated significant total effect of paracellular permeability on RVM (-0.603 ± 0.134 , $p < 0.001$), in line with our resting state DMN connectivity findings, as well as significant direct effect (-0.399 ± 0.174 , $p < 0.022$). The other component of the total effect was a trend-level indirect effect (-0.204 ± 0.11 , $p < 0.062$), almost solely driven by the CSQ-pain behavior (-0.203 ± 0.090 , $p < 0.024$), suggesting that this coping strategy, instead for somatic symptom severity or visceral specific anxiety, may modulate the correlation between barrier function and DMN-RVM connectivity.

Figure 15. Path model used to assess the mediating effects of the IBS-SSS, CSQ and VSI questionnaires on the effect of permeability on resting state functional connectivity in the RVM for the IBS patients only. Solid arrows indicate direct effects; dashed arrows indicate indirect effects. Significant and trend-level ($p < 0.01$) coefficients are shown in standardized form.

Quantitative Magnetic Resonance Spectroscopy data

aINS neurotransmitter concentrations

qMRS data analysis showed significantly lower concentrations of excitatory neurotransmitter Glx in aINS in IBS patients compared to HC. Differences were more robust for the right than the left hemisphere (right aINS $U = 170$; $p < .001$, left aINS $U = 228.5$; $p = .017$; Figure 16) although significant for both sides. However, no significant differences between IBS and HC were found in GABA+ concentrations in either of the hemispheres.

Figure 16. Results from group comparisons of Glx concentrations in (A) left and (B) right aINS in HC and IBS patients. All data are given as median and interquartile range (IQR). Significantly lower bilateral aINS Glx concentrations were observed in patients with IBS compared to HC. * $p < .05$; *** $p < .001$. Glx, glutamate and glutamine.

aINS neurotransmitter concentrations and questionnaire data

Stepwise regression analyses performed in IBS patients group showed hemisphere-specific predictors of Glx concentrations. For the right aINS, longer abdominal pain duration significantly predicted lower Glx concentrations ($R^2 = .354$; $F = 15.32$; $\beta = -.624$; $t = -4.44$; $p < .001$; Figure 17A); as well as less frequently used coping strategy pain behaviors, however in a less robust way ($R^2 = .117$; $F = 5.99$; $\beta = .344$; $t = 2.45$; $p = .021$; Figure 17B).

For the left aINS, less frequent use of the coping strategy diverting attention significantly predicted lower Glx concentrations ($R^2 = .228$; $F = 8.28$; $\beta = .478$; $t = 2.88$; $p = .008$; Figure 17C).

GABA+ concentrations were comparable in both groups. However, regression analyses revealed that less frequently used pain coping strategy calming self statements predicted higher concentrations of the inhibitory neurotransmitter in the left aINS ($R^2 = .187$; $F = 6.46$; $\beta = -.433$; $t = -2.54$; $p = .017$; Figure 17D). No model for the right aINS GABA+ was found.

Figure 17: Scatterplots with regression curves and 95% confidence intervals depicting significant results from stepwise regression: (A) abdominal pain duration and (B) CSQ-pain behavior as predictors of Glx concentration in the right aINS, (C) CSQ-diverting attention as a predictor of left aINS Glx and (D) CSQ-calming self statements (self-talk) as a significant predictor of GABA+ concentrations in the left aINS. CSQ, Coping Strategies Questionnaire; Glx, glutamate + glutamine; GABA+, γ -aminobutyric acid + co-edited macromolecular signals.

DISCUSSION

Irritable bowel syndrome is a flagship disease of all the disorders of brain-gut interaction, mostly due to its prevalence and substantial economic and individual disease burden. The bidirectional and multilayered brain-gut axis is a well-established paradigm in the still not completely understood disease pathology. This thesis aimed to elucidate mechanisms that contribute to both peripheral and central factors along the brain-gut axis and to investigate possible interactions, which might be crucial to broaden our understanding of IBS pathophysiology (Figure 18).

Figure 18. Overview of the main findings of the thesis.

Brain-gut axis: gut aspects

Disturbances in the intestinal mucosa function, as a result of gastrointestinal infection and impaired mucosal barrier, are among the first findings in IBS research. The idea of a "leaky gut" has been discussed for several decades and still, new studies confirming disruption of intestinal barrier function in IBS arise, yet mainly considering specific subgroups of patients, i.e., in IBS-D or PI-IBS. Recently, however, evidence of intestinal barrier dysfunction also in other IBS subgroups has been emerging [58,76,147,195,214,227,271].

Our results confirm the alterations in the structure and the function of colonic mucosa barrier in IBS patients, regardless of IBS subgroup. Since activated MC are known to modify mucosal permeability [101,111,259], we ascertained the absence of allergy and no use of NSAID in our cohort in order to avoid bias. We found decreased levels of TJ protein occludin in IBS irrespective of a consistency subgroup in comparison to HC at the baseline, further diminished after adding *Salmonella*, but restored after blocking VIP receptors or MC. This implicates the importance of immune activation in intestinal barrier disruption. Moreover, we did not detect the bacteria to enter the mucosa through the paracellular space, thus disruption of TJ appears not to be explained by a direct bacterial effect. Finally, addition of bacteria did not influence the paracellular permeability measured as permeability to ^{51}Cr -EDTA, despite the observed substantial effect of *Salmonella* on TER.

Interactions between intestinal mucosa and microbiota have previously been demonstrated, however, these approaches have applied killed bacteria strains, which likely does not mimic the in vivo pathological processes [113,110,111]. Moreover, there are very few studies employing Ussing chamber experiments for ex-vivo human studies [75,113,185], a technique that gives the undeniable advantage of meticulous investigation of live human tissue. Here, we studied mucosal permeability for live bacteria, both commensal and pathogenic, and found markedly increased bacterial passage as well as significantly increased paracellular permeability. Despite the absence of PI-IBS cases in our cohort, our findings show almost no overlap between IBS and HC in bacterial passage for both bacterial strains, which highlights the significance of the well-established interaction between intestinal mucosa and microbial flora in IBS. In fact, the role of microbiota in IBS pathophysiology has lately been deemed of interest. Namely, evidence supports the impact of bacteria and its metabolites on mucosal barrier and immune function, motility, visceral sensitivity and interactions with the enteric and central nervous system, in-

cluding emotional behavior and pain modulation [154]. On the other hand, gut microbiota composition is subject to parasympathetic modulation of the ANS. The ANS can modify intestinal motility by either compromising ENS-generated migrating motor complex, which can lead to bacterial overgrowth, or by mediating stress-induced transit acceleration, which can result in diminished bacterial richness [154]. Furthermore, ANS modulates mucus secretion, changing the thickness and quality of the mucus layer, thus modifying the habitat of the gut biofilm [143]. The lack of mucus on colonic biopsies, due to the circulation in Ussing chambers, may contribute to the observed impactful susceptibility of examined mucosa to bacterial passage, probably significantly tempered in vivo by adequate mucus layer. Another important effect of the ANS on microbiota is conducted through the interaction between the ANS and the intestinal immune system. Neuronal and neuroendocrine signaling from neurons and immune cells can change the microbial flora composition and activity [154]. In our study, impaired intestinal permeability was restored both in IBS and HC by blocking VIP receptors and stabilizing MC. The reciprocal interaction between enteric neurons and MC requires previous MC activation, which is corroborated by our findings of enhanced MC degranulation and VPAC1 expression in IBS, as well as by elevated levels of tryptase in biopsy lysates from IBS patients. Despite the elevated MC counts in our cohort, there was no association with IBS symptoms. The substantial influence of MC on intestinal barrier is well recognized [15,16,29,35,42,50,83,120,149,150,263], and stabilizing of MC with ketotifen has proven not only to restore intestinal permeability, but also to alleviate IBS symptoms [120]. However, the decrease of bacterial passage after blocking MC in our study was comparable to the effect obtained by blocking VIP receptors. VIP is a neurotransmitter produced by different cells, including MC, although mainly derived from neurons, and is also released duringin response to stress [112], particularly chronic, psychological stress. Our group has previously demonstrated pro-inflammatory effects of VIP both in animals and humans [34,112], in line with the observations made herein that showed tightening of mucosal barrier after blocking VIP both in a physiological health situation and a disorder of brain-gut axis strongly associated with chronic stress. We observed increased VIP serum levels and numerically but not statistically higher concentrations of VIP in biopsy lysates in IBS. This could implicate increased activity of the central (and probably the peripheral) nervous system in IBS, as the CNS is known to release the majority of VIP measured in the blood [87].

In order to understand the clinical relevance of the demonstrated alterations in colonic mucosal barrier function in IBS, we attempted to find associations with either somatic or psychological symptoms reported by the patients. Despite unprecedented findings on increased bacterial translocation in colonic mucosa in IBS, no correlation was found between bacterial passage and any of the IBS symptoms. Neither VIP nor MC density were connected to any of the symptoms. Paracellular permeability, however, was negatively associated with IBS symptom severity, symptom-specific anxiety and coping strategies. Our correlational findings clearly indicate that IBS symptomatology cannot be explained merely with the peripheral alterations and needs to be elucidated in a bigger perspective of the whole brain gut axis.

Brain-gut axis: brain aspects

Pain is the pivotal symptom of IBS [159]. Whatever type of pain in question, the brain is definitely the last relay to expose the pain sensation. Brain imaging indeed substantiates the patient's subjective assessments of pain and other psychological factors known to shape the disease. There is accumulating evidence from fMRI studies pointing out altered neural responses to expected or delivered rectal distensions in patients with IBS, particularly involving brain regions forming the brain's salience, emotional arousal and central executive networks [128,137,211,242]. As we observed association between mucosal barrier function and several somatic IBS symptoms and psychological factors, the question appeared if and how brain imaging might support these findings. We aimed to link the peripheral findings with the intrinsic brain activity during rest, focusing on the DMN as the most important task-negative brain network, known to be associated with self-related processing and tightly coupled with monitoring of internal organs [9]. rs-fMRI FC, resembles momentary brain activity changes, thus we expanded our observation using DTI, to determine microstructural integrity of the white matter and its possible correspondence with FC fMRI findings. Although scarce, IBS studies applying DTI demonstrate microstructural changes within sensory processing brain regions [39,61]. While our peripheral findings of bacterial passage were more robust than the ones referring to paracellular permeability, it was the paracellular permeability that showed associations with IBS symptoms. Also, associations between permeability and resting state functional connectivity of the DMN were more pronounced for the paracellular permeability. Specifically, mucosal permeability for *Escherichia coli* showed no association with rs-fMRI FC of the DMN, whereas the cor-

relations for the passage of *Salmonella* demonstrated substantial overlap between the findings in HC and IBS.

In HC, increasing paracellular permeability was related to increased FC between the DMN and the RVM and decreased paracellular permeability was associated with increased FC between the DMN and the PAG and SN. That would indicate that in health, when the gut barrier is tight, the ascending pain processing accords with the well-known visceral pathway and employs the right insula to judge the salience of the afferent signals, as well as the PAG, the brain region of descending pain inhibition [74,251,266], to subordinate visceral stimuli. On the other hand, when the gut barrier is more disrupted, increasing afferent input comes up to the brain. Then, the brainstem-derived pain inhibition occurs via the final relay of pain output, the RVM, without engaging higher-order pain structures.

Nevertheless, that seems not to be the case in IBS. Here, we observed a correlation between decreased paracellular permeability and increased connectivity of the DMN with the RVM and the right amygdala.

In an attempt to explicate these associations, the paracellular findings were placed under thorough scrutiny. When looking closely at the paracellular permeability, considerable overlap was seen between IBS and HC, with a small subgroup of IBS patients showing higher paracellular permeability than HC. Further analyses revealed that this subgroup with heightened permeability reported less severe IBS symptoms and less symptom-specific anxiety than the subgroup with paracellular permeability comparable to that of HC. While inspecting the rs-fMRI – permeability data in these subgroups, our findings suggested that in IBS patients with “normal” gut barrier function, self-referential processing of visceral input engages the hub of pain and emotion processing, the amygdala, as well as the descending pain facilitation via the RVM. IBS patients with increased permeability, however, displayed DMN connectivity corresponding with the pattern seen in HC.

The amygdala has a pivotal role in decoding the emotional significance of afferent stimuli and in shaping behavioral responses and emotional memories [92]. It is also known to be an important center of pain processing and modulation, being able to switch off and on nociceptive pain perception [198]. We found the right amygdala to be activated, which is in line with previous evidence of lateralization of the amygdala in somatic and visceral pain, indicating pronociceptive function of the right amygdala [10,200].

On the other hand, none of the abovementioned IBS subgroups, neither the patients with "normal" nor the ones with increased paracellular permeability, exhibited significant correlations between mucosal function and FC between the DMN and the PAG, a key hub of the descending pain inhibition system [176]. Neither did the bacterial permeability show any DMN-PAG correlations. This would suggest impaired central pain inhibition in IBS. As the RVM, however, may exert both pain facilitating and inhibiting effects [176], it could be hypothesized that the IBS subgroup with higher paracellular permeability activates more effectively RVM-driven pain inhibition, due to increased peripheral input, while the subgroup with more "normal" paracellular permeability engages RVM-derived pain facilitation, possibly prompted by emotional modulation in the right amygdala.

In Paper II, the paracellular permeability correlations with brain FC were not identical with the bacterial permeability- brain FC correlations and much more robust for the paracellular findings, advocating perhaps different mechanisms that support those associations. One could speculate that changes in paracellular permeability, as a more enduring process than disruption in mucosal barrier due to bacterial invasion, exert a predominant impact on brain function, directly or indirectly. Moreover, both for the bacterial and paracellular permeability, DTI findings confirmed enhanced structural fiber organization among many of the abovementioned brain regions depicted using FC fMRI, implicating a persistent character of the observed associations. Together, these findings suggest that mucosal barrier function might be associated with self-referential processing attributable to the DMN, as well as to compromised descending pain modulation pathways, known to contribute to central sensitization [7,260].

The sensation of pain, however, is experienced only when considered salient and made conscious. This process of switching between the self-related DMN and the cognitive CEN depending on the saliency and emotional load of the stimulus is conducted by the salience network's core node, aINS, constituting the interface between sensory input from posterior insula and emotional input from the amygdala [161]. This brain region, one of the most often activated brain regions in functional neuroimaging studies [38], is widely recognized as a hub of interoceptive awareness and cognitive and emotional pain modulation and thus a highly important component of brain-gut visceral pain pathways [45,48]. Alterations of functional connectivity between SN, DMN, CEN and the INS have been reported in IBS [95,98,253], however little is known about the un-

derlying changes in brain neurotransmitters in this brain region. Glu, the main excitatory neurotransmitter, is involved in modulation of visceral sensitivity and motility at the level of the ENS, as well as in the regulation of both the afferent and efferent pathways of the brain-gut axis [69]. qMRS enables measurement of both excitatory and inhibitory neurotransmitters in the brain tissue, however MEGA-PRESS technique used in our study is specifically targeted for the quantification of GABA+ concentrations, hence allowing detection of Glx as a combined measure of Glu and Gln [173,194]. We observed reduced Glx concentrations in bilateral aINS with more robust results for the right aINS, which indicates alterations in excitatory neurotransmission in this brain region. This may support the observed aberrations in cognitive and emotional pain processing and the disability to engage descending pain inhibitory pathways in chronic visceral pain conditions [243,244,267]. To our best knowledge there is only one existing qMRS study in IBS, showing reduced hippocampal Glu level [170] interpreted as a potential hypofunction of hippocampal glutamatergic neurotransmission in IBS. Studies in other chronic pain conditions are inconsistent, demonstrating unchanged Glu concentrations in aINS [68,67,89,88] or increased levels in pINS [8,88] in fibromyalgia as well as increased Glu in aINS in endometriosis-associated chronic pelvic pain [8]. That discordance may result from for instance differences in applied MRS techniques, different distribution of grey matter versus white matter and cerebro-spinal fluid in the voxel, varying disease types, or intensity and duration of the disease.

In our study, we found that lower Glx levels in the right aINS were predicted by more pain, while in the left aINS lower Glx levels were related to less use of adaptive pain coping strategies, which is in line with a presumed functional asymmetry of aINS [45,47,268]. Studies on functional connectivity between aINS and subcortical and cortical areas support the notion of hemispheric lateralization both in health and chronic pain conditions [107,268]. Kann and colleagues demonstrated in their FC study that the right aINS, receiving input from sympathetic afferents, projects to cortical brain areas associated with attention and arousal, whereas the left aINS, encoding parasympathetic input, projects to brain regions involved in cognitive control and perspective taking [107]. Zhou et al confirmed a correlation between increased neural activity in the right aINS and disease severity in functional dyspepsia patients [270]. Moreover, Lowén et al showed that BOLD response to cued rectal distension in the right aINS may be attenuated as a result of successful IBS treatment with hypnotherapy or educational intervention [142]. On the other hand, augmented parasympathetic input induced by slow breathing increased acti-

vation of the left aINS, which resulted in decreased acute pain intensity and unpleasantness scores in HC, an effect that was also detected in visceral pain patients suffering from fibromyalgia, albeit to a lower extent [268]. Altogether, there is mounting evidence that confirms hemisphere-specific associations between aINS and visceral pain processing and regulation, further corroborated by our findings, providing additional evidence on altered brain biochemistry in aINS as a possible biochemical underpinning of the observed aberrant neuroactivation.

Brain-gut axis: psychological aspects

The impact of mood and emotional states on pain perception is widely recognized, both for chronic and acute pain states [243]. Similarly, the influence of adaptive and maladaptive coping is pivotal in IBS pathophysiology and treatment [56,118,209,212,213,247,261].

Although anxiety and depression are clearly overrepresented in IBS and commonly proven to exert significant influence on disease severity [62,126,130,256], we did not observe association between anxiety and depression assessed with HADS and peripheral measures. We demonstrated a negative correlation between paracellular permeability and disease specific anxiety, assessed with VSI, which however failed to show any significant effect on modulating the correlation between the barrier function and DMN-RVM connectivity.

Nevertheless, it is noteworthy that in all of our studies we observed significant association between coping strategies assessed with CSQ and both peripheral and central measures. Namely, we demonstrated a negative correlation between pain behaviour coping strategy and paracellular permeability. This coping strategy had also a significant negative indirect effect on the correlation between intestinal barrier function and DMN-RVM connectivity. This suggests that the influence of paracellular permeability on connectivity between DMN and top-down pain control might be modulated by the utilization of pain coping strategies. On the other hand, less use of diverting attention coping strategy proved to predict lower Glx concentrations in the left aINS. One could speculate that engagement of this coping strategy might influence the activation of the left aINS, commonly recognized to corroborate in attenuation of pain sensations. As mentioned before, slow breathing, that activates parasympathetic afferents, which terminate in left aINS, resulted in HC in decrease of affective responses to acute pain stimulation [268].

The significance of cognitive and behavioral coping is well established in IBS pathophysiology and its relevance in IBS treatment is well

acknowledged [64] and fully corroborated by our findings. Indeed, treatment strategies that target psychosocial factors in IBS, such as cognitive behavioral therapy, mindfulness-based therapy or gut directed hypnotherapy, incorporating the facilitation of adaptive coping strategies, augmenting parasympathetic afferent input and modulating activation of cortico-subcortical brain regions, have an overall NNT (number needed to treat) of four patients (95% CI: 3–5), which is better than most of the drug therapies available in IBS [70].

CONCLUSIONS

In the studies summarized in this thesis, we aimed to assess and bring together some of the peripheral and central mechanisms in IBS and elucidate possible mutual associations.

Exploratory investigations of the peripheral mechanisms revealed significantly increased paracellular permeability as well as passage of commensal and pathogenic live bacteria in IBS compared with HC, engaging MC and VIP as regulating factors. Despite the clearly altered barrier function in IBS, no correlation was found between bacterial passage and IBS symptoms; however, a negative association between paracellular permeability and some somatic and psychological IBS symptoms was detected.

Moreover, we aimed to determine associations between paracellular colonic barrier and structural and resting-state functional brain connectivity. This novel approach resulted in the identification of different patterns of correlation between colonic mucosa permeability and functional and anatomical brain connectivity in IBS compared to HC. Specifically, in patients with permeability within the range of HC, an association was detected not only with more severe IBS symptoms but also with increased functional and structural connectivity between DMN and brain regions involved in pain modulation.

Finally, using qMRS, we aimed to shed light on putative biochemical underpinning of altered function of the aINS as a key node of the SN, pivotal in saliency switching, as well as in visceral pain processing and modulation. We showed decreased concentrations of the excitatory neurotransmitter Glx in bilateral aINS in IBS compared to HC, while inhibitory neurotransmitter GABA+ levels were comparable. More pain predicted lower Glx levels in right aINS while less use of adaptive pain coping strategies were correlated with lower Glx concentrations in the left aINS, which is in line with a previously proposed functional asymmetry of aINS. Our findings suggest that glutamatergic deficiency might be involved in a failure to adaptively engage aINS in pain processing and its cognitive modulation which could substantiate enhanced pain symptoms and increased symptom burden in patients with IBS.

This thesis broadens the knowledge on peripheral and central mechanisms in IBS, showing evidence on disturbed intestinal barrier function,

regulated by MC and VIP as well as altered biochemistry of the core brain region of SN, correlated to somatic and psychological IBS symptoms in a hemisphere-specific way. Moreover, we present novel findings that outline some of the mechanisms of the brain-gut axis, by showing association between mucosal permeability, IBS symptoms and resting-state functional and structural connectivity that engage brain regions involved in emotion and pain modulation. Our findings thereby complement and extend a bio-psycho-social disease model of IBS, corroborating the essential role of coping strategies in IBS pathophysiology and thus supporting the relevance of psychological treatment strategies in IBS.

FUTURE DIRECTIONS

Although IBS is definitely the most studied disorder of gut-brain interaction, the complexity of this disease offers boundless possibilities for future research. The unquestionably crucial role of psychological aspects in the disease pathophysiology limits the validity of animal models in IBS research. Instead, in order to study peripheral mechanisms, such as the enteric nervous system and its regulation by the gut microbiota, human organoid engineering to create better in vitro models could be employed. The effect of microbiota on brain function has been lately deemed of interest [154], however still little is known about the mechanisms of interaction between the gut microflora, the intrinsic and the extrinsic primary afferent neurons. On the other hand, the lateralization of the central findings and the common knowledge of the termination of the parasympathetic afferents in the left aINS, make it highly interesting to study the influence of vagal nerve stimulation on pain perception and modulation in IBS.

Ultimately, it is the brain that is responsible for creating the feeling of pain, thus clearly brain research is well established in IBS. BOLD fMRI as well as functional connectivity are cornerstones in brain imaging; however, those techniques do not provide any information of the neurochemical underpinnings of the observed blood flow changes. qMRS used in our study enables the measurement of neurotransmitter concentrations providing important insight in biochemistry potentially underlying aberrant brain function. Novel developments in brain imaging techniques are emerging particularly in the field of spectroscopy, allowing a more fine grained detection of neurotransmitters in much smaller voxels with higher resolution and also in deeper brain tissue, such as amygdala, which is now inaccessible for qMRS studies. One of the new technologies is functional ^1H MRS (fMRS) [229] able to detect task-induced changes in Glu concentrations, both in a single voxel and in multiple voxels. Multi-voxel fMRS, called Magnetic Resonance Spectroscopy Imaging (MRSI) is a technique, where a 2D or even 3D voxels can cover whole brain with a high spatial resolution. In fact, proof-of-concept studies using simultaneous acquisition of both BOLD-fMRI and glutamate changes with MRSI [99] demonstrate significant correlation and spatial accuracy between the hemodynamic and neurochemical responses. Combining those techniques

with DTI could even provide data on sustainability of the observed alterations.

I strongly believe that multimodal brain research may help to detect new disease biomarkers, as well as different disease fingerprints, enabling individualized treatment. Although the present IBS subgrouping based on Rome criteria is still justifiable from a clinical point of view, it may not fully reflect the disease pathophysiology, which we also confirmed in our studies. However, what we did repeatedly observe in our studies was the correlation of our findings with psychological aspects, specifically coping strategies. As cognitive behavioral therapy constitutes an established type of IBS treatment, this thesis further corroborates the importance of individualized psychological interventions as an evidence-based instrument in the management of IBS. A multimodal brain imaging study investigating changes in brain responses before and after a successful psychological intervention, as well as sustainability of these responses, would be highly interesting.

Finally, future research should pay attention to two major aspects, which are likely to significantly impact the generalizability. One of them is the power, especially as far as brain imaging is concerned. In order to avoid this issue, networking and centers of excellence may be a solution. Next is the sex and gender aspect as it is well known that IBS symptomatology and pathophysiology differs substantially between women and men [37,125]. Even in this case, collaboration between different secondary and tertiary care centers, as well as establishing quaternary centers, may help to surpass this issue. Chronic pain is an epidemic worldwide. We will never solve it unless we work together, beyond our urge to stand out in the research world.

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