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Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

Lehrstuhl für Humanbiologie

Effect of sera from patients with inflammatory bowel disease on guinea pig enteric neurons

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Aim for the moon. If you miss, you may hit a star.

W. Clement Stone

For my family

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Publications

Peer-reviewed Publications

A fresh look at IBS-opportunities for systems medicine approaches.

Albusoda A, Barki N, Herregods T, Kamphuis JB, Karunaratne TB, **Lazarou M**, Lee I, Mazurak N, Perna E, Polster A, Pribic T, Uhlig F, Wang H, Enck P

Neurogastroenterol Motil. 2017 Mar; 29(3). DOI: 10.1111/nmo.12989. Epub 2016 Nov 7.

Targeting nNOS ameliorates the severe neuropathic pain due to chronic pancreatitis

Demir I E, Heinrich T, Carty D,Saricaoglu O S, Klauss S, Teller S, Kehl T, Reyes C M, Tieftrunk E, **Lazarou M**, Bahceci D H, Gökcek B, Ucurum B E, Diakopoulos K, Lesina M, Schemann M, Erkan M, Krüger A, Algül H, Friess H, Ceyhan G

EBioMedicine. 2019 Aug; 46:431-443. DOI:10.1016/j.ebiom.2019.07.055. Epub 2019 Aug 8.

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Sera from Crohn's disease patients excite enteric neurons

Lazarou M, Buhner S, Pehl C, Bourreille A, Neunlist M, Schemann M

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Summary

The enteric nervous system (ENS), which is located in the gut wall, is responsible for the normal gut function. It regulates motility, secretion and immune cell functions. Alterations in neuronal function and morphology of cells in the gut wall are often associated with gut pathologies, such as inflammatory bowel disease (IBD). IBD is a group of chronic conditions characterized by mucosal or transmural inflammation in the gastrointestinal wall. The two main types of IBD are Crohn's Disease (CD) and Ulcerative Colitis (UC). UC affects the innermost lining of the colon and the rectum. The inflamed areas develop ulcers. In contrast, CD has a patchy spread of inflammation and it may affect the whole gastrointestinal tract from the mouth to the anus. Patients in both groups usually experience motility and secretion problems as described by abdominal pain, discomfort and diarrhoea. The symptoms suggest an effect of inflammation on enteric neurons since the enteric nervous system regulates all GI functions.

Furthermore, cytokines and antibodies (autoantibodies and microbial antibodies) were shown to be upregulated in the serum of those patients (Mitsuyama et al., 2016). Therefore, our aim was to study the activation profile of enteric neurons upon serum application. In particular, we wanted to delineate the influence of blood-borne factors on neural excitability.

Experiments were performed on guinea pig submucous neurons. Given the close proximity of submucosal neurons and immune cells, we expected the neuronal activation to be primarily a feature of the submucous plexus (Buhner et al., 2012). To investigate the activation of the enteric neurons upon stimulation with sera we used the neuroimaging technique with voltage-sensitive dye. This technique allows high temporal and spatial resolution. A high number of neurons activated by the serum at the same time can be visualised.

Our results revealed that sera from CD patients activate enteric neurons in the submucous plexus. The sera from CD patients activated a higher number of neurons. Moreover, these neurons fired with a higher frequency compared to the neurons treated with the sera from healthy controls. Furthermore, activation of enteric neurons by the sera was independent of the disease stage. Enteric neurons responded equally strong to the application of sera

from CD-active and CD-remission patients. Thus, these results suggested that neuronal activation might be a general feature in CD patients.

TNF-α has a role in ongoing neuronal activation. Inhibiting TNF-α activity in the sera of CD patients with a specific antibody reduced the number of action potentials significantly. In parallel, these functional experiments proposed the importance of other cytokines residing in the serum as the neuronal activation was not completely abolished by blocking TNF-α activity.

Moreover, the study pointed to differences in neuronal activation between sera from CD patients and sera from UC patients. The evoked action potential discharge and the number of neurons responding to sera from UC patients (active and remission) were reduced compared to the ones from CD patients. The activation caused by UC patient sera was comparable to the activation triggered by the sera from healthy controls.

In conclusion, our results emphasize the importance of serum factors for the excitability of enteric neurons and suggest the functional relevance of a blood-ENS axis.

Das enterische Nervensystem in der Darmwand ist für die normale Darmfunktion verantwortlich. Es reguliert die Beweglichkeit, die Sekretion und die Funktionen der Immunzellen. Veränderungen in der neuronalen Funktion und Morphologie sind oft mit Darmpathologien, wie der Entzündlichen Darmerkrankung (CED) verbunden. CEDs sind eine Gruppe von chronischen Erkrankungen, die durch Schleimhaut- oder transmurale Entzündungen in der Magen-Darm-Wand gekennzeichnet sind. Die beiden Haupttypen der CEDs sind Morbus Crohn (MC) und Colitis ulcerosa (CU). CU beeinflusst die innerste Auskleidung des Dickdarms und Rektums und verursacht Entzündungen. Diese entzündeten Stellen entwickeln Geschwüre. Im Gegensatz dazu hat die MC eine ungleichmäßige Entzündungsausbreitung und kann den gesamten Magen-Darm-Trakt vom Mund bis zum Anus betreffen. Patienten in beiden Gruppen leiden in der Regel an Motilitäts- und Sekretionsproblemen, die durch Bauchschmerzen, Unwohlsein und Durchfall beschrieben werden. Diese Symptome deuten auf eine Entzündung der enterischen Neuronen hin, da alle GI-Funktionen durch das enterische Nervensystem (ENS) reguliert werden.

Darüber hinaus konnten erhöhte Zytokine und Antikörper (Autoantikörper und mikrobielle Antikörper) im Serum dieser Patienten nachgewiesen werden (Mitsuyama et al., 2016). Das Ziel dieser Arbeit war es, das Aktivierungsprofil von enterischen Neuronen bei der Serumanwendung zu untersuchen und den Einfluss des durch das Blut übertragenen Faktors auf die neuronale Erregbarkeit darzustellen.
Hierfür wurden Experimente an submukösen Neuronen von Meerschweinchen durchgeführt. Aufgrund der unmittelbaren Nähe von submukösen Neuronen und Immunzellen erwarteten wir, dass die neuronale Aktivierung durch Mediatoren in erster Linie das Merkmal des submukösen Plexus ist (Buhner et al., 2012). Um die neuronale Aktivierung der Neuronen bei Stimulation mit Seren zu untersuchen, wurde die Neuroimaging-Technik mit spannungssensitiven Farbstoffen eingesetzt. Diese Technik ermöglicht eine hohe zeitliche und räumliche Auflösung. Somit können eine Reihe von Neuronen visualisiert und gleichzeitig durch das Serum aktiviert werden.

Die Studie ergab, dass Seren von Morbus Crohn Patienten enterische Neuronen aktivieren. Sie aktivieren mehr Neuronen mit einer höheren Aktionspotenzialfrequenz, als Seren aus gesunden Kontrollen. Darüber hinaus war die Aktivierung der enterischen Neuronen durch die Seren unabhängig vom Krankheitsstadium. Seren von Patienten mit

aktiver Entzündung und im Remissionsstadium aktivierten enterischen Neuronen mit gleicher Frequenz. Diese Ergebnisse deuten darauf hin, dass die neuronale Aktivierung ein allgemeines Merkmal bei MC-Patienten sein könnte.

Die Hemmung der TNF-α-Aktivität in Seren von MC-Patienten in beiden Krankheitsstadien mit einem spezifischen Antikörper gegen sie (Adalimumab) verwies auf ihre Rolle bei der laufenden neuronalen Aktivierung, da die Frequenz des Aktionspotenzials signifikant reduziert wurde. Parallel dazu zeigten diese funktionellen Experimente die Bedeutung anderer Zytokine im Serum auf, dadurch dass die neuronale Aktivierung durch den TNF-α Antikörper nicht vollständig blockiert wurde.

Darüber hinaus verdeutlichte die Studie Unterschiede in der neuronalen Aktivierung zwischen Morbus Crohn und Colitis ulcerosa. Die evozierte Aktionspotentialentladung und die Anzahl der Neuronen, die auf das Serum von CU-Patienten ansprechen (aktiv und Remission), waren geringer als bei MC-Patienten. Die durch Seren von CU-Patienten verursachte Aktivierung war vergleichbar mit der durch Seren verursachte Aktivierung aus Kontrollen von gesunden Patienten.

Abschließend hebt die Studie die Bedeutung von Serumfaktoren für die Erregbarkeit von enterischen Neuronen und die funktionelle Bedeutung einer Blut-ENS-Achse hervor

Abbreviations

5-HT_3	Serotonin
AH neuron	After-Hyperpolarizing Neuron
AJC	Apical Junction Complex
AP	Action Potential
APC	Antigen Presenting Cells
ATP	Adenosine Triphosphate
CCD	Charged – Coupled Device
CD	Crohn's Disease
CDAI	Crohn's Disease Activity Index
ChAT	Choline Acetyltransferase
CRP	C-Reactive Protein
Di-8-ANEPPS	(1-(3-Sulfanato-Propyl-4-[B-[2-(Di-N-Octylamino)-6-Naphthyl] Vinyl] Pyridinium Betaine
DIV	Division Day
DC	Dendritic cells
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNBS	Dinitrobenzene Sulfonic Acid
DRG	Dorsal Root Ganglia
DSS	Dextran Sodium Sulfate
EGC	Enteric Glia Cells
ENCCs	Enteric Neural Crest Cells
ENS	Enteric Nervous System

EPSP	Excitatory Postsynaptic Potential
FODMAP	Fermentable Oligo-, Di-, Mono-saccharides And Polyols
FBS	Fetal Bovine Serum
GALT	Gut Associate Lymphoid Tissue
GB	Leucocytes
GI	Gastrointestinal
GP	Guinea Pig
GWAS	Genome Wide Association Study
H1	Histamine Receptor 1
H2	Histamine Receptor 2
HC	Healthy Control
HCT	Haematocrit
Hg	Haemoglobin
HRP	Horseradish Peroxidase
HS	Horse Serum
HuD	HuD Protein
IBD	Inflammatory Bowel Disease
IBS	Inflammatory Bowel Syndrome
ICC	Interstitial Cells Of Cajal
IHC	Immunohistochemistry
IL	Interleukin
INF	Interferon
LP	Lamina Propria

LPMC	Lamina Propria Mononuclear Cells
MSORT	Multisite Optical Recording Technique
MC	Mast Cell
NMDA receptor	N-methyl-D-aspartate receptor
NK	Natural Killer
NO	Nitric Oxide
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline –Tween
S neuron	Synaptic Neuron
SCD	Specific carbohydrate diet
SCFA	Short Chain Fatty Acids
SMP	Submucous Plexus
SNP	Single Nucleotide Polymorphism
SP	Substance P
Th-cells	Tumour helper cells
TNBS	2,4,6-Trinitrobenzene Sulfonic Acid
TNF	Tumour Necrosis Factor
Treg	Regulatory T Cell
TRPA1	Transient Receptor Potential Ankyrin
Tuj-I	Tubulin
TX	Triton-100
UC	Ulcerative Colitis

VIP	Vasoactive Intestinal Peptide
WT	Wild Type

1. Introduction

1.1 Enteric nervous system

The enteric nervous system (ENS) is embedded in the lining of the gastrointestinal tract (Bayliss and Starling, 1899). The human ENS consists of >120.000.000 neurons. In rodents neuron numbers can range from 2.000.000 neurons in the mouse to ~ 15.000.000 neurons in the guinea pig (Dr Klaus Michel, 2019, unpublished data). This extensive intrinsic nervous system regulates motility, secretion, local blood flow and immune responses (Costa et al., 2000).

The enteric neurons are organised in ganglionated plexuses. The two plexuses are the myenteric plexus (Auerbach's plexus) and the submucous plexus (Meissner's plexus). The myenteric plexus is located between the longitudinal and circular muscle layers while the submucous plexus between the mucosa and the circular muscle layer (Costa et al., 1996, 2000; Furness, 2000; Timmermans et al., 1992). The submucous plexus is found only in the small and large intestines. The neuronal density of submucous plexus is lower than the one of myenteric plexus (Wood et al., 1999). Also, the intraganglionic fibre tracts are finer and the ganglia are smaller in the submucous plexus compared to those of the myenteric plexus (Timmermans et al., 2001).

In contrast to the other cell types of the digestive tract, the ENS arises from the neural crest during development (Obermayr et al., 2013). Enteric neural crest cells (ENCCs) undergo an inward radial migration resulting in the formation of a myenteric and a submucous plexus. Initially, ENCCs colonize the outer half of the mesenchyme (myenteric plexus) and then they move inwards to colonize the submucosal regions (Jiang et al., 2003).

In response to different guidance factors and morphogens, the ENCCs differentiate into neurons and glial cells. Thus, they are shaping the ENS. This process is complex and asynchronous with the neurons being formed earlier than the glia cells (Young et al., 2003). Furthermore, different subtypes of neurons exit the cell cycle at distinct developmental stages and express specific neurochemical markers (Obermayr et al., 2013). Submucous neurons express neurotransmitters and neurotransmitter synthetic enzymes later than myenteric neurons (Pham et al., 1991).

Besides their chemical coding, neurons can be further classified according to their morphology, electrical properties and function.

Neurons were classified by Dogiel based on the different shapes, the number of axons and lengths of their dendrites (Dogiel, 1985). There are seven different categories, Dogiel I – VII (Brehmer et al., 1999; Furness, 2000; Lomax et al., 1999; Portbury et al., 1995; Stach, 1979).

Enteric neurons can be classified further into AH- or S-neurons based on their electrophysiological properties (Hirst et al., 1974; Nishi and North, 1973). AH-neurons are characterised by the presence of a slow afterhyperpolarization (3-10s) following an action potential, while S-neurons have either fast (20-60ms) or completely lacking afterhyperpolarization (Messenger et al., 1994).

An additional categorization of enteric neurons is based on their function. They are classified to sensory neurons, interneurons and motor neurons (Costa et al., 2000).

1.2 Inflammatory Bowel Disease

ENS structural and functional alterations have been associated with pathological conditions as they can alter motility, secretion and immune cell function. Changes of the morphology and function of enteric neurons were described in gastrointestinal inflammatory diseases such as Inflammatory Bowel Disease (IBD) (Lomax et al., 2005a; Mawe et al., 2004; Steinhoff et al., 1988).

IBD is a group of chronic inflammatory conditions affecting the gastrointestinal tract. It is characterised by relapsing and remission phases. The two main types are Crohn's disease (CD) and Ulcerative colitis (UC) (Crohn et al., 1952; Wilks S., 1859). IBD affects approximately 2.2 million Europeans and 1.5 million Americans. Over the years the expanding urbanization of developing countries led to increased disease incidence of the previously low-risk populations of Asia, South America and Middle East (Cosnes et al., 2011; Molodecky et al., 2012).

The exact cause of IBD is unclear. It is considered a multifactorial disease where genetic factors, external environment and microbiota together with an inappropriate immune

response act in synergy towards its development (Buhner, 2006). Neurogenic inflammation is another important contributing factor.

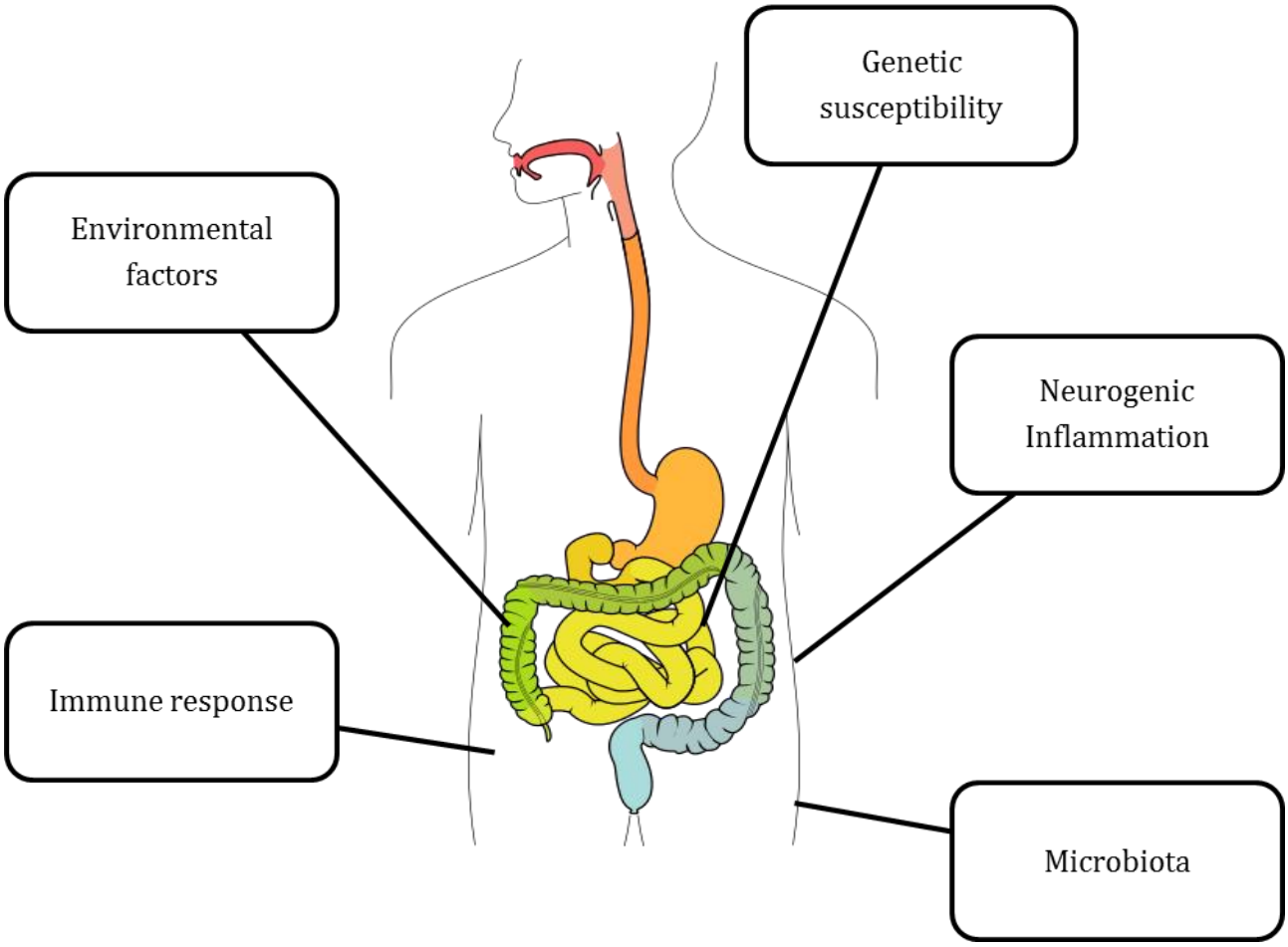


Figure 1.1: Risk factors of IBD

A combination of genetic and environmental factors together with the microbiome and an inappropriate immune response are associated with IBD development and progression. Neurogenic inflammation also affects IBD.

1.3 Genetics

Epidemiological data strongly support a genetic contribution to the pathogenesis of IBD. Geographical location and racial or ethnic background are associated with disease prevalence. The prevalence rates for CD per 100,000 people were 43.6 among whites, 29.8 among blacks, 4.1 among Hispanics, 5.6 among Asians and 8.4 among ″others″ (Kurata et al., 1992).

CD shows a higher heritability compared to UC. Studies on European population indicated that 64% of CD concordance pairs were concordant for disease location and 57% were diagnosed within 2 years. Combined study concordance rates were 36% for CD and 16% for UC monozygotic twins (Russell and Satsangi, 2004).

Genome-wide association studies (GWAS) revealed a link between genetic variants and IBD. IL23R, a gene encoding a subunit of the receptor for the pro-inflammatory cytokine IL-23, was the first to associate with both UC and CD (Duerr et al., 2006). Polymorphisms in this receptor are associated with CD in the Caucasian population but not in Asian (Duerr et al., 2006; Naser et al., 2012; Xu et al., 2016).

Nowadays, 163 loci have been identified with 110 being related to both types of IBD, 30 being specific for CD and 23 for UC (Jostins et al., 2012).

1.4 Microbiota

With the term microbiota, we refer to bacteria, archaea, viruses, fungi and protozoa which inhabit the surfaces and the cavities of the host open to the environment (Berg, 1996). Under physiological conditions, the microbiota is involved in immunologic, hormonal and metabolic homeostasis of the host (Koboziev et al., 2014). Alterations in diversity, composition and functionality of the intestinal microbiota can result in increased immune stimulation, epithelial dysfunction, or enhanced mucosal permeability, and consequently, IBD (Sartor, 2008).

Epidemiological studies revealed the first link between IBD and microbiota. Early life parameters such as family size, exposure to animals during childhood and breastfeeding were inversely related to IBD. These parameters can determine intestinal microbiota during adulthood, thus suggesting the importance of micro-organisms (Castiglione et al., 2012; Ng et al., 2015; Timm et al., 2014).

Furthermore, there is growing evidence that dysbiosis, both in faecal and mucosal communities, is related to IBD (Joossens et al., 2011; Manichanh et al., 2006; Marchesi et al., 2007; Qin et al., 2010; Walker et al., 2011). Biodiversity of bacteria changes with a decrease of commensal bacteria (i.e. *Clostridium*, *Bacteroides*, *and Bifidobacterium*) and with an increase of 30% of normally outcompeted species in healthy individuals. These outcompeted species become the dominant species in CD microbiome (i.e. sulphate-reducing bacteria, *Escherichia coli*) (Frank et al., 2007; Gophna et al., 2006; Manichanh et al., 2006; Seksik et al., 2003).

Clostridium and *Bacteroides* species are the main producers of short-chain fatty acids (SCFA) in the human colon (Fleming and Floch, 1986). Decrease of their population can be linked to the decrease of SCFA concentrations in faecal samples of IBD patients (Mariadason et al., 1997). Clostridia of groups IV and XIVa are the main butyrate-producing bacteria in the gut. Butyrate is used by colonic epithelial cells as a source of energy. It further inhibits pro-inflammatory cytokine expression in the intestinal mucosa by histones hyper-acetylation and suppression of NF-κB signalling (Segain et al., 2000). Additionally, butyrate affects intestinal barrier integrity in a paradoxical way (Mariadason et al., 1999; Peng et al., 2007). On one hand, low concentration of butyrate may be favouring mucosal barrier function as it can induce the production of mucin and

antimicrobial peptides and increase the expression of tight junction proteins (Vanhoutvin et al., 2009). However, too low concentration contributes to a down-regulation of tight junction protein expression and disruption of mucosal barrier function. On the other hand, excessive butyrate increases intestinal epithelial cells apoptosis and may cause an increased intestinal permeability (Peng et al., 2007).

1.5 Environmental factors

Numerous environmental exposures such as smoking, diet, appendectomy, vaccination/ antibiotics and childhood hygiene have been postulated as risk factors for IBD.

1.5.1 Tobacco smoking

Smoking is an important environmental factor related to both CD and UC. It has a divergent effect as it increases the risk for the development of CD and reduces it for UC (Harries et al., 1982; Somerville et al., 1984).

Single nucleotide polymorphism (SNP) refers to a variation in the DNA and is often associated with diseases. Approximately 45% of SNPs interacting with smoking were in close vicinity with IBD associated SNPs. These SNPs are located within genes regulating immune tolerance and mucosal barrier function (Yadav et al., 2017).

Moreover, smoking exerts an effect on the microbiota. Studies on smoking cessation revealed a shift in the microbiome with an increase of commensal bacteria *(Firmicutes, Actinobacteria)* and a decrease of *Bacteriodetes* and *Proteobacteria* (Biedermann et al., 2014; Mahid et al., 2006; Mondot et al., 2011).

The colonic mucus layer is thicker in CD compared to UC patients. In sites of inflammation, the mucus layer of UC patients was absent or thinner (Pullan, 1996). Culture of biopsy specimens from UC smokers showed similar mucus production compared to controls (Cope et al., 1986). Therefore, smoking may be associated with mucus synthesis and this may explain the discrepancy between the two diseases. More precisely, nicotine can affect the thickness of the adherent mucus layer in colonic mucosa through its action on mucosal eicosanoids (Zijlstra et al., 1994).

A role of nicotine on intestinal permeability has also been proposed. Smoking tightens gut junctions by exerting a role in the paracellular junctions in the intestinal epithelium or by

decreasing the permeability in the distal small bowel and the colon (Prytz et al., 1989; Suenaert et al., 2000). Furthermore, nicotine decreases pro-inflammatory cytokines (IL-1β, IL-8, TNF-α) and up-regulates anti-inflammatory cytokines (IL-10, IL-4, IL-15, IL-17) in the colonic mucosa (Eliakim and Karmeli, 2003; Madretsma et al., 1996; Sher et al., 1999; Van Dijk et al., 1995).

1.5.2 Hygiene Hypothesis

The hygiene hypothesis supports that lack of exposure to enteric pathogens or microorganisms during childhood correlates with a higher susceptibility of developing immunological disorders later in life. Exposure to certain enteric microorganisms primes an immunoregulatory response. The innate immune system causes antigen-presenting cells (APC) to mature into regulatory APC (APC_{reg}) that drive regulatory T-cell (T_{reg}) (Rook and Brunet, 2005).

A case-control study comparing subjects whose first houses had a hot water tap and separate bathroom revealed a link between household amenities in infancy and Crohn's disease. Ulcerative colitis was not correlated (Buhner, 2006; Gent et al., 1994). Furthermore, a study on the adolescent population of Israel linked IBD to living in an urban environment, a small number of siblings in the family and higher birth order (Klement et al., 2008).

1.5.3 Diet

Several diets have been associated with mucosal immunity as well as the modulation of gut microbiota and epigenetic changes, thus creating the concept of immunonutrition. Immunonutrition relies on the importance of vitamins and trace elements such as zinc, selenium, manganese and iron to manipulate immune response (Aleksandrova et al., 2017; Philpott and Ferguson, 2004). In Japan, CD patients follow enteral nutrition as first-line and maintenance therapy after induction of remission and have lower mortality rates compared to European patients (Hiwatashi, 1997; Matsui et al., 2005). Specific carbohydrate diet (SCD), low FODMAP (Fermentable Oligo-, Di-, Mono-saccharides And Polyols) and Mediterranean diets have been advertised to alleviate intestinal inflammation and thereby, are suitable for IBD patients. Studies in rodents have shown damage of intestinal epithelial barrier with diets high in saturated fats. An increased concentration of dietary fat at the intestinal mucosa can induce changes in host bile acid composition, alter

gut microbiota and consequently compromise barrier function (Devkota et al., 2012; Wit et al., 2012). Increased intestinal permeability by high-fat diet leads to enhance dendritic cells recruitment and promotes Th17 immune responses, hence accelerating disease onset of small intestinal inflammation in Crohn's disease mouse model (Gruber et al., 2013).

1.5.4 Neurogenic Inflammation

The nervous and the immune system regulate physiological homeostasis and protect against threats. They can perceive and respond to both environmental and internal cues. In addition, they can develop memory responses to adapt to ever-changing conditions. The two systems act simultaneously to achieve a tightly integrated response. The cross-talk between them is achieved by the close proximity of immune cells with extrinsic nerves and intrinsic neurons, as well as by the expression of specific neurotransmitters and receptors. Evidence for neuro-immune crosstalk comes from the Peyer's patches, an important component of the gut associate lymphoid tissue (GALT). An immune response is initiated there. They are innervated by intrinsic neurons and in parallel, they are abundant with immune cells (Vulchanova et al., 2007).

Mast cells (MCs) are considered an important player for neuroimmune interactions in the gut. They are anatomically close to nerves and they are responsible for maintaining mucosal homeostasis. There are evidences for a nerve-MC interaction (Buhner and Schemann, 2012). This interaction is also evident in IBD as mast cell mediators are secreted during active inflammation in CD and UC (Raithel et al., 2001). Synchronous recording of MCs and nerve activity after their selective stimulation revealed that the interaction is bidirectional with the nerve to MC signalling being more prominent compared to the MC to nerve signalling (Buhner et al., 2017).

Moreover, electrical stimulation of dorsal root ganglia (DRG) induces skin vasodilation, proposing the concept of neurogenic inflammation (Bayliss W. M. and Starling E. H., 1901; Chiu et al., 2012; Freusberg, A. and Goltz, F., 1874). The term refers to the release of neuropeptides from the sensory nerves and the induction of vasodilation, plasma extravasation and leucocyte migration. Experiments in murine models suggested the involvement of capsaicin-sensitive afferents in the inflammation of their small intestine (Lördal et al., 1996; Maggi, 1995; Sann et al., 1996). Neuropeptides can also directly

attract and activate innate and adaptive immune cells (Chiu et al., 2012; Maikami et al., 2011; Rochlitzer et al., 2011).

Furthermore, in a case report study, a UC patient in remission experienced relapses after spinal cord stimulation (Kemler et al., 1999). Based on this observation it was proposed that the case reflected neurogenic inflammation as a result of tachykinin substance P (SP) release from afferent nerve fibres within the colon (Barbara et al., 1999). SP was the first identified immunomodulatory neuropeptide shown to regulate the production of IL-1, TNF-α and IL-6 in human monocytes (Lotz et al., 1988). SP is linked to the regulation of immune and inflammatory responses. High concentrations of SP receptor binding sites are expressed by arterioles and venules located in the submucosa, muscularis mucosa, external circular muscle, external longitudinal muscle, and serosa in colon tissue from Ulcerative Colitis and Crohn's disease patients (Mantyh et al., 1988).

The notion of neurogenic inflammation was further supported by the case report study of a UC patient experiencing complete remission of his colitis after a spinal cord injury (Peck and Wood, 2000).

A brain-gut connection was evident in the cotton-top tamarin. It is a non-human primate model that develops idiopathic colitis when it is held in captivity due to environmental stress (Wood et al., 1998). Treatment with a non-peptide neurokinin-1 antagonist suppressed the acute inflammatory response in the colon of cotton-top tamarins with colitis supporting the role of SP (Wood et al., 1996).

The role of sensory neurons in inflammatory conditions was also demonstrated by the experiments on rats, who underwent surgical denervation around the inferior mesenteric artery and showed ameliorated Trinitrobenzene Sulfonic Acid (TNBS) induced colitis (Takami et al., 2009). Furthermore, TNBS could induce TRPA1-dependent release of colonic substance P and CGRP initiating and increasing the activation of innate and adaptive immunity, thus prompting neurogenic inflammation (Engel et al., 2011a, 2011b).

1.6 Symptoms and classification

Symptoms of IBD patients are heterogeneous. They can be gastrointestinal, systemic and extra-intestinal. The most common symptoms are abdominal pain accompanied by

diarrhoea. Location, severity and behaviour of the disease affect the clinical presentation. Therefore, categorizing patients in different cohorts may illustrate similar genetic and serological markers among them and lead to better treatment. In this respect, IBD patients are grouped based on the Montreal classification (Torres et al., 2016).

CD patients are grouped based on age at diagnosis (below 16 years, 17-40 years, >40 years; location (L1 ileal, L2 colonic, L3 ileocolonic, L4 isolated upper disease) and behaviour (B1 non-stricturing, B2 stricturing, B3 penetrating, p perianal disease modifier) (Satsangi et al., 2006) (Table 1). Symptoms are classified with the Crohn's disease activity index (CDAI). Index values ≤ 150 are associated with the quiescent disease state, while values above that with the active disease state (Best et al., 1976) (Table S6).

UC is defined based on the extent (E1 Ulcerative proctitis, E2 left-sided, E3 pancolitis) and severity of the disease (S0 clinical remission, S1 mild, S2 moderate, S3 severe) (Satsangi et al., 2006) (Table 2). Symptoms are classified based on Lichtiger score. Lichtiger scale ranges from 0 (no symptoms) to 21 (severe symptoms) (Lichtiger and Present, 1990) (Table S8).

Endoscopy is a central part of IBD diagnosis. The Simple Endoscopic Score for Crohn's Disease is calculated based on the evaluation of rectum, sigma-descending colon, transverse colon, ascending colon and terminal ileum. In these segments the presence and size of ulcerations and the extent of the inflammatory area and stenosis are assessed (Daperno et al., 2004) (Table S7).

The Ulcerative Colitis Endoscopic Index of Severity assesses vascular pattern, bleeding and erosions/ulcerations (Travis et al., 2012) (Table S9).

Table 1: Montreal classification for CD is based on age at diagnosis, disease location and behaviour

Age at diagnosis	A1 below 16 years A2 between 17 and 40 years A3 above 40 years
Location	L1 ileal L2 colonic L3 ileocolonic L4 isolated upper disease
Behaviour	B1 non-stricturing, non-penetrating B2 stricturing B3 penetrating p perianal disease modifier

(Satsangi et al., 2006)

Table 2: Montreal classification for UC is based on the extent of the inflammation and the severity of the disease

Extent	E1	Ulcerative proctitis	Involvement limited to the rectum (that is, proximal extent of inflammation is distal to the rectosigmoid junction)
	E2	Left-sided UC (distal UC)	Involvement limited to a proportion of the colorectum distal to the splenic flexure
	E3	Extensive UC (pancolitis)	Involvement extends proximal to the splenic flexure
Severity	S0	Clinical remission	Asymptomatic
	S1	Mild UC	Passage of four or fewer stools/day (with or without blood), absence of any systemic illness, and normal inflammatory markers (ESR)
	S2	Moderate UC	Passage of more than four stools per day but with minimal signs of systemic toxicity
	S3	Severe UC	Passage of at least six bloody stools daily, pulse rate of at least 90 beats per minute, temperature of at least 37.5°C, haemoglobin of less than 10.5 g/100 ml, and ESR of at least 30 mm/h

(Satsangi et al., 2006)

1.7 Biomarkers in the serum of IBD patients

IBD diagnosis and management is based on the patient's history and physical examination, along with findings from laboratory tests, endoscopy and histology evaluation (Travis et al., 2006). Since these are invasive procedures, research focuses on identifying laboratory markers suitable for the disease evaluation.

During inflammation, leucocytes migrate to the gut promoting the production of inflammatory proteins which are detectable in the serum and stool (Tibble et al., 2000, 2002). While these markers have been shown to correlate with disease activity and prognosis of IBD, their specificity is insufficient. As a result, a differentiation between IBD types or other intestinal diseases is not possible (Meuwis et al., 2007; Tibble and Bjarnason, 2001; Vermeire et al., 2004).

In this study, we investigated the mediators residing in the serum of CD and UC patients and healthy controls. In addition, we examined the differences in mediators' level active and remission phase of each disease.

Whereas CD and UC patients demonstrate overlapping genetic profiles, they are characterised by very different T-cell responses. T-helper (Th) cells can differentiate into Th1 and Th2 producing IFN-γ, IL-2 and IL-4, IL-13, IL-5, respectively (Fuss et al., 1996; Mosmann et al., 1986). CD is a Th1 cytokine-mediated disease and UC is a Th2 "like" disease since the signature Th2 cytokine IL-4 was not increased in UC patients (Strober and Fuss, 2011). Both diseases are mediated as well by Th17 cells, another subset of Th cells, and their related cytokines (Feng et al., 2011). Th17 cells are characterised by their plasticity to produce both IL-17 and IFN-γ (Annunziato et al., 2007).

Besides the characteristic Th1/Th2 and Th17 cytokines, there are additional cytokines such as TNF-α, IL-1β and IL-6 associated with both types of IBD. Th1, Th2 and Th17 stimulate primarily innate immune cells (macrophages, epithelial cells, mast cells) which then stimulate the other cytokines. Also, TNF-α, IL-1β and IL-6 can activate NF-κB and the MAP kinases and thus inducing "downstream" pro-inflammatory effects resulting in tissue and organ pathology in IBD. Additionally, these cytokines can act on "upstream" pathways. IL-6 and possibly IL-1β can manifest the induction of Th17 responses (Dienz and Rincon, 2009; Santarlasci et al., 2013). TNF-α is multifunctional cytokine with an

elaborated role in IBD pathogenesis. It is a co-factor in Th1 responses as it can enhance the production of IL-12 (Shibuya et al., 1998).

Cytokines play a significant role in IBD pathogenesis. The balance between proinflammatory and anti-inflammatory cytokines defines the effect of an inflammatory response. Hence in our study, we examined the effect exerted by cytokines present in the serum of IBD patients in the neuronal activity.

1.7.1 TNF-α

Tumour necrosis factor (TNF-α) is one of the major pro-inflammatory cytokines involved in the pathogenesis of IBD. Many studies assessing its presence in the serum and/or in the mucosa of IBD patients have been contacted over the years yielding contradictory results. Intestinal mucosal biopsies from CD patients showed an increased number of TNF-secreting cells in the submucosa and lamina propria of CD patients compared to specimens from UC and control tissues (Murch et al., 1993). Furthermore, TNF-α mRNA transcripts were significantly elevated in the inflamed mucosa of CD patients pointing to a role of TNF-α in mucosal cytokine regulation (Dionne et al., 2000a). Lamina Propria Mononuclear Cells (LPMC) isolated from inflamed IBD mucosa spontaneously secrete increased amounts of TNF-a (Reinecker et al., 1993). *In vitro* study on LPMC showed that TNF-α augmented the Th1 cytokine production (Plevy et al., 1997). However, TNF-α had a different effect on peripheral blood mononuclear cells (PBMC) (Plevy et al., 1997).

To correlate the effects of TNF-α in the peripheral immune compartment with the effects on the local level, studies measuring TNF-α in serum were performed. TNF-α concentration in serum determined with immuno-PCR pointed towards a higher concentration in the active stage compared to the inactive stage in both CD and UC patients. Also, CD patients had higher TNF-α concentration compared to UC and HC sera (Komatsu et al., 2001). On the contrary, studies utilizing ELISA method detected a lower concentration of TNF-α and also reported no significant difference in the TNF-α concentration in the serum of CD and UC patients related to the disease stage (Avdagić et al., 2013).

Another line of evidence for the role of TNF-α in IBD comes from animal modes. Anti-TNF treated or TNF knockout rodents showed significant amelioration of the mucosal inflammation. Macrophage-enriched LPMC from mice with TNBS-induced colitis produced

higher levels of TNF-α mRNA and protein than cells from control mice. When these mice were treated with TNF-α antibody there was an improvement of the clinical and histopathologic signs of colitis. In addition, isolated macrophage-enriched LP cells from antibody-treated mice produced less pro-inflammatory cytokines such as IL-1 and IL-6 in cell culture. In *in vivo* studies, colonic inflammation and lethal pancolitis were induced in TNF-α -transgenic mice treated with TNBS. On the contrary, in mice deficient for TNF-α gene TNBS-induced colitis could not be developed (Neurath et al., 1997).

The role of TNF-α in the pathogenesis of mucosal inflammation in IBD is strongly highlighted by the improvement of symptoms in CD patients after treatment with a TNF-α antibody (Targan et al., 1997). Anti-TNF-α treatment maintained clinical remission and response in patients with moderate to severe active CD and UC for up to 4 years (Colombel et al., 2014; Panaccione et al., 2013). The first TNF-α antibody approved for the treatment of IBD was infliximab (Remicade®). This antibody is a chimeric monoclonal antibody with 25% murine and 75% human sequence and is administered intravenously. The second antibody is Adalimumab (Humira®), which is produced by CHO cells. It is a fully-humanized monoclonal antibody, which is administered subcutaneously. The exact mechanism of action of these antibodies is still unknown. TNF neutralization, outside to inside signalling, Fc-dependent apoptosis, direct or indirect apoptosis and modulation of the immune system have been proposed as mechanisms of action of anti-TNF antibodies in inflammatory conditions (Billmeier et al., 2016).

An association of impaired epithelial barrier, TNF-α and CD may also be drawn as intestinal permeability of active CD patients is restored and inflammation is reduced after treatment with TNF-α antibody (Gibson, 2004; Suenaert et al., 2002).

TNF-α also induces the secretion of cytokines and chemokines from endothelial, and mucosal mononuclear cells and epithelial cells. It contributes to the recruitment of inflammatory cells to the epithelium and the submucosa (Nilsen et al., 1998; Papadakis and Targan, 2000; Van Deventer, 1997).

Dendritic cells (DC) are antigen-presenting cells residing in the lining of the stomach and the intestine. TNF can trigger their activation in an autocrine manner. Fully mature DC can initiate T cell proliferation. When they fail to migrate to the lymph nodes, DC may act as

nucleation sites to establish a lymphoid structure and thereby sustaining inflammation (Hawiger et al., 2001; Sallusto and Lanzavecchia, 1999). Increased number of mature DC were present in colonic tissue from CD patients (Middel et al., 2006).

While the above studies are highlighting the role of TNF-α in the pathophysiology of IBD, data regarding its direct effect on enteric neurons are lacking. There is only one study on cultured myenteric neurons suggesting that TNF-α hyperpolarizes membrane potential and potentiates the response to nicotinic receptor stimulation (Rehn et al., 2004).

Therefore in our study, we aim to characterize *in vivo* the evoked neuronal response upon TNF-α treatment. Moreover, we investigate the correlation of the neuronal activity with the circulating levels of TNF-α in CD and UC patients.

1.7.2 IL-1β

Proinflammatory IL-1β along with TNF-α, are involved in IBD as they can initiate a cascade of inflammatory response. IL-1β is produced from blood monocytes, tissue macrophages, dendritic cells, B lymphocytes and natural killer (NK) cells (Durum et al., 1985; Santarlasci et al., 2013). The close proximity of immune cells to neuronal varicosities suggests that secreted cytokines may affect neuronal cells. Increased levels of IL-1β were found in the colonic mucosa of IBD patients as well as, in the colonic tissue of a rabbit model of experimental colitis (Cominelli et al., 1990; Ligumsky et al., 1990). Increased IL-1β levels were also present in an infection animal model of Trichinella spiralis. In this model, IL-1β induce an increase in SP in rat myenteric neurons (Hurst et al., 1993). Additionally, the suppressed release of acetylcholine was noted in the inflamed intestine (Main et al., 1993). IL-1β reduced contractile response to acetylcholine in the colonic mucosa of rat colitis model (Aubé et al., 1996).

Studies in Aplysia have shown that IL-1β hyperpolarizes neural membranes by inducing a decrease in Na^+ conductance (Sawada et al., 1991). While this study highlights IL-1β direct effect on the nervous system later studies on the ENS of guinea pig argued for an excitatory role of IL-1β. This cytokine can directly excite secretomotor neurons by evoking secretion from mucosal crypts. In parallel, it can suppress the release of norepinephrine at sympathetic synapses on submucous neurons. Additionally, inactivation of sympathetic inhibitory action on secretor motor neurons facilitates secretion and thus causes diarrhoea (Xia et al., 1999). An excitatory effect after application of IL-1β on guinea pig ileum

myenteric neurons was also noted, further highlighting its importance as a neuromodulator. Depolarizing action of IL-1β reflected mainly the closure of K^+ channels. This resulted in presynaptic inhibition of cholinergic transmission which causes the motility changes seen during intestinal inflammation (Kelles et al., 2000). IL-1β specifically activates certain neurochemical defined neural pathways. Immunohistochemistry of isolated guinea pig ileum and colon showed nitric oxide synthase (NOS) or encephalin immunoreactivity for the majority of neurons activated in the myenteric plexus of the ileum. Neurons in the colon expressed only NOS. In the submucosal plexus of both ileum and colon, neurons were stained positive for vasoactive intestinal polypeptide (VIP) (Tjwa et al., 2003).

IL-1β can also act on enterocyte level to promote diarrhoea as it can induce HCO_3-secretion after the subepithelial release of PGE2 in the human distal colon (Bode et al., 1998). Whether ENS is involved in this effect is contradictory. While the study of Bode et al. excluded any involvement, Theodorou et al. (Theodorou et al., 1994) argued for an IL-1β-induced water movement mediated via the ENS.

An indirect effect of IL-1β on the ENS through prostaglandins was also proposed. A direct effect of PGE in the activation of enteric neurons was extensively studied (Frieling et al., 1994, 1995, 1997). PGE_2 induces Fos expression in non–cholinergic secretomotorneurons. Upon incubation of guinea pig tissue with IL-1β an increase in neural Fos expression was noted, highlighting an indirect of IL1β on non- cholinergic activation (Sharkey and Kroese, 2001).

IL-1β can affect enteric glia proliferation by suppressing their proliferation and therefore exert a role in regeneration/repair of the ENS *in vivo* (Rühl et al., 2001).

1.7.3 IL-6

IL-6 is pleiotropic cytokine acting in pro- and anti-inflammatory way. Anti-inflammatory signalling is mediated by *classic signalling* whereas pro-inflammatory one is rather mediated by *trans-signalling* (Hibi et al., 1990; Scheller et al., 2011). IL-6 is elevated in the colonic mucosa of IBD patients as well as in their serum (Mitsuyama et al., 1991). Increased IL-6 mRNA levels were measured in tissues from active IBD patients, indicating that IL-6-mediated immune processes are ongoing in the inflammatory mucosal

environment of CD and UC (Stevens et al., 1992). Serum levels of IL-6 at baseline were significantly higher in active than in remissive Crohn's disease (Ogawa et al., 2012).

Trans-signalling was shown to rescue mucosal T cells from entering apoptosis. Therefore *trans-signalling* is contributing to chronic intestinal inflammation making it relevant for CD patients (Kemseke et al., 2000). A neutralizing antibody against IL-6R induced apoptosis of lamina propria T cells, hence suppressing established experimental colitis in animal models of CD. Likewise, specific neutralization of sIL-6R *in vivo* by a gp130–Fc fusion protein caused suppression of colitis activity and induction of apoptosis, demonstrating that sIL-6R prevents mucosal T-cell apoptosis (Atreya et al., 2000).

A direct excitatory effect of IL-6 on enteric neurons was proposed by Xia et al showing that IL-6 can suppress the slow IPSPs and the nicotinic fast EPSPs. Along with IL-1β they synergistically block norepinephrine release at excitatory synapses to prevent sympathetic inactivation of enteric microcircuits responsible for the secretion from mucosal crypts which is associated with the diarrheal symptoms in IBD (Xia et al., 1999).

IL- 6 acts as a putative mediator in the regulation of VIP expression during CD. Colonic biopsies from IBD patients showed an increased expression of IL-6 even in non-inflamed areas. ENS primary cultures were incubated with IL-6 for 12 days and showed reduced expression of VIP mRNA and protein. CD supernatants applied to the ENS cultures in the presence of anti-IL-6 blocking antibody showed an increase in the intracellular VIP concentration (Soufflet et al., 2018).

Preliminary data also support IL-6 mRNA expression from enteric neurons and subsequent activation of bone-marrow-derived DCs supporting the concept of neurogenic inflammation (Ogata et al., 2017).

1.7.4 GM-CSF

Granulocyte macrophage-colony stimulating factor (GM-CSF) is considered a major regulator governing the functions of granulocyte and macrophage colonies at all stages of maturation (Burgess and Metcalf, 1980). GM-CSF is elevated in the serum of adults CD patients independent of the disease stage but not in juveniles (Vasilyeva et al., 2016). Increase secretion of GM-CSF was measured in mucosal lesions of IBD (Noguchi et al.,

2001). Polymorphonuclear neutrophils accumulate in the mucosa of IBD patients as their apoptosis is delayed by GM-CSF (Ina et al., 1999).

GM-CSF is inducing clinical remission when administered to CD patients and improves the quality of life (Dieckgraefe and Korzenik, 2002; Korzenik et al., 2005). However, the exact mechanism by which it exerts its action is not known.

Studies in colitis mice models provide evidence regarding its action. In this model macrophage-derived proinflammatory cytokines (GM-CSF, IL-1β and IL-6) were elevated at the sites of lesions (Dieleman et al., 1994). In dextran sodium sulfate (DSS) colitis mice models GM-CSF therapy reduced clinical signs of colitis and the release of inflammatory mediators (Sainathan et al., 2008). Treatment of colitis mice with GM-CSF affected ulcers by improving mucosal repair with faster ulcer re-epithelialization, accelerating the hyperproliferative response of epithelial cells in ulcer-adjacent crypts, and lowering colonoscopic ulceration scores. Its effect was timely associated with a reduction in neutrophil numbers and increased accumulation of CD11b(+) monocytic cells in colon tissues. Additionally, transfer of splenic GM-CSF-induced CD11b(+) myeloid cells into improved colitis. GM-CSF-induced CD11b(+) myeloid cells were shown to promote *in vitro* wound repair (Bernasconi et al., 2010).

Mice deficient for GM-CSF developed severe colitis in response to enteric exposure to DSS. Infiltrating macrophages and increased colonic levels of IL-17, TNF-α and IL-6 were observed in GM-CSF (-/-) mice suggesting that they are more susceptible to acute DSS-induced colitis, probably because of an impaired gut innate immune response (Egea et al., 2010; Xu et al., 2008).

1.7.5 IL-17A

Studies on T cell-mediated colitis animal model have generated ambiguous results regarding Th17 cells in intestinal inflammation. In a number of studies, activation of Th17 cells and production of IL-17 is considered responsible for chronic intestinal inflammation, or at least redundant but highly pathogenic. Other studies argue against it as inflammation also develops in mice with deficient IL-17 production in their T-cells (Izcue et al., 2008; Leppkes et al., 2009; Yen et al., 2006).

Intestinal mucosal biopsies from active IBD patients showed an increased expression of IL-17A mRNA. Increased levels of IL-17A were also detected in the serum of patients compared to healthy controls (Fujino et al., 2003). Higher IL-17A serum levels at baseline were measured in active CD compared to remissive disease (Ogawa et al., 2012). A 2–3 fold long-term rise in the plasma levels of IL-17A and IL-21-producing CD45RO+CD4 + T cells was measured in quiescent CD compared to active CD (Dige et al., 2013).

An anti-IL17A antibody, Secukinab® was ineffective in patients with CD and even exacerbated CD in a subgroup of patients with objective inflammatory indications (Hueber et al., 2012). Increased IL-17A levels might be an adaptive response to the mucosal inflammation rather than being the actual cause of intestinal inflammation (Caprioli et al., 2008).

1.7.6 IL-22

IL-22 is also expressed by Th17 cells and cells of the innate immune system (Cella et al., 2009; Liang et al., 2006). Its primary structure is comparable to that of IL-10 and IFN-*γ,* hence they share receptor subunits (Kotenko et al., 2001).

IL-22 has a protective and pathophysiological role in parallel. It is produced at sites of inflammation and it can mediate a physiological response to repair local tissue damage. It was shown to protect and regenerate epithelial tissue in an experimental model of colitis (Pickert et al., 2009). Along with IL-17, they induce the production of antimicrobial peptides expression such as defensins (Liang et al., 2006). In parallel, IL-22 can induce expression of pro-inflammatory molecules such as IL-1, IL-6, IL-8, IL-11 and GM-CSF (Andoh et al., 2005).

IL-22 mRNA expression was increased in inflamed colonic lesions of patients with CD and murine DSS-induced colitis mouse model. The IL-22 increase was associated with increased expression of proinflammatory cytokines and IEC migration (Brand et al., 2006). Moreover, IL-22 serum levels correlate with CD activity since active patients exhibit higher levels of this cytokine compared to remission patients (Schmechel et al., 2008).

Overall, IL-22 is a two-faced cytokine that has protective but also deleterious roles in the intestinal inflammation depending on the cytokine environment. Treatment with a recombinant cytokine or gene therapy involving IL-22 are promising in IBD treatment

(Mizoguchi, 2012; Seiderer and Brand, 2009). Also, activation of IL-22 pathway reduces intestinal inflammation in UC patients (Chebli et al., 2017). On the other hand, continuous activation of IL-22 has been linked with colitis-associated cancer (Huber et al., 2012; Mizoguchi et al., 2017).

1.7.7 IL-10

IL-10 is an anti-inflammatory cytokine. It can inhibit the production of IL-2, IL-3, IFN-γ, and GM-CSF by Th1 cells but not Th2 cells (Kucharzik et al., 1995).

Elevated levels of circulating IL-10 were described in active CD and UC patients and were correlated with the disease activity (Kucharzik et al., 1995). A later study, argued for higher IL-10 serum levels only in UC patients (Szkaradkiewicz et al., 2009). Since IBD is characterised by an imbalance of pro-inflammatory and anti-inflammatory cytokines the presence of IL-10 in the serum of patients is not surprising. Although IL-10 is upregulated in response to pro-inflammatory cytokines the immunosuppressive effect that exerts is not strong enough to alleviate inflammatory activity (Kucharzik et al., 1995; Szkaradkiewicz et al., 2009).

1.7.8 IL-12p70

IL-12 is produced by monocytes, macrophages, B-cells and dendritic cells in response to infection (Ma and Trinchieri, 2001). IL-12 is a heterodimeric protein composed of p35 and p40 subunits (Podlaski et al., 1992). When the two subunits are combined they form the bioactive IL-12p70.

The correlation of IL-12 with IBD was demonstrated in a TNBS-colitis model, as well as in IL-2-/- mice, where LP T-cells isolated from inflamed bowel showed increased secretion of IFN-γ. Treatment with an anti-IL-12 antibody attenuated the disease (Ehrhardt et al., 1997; Neurath et al., 1995). An abundance of IL-12-containing macrophages and IFN-γ-expressing T cells was shown in the intestinal lamina and muscularis propria of CD patients. Additionally, culturing T-cells from gut specimens of those patients in the presence of anti-IL-12 antibody downregulated the development of IFN-γ-expressing T cells (Parronchi et al., 1997). Administration of an anti-IL-12 antibody is proposed as a therapeutic approach of the dysregulated Th1 responses underlying CD (Fuss et al., 2006)

Serum IL-12 levels were increased in adult CD patients throughout the disease (Vasilyeva et al., 2016). IL-12 along with IL-23 and IL-17A were proposed as possible predictive markers for a poor therapeutic response to IFX (Ogawa et al., 2012).

1.7.9 IL-8

IL-8 is a pro-inflammatory chemokine secreted by macrophage and intestinal epithelial cells (Arai et al., 1998). It acts as a potent chemoattractant and activator for neutrophils thus mediating neutrophil infiltration of the gut wall in IBD (Mitsuyama et al., 1994; Raab et al., 1993). It was elevated in the mucosa of UC patients (Izzo et al., 1993; Mahida et al., 1992). The number of cells expressing IL-8 gene correlated with the histological grade of active inflammation. Therefore, IL-8 expressing cells were diffusely distributed over the entire affected mucosa; in the epithelium and the LP of UC patients (Arai et al., 1998; Mazzucchelli et al., 1994).

Circulating chemokine levels were also higher in the UC group of patients compared to CD and controls (Jones et al., 1993). IL-8 was proposed as a biomarker for UC as is closely related to disease activity and associated with endoscopic and histological severity. The high IL-8 concentrations support neutrophil chemotaxis as a crucial mechanism for UC (Rodríguez-Perlvárez et al., 2012).

1.7.10 IFN-γ

Interferon γ is a pro-inflammatory cytokine with a role in innate immunity as is produced primarily by NK cells, as well as in adaptive immunity through CD8 and CD4 Th1 effector T cells (Schoenborn and Wilson, 2007).

Elevated levels of this cytokine were found in the serum and organ culture medium of colonic mucosal tissue specimens from CD patients, but not in PMBC suggesting that IFN-γ is produced by intestinal mononuclear cells. IFN-γ serum levels were significantly higher in active than in remission CD patients. This may attribute to the larger production of IFN-γ by stimulated cells in the inflamed intestine (Ogawa et al., 2012; Sasaki et al., 1992). LPMC from CD patients were spontaneously releasing IFN-γ contributing to the increased epithelial expression of MHC-II antigens (Fais et al., 1991; Noguchi et al., 1995). Furthermore, treatment of colitis models with an anti-IFN-γ antibody attenuated chronic intestinal inflammation (Obermeier et al., 1999; Powrie et al., 1994). IFN-γ $^{-/-}$ mice did not

develop colitis when treated with DSS in contrast to WT ones suggesting the role of this cytokine in the initiation of colitis (Ito et al., 2006).

Nevertheless, the role of IFN-γ in induction and progression of IBD is controversial as other studies showed decreased levels of IFN-γ present in intestinal mononuclear cells (Lieberman et al., 1988) Contradictory were also the studies in animal models. Administration of anti-IFN-γ neutralization antibody had a week effect on DSS-induced colitis (Hans et al., 2000). Moreover, IFN-γ receptor$^{-/-}$ mice developed inflammatory colitis after administration of TNBS to an analogous degree as WT mice (Tozawa et al., 2003).

1.8 Aim

Gut function is compromised upon inflammation. Numerous enteric neurons, particularly those in the submucosal plexus, are in close contact with immune cells (Buhner et al., 2009). A number of clinical and experimental studies suggested a role of inflammatory mediators on enteric neurons leading to the generation of IBD symptoms (Lakhan and Kirchgessner, 2010). Hence, direct evidence for activation of human enteric neurons in response to mediators residing in the serum of IBD patients is lacking.

1. Therefore, our first aim was to determine whether serum samples from patients with CD and UC can activate guinea pig enteric neurons.

IBD is a group of chronic, relapsing diseases. Patients are characterised as active or remission based on clinical and endoscopic findings.

2. Thus, our second aim was to delineate whether sera from patients in different disease stage could have a diverse effect on enteric neurons.

Cytokines play a key role in orchestrating the inflammatory mechanisms. In terms of IBD, they are involved in the pathogenesis as well as in the disease progression (Neurath, 2014).

3. Therefore, the levels of ten different cytokines in the serum samples of the CD/UC patients were evaluated.

TNF-α is proposed as an important component in the pathophysiology of IBD. Genetic association studies between TNF-α and CD, as well as increased levels of TNF-α in CD supernatant samples, support its role in the disease progression.

4. To determine whether TNF-α has an effect on the serum evoked neuronal activation we pharmacologically inhibited TNF-α using the antibody Adalimumab.
5. Furthermore, we investigate the effect of TNF-α on neuronal sprouting and cell death in enteric neuron culture.

2. Materials and Methods

2.1 Krebs solutions

All substances for the preparation of Krebs solutions were obtained from Sigma- Aldrich (Schnelldorf, Germany). The pH of Krebs solutions was adjusted to 7.4 at a temperature of 37.0°C.

Table 2.1: Krebs solution for tissue preparation

Substance	g/l
$MgCl_26H_2O$	0.244
$CaCl_22H_2O$	0.368
NaH_2PO_4	0.144
NaCl	6.84
$NaHCO_3$	2.10
Glucose	1.98
KCl	0.350

Table 2.2: Krebs solution for experiment

Substance	g/l
$MgCl_26H_2O$	0.244
$CaCl_22H_2O$	0.368
NaH_2PO_4	0.144
NaCl	6.84
NaHCO3	1.68
Glucose	1.98
KCl	0.35

2.2 Samples and Tissue Preparation

2.2.1 Serum samples

Serum samples were collected in collaboration with our clinical partners at the INSERM Nantes and the Vilsbiburg Clinical Hospital. All procedures performed on human samples were approved by the appropriate ethics committees (Technische Universität München Prot.No 906/, Ministère de l'Enseignement Supérieur et. de la Recherché (France) Prot.No.DC-2008-402).

Informed written consent was obtained from all subjects. Characteristics of study participants in regards to age gender, medication and sample collection are summarized in S1 Table. The IBD patients were endoscopically diagnosed and were classified to active and remission based on their clinical disease activity index (CDAI). A CDAI score >150 classify the person as an active CD patient. The questionnaire for CDAI is summarized in S6 Table. Sera of active CD patients were collected before the administration of the TNF treatment (referred as S0 phase). Sera of patients in remission were collected 3 months after the first treatment (referred as S14 phase).

UC patients were classified using the Lichtiger index, with a score of >5 they were considered active.

Healthy controls (HC) were subjects with no abdominal symptoms.

2.2.2 Guinea Pig

Neuroimaging and immunochemistry studies were performed on male Dunkin Harley guinea pigs (Envigo RMS GmbH, Horst, Netherlands).

Guinea pigs were kept in airflow cabinets (UniProtect, Ehret, Emmendingen) at 24.2°C and 60% relative humidity with a day/night cycle of 12 h/12 h. In each cage, they were 2-3 guinea pigs. At the time of arrival, they weighted 200-250kg. They were allowed free access to food (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) and water *ad libitum*. Animal weight at the time of experiments was 359 ± 3.6 g (mean ± SEM).

All procedures involving guinea pig were conducted according to the German guidelines for animal care and welfare (German Animal Protection Law, revised according to the Directive 2010/63/EU) and approved by the Bavarian state ethics committee (Regierung

Oberbayern, which serves as the Institutional Care and Use Committee for the Technische Universität München) according to §4 and §11German Animal Protection Law under the reference number 32-568-2.

2.2.3 Rat

Studies on rat cultured neurons were performed in accordance with French standard ethical guidelines for laboratory animals (Agreement no. 02476.01).

Pregnant Sprague-Dawley rats were obtained on gestational day 15 (Janvier Labs, Le Genest-Saint-Isle, France) and were individually housed in cages on a day/night cycle of 12 h/12 h with free access to food and water. Mothers and their pups (10–14 pups/litters) were kept in the same conditions during the whole experiments. Day of birth was considered as postnatal day (P) 0. Pups were killed at P1, P7, P21 and P36. Pups were killed by decapitation (P1 and P7) or were anaesthetised with isoflurane (5 min; Abbot, Maidenhead, UK) and killed by cervical dislocation (P21 and P36). For cell culture, pregnant rats were anaesthetised using isoflurane and killed by cervical dislocation at gestational day 15, and the embryos were collected to proceed to cell culture.

2.2.4 Tissue preparation

Neuroimaging experiments were performed on male Dunkin Harley guinea pigs. Guinea pigs were sacrificed using cervical dislocation followed by exsanguination. Colon was removed and placed in ice-cold Krebs aerated with carbogen (95% O_2, 5% CO_2; Linde Gas, Unterschleissheim, Germany; pH=7.4). A two cm segment of the distal colon was cut-opened along the mesenteric border and pinned out flat (mucosal surface facing up) in a petri dish coated with Sylgard (Sylgard 184, Dow Corning, Midland, USA). During dissection, the segment was constantly perfused with oxygenated ice-cold Krebs solution. The mucosa was scraped off gently with rounded forceps (Dumont #7, FST). Muscle layer and myenteric plexus were removed, using a fine spring scissor (15370-52, FST) and forceps (Dumont #5, FST), in order to obtain the preparation of the submucous plexus. The tissue was then pinned under light stretch over a window on a Sylgard ring with a size of 10 × 20 mm. The ring was then transferred into a recording chamber (approximately seven ml volume) with a 42 mm diameter glass bottom (130 – 170 µm thickness; Sauer, Reutlingen, Germany).

2.3 Ganglia labelling with voltage-sensitive dye

The recording chamber was mounted onto an epifluorescence Olympus IX 50 microscope (Olympus Corporation, Tokyo, Japan) equipped with a 3W green LED (LET A2A true green (521 nm) 700mA; OSRAM GmbH, Munich, Germany) which was connected to a self-made stabilized power supply. Ganglia were visualized at 10x magnification using Hoffmann modulation contrast optics.

The tissue preparation was continuously perfused with Krebs solution (pH=7.4; 37 °C) gassed with carbogen (5% CO 2 and 95% O2; Linde Gas, Unterschleissheim, Germany) at a flow rate of seven ml/min. Individual ganglia were stained with the voltage-sensitive dye 1-(3-sulfanatopropyl)-4-[β-[2-(di-*n*-octylamino)-6-naphthyl]vinyl] pyridinium betaine (Di-8-ANEPPS) (Invitrogen, Carlsbad, CA, USA). The concentration of the dye was 20µM dissolved in Krebs solution containing 0.0135% Pluronic F-127 (Invitrogen, Carlsbad, California, United States of America) and 0.135% DMSO (Acros Organics, Geel, Belgium). Dye was injected into a ganglion (1 bar) through a fine-tipped micropipette loaded with 20 µmol/L Di-8-ANEPPS solution (Neunlist et al., 1999). The micropipette was placed within an intraganglioning fibre tract and the pressure was applied for 200-400ms. After several minutes the dye incorporated into the outer membrane of the cells. An interval of 20 minutes was held between staining and the first neuronal recording allowing sufficient time for the dye to bind to the lipid bilayer of the cell membrane.

Multisite optical recording technique (MSORT) was used to assess the neuronal activity of guinea pig submucous neurons stained with the voltage-sensitive dye Di-8-ANEPPS. The neuroimaging technique employed in this study has been previously described in detail (Neunlist et al., 1999; Schemann et al., 2002).

Recording of a given ganglion can be performed with a high spatial and temporal resolution (Schemann et al., 2002). Ganglia are stained with the voltage-sensitive dye. The potentiometric fluorescent dye is incorporated into the cell membrane. Based on electrochromism, absorption and emission spectra of the dye alters according to the membrane potential (Neunlist et al., 1999; Schemann et al., 2002). In the present study, neuronal recordings of stained ganglia were performed with an epifluorescence Olympus IX 50 microscope (Olympus Corporation, Tokyo, Japan) equipped with a 40x oil immersion

objective (UApo 340nm, Olympus Corporation, Tokyo, Japan), numerical aperture of 1.35 – 0.65. The filter cubed used contained a dichroic mirror with a separation wavelength of 565 nm, a 545± 15 nm excitation filter and 580nm barrier filter (AHF Analysetechnik AG; Tübingen, Germany) In the initial experiments signals were acquired with a frequency of 1.6 kHz and detected with an array of 464 photodiodes (RedShirt Imaging, LLC Decatur, GA, USA) (Figure 2.1). Changes in the fluorescence were processed with a computer at a frame rate of 1.6 kHz allowing the detection of an action potential. In the later experiments, a cooled charge-coupled device (CCD) camera was used (NeuroCCD-SMQ imaging system, RedShirt Imaging LLC Decatur, GA, USA) for signal detection (Figure 2.1). The microscope and the camera were mounted on an optical table (TMC, Technical Manufacturing Corporation, Peabody, USA) to control noise from vibration. All equipment was shielded by a Faraday cage to regulate noise caused by electromagnetic fields.

Drug application, as well as staining of ganglia, was achieved by local pressure application (PDES-2L, npi electronic GmbH, Tamm, Germany) via a glass microejection pipette (Science Products, Hofheim, Germany). The tips of the pipettes were pulled with a Flaming/Brown micropipette puller (Sutter Instrument Co., Novato, CA, USA). Tips diameter was approximately five µm. The viability of neurons was assessed with electrical stimulation or nicotine application. Electrical stimulation was achieved with a Teflon TM coated platinum electrode with a diameter of 25 µm (Science products GmbH, Hofheim, Germany) connected to a pulse generator (Master 8, Science products GmbH, Hofheim, Germany) and a constant current stimulus-isolating unit (A360, WPI; Berlin, Germany). Duration of an electrical pulse was 400µs and amplitudes varied between 10-100 µA. The ground electrode was placed in the chamber, which was continuously perfused with Krebs solution.

2.4 Analysis of neuroimaging data

Data were processed using Neuroplex 10.1.02 software (RedShirtImaging, Decatur, GA). The voltage-sensitive dye incorporates into the outer membrane of the cells allowing their identification and moreover the calculation of the total number of neurons per ganglion (Breunig et al., 2007; Michel et al., 2005, 2011). After the neuroimaging recording, a photomicrograph of the ganglion was taken using a high-resolution video camera. The

photomicrograph and the traces of photodiodes were overlayed in order to identify the response of individual neurons (Figure 2.1).

The statistical analysis was performed with the responding cells. For analysing the frequency of action potentials, firing activity at the baseline was subtracted from the activity evoked by the application of serum samples. The percentage of neurons was calculated as the number of neurons responding to the stimulus to the total number of stained neurons. Also, the total number of neurons in each ganglion was confirmed by the response to nicotine or electrical stimulation. Neuroindex is the product of the percentage of responding neurons and their spike frequency and was calculated as a measure of evoked neuronal activity in the ganglion (Buhner et al., 2014).

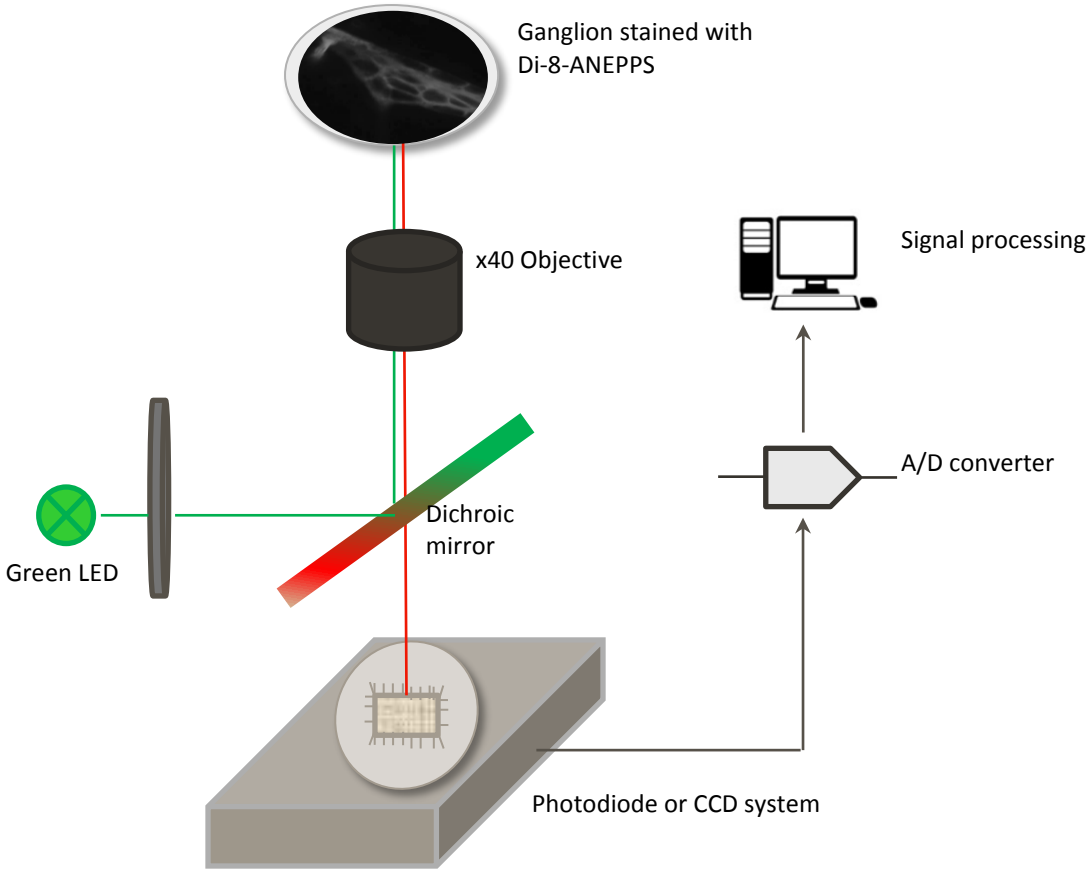


Figure 2.1: Schematic illustration of the experimental setup

Submucous enteric neurons were stained using the voltage-sensitive dye Di-8-ANEPPS. A green LED lamp was used to excite the Di-8-ANEPPS stained tissue. Changes in the fluorescence were detected by the photodiode system or the CCD system. Optical signals were processed and analysed with a computer enabling the detection of action potentials.

Photodiode system: an array of semiconductors converting light into electrical current. CCD: a charge-coupled device where photons can generate a charge, which is converted into a digital value. A/D converter: analogue-to-digital converter.

2.5 Viability of the enteric neurons

To assess the viability of enteric neurons intraganglionic fibre tracts were electrically stimulated to induce fEPSPs. Duration of the electrical pulse was 400µs and amplitudes varied between 10-100 µA.

Nicotine was also used to evoke action potential discharge. Aliquots of 100µM were prepared new every week from 10mM stock solution. Nicotine was applied to stained ganglia through a microejection pipette (1 bar, 200 ms duration).

2.6 Application protocols

According to previously published calibration curves, any substances applied by pressure pulse ejection will be diluted by approximately 1:10 in the perfused Krebs once it reaches the ganglion (Breunig et al., 2007). Application of substances during neuroimaging experiments was through a pressure-controlled picospritzer.

2.7 Reproducibility of neuronal responses

Reproducibility of responses is required in order to proceed with a pharmacological approach. To determine the reproducibility of the neuronal responses, serum was applied two consecutive times on the same ganglion. An interval of 20 minutes was held between each neuronal recording allowing sufficient time for the neurons to return to the basal activity. Reproducibility experiments were also performed using CD and UC sera samples.

2.7.1 Application of serum samples

All sera were freshly prepared prior to experiments from aliquots stored at -80°C. Sera were diluted (1:1) with Krebs solution. They were applied to ganglia by pressure ejection (1 bar, 200 ms duration) from microejection pipettes that were placed close to the ganglion (approximate distance: 100-200 µm) using a micromanipulator. Sera were applied in pairs. Serum from HC was applied with serum from an active or remission patient. Gender and age of applied sera were matched. A comparison regarding the disease stage was performed by applying sera from the same patient in an active and remission stage on the same ganglion. The order of application was randomised.

2.7.2 Application of TNF-α

TNF-α is associated with the pathogenesis of IBD. Therefore TNF-α (Biomol GmbH, Hamburg, Germany) was spritzed onto the ganglia for 200ms at the concentrations of 2 pg/ml, 10 ng/ml and 100 ng/ml. Concentration reaching the ganglia was diluted 1:10 in the perfused Krebs, thus the final concentration was 0.2pg/ml, 100pg/ml and 10ng/ml (Breunig et al., 2007).

For the cell culture experiments, neurons were incubated with 20pg/ml, 1ng/ml, 10ng/ml and 50ng/ml of TNF-α for 48, 24 and 3 hours.

2.7.3 Application of Adalimumab (Humira®)

Adalimumab (Abbott GmbH & Co.KG, Germany) is a fully human anti-TNF-α monoclonal antibody. It is administered by subcutaneous injection. Each 0.8ml pre-filled syringe contained 40mg of Adalimumab and was stored at 4°C. The solution was extracted from the syringes under sterile conditions and was stored in aliquots of 50µg/ml. Adalimumab was applied onto the ganglia with spritz application in concentrations of 500µg/ml, 50µg/ml and 5µg/ml without any effect (3/4/20). Adalimumab was pre-incubated with the serum at a concentration of 5µg/ml resembling the concentration patients have in their blood after they injected the 40mg of Adalimumab contained in one spritz (Baert et al., 2016; Silva-Ferreira et al., 2016). The mixture of adalimumab-serum was applied to the ganglia through microejection pipettes (1 bar, 200 ms duration).

2.7.4 Application of cilansetron

Cilansetron (Solvay Pharmaceuticals, Brussels, Belgium) is a 5-HT_3 antagonist usually indicated for the treatment of diarrhoea-predominant irritable bowel syndrome (IBS) (Ford et al., 2009). Cilansetron was perfused with the experimental Krebs at a working concentration of 10^{-6} M for 20 minutes. Wash-out was achieved after 60 minutes but often complete wash-out was not possible.

2.7.5 Application of pyrilamin/ranitidine

H_1 receptor antagonist pyrilamin (1 µM) and H_2 receptor antagonist ranitidine (10 µM) (Sigma, Schnelldorf, Germany) were dissolved in experimental Krebs (Breunig et al., 2007). Tissues were perfused with H1/ H2 receptor antagonists for 45 minutes. Wash-out was achieved after 20 minutes.

2.8 Immunohistochemistry

Fresh guinea pig preparations were fixed overnight at 4ºC in 4% Paraformaldehyde solution followed by washing steps (3 x10min) with Phosphate Buffer (0.1 M). The tissue specimens were stored at 4°C in Phosphate Buffer Saline (PBS, 0.1M) solution containing 0.1% NaN_3.

To avoid unspecific binding the preparations were incubated for 1h at room temperature in blocking serum (0.1% NaN_3, 4% horse serum (HS), 3% Triton X-100 (TX) in PBS (PBS/NaN_3 /HS/TX) under constant agitation. Tissues were incubated with 200µl solution containing the primary antibody (Table 3) for 12-16h at room temperature followed by washing steps (3 x10min) with PBS. They were then incubated in 200µl solution with the species-specific secondary antibody for 2h at room temperature (Table 4). After a final wash (3 x 10min), preparations were mounted on a coverslip in Cityofluor or in a solution of PBS (pH= 7.0) / NaN_3 (0.1%) / glycerol (80%).

For the assessment of the tissue, an Olympus microscope (BX61 WI; Olympus Corporation, Tokyo, Japan) with appropriate filter blocks was used. The filters were Cy2 with a dichroic mirror of 505nm (Olympus Corporation, Tokyo Japan); Cy3 with a dichroic mirror of 565nm (AHF Analysetechnik AG Tübingen, Germany) and Cy5 with dichroic mirror of 660m (AHF Analysetechnik AG Tübingen, Germany) The microscope was equipped with an SIS view II charge-coupled device (CCD) camera and an analysis Software Cell^P v.3.4 (Olympus Corporation, Tokyo, Japan) for image acquisition, processing and editing.

Table 3: List of primary antibodies used for immunofluorescence

Primary antibody	**Dilution**	**Supplier**
Serum	1: 500	University of Nantes, Vilsbiburg Academic Hospital
Rabbit anti-VIP	1: 1000	Peninsula Laboratories, (California, USA)
Goat anti-ChAT	1: 100	Merck- Millipore, (Massachusetts, USA)
AI 01c P.R. HU	1: 10000	Serum of patient #27
Mouse Anti-β-Tubulin III (Tuj-1)	1:300	Sigma-Aldrich, (Missouri, USA)
Rabbit Anti-HuD	1:500	Santa Cruz Biotechnologies (Texas, USA)

Table 4: List of secondary antibodies used for immunofluorescence

Secondary antibody	**Dilution**	**Supplier**
Donkey anti-rabbit Cy5	1: 500	Dianova, (Hamburg, Germany)
Donkey anti-goat Cy2	1: 200	Dianova, (Hamburg, Germany)
Donkey anti-human IgG Cy3	1: 500	Dianova, (Hamburg, Germany)

Anti-mouse FP488	1: 200	Interchim, (Montluçon, France)
Anti-rabbit Cy3	1: 500	Jackson ImmunoResearch, (Pennsylvania, USA)

2.9 Analysis of distance between ganglia and blood vessels

Substances can be diffused from blood vessels to ganglia (Zoli et al., 1999). Also, blood vessels in the ENS are permeable to macromolecules (Kiernan, 1996). Therefore, their proximity might affect neuronal responses.

At the end of neuronal recording from a given ganglion, a bright-field picture was obtained with a 10x objective (UApo 340nm, Olympus Corporation, Tokyo, Japan). These pictures were used to determine whether the distance of ganglia to blood vessel could affect the neuronal response. The software ImageJ was used to calculate the distance between blood vessels and stimulated ganglia. There was no selection between veins and arteries. Vertical lines were drawn from the stimulated ganglion to the middle of the adjacent blood vessel (Figure 2.2). The distance was calculated as the mean value of the vertical lines. In cases where no blood vessel was visible in the field of view, the distance was assigned to 600 µm. In situations where blood vessels were crossing over or under the ganglia, the distance was assigned to 0 µm.

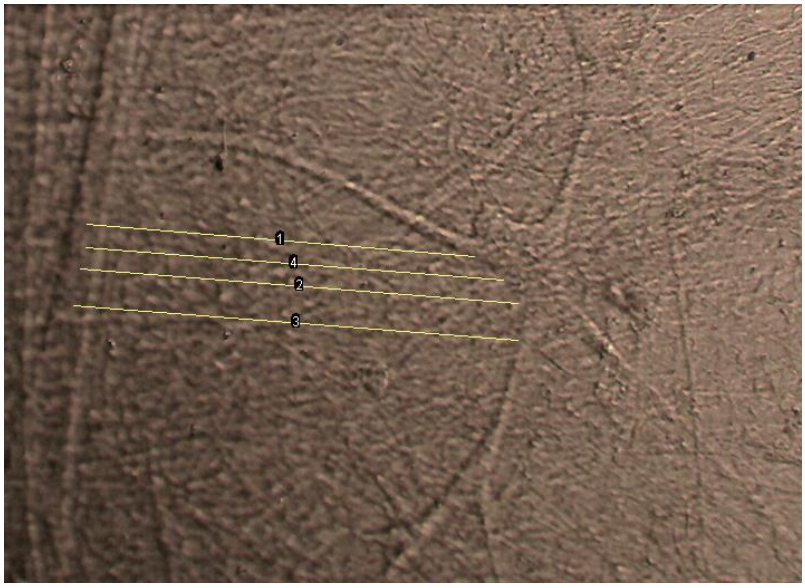


Figure 2.2: Determination of proximity of ganglia and blood vessels

Distance between ganglia and blood vessel. The distance is calculated as the mean value of the four yellow vertical lines (1-4).

2.10 Neuro-glial co-culture

A glial feeder layer was made up of EGCs which were derived from ENS primary cultures (Van Landeghem et al., 2011). The ENS cultures were trypsinised on DIV13 and seeded in a serum-containing media after differential centrifugation. They were further cultured for 7 days. Then, morphological glial-like cells were trypsinised using a cloning cylinder and seeded in culture flask in serum-containing media. To constitute the glia feeder layer EGCs were plated at a density of 7500 cells/cm^2 in a 24-well plate and maintained for 4 days in DMEM containing 10% FBS, 2 mm glutamine, 50 µg/ml streptomycin and 50 µl/ml penicillin. The medium was replaced with serum-free Neurobasal/B27 medium (Gibco, Waltham, MA, USA) 3h before neuron culture.

Rat enteric neuron culture was prepared from the intestine of E15 rat embryos and the dissociated cells were plated at 175,000 cells cm^2 on glass coverslips coated with poly-l-lysine (1 mg/ml, Sigma) in DMEM high glucose containing 10% FBS, 2 mm glutamine, 50 µg/ml streptomycin and 50 µl/ml penicillin. After 3h the coverslips were transferred to the wells containing the glial feeder layer. After 24h, 3µM 1-β-d-arabinofuranosylcytosine

(AraC, 5 µM, Calbiochem, Billerica, MA, USA) were added to prevent overgrowth of EGCs and myofibroblasts (Figure 2.3). The cells were maintained for up to 12 days. Experiments with TNF-α were performed on DIV4.

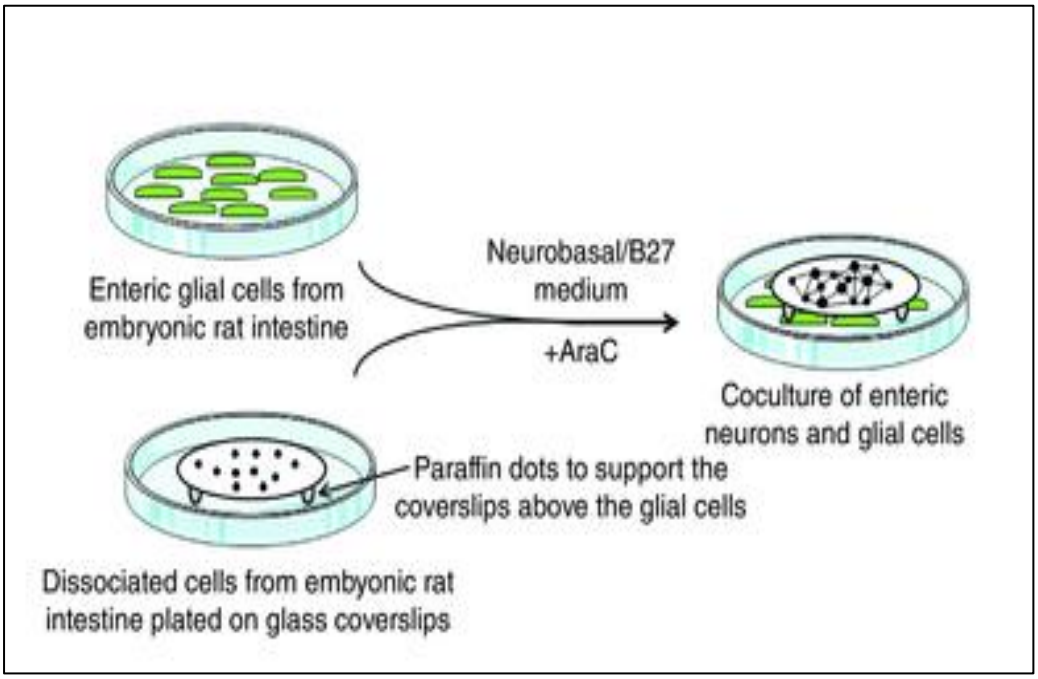


Figure 2.3: Schematic representation of the enteric neuron/glia co-culture model.

The two cell types (enteric glial cells and dissociated cells from embryonic rat intestine) are first prepared separately and are then co-cultured once the neurons have attached to the coverslips. The neurons and the glia remain separated by a narrow gap provided by the paraffin dots stuck on the glass coverslip. Adapted according to: (Le Berre-Scoul et al., 2017).

2.11 Immunocytochemistry

Rat ENS cells were fixed in 4% formaldehyde solution in PBS for 15 minutes. Then they were washed once with PBS for 15 min and permeabilized for 5 min at room temperature in 0.25% Triton-X100 in PBS/NaN_3. Cells were washed with PBS for 5 minutes and incubated for 30 min at 37°C in 10% BSA/ PBS. They were then incubated with the primary antibody (diluted in PBS containing 0.02% azide and 3% BSA) (Tuj-1/Hu; Table 3) for 12-16h at 4°C followed by washing steps (3 x 5 min) with PBS. The next step was incubation with the secondary antibody for 2h at room temperature (FP488/Cy3; Table 4). After a final wash with PBS (3 x 5min), paraffin residues were removed, slides were dipped in water and mounted on a coverslip with ProLong Gold Antifade Reagent with DAPI (Molecular Probes).

The number of neurons per field (Hu-positive cells) was counted in the field of view from a ×20 objective for each experiment (n=5-7). The total surface of the coverslip was scanned.

Images of Tuj-1 labelled cells were acquired with a 20x objective. Tuj-1 stains neurons (cell bodies and axons) and therefore allows the visualisation of length and arborisation. Images were processed with Sholl analysis using the ImageJ-Win32 software. Sholl analysis creates a series of concentric circles around the soma of the neuron. For this study, a template of concentric circles of 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200µm from the ganglion centre was overlaid on the ganglion (Figure 2.4). The number of axons crossing each circle was counted. For each experimental condition, five ganglia were analysed.

Figure 2.4: Schematic representation of Sholl analysis

The ganglion is labelled with Tuj-1 (green) and neuron nucleus with DAPI (blue). The template of concentric circles from the ganglion centre overlaid on the ganglion is displayed. The number of axons crossing each circle was calculated.

2.12 IgG isolation

2.12.1 Denaturation of antibodies

Autoantibodies can excite human and guinea pig enteric neurons (Li et al., 2016; Piepgras et al., 2015). To examine whether autoantibodies in the sera of CD patients have an effect, we proceed with a thermal denaturation/ deactivation of antibodies. Serum was incubated in a water bath at 71°C for 30 minutes.

2.12.2 Co-Immunoprecipitation with magnetic beads

Since thermal denaturation is not specific, Protein A/G Microbeads (µMACS, Miltenyi Biotec, Bergisch Gladbach, Germany) were used for the isolation of the IgG, IgA antibodies. Different protocols were followed.

Protocol 1:

Serum was incubated with µMacs Protein A/G Microbeads (1:20) on ice for 30 minutes. The mixture was then transferred into a specialised column for molecule isolation (µ Column) placed already in a magnetic holder. The column was washed with 200µl Krebs and flow-through was collected (F1). This step was repeated once more (F2). The column was then removed from the magnetic holder and by using the plunger beads with the bound protein were collected (Ft).

We expected the beads to bind IgG and visualize neuronal immunostaining when they were applied onto tissue preparations. This was not the case, therefore, we try to break the bound of IgG –magnetic beads.

F1 and F2 flow-through were collected as described above. The column was removed from the magnetic holder and treated with 200µl High Salt Buffer (20mM Tris HCl (pH 7.5), 400nM NaCl) and flow-through (Ft1) was collected. The step was repeated (Ft2). In the final step, the column was removed from the magnetic field and beads were collected (Fb).

Recording of neuronal activity was not possible with this method. Applying the Ft1 flow through onto the submucosal neuron reduced the ANEPPS staining intensity.

Protocol 2:

Serum was incubated with µMacs Protein A/G Microbeads (1:20) on ice for 30min. The column was pre-treated with 1% Triton x+ experimental Krebs. The magnetisable immune complex was then passed over the pre-treated column and the first flow-through was collected. The column was then washed 5x with 200µl experimental Krebs and the flow-through was discarded. The column was removed from the magnetic field of MACS Separator followed by centrifugation for 1min at 300g. The flow-through was collected and applied to the submucosal neurons. No results were obtained as Triton-X might interfere with the cell membrane and distorts ANEPPS intensity.

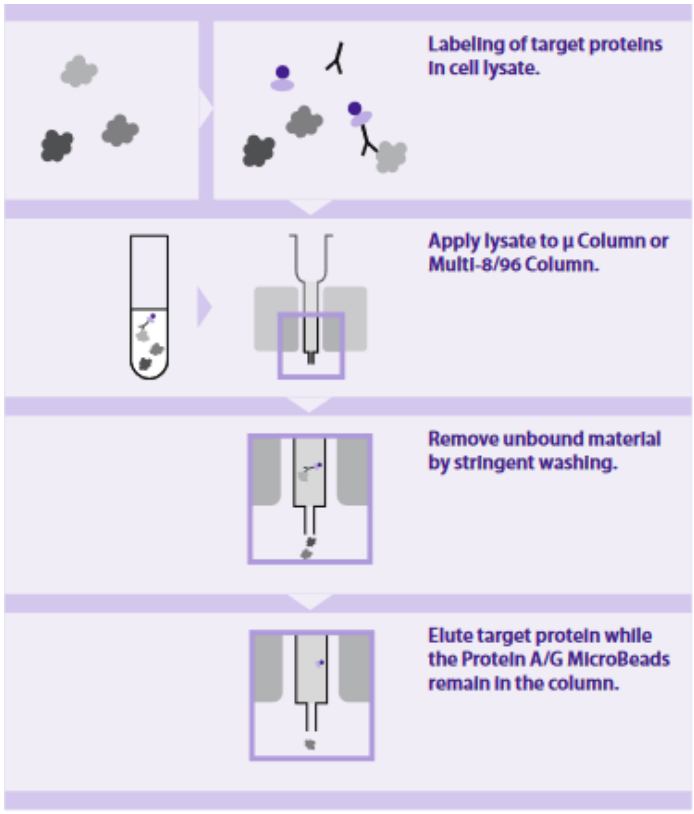


Figure 2.5: Isolation of IgA/ IgG using Protein A/G microbeads

Schematic representation of antibodies isolation with magnetic beads. The magnetisable immune complex was passed over a column and eluted proteins were collected for further application on enteric neurons (Miltenyi Biotec, 2006).

2.13 Biomarker analysis

To analyse cytokines residing in the serum samples U-PLEX (Meso Scale Diagnostics, Maryland, United States of America) immunoassay was performed. U-PLEX is based on multi-array technology as it allows the analysis of several biomarkers per well. In this study, 10 cytokines were analysed (TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-22, IFN-γ, and GM-CSF).

Biotinylated capture reagents are coupled to U-PLEX Linkers. These linkers then self-assemble onto unique spots on the U-PLEX plate. Cytokines present in the serum samples bind to the respective capture reagents which are then bound to the detection antibodies

conjugated with electrochemiluminescent labels (MSD GOLD SULFO-TAG), thus completing the sandwich immunoassay.

At first, to couple the biotinylated capture reagents to U-PLEX Linkers, 200µl of each antibody were added to 300µl of the respective linker and incubated at room temperature for 30 minutes. Afterwards, 200µl Stop Solution was added and incubated at room temperature for 30 minutes. All the U-PLEX coupled antibodies were mixed by adding 600µl of each of them into a single tube and were vortexed. Ten U-PLEX coupled antibodies were pooled together forming the multiplex coating solution. From this solution, 50µl were added in each well. The plate was sealed with an adhesive seal and shook for 1 h at room temperature. The plate was further washed three times with 200µl/well of PBS-T (Phosphate-buffered saline (PBS) plus 0.005% Tween-20.

After the plate was coated, 25µl of Diluent 43 was added to each well and the plate was tapped gently on the sides. Then 25µl of the Calibrator Standard or the sample were added to each well. The plate was covered with an adhesive plate seal and incubated at room temperature with shaking for 1 h. The plate was washed three times with 200µl/well of PBS-T and 50µl of detection antibody solution were added to each well. The plate was sealed again with an adhesive plate seal and incubated at room temperature with shaking for 1 h, followed by three times washing with 200µl/well of PBS-T.

For the final step 150µl of 2X Read Buffer T were added to each well. The plate was then placed on an MSD instrument. Voltage was applied to the plate electrodes causing the capture labels to emit light. Based on the intensity of the emitted light the quantity of each analyte was defined.

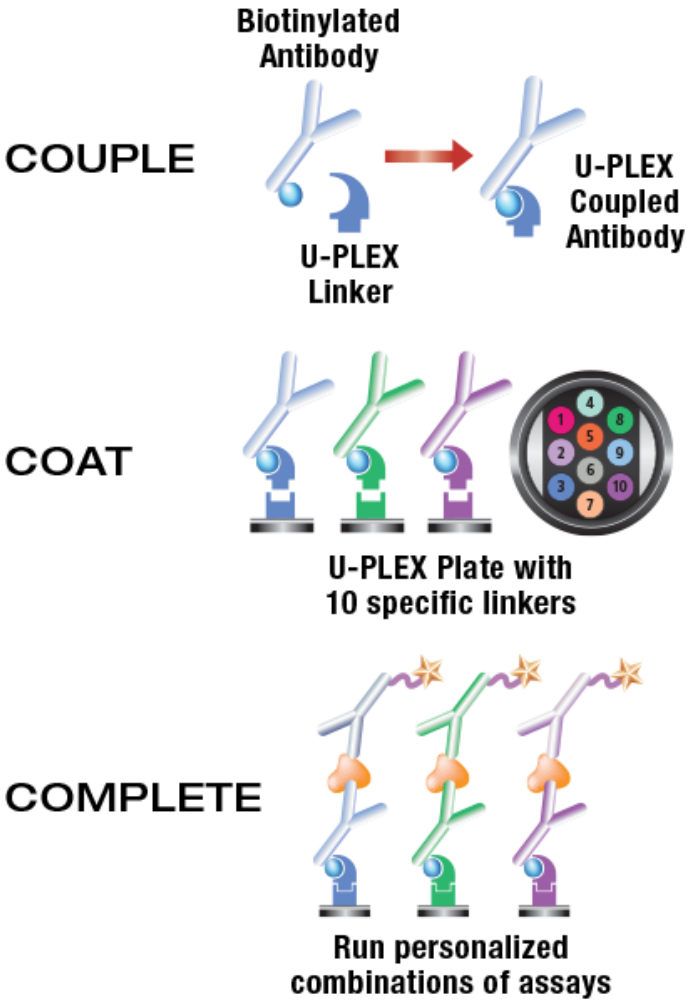


Figure 2.6: Schematic representation of the biomarker analysis

The biotinylated antibody binds to the U-PLEX linker. The U-PLEX coupled antibody binds to the captured reagents on the plate. Afterwards, detection antibodies conjugated with electro-chemiluminescent labels bind to the coupled antibodies, thus completing the sandwich immunoassay (Meso Scale Diagnostics, 2018).

2.14 Data analysis and statistics

Data from neuroimaging experiments were analysed with the Neuroplex 10.1.02 (RedShirtImaging, Decatur, GA, USA) software. Traces of the spike discharge were obtained by averaging the relative change in fluorescence from the pixels on the neuronal cell body. Traces were extracted using the Igor Pro v.6.3.4.1 software (WaveMetrics, Lake Oswego, Oregon, USA). Frequency and number of neurons data were collected in Microsoft Office Excel 2016 (Microsoft Corp., Redmont, WA, USA). Statistical analysis was performed using the software GraphPad Prism 6 (GraphPad Software Inc., La Jo, CA, USA) and R version 3.4.4 (R Foundation for Statistical Computing, Vienna, AT).

The spontaneous activity recorded at basal recording was substracted from the frequency of action potential discharge following application of serum samples. At least a difference of two action potentials was calculated, in order to consider the recording a genuine response to the substance.

As a parameter for the serum-evoked activation of submucous neurons, neuroindex was calculated by multiplying the percentage of activated neurons and their average frequency of action potential discharge (Hz) (Buhner et al., 2014).

Labelling of enteric neurons was assessed using the open-source program Fiji / ImageJ v.1.5.1 (National Institutes of Health, Bethesda, Maryland, USA). To analyse axonal complexity, images were processed for Sholl analysis using the ImageJ-Win32 software (National Institutes of Health, Bethesda, Maryland, USA).

To detect any significant difference various statistical tests were used, depending on the distribution of data and the study design. Normal distribution was assessed using the Shapiro Wilk test. Normally distributed data were analysed using parametric tests, like paired t-Test and are presented with mean values and standard deviation (SD). Multiple comparisons were performed with the Bonferroni t-test. Not normally distributed data were analysed with the Wilcoxon Signed Rank Test, the Kruskal -Wallis One Way Analysis of Variance of Ranks, the one-way ANOVA and the two-way ANOVA tests. These data are expressed as median with the 25th and 75th percentiles (25th–75th; interquartile range; IQR). The multiple comparisons were achieved with Dunn's Method and Tukey Test. Homogeneity of variance was assessed using the Fligner- Killeen- Test. Correlations were

performed with the Spearman non-parametric correlation test. The level of statistical significance was set to $p < 0.05$.

3. Results

3.1 Neuroimaging studies

Neuroimaging studies were performed on 132 adult male Dunkin Hartley guinea pigs resulting in the recording of 1626 neurons from 144 submucosal ganglia (mean number of neurons per ganglion was 11). Recordings were performed for 2s with a sampling rate of 1.6 kHz.

For all neuroimaging experiments action potential frequency, percentage of responding neurons per ganglion and neuroindex were calculated. Basal firing activity was accessed before the application of any serum sample and was subtracted from the total firing frequency after the serum application. The neuroindex is defined as the product of the percentage of responding neurons and their spike frequency and was used as a measure of evoked neuronal activity in the ganglion.

Through this thesis, the number of guinea pig preparations, ganglia and neurons is given in sequence without further specification, e.g. experiments on 4 guinea pig tissue, 10 ganglia and 126 neurons are presented as (4/10/126).

3.2 Reproducibility of neuronal responses

Previous studies showed that the recording period of 2s is ideal for guinea pig experiments as it does not cause significant bleaching or phototoxicity (Qin Li, 2013). Sera were applied with 20 minutes intervals. Responses to serum were reproducible between applications. Therefore, the pharmacology was performed on the same ganglion.

To test for reproducibility serum was administered as two consecutive applications on 4 preparations, 4 ganglia and 11 neurons. No statistical difference was noted between the two applications. Viability of neurons was assessed using nicotine (Figure 3.1).

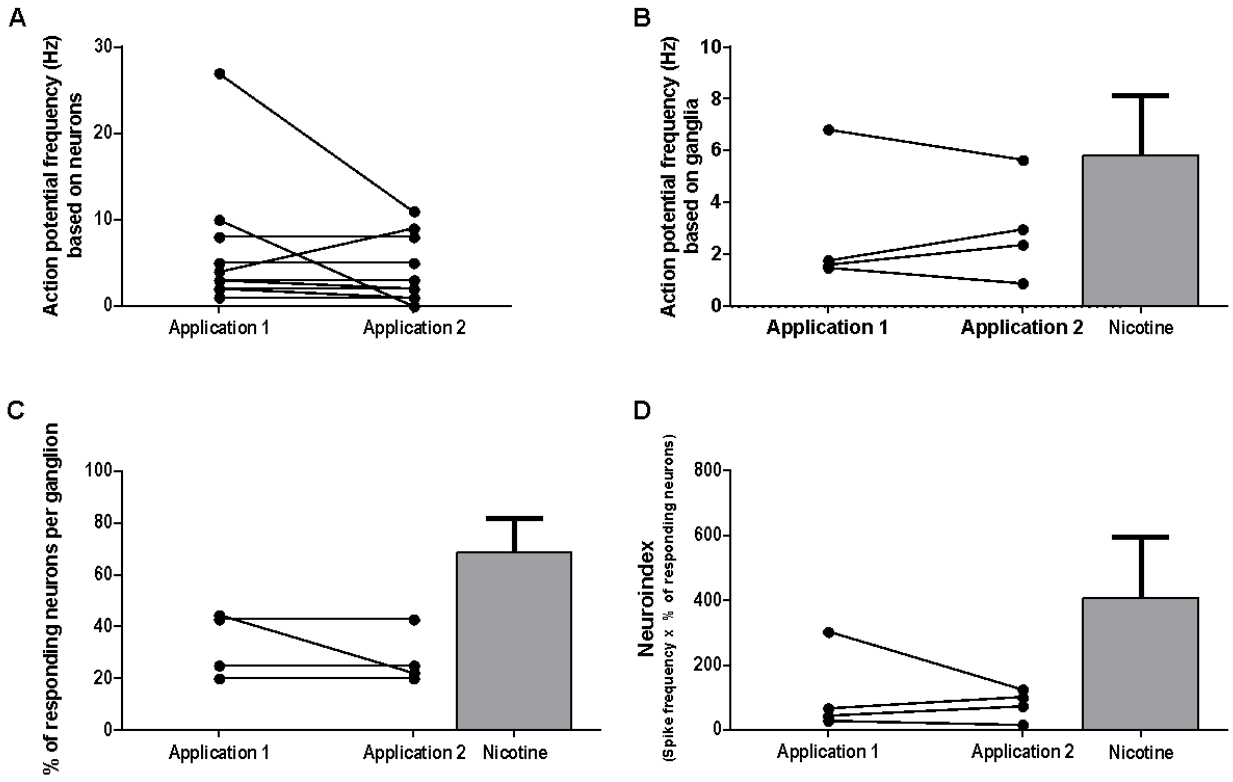


Figure 3.1: Reproducibility of two consecutive serum applications

(A) The response of individual neurons to the two applications. 8 out of 11 neurons retain the same discharge upon consecutive serum applications (paired t-test; p=0.42). (B) The evoked spike discharge per ganglion (p=0.93) is indicated, n=4. (C) The percentage of responding neurons was similar between the two applications (p=0.39), (D) as well as the neuroindex (p=0.57), n=4. Nicotine application serves as a tool to assess the viability of the neurons (4/4/42).

3.3 Osmolarity of serum samples

A prerequisite of any recording experiment is to adjust the osmolarity of the sample applied as fluctuations in osmolarity can masque neuronal responses generated from mediators in the serum. Unfortunately, in this study, this was not possible since the available quantities of serum samples were too small to measure osmolarity. Furthermore, freezing – thaw cycles can affect the osmolarity of the sample (Seifarth et al., 2004)

3.4 pH of serum samples

The pH varied between the serum samples. The optimum pH is 7.4. Some samples had a pH close to 8 (ranging from 7.80 to 8.01) before aliquoting. Measuring the pH after aliquoting was not possible due to the small volume of the aliquots. Nevertheless, to exclude the possibility that any effect observed was due to pH shifts, experimental Krebs at pH 8 was applied to the ganglia in the same way with serum application (1 bar, 200 ms duration). Krebs at pH 8 did not affect neuronal excitability (1/3/31).

3.5 Complete blood cell counts

Complete blood cell count is a routine blood test to evaluate the overall health as an abnormal increase or decrease of cell count can indicate an underlying medical condition. It measures red blood cells, white blood cells, haemoglobin, haematocrit, and platelets. Upon inflammation, there is an increase in the count of leucocytes and platelets as is evident in the CD patients (Figure 3.2).

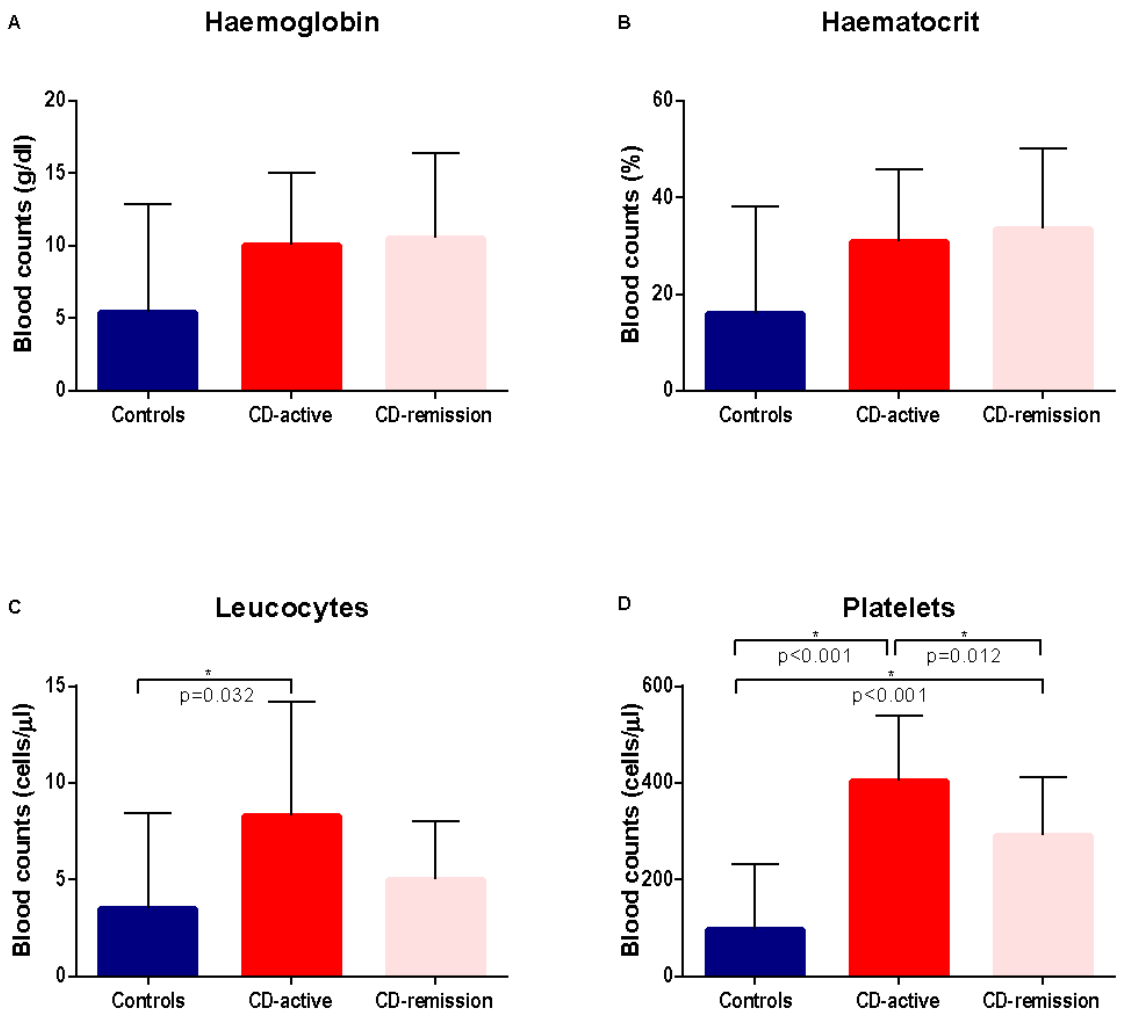


Figure 3.2: Complete blood cell count in control, CD-active and CD-remission samples

An increase in leucocytes and platelets is noted in CD-active patients compared to control and CD-remission samples. The normal ranges are for haemoglobin: 14-17 g/dl, haematocrit: 36-50%, leucocytes 4-5 million cells/µl and platelets 140,000-450,000 cells/µL. Differences in the mean values among the treatment groups were calculated with one-way ANOVA on Ranks. Pairwise multiple comparisons for leucocytes and platelets were performed with Bonferroni t-test.

3.6 Neuroimaging experiments with sera from Crohn's disease patients

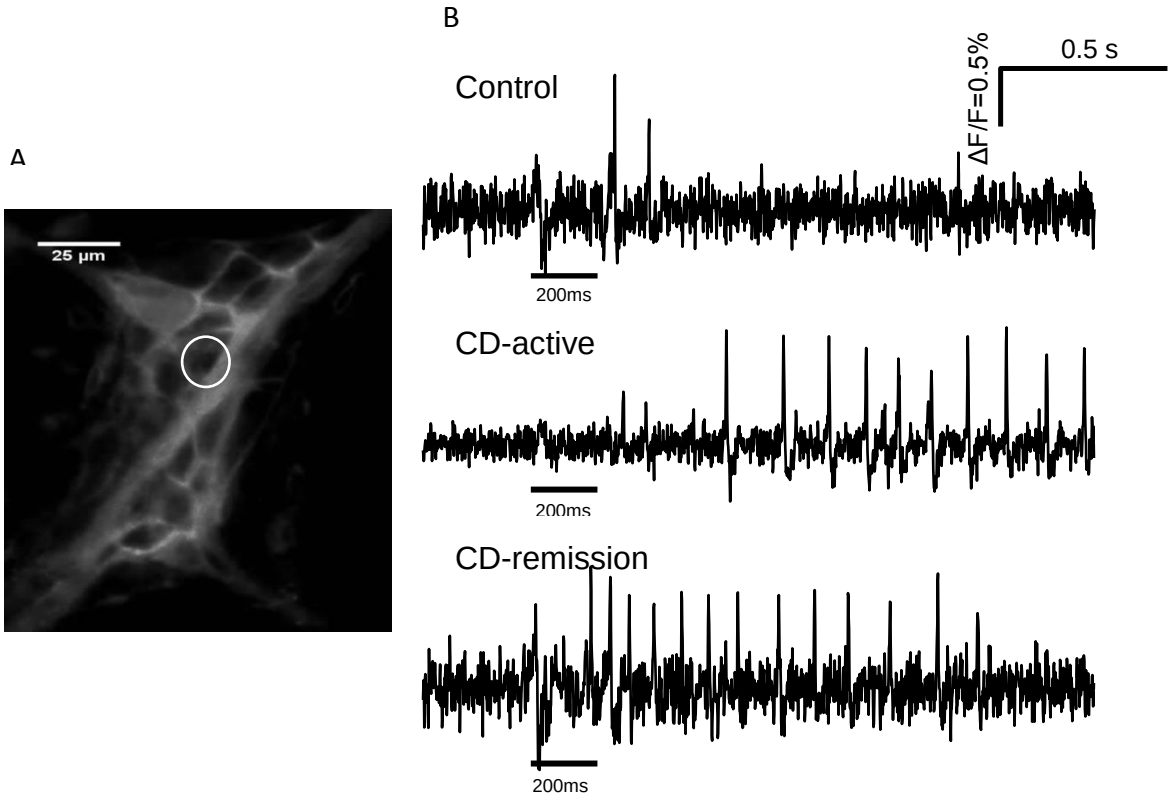


Figure 3.3: Activation of guinea pig submucous neurons by sera from healthy controls and CD-patients

(A) The picture on the left side illustrates a Di-8-ANEPPS stained guinea pig ganglion. Individual neurons in the ganglion of the submucous plexus can be seen by the bright outline of the outer membrane. (B) The response of the encircled neuron in the serum from healthy control and CD patient (#002) in the active and the remission stage are shown on the right side. Neuron showed an immediate onset of spike discharge after the application of the sera.

3.6.1 CD patient sera cause stronger neuronal activation than control sera

We applied sera from patients with Crohn's disease (CD) (n=12 CD-remission, n=20 CD-active) and healthy controls (HC) (n=20) on guinea pig submucous neurons and analyse their spike discharge in 44 submucous plexus preparations (50 ganglia, 574 neurons).

In each ganglion two different sera were applied, one serum from a CD patient and another one of control. Sere were matched based on the age, gender, and were applied in random order. Sera from CD-active or CD-remission patients caused a fast onset spike discharge of 3.7(2.4/4.6) Hz or 3.9(2.9/4.9) Hz, respectively, which was significantly higher than the one observed after HC-serum 1.7(0.1/2.5) Hz; p=0.003 and 1.8(0.2/2.3); p=0.03, respectively) (Figure 3.4A). Moreover, the proportion of neurons responding to CD-active or CD-remission sera was 36.8% (22.9/44.9) or 25.0% (16.6/37.5), respectively, and both thereby, significantly higher than the proportion sensitive to HC-serum (11.1%(0.7/25.7); p<0.0001 and 13.6%(1.6/21.5);p=0.0008) (Figure 3.4B). The neuroindex was 92.4(50.6/174.8) for CD-active and 94.2(79.5/142.7) for CD-remission samples. The neuroindex for HC paired with CD active was 21.5(1.6/49.9) and 17.8(5.4/51.5) for those paired with CD remission. Neuroindex was higher for CD samples compared to HC (p<0.05) (Figure 3.4C).

To demonstrate the difference in neuronal activation among sera from CD patients and controls we subtracted the neuroindex of controls from the neuroindex evoked by sera from CD-active patients. Also, delta (Δ) neuroindex was calculated for CD-remission response deducting the response to the control sera (Figure 3.4D).

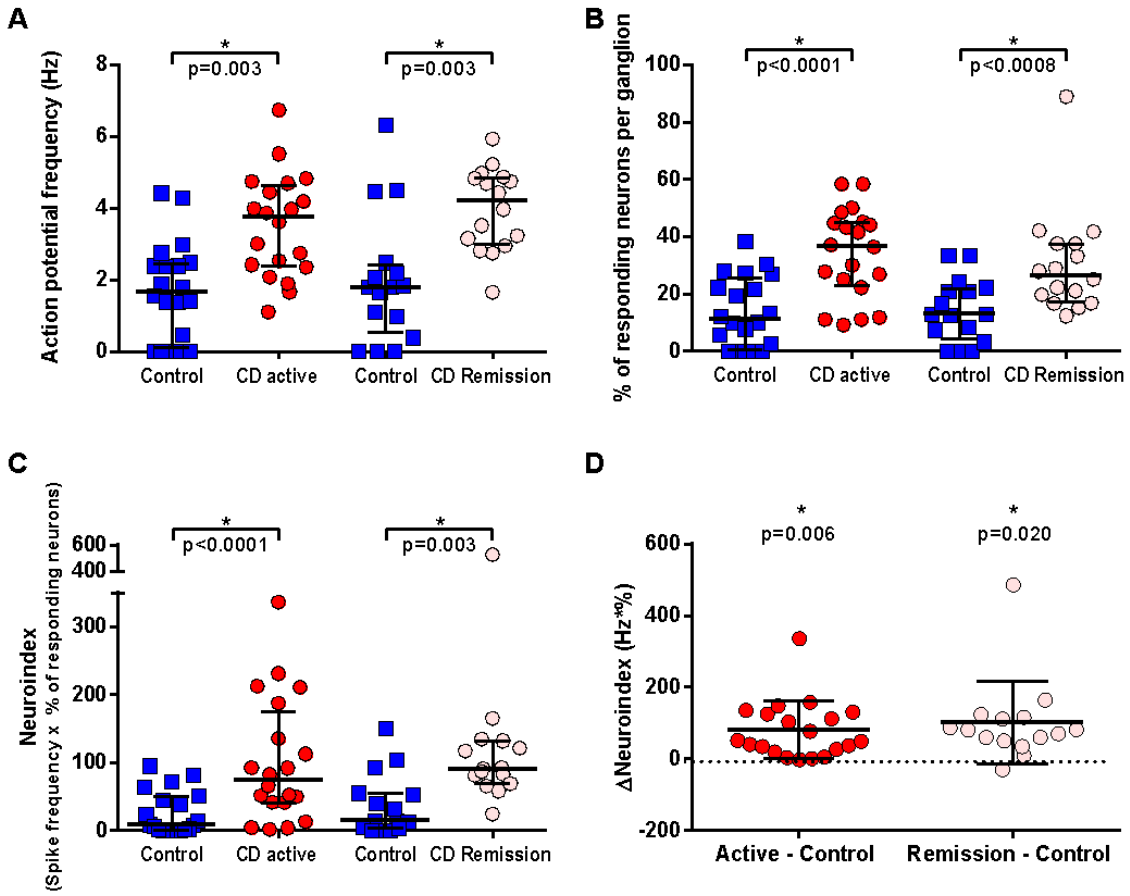


Figure 3.4: Patient sera cause stronger neuronal activation than control sera

(A, B) Sera from CD-patients and healthy controls evoked activation of guinea pig enteric neurons to a different degree. Patient sera evoked a significantly stronger spike discharge in a larger proportion of GP enteric neurons. (C) As a result, the neuroindex was also significantly higher. (D) The delta values clearly show the difference between responses to active sera subtracting the paired controls and to remission sera substracting the paired controls, Wilcoxon test; Median values with interquartile range (25 quartile / 75 quartile) are shown with black lines. Controls are shown in blue; CD-active in red, CD-remission in pink. Each dot represents a different patient serum, CD-active n=20; CD-remission n=17 (44/50/574).

3.6.2 Neuronal activation is independent of the disease stage in CD patients

We tested in a pairwise manner the response to serum from CD patients before and after they achieved remission (n=8). Strikingly, sera from CD patients in the active stage of the disease activated neurons to a similar degree with sera from CD patients in the remission stage. The fast onset spike discharge was 3.4±1.1 Hz for CD-active and 2.6±1.6 Hz for CD-remission serum (Figure 3.5A). Besides, the proportion of neurons responding was 37.8%±20.6 and 36.4%±25.6 for CD-active and CD-remission sera, respectively (Figure 3.5B). The neuroindex was 139.1±112.7, 125.5±156.7 % x Hz for CD-active and CD-remission, respectively. None of the parameters yielded any statistical significance (Figure 3.5C).

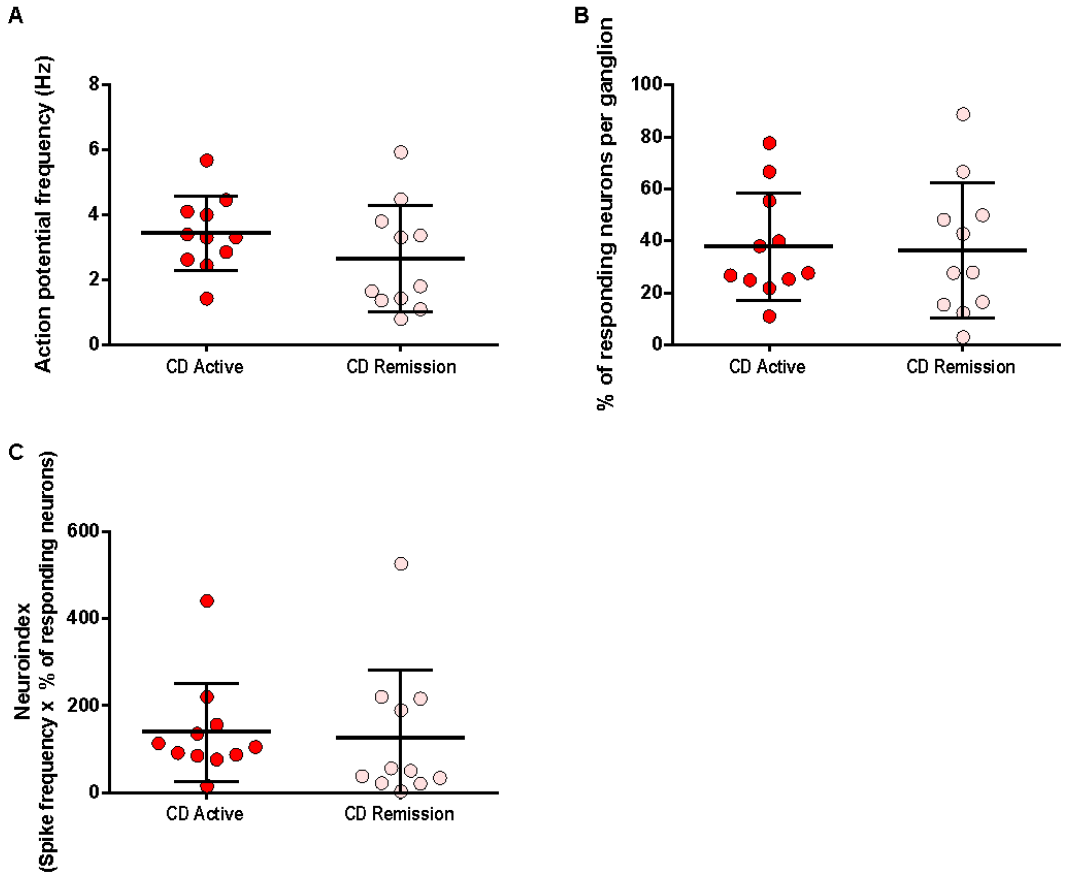


Figure 3.5: Neuronal activation is independent of the disease stage

(A) Sera from CD patients before and after they achieved remission evoked similar spike discharge (p=0.28). (B) Also, the percentage of activated enteric neurons was similar (p=0.77) (C) Therefore, no difference was observed for the neuroindex either (p=0.63). Paired t-test; Results are shown

as mean with SD. CD-active in red; CD-remission in pink. Each dot represents a different patient serum, CD-active n=11; CD-remission n=11 (14/24/263).

3.6.3 Disease score (CDAI) does not correlate with neuronal activation

Patients are classified as active and remission based on their CDAI score and/or endoscopic indications. A CD patient with CDAI score less than 150 is classified in the remission stage. There was a no correlation of CDAI score and the neuronal activation evoked by the CD serum. A non-parametric Spearman correlation coefficient rs > 0 suggests that the two compared values can increase or decrease together (Figure 3.6).

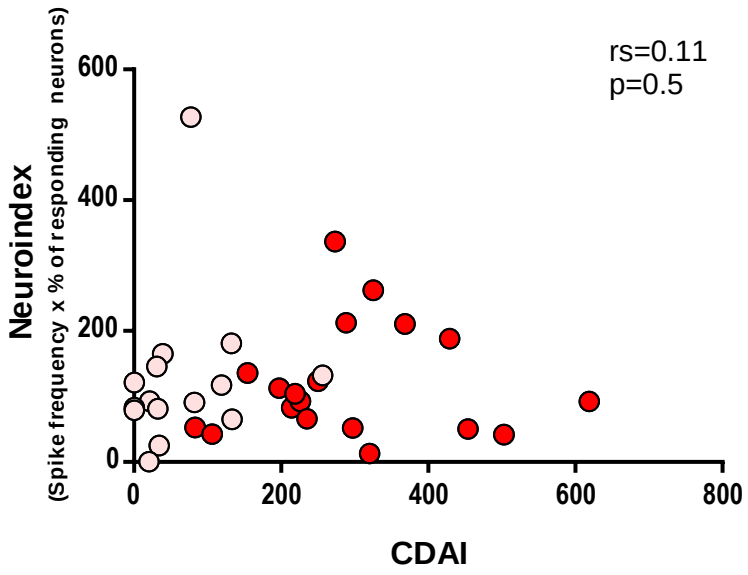


Figure 3.6: Neuronal activation is independent of the CDAI score

There was a no correlation between the CDAI score and the neuroindex. Sera from CD-active patients are shown in red and CD-remission in pink. Each dot represents a different patient serum. The Spearman correlation test was used to analyse correlations between disease score and neuroindex (rs: non-parametric Spearman correlation coefficient).

3.6.4 CRP levels in CD patients

C-reactive protein (CRP) is an annular protein produced in the liver. Along with platelet counts, it is used as blood test marker of inflammation. Under physiological conditions, CRP is not detected in the blood. Every increase of CRP above normal values (i.e. > 5mg/L) demonstrates significant activity in CD. In case of severe attacks or strong activity, CRP can be higher than 25mg/L. To further study whether there is an association between the strength of the disease and the neuronal activation we classified the patients based on their CRP levels. There was no correlation between the levels of CRP and the evoked neuronal activity of enteric neurons.

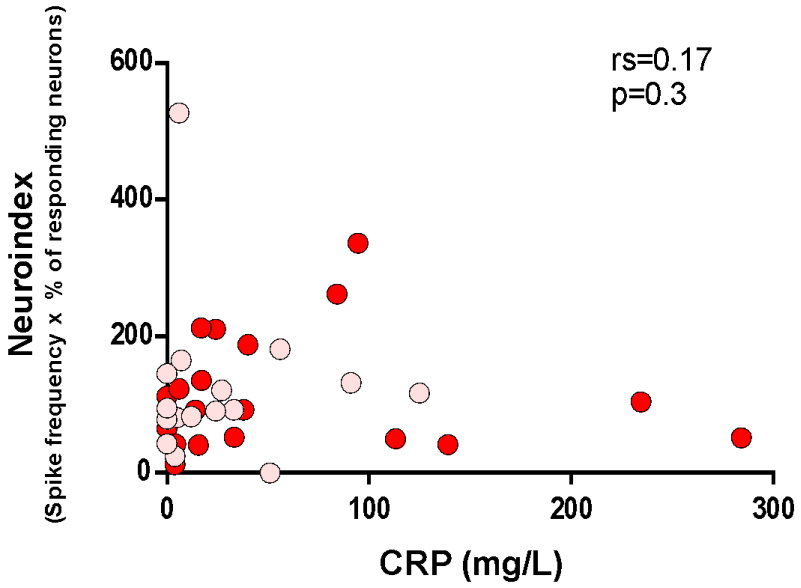


Figure 3.7: Neuronal activation is independent of the disease stage as shown by CRP

There was no correlation between the neuronal response to patient serum and the comparing CRP values of patients. This was the case for both CD-active and CD-remission patients. Samples from CD-active are shown in red and CD-remission in pink. Each dot represents a different patient serum, CD-active n=20; CD-remission n=17. The analysis was performed with the Spearman correlation test.

3.7 Analysis of cytokines residing in the serum

Several cytokines were proposed to be involved in IBD pathology. To identify whether some of these reside in the tested sera we measured the level of ten cytokines (TNF-α, INF-γ, IL-1β, IL-17A, IL-6, IL-8 IL-12p70, IL-10, GM-CSF, IL-22) by multiplex immunoassay.

Cytokines in control and remission patients, both CD and UC, are mainly clustering together in the PCA analysis. Mediators in these sera were in lower concentrations compared to the sera from patients in an active disease stage (Figure 3.8). More precisely, INF-γ and IL-22 were elevated in all of the patient groups compared to the control group. A significantly higher concentration of IL-1β was measured in the serum of CD active patients compared to the CD remission patients. In the UC active patients, a high concentration of IL-8 was measured (Figure 3.9).

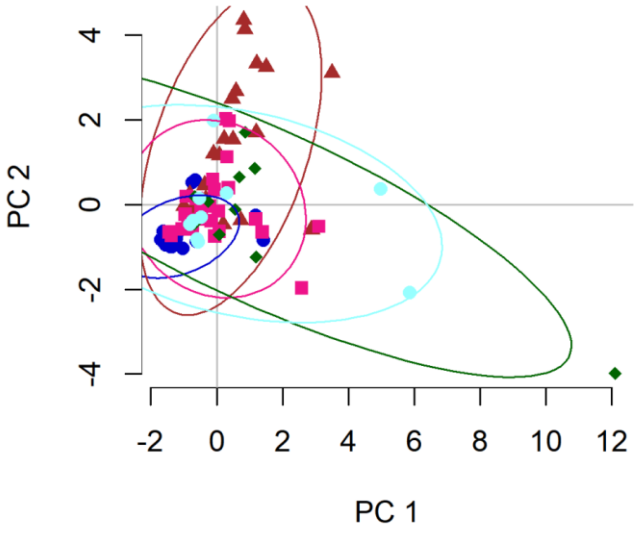
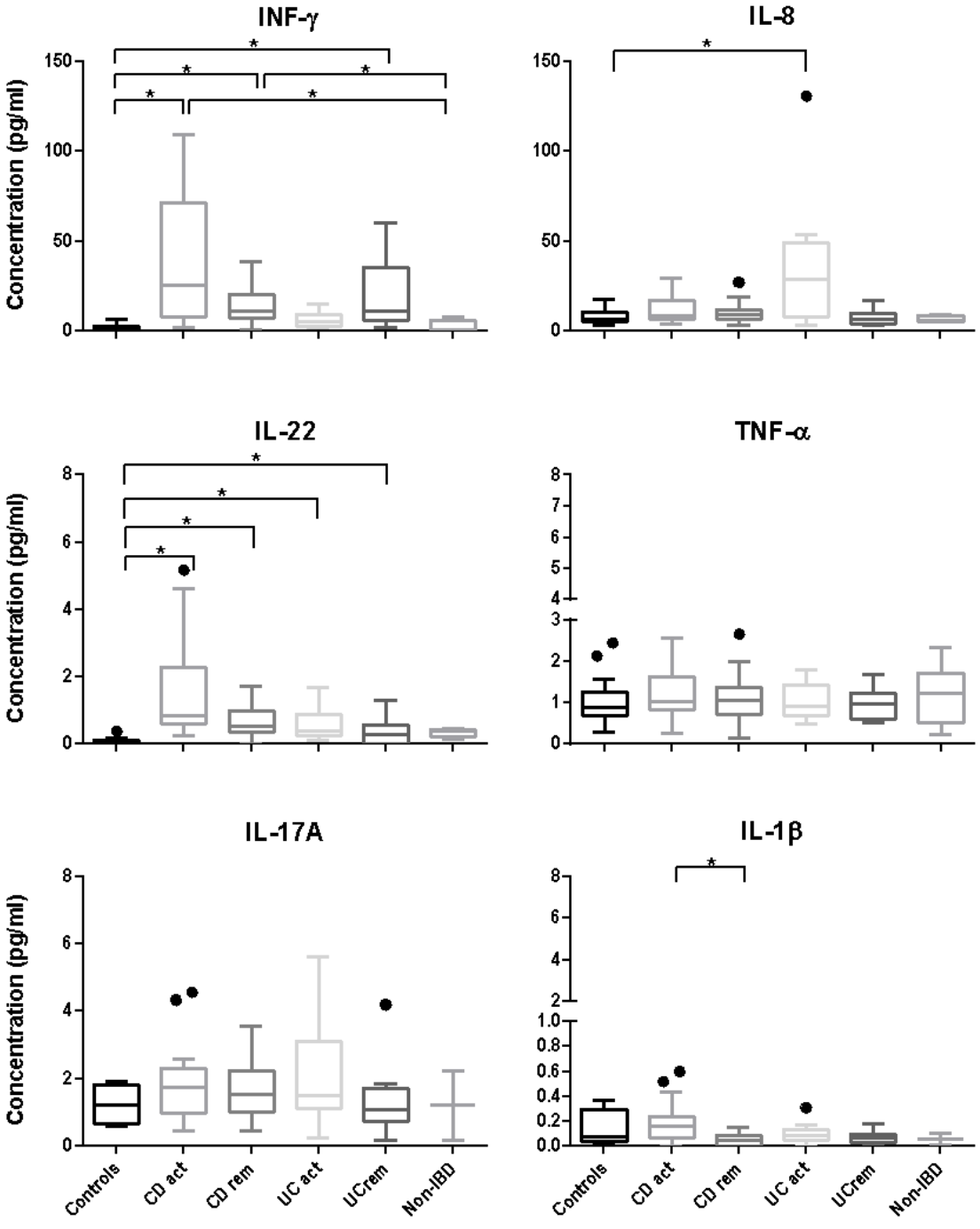


Figure 3.8: Cytokines analysis

Principal component analysis of all 102 serum samples regarding ten of the cytokines residing in their serum. Controls are shown in blue, CD active in red, CD remission in pink, UC active in green and UC remission in light blue. Ellipses around each defined group show 95% confidence interval.


INF-γ
IL-8
IL-22
TNF-α
IL-17A
IL-1β
Concentration (pg/ml)
Controls
CD act
CD rem
UC act
UCrem
Non-IBD

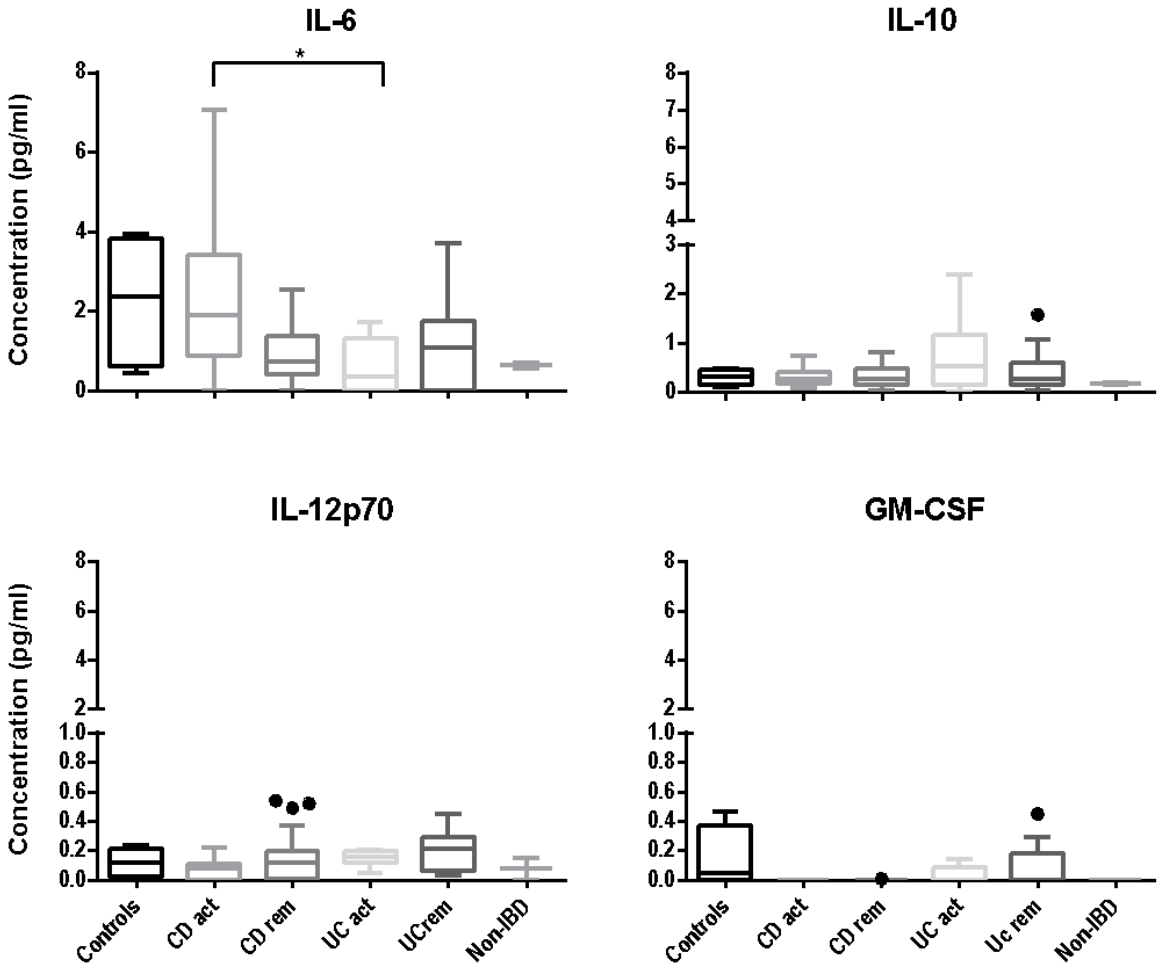


Figure 3.9: Cytokines concentration in serum

Box plots of the serum cytokines concentration measured by multiplex immunoassay in Controls (n=29), CD-active (n=24), CD-remission (n=25), UC-active (n=12), UC-remission (n=12), Non-IBD (n=2) groups. Kruskal-Wallis test; median with interquartile range. Outliers are displayed as circles.

3.8 Effect of TNF-α on guinea pig enteric neurons

TNF-α is extensively described as a potent inflammatory mediator (Sedger and McDermott, 2014). Nevertheless, the effect of TNF-α on spike frequency and the proportion of responding neurons are not reported yet. Therefore, we tested the effect of various TNF-α concentration on the evoked response in guinea pig submucous neurons. Increasing concentrations of TNF-α correlated with an increase of neuronal responses, R^2= 0.4, p=0.0009 (Figure 3.10 A, B).

Adalimumab is a fully human-derived antibody specifically directed against TNF-α. It binds with high affinity to TNF-α thus blocking it from interacting with the p55 and p75 cell-surface TNF receptors. To study the effect of adalimumab on TNF-α induced spike discharge and number of responding neurons, we applied it directly on enteric neurons. Application of adalimumab in different concentrations (5, 50, 500 µg/ml) did not affect enteric neurons.

TNF-α was pre-incubated with adalimumab (Concentration: 1:10; adalimumab: TNF-α) for 20 minutes. Incubation of TNF-α with adalimumab minimized the excitatory effect of TNF-α onto submucosal enteric neurons. TNF-α alone yielded a response of 99.2± 51.3 which was reduced to 18.1 ± 25.0 % x Hz after incubation with adalimumab (Figure 3.10B).

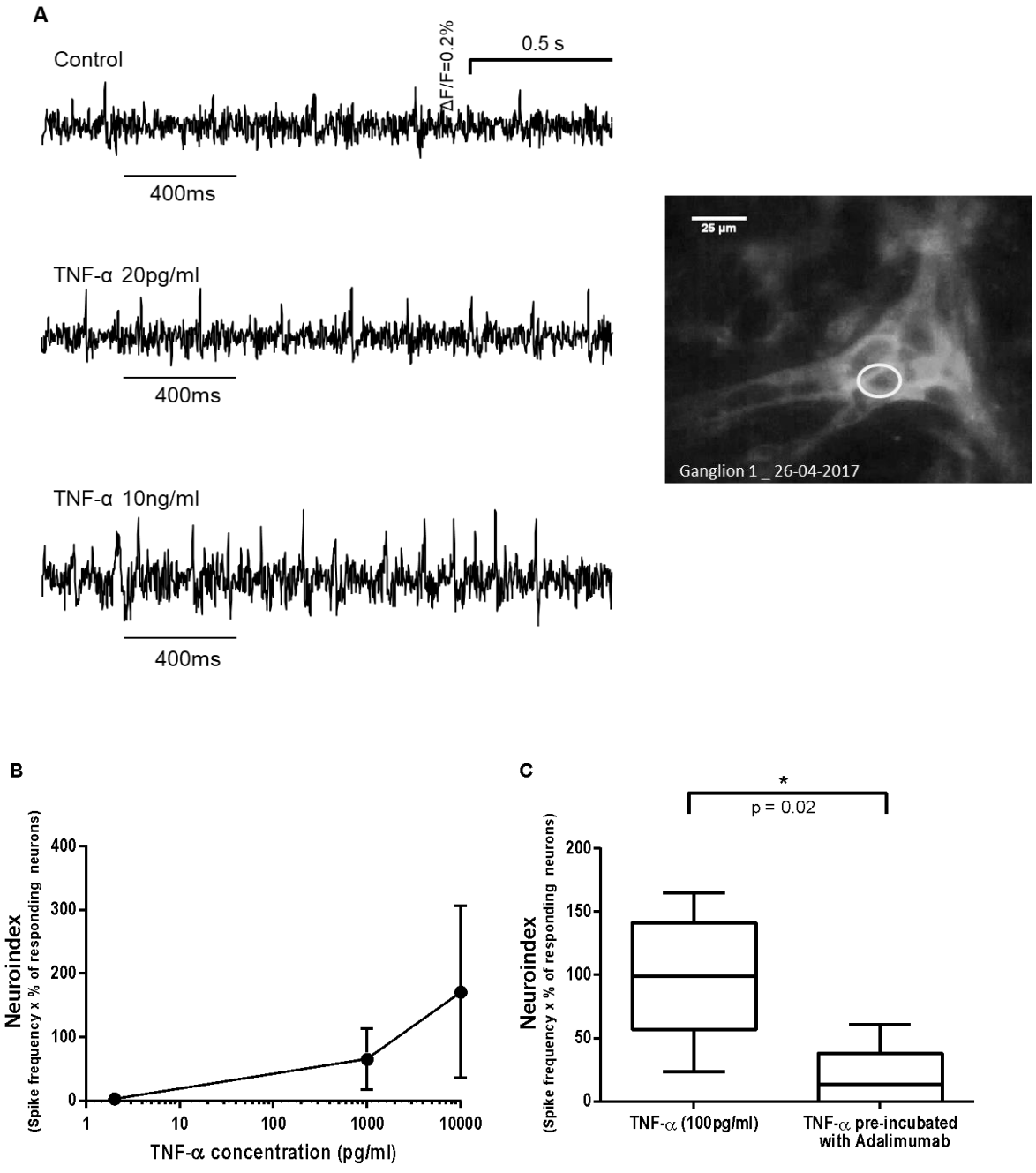


Figure 3.10 TNF-α can excite enteric neurons

(A) Traces showing the neuronal activation upon application of TNF-α. The upper trace shows the basal activity of the encircled neuron while the two following traces show the evoked neuronal response after application of 20pg/ml and 10ng/ml of TNF-α. Ganglion was adjusted for brightness (59%) and contrast (20%). (B) Excitation of enteric neurons correlates with the concentration of TNF-α. (C) Adalimumab, a TNF-α specific antibody, can reduce or completely abolish the effect of TNF-α.

3.8.1 CD sera effect is reduced upon incubation with Adalimumab

We followed a pharmacological approach to determine whether the evoked neuronal activation is correlated with certain cytokines residing in the serum of CD patients. We targeted TNF-α since the patients were already receiving medication towards it.

Pre-incubation of serum from CD-active and CD-remission patients with adalimumab showed a reduction of neuronal activity (neuroindex: $p<0.05$) (Figure 3.11). Patient serum #23 did not follow the same pattern; in contrast, evoked neuronal activity was higher after incubation with adalimumab (Figure 3.11).

The fast onset spike discharge was 3.8±3.1 Hz for CD-active before the incubation with Adalimumab and 2.5±3.4 Hz afterwards ($p=0.04$). For CD-remission, it was 3.6±2.1Hz before and 1.9±1.4Hz after the incubation (Figure 3.12A). The proportion of responding neurons was 35.5%±15.6 for CD-active before and 17.7%±15.3 after treatment with Adalimumab ($p=0.01$). Similarly, the percentage of neurons responding to serum from CD-remission patients was 29.3%±16.9 and 13.8%±12.5 before and after treatment, respectively ($p=0.02$) (Figure 3.12B). The neuroindex evoked by sera from CD-active samples was 156.6±96.7%^{x}Hz and 70.9±71.9%^{x}Hz before and after incubation with Adalimumab, respectively ($p=0.04$). The neuroindex for serum from CD-remission samples was 144.0±133.7%^{x}Hz before and 41.55±45%^{x}Hz after incubation with Adalimumab ($p=0.02$) (Figure 3.12C). The neuroindex was positively correlated with the concentration of TNF-α in the serum samples. 58% of the variance is shared between neuroindex and TNF-α concentration (Figure 3.12D).

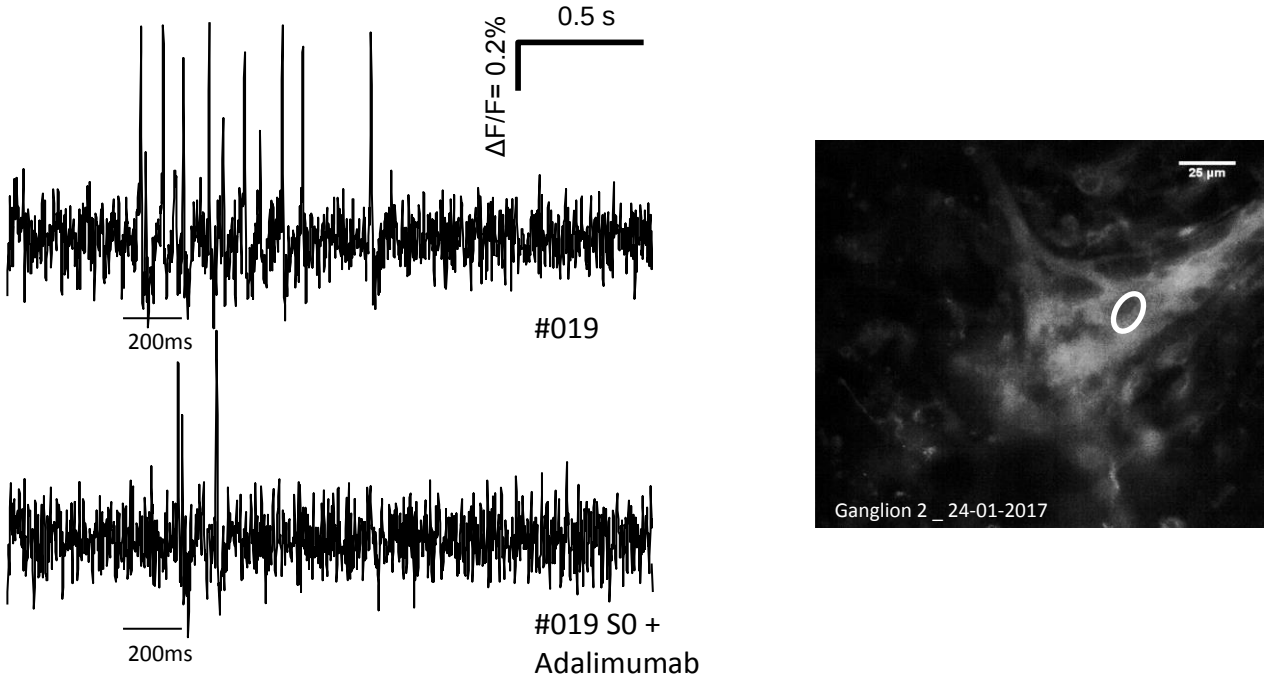


Figure 3.11: Traces showing the reduced neuronal activation upon pre-incubation of serum with TNF-α antibody

The upper trace shows the response of the encircled neuron to the serum from CD active patient #019 and the bottom trace the response to the serum #019 incubated with Adalimumab. Ganglion was adjusted for brightness (53%) and contrast (21%).

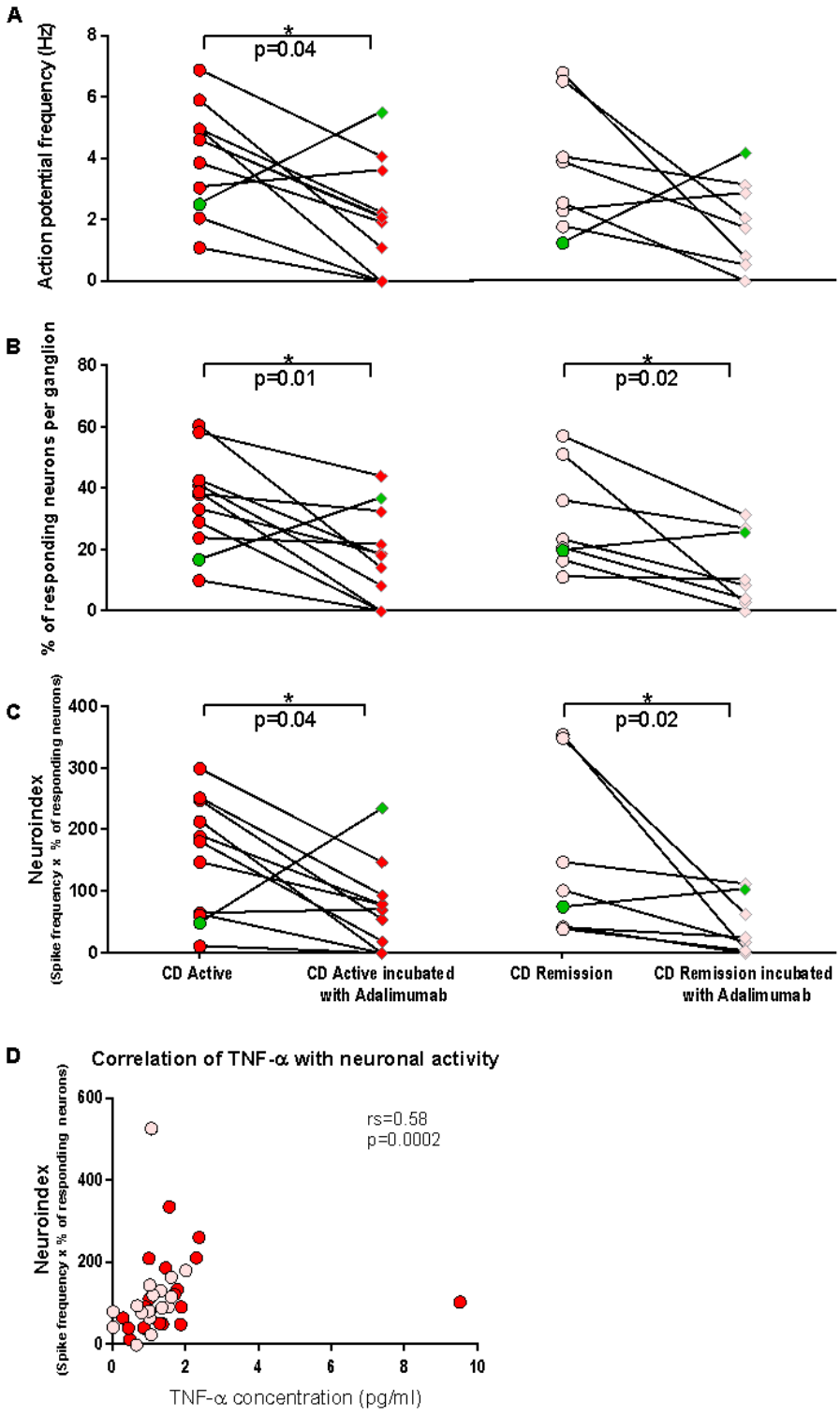


Figure 3.12: Serum evoked neuronal activation is reduced upon pre-incubation with Adalimumab

(A, B) Incubation of serum with Adalimumab resulted in reduced neuronal activation in a lower proportion of enteric neurons. (C) Correspondingly, the overall activity in the ganglion was reduced

as shown here by the neuroindex. Experiments were performed on 13/26/274 for CD-active and 11/17/222 CD-remission sera. Analysis with paired t-test. (D) TNF-α concentration correlates with neuronal activity based on the Spearman correlation test. CD-active in red; CD-remission in pink. Each dot represents a different patient serum. CD-active n=11; (13/26/274) CD-remission n=8 (11/17/222). Serum of patient #23 is marked in green as is not following the same pattern with the rest of the sera.

3.9 Neuroimaging experiments with sera from Ulcerative Colitis patients

3.9.1 UC Patient sera cause similar neuronal activation as control sera

We applied the sera from patients with UC (n=4 UC-remission, n=4 UC-active) and HC (n=4) on guinea pig submucous neurons and analysed the evoked spike discharge (11/20/218) (Figure 3.13).

Sera from UC-active or UC-remission patients caused a fast onset spike discharge of 1.3 (1.2/2.7) Hz and 2.2 (1.7/2.6) Hz, respectively, which was similar to the one observed after application of serum from HCs (1.6 (1.0/2.4) Hz) (Figure 3.14A). The percentage of neurons responding to serum from UC-active, UC-remission patient and HC was 16.9 % (12.4%/19.7%), 22.7 % (19.4%/25.5%) and 15.1 % (11.5%/19.0%), respectively (Figure 3.14B). The neuroindex was 35.8 % (25.0%/45.0%) x Hz, 72.4 % (49.0%/82.8%) x Hz and 38.9 % (8.3%/88.4%) x Hz for CD-active, CD-remission and HC, respectively (Figure 3.14C). None of the parameters yielded any significant differences between the sera from UC patients and sera from healthy controls.

Additionally, patient sera caused a similar degree of neuronal activation independently of the disease stage of UC. Sera from UC patients, after they achieved remission, showed a slightly higher spike discharge in more neurons compared to sera from patients in an active stage.

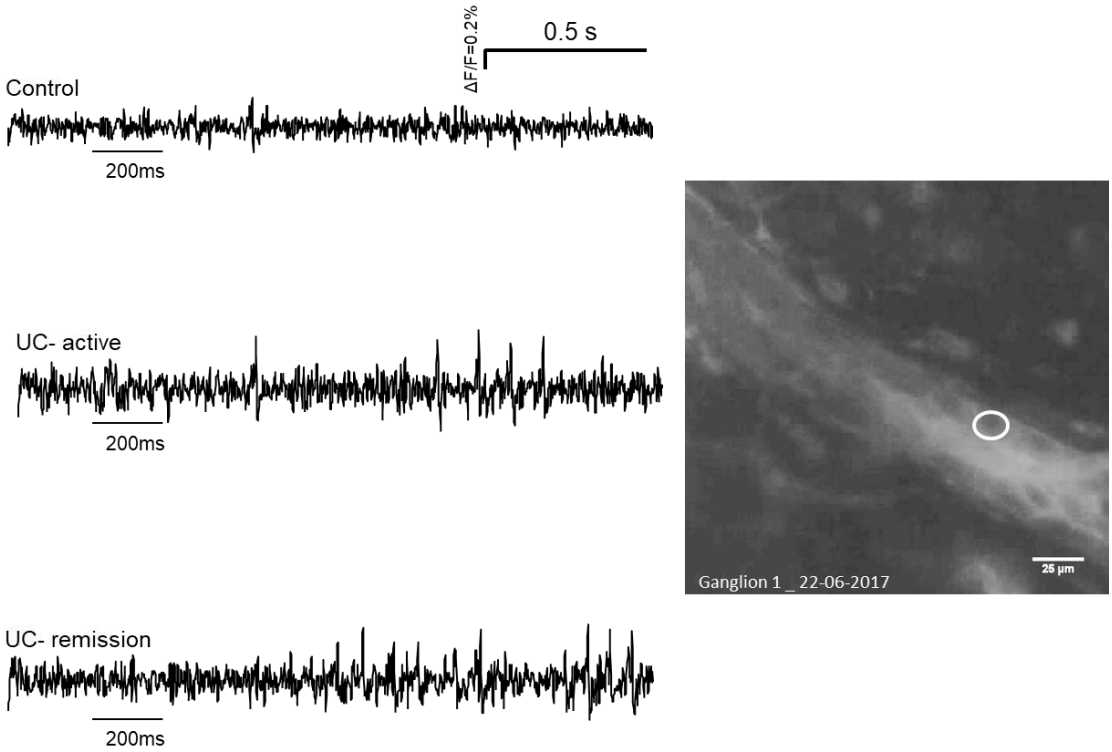


Figure 3.13: Activation of guinea pig submucous neurons by sera from healthy controls and UC patients

The upper trace shows the basal activity of the encircled neuron. The following two traces show the evoked action potential discharge after application of UC serum from the same patient before (#5323) and after remission (#6424) respectively. Ganglion was adjusted for brightness (59%) and contrast (39%).

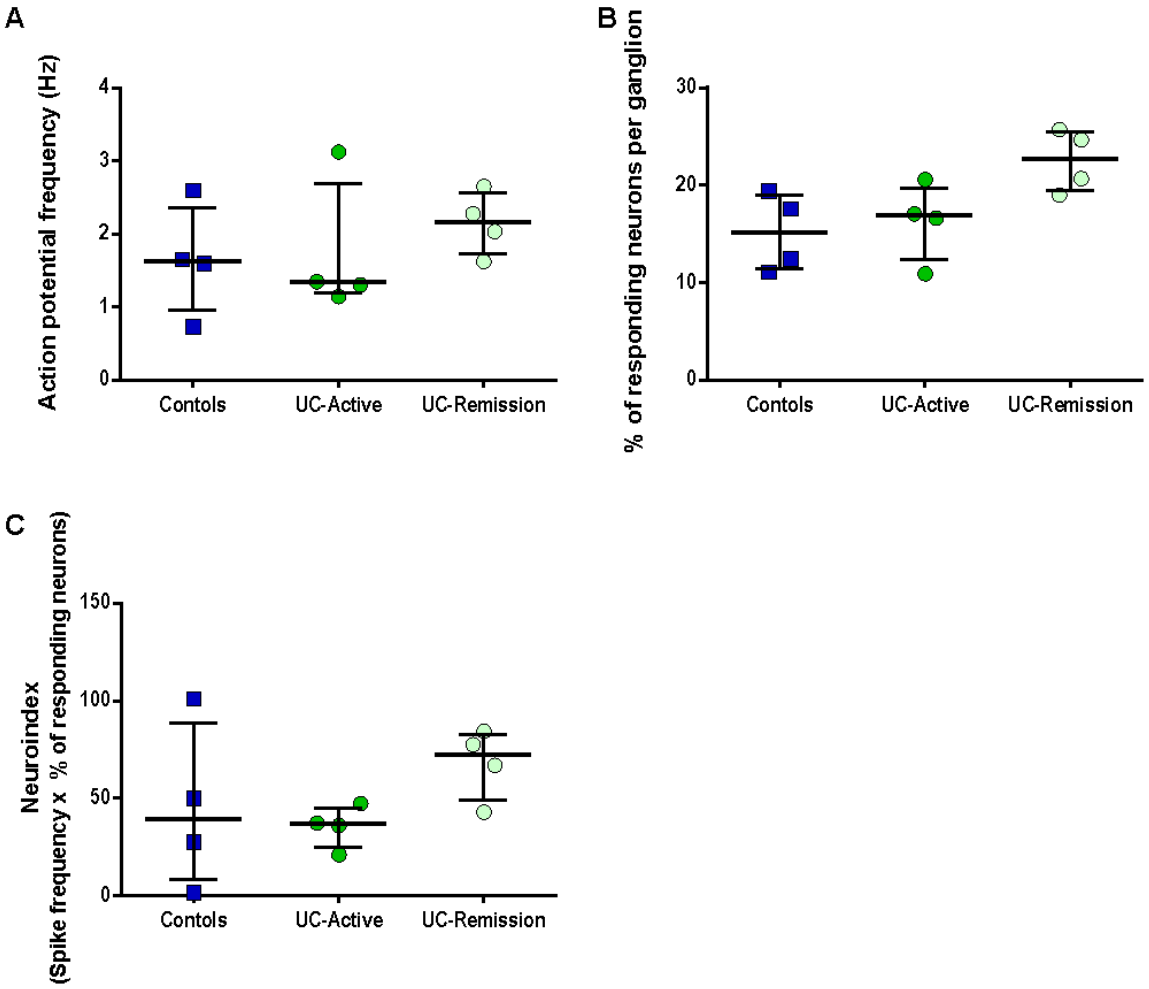


Figure 3.14: UC-patient sera cause similar neuronal activation with control sera independent of disease stage

(A, B) There was no significant difference of the evoked action potential discharge or the proportion of activated GP enteric neurons between effects of sera from UC-patients and healthy controls. (C) As a result, the neuroindex was also not statistically different among the three groups. The analysis was performed with one-way ANOVA test; data are represented with median value and interquartile range (25 quartile / 75 quartile). Controls are shown in blue; UC-active in dark green and UC-remission in light green. Each dot represents a different patient serum, UC-active n=4; UC-remission n=4; control n=4 (11/20/218).

3.10 Neuronal responses to non-IBD serum

3.10.1 Functional gastrointestinal disorders

Anti-enteric antibodies are frequently found in IBD patients and are associated with bowel secretion (Li et al., 2016; Lütt et al., 2018). To assess whether staining of anti-enteric antibodies is associated with neuronal activation, we additionally tested the evoked responses of two non-IBD patient sera showing staining of the enteric plexuses (Figure 3.16A). These two patients had no defined clinical diagnosis.

Serum from a 34-year-old female patient diagnosed with slow transit constipation stained the submucous plexus (Figure 3.15A) and more clearly the cell bodies of myenteric neurons (Figure 3.15B). Application of this serum did not evoke any spike discharge in either of the plexuses.

MF serum belongs to a female patient diagnosed with abdominal adhesions who suffered from a number of food allergies and intolerances. It stained both submucous and myenteric plexuses (Figure 3.15 C, D). When applied onto the enteric neurons it evoked an action potential discharge of 6.9 Hz in 16.6% of the neurons.

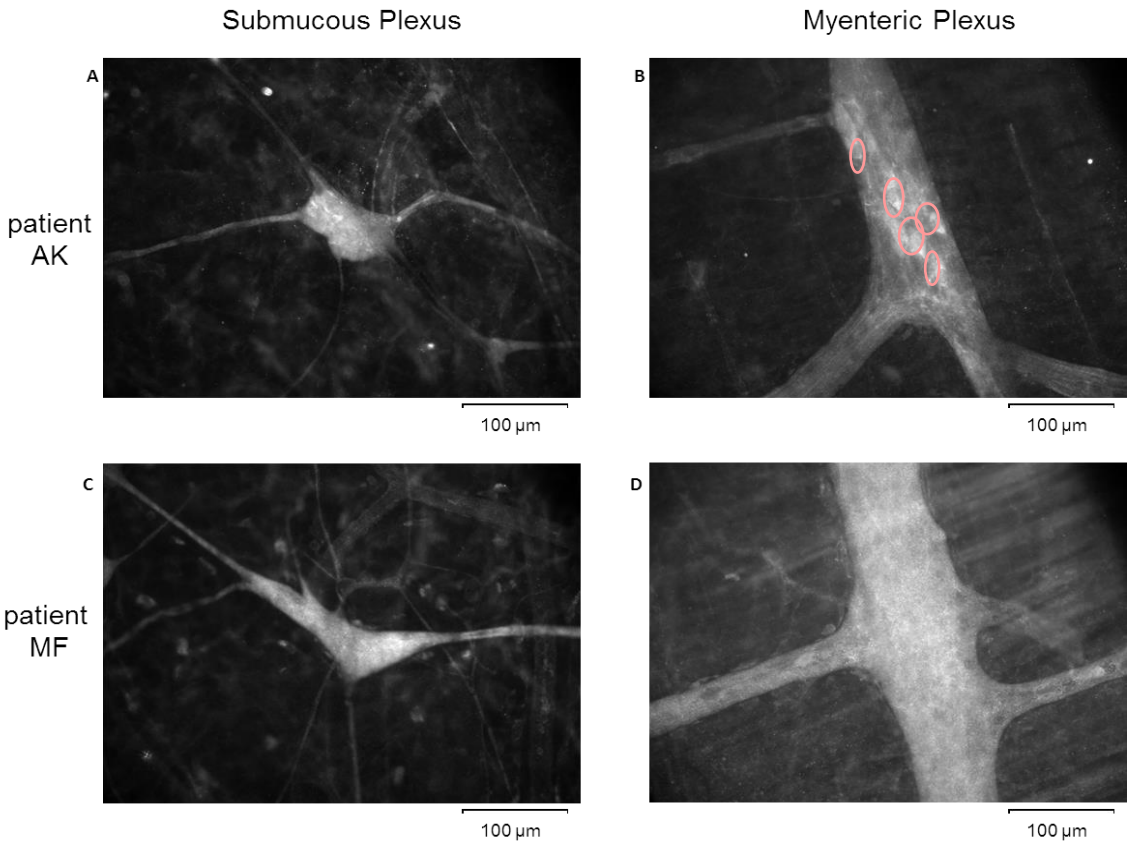


Figure 3.15: Immunostaining of the non-IBD sera in guinea pig neurons

The upper panel shows ganglia labelled with antibodies residing in the serum of MF patient and lower panel with antibodies occur in the AK patient serum. (A) Immunostaining on the guinea pig ileum submucous plexus. (B) Encircled are the cell bodies of myenteric neurons stained. (C, D) The ganglia at both myenteric and submucous plexus are stained.

To determine the neuroactive components contained in the MF serum, we used a pharmacologic approach. We tested for TNF-α by incubating the samples with Adalimumab. We further test whether receptors for serotonin and histamine were involved by using receptor-specific antagonists. Histamine and serotonin were shown to be the main mediators released from mucosal biopsies of IBS patients capable to induce neuronal excitation (Buhner, 2009). Therefore, we consider plausible testing for mediators present in common GI disorders in these two sera. The relevant pharmacology was performed in the same ganglion to analyse recovery after washout of the blockers.

To demonstrate the involvement of serotonin, we reapplied MF serum after 20 minutes perfusion with the 5-HT_3 receptor antagonist cilansetron. Cilansetron had no effect on the spike discharge evoked by MF serum (Figure 3.16 B-D).

To check for a histaminergic component, we perfused the tissue for 20 minutes with a combination of the H_1 and H_2 histamine receptor antagonists' pyrilamin and ranitidine, respectively. After perfusion, we could not record any action potential. Therefore, the histamine receptor antagonists eliminated the serum-evoked spike discharge or the neurons were no longer alive. This effect did not recover even after 1-hour washout period (Figure 3.16 E-G).

We incubated MF serum with the Adalimumab for 20 minutes before applying it on neurons. Although a tendency to lower neuronal responses after incubation with TNF-α antibody was observed, the effect was not significantly different from the application of MF serum alone (Figure 3.16 H-J).

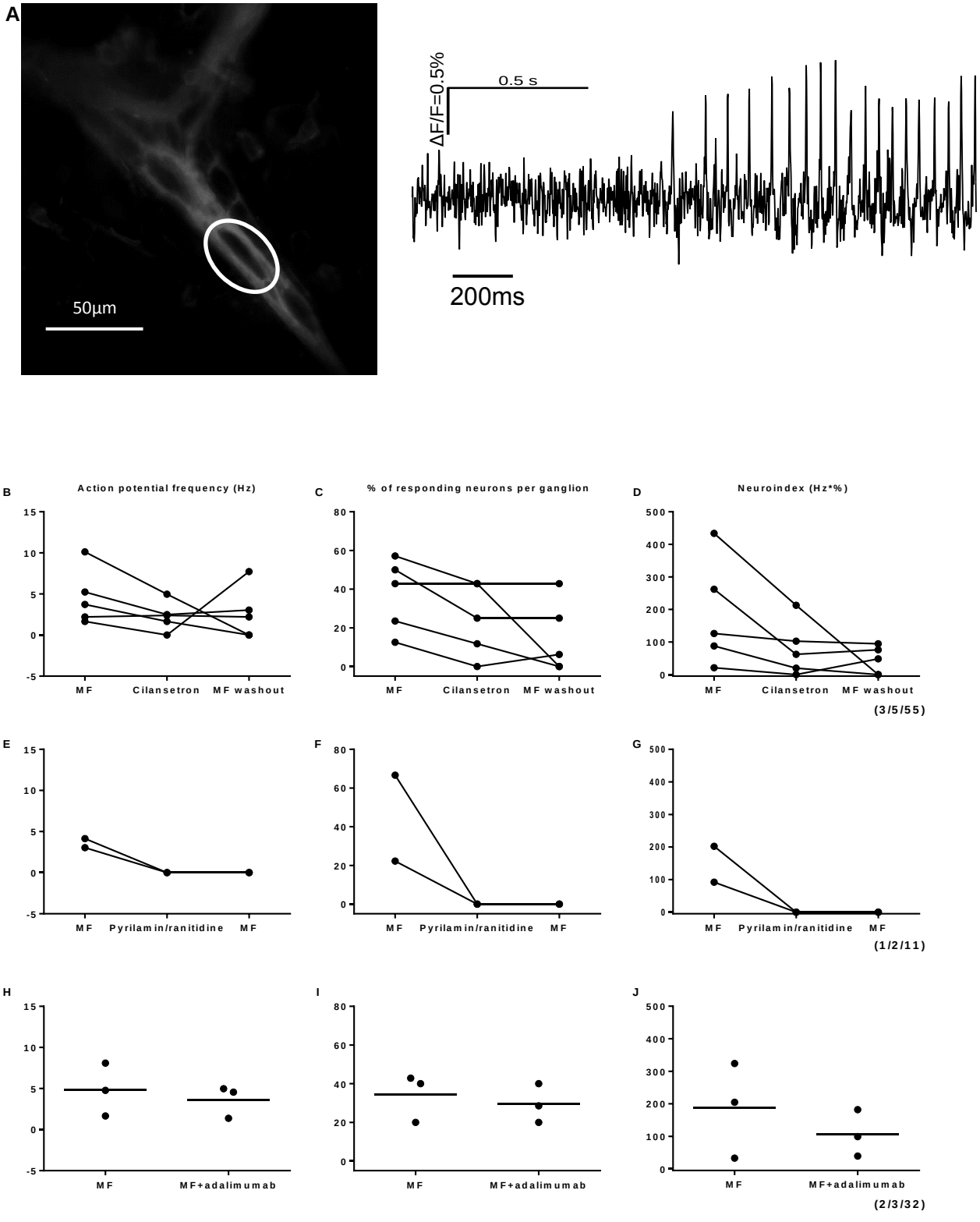

A
50µm
0.5 s
ΔF/F=0.5%
200ms
B
Action potential frequency (Hz)
MF
Cilansetron
MF washout
C
% of responding neurons per ganglion
MF
Cilansetron
MF washout
D
Neuroindex (Hz*%)
MF
Cilansetron
MF washout
(3/5/55)
E
MF
Pyrilamin/ranitidine
MF
F
MF
Pyrilamin/ranitidine
MF
G
MF
Pyrilamin/ranitidine
MF
(1/2/11)
H
MF
MF+adalimumab
I
MF
MF+adalimumab
J
MF
MF+adalimumab
(2/3/32)

Figure 3.16: Neuronal responses to a non-IBD serum (patient MF)

(A) High-resolution image of a ganglion in the guinea pig submucous plexus after incubation with the voltage-sensitive dye Di-8-ANEPPS. Example trace of a neuron (encircled) shows a fast onset spike discharge after brief spritz application (200ms) of MF serum. (B) 5-HT_3 receptor antagonist cilansetron did not affect the frequency of action potentials (p=0.44), (C) the percentage of responding neurons (p=0.06) or (D) the neuroindex (p=0.14). One-way ANOVA. (E) The effect of MF serum was abolished after perfusion with the H_1 antagonist pyrilamine and H_2 antagonist ranitidine but did not recover after washout of the antagonists. No statistical analysis was noted for the frequency of action potentials, (F) the percentage of responding neurons and (G) the neuroindex. Incubation of MF serum with Adalimumab reduced the evoked neuronal response compare to application to application of MF serum alone. No statistical significance was observed for the (H) frequency of action potentials (p=0.50), (I) the percentage of responding neurons (p>0.99) and (J) the neuroindex (p=0.50); Wilcoxon test.

3.10.2 Acute appendicitis

About 25% of patients with ileal CD and 50% of those with colonic CD have appendiceal involvement (Ottinger and Ozdemirli, 1996). Although, appendiceal involvement is rarely the first manifestation of the disease a clinical picture similar to acute appendicitis is not an uncommon presentation of Crohn's disease (Shaoul et al., 2005). To determine whether there are differences between the effects of sera from patients with an acute inflammatory and a chronic inflammatory condition, sera from two patients with acute appendicitis were tested (Figure 3.17). Responses after application of those sera were not reproducible. Therefore, paired experiments were not possible.

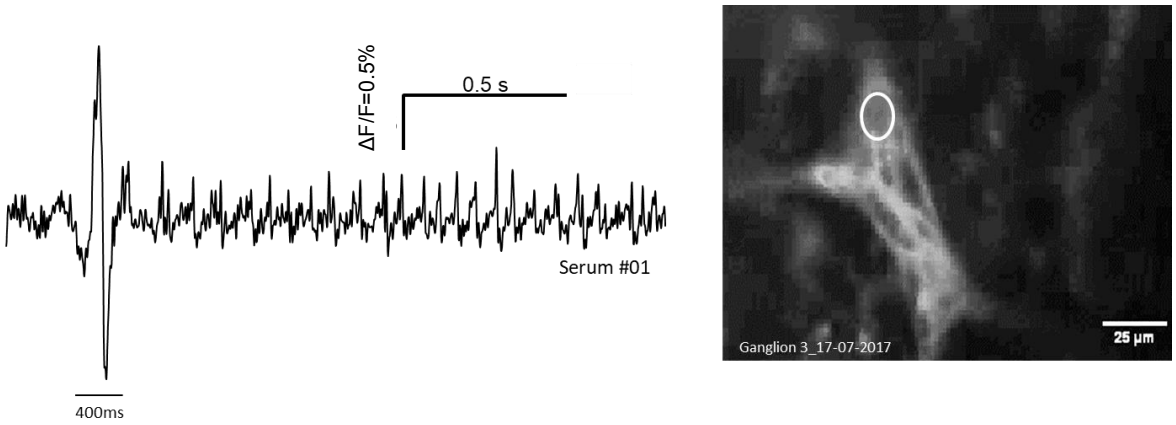


Figure 3.17: Neuronal response to serum from a patient with an acute inflammatory condition (acute appendicitis)

Example trace of a neuron displaying fast onset spike discharge after application of serum (400ms) from patient #01 diagnosed with acute appendicitis. Ganglion was adjusted for brightness (66%) and contrast (34%).

3.11 Anti-enteric neuronal antibodies in the serum

3.11.1 Screening of serum for IgG antibodies

Antineuronal antibodies were identified in the serum of patients with diverse gastrointestinal diseases (Moses et al., 2003; Törnblom et al., 2007). Therefore, we assessed the ability of IgG residing in the serum of patients and healthy controls to bind to targets in the myenteric and submucous plexus of the guinea pig colon (Figure 3.18, Figure S1). 20 out of 70 patient sera contain circulating anti-neuronal antibodies targeting molecules expressed by neurons (Table 5).

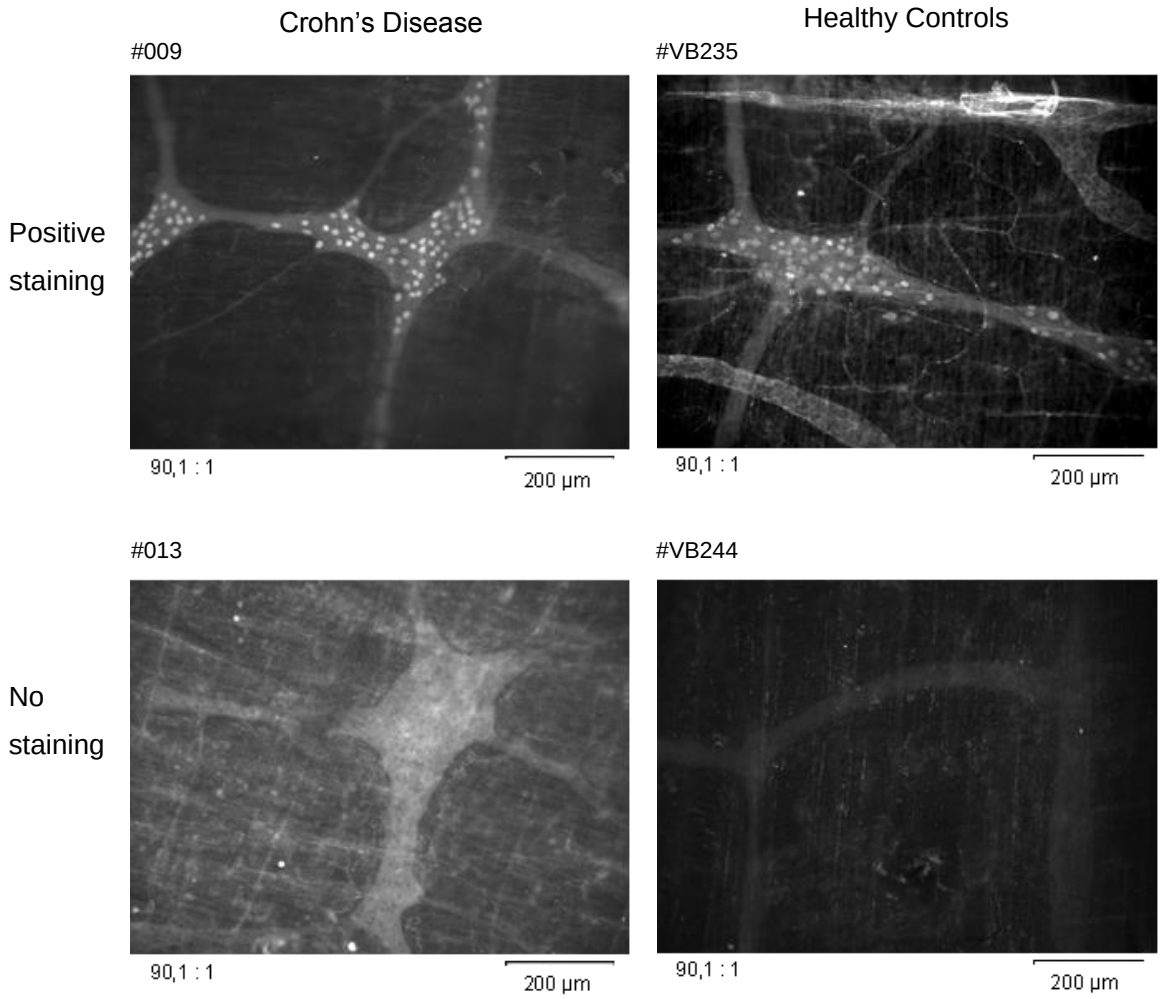


Figure 3.18: Immunostaining of serum autoantibodies in the guinea pig myenteric plexus neurons

The upper panel shows immunostaining of autoantibodies residing in the subject sera in the guinea pig myenteric plexus neurons. The autoantibodies labelled the cell bodies of myenteric neurons. The bottom panel refers to non-stained ganglia.

Table 5: Number of sera samples with and without autoantibodies

	Crohn's Disease	Ulcerative Colitis	Healthy Controls
Positive staining	11	2	7
No staining	15	8	27

3.11.2 De-activation of IgG

Previous work in our lab showed that IgG isolated from sera of patients with paraneoplastic neurological autoimmunity labelled enteric neurons and caused an immediate spike discharge in enteric neurons (Li et al., 2016; Piepgras et al., 2015). Since several sera from CD-patients labelled enteric neurons, we were interested in their effect on enteric neurons.

Evoked neuronal activity was similar between sera from CD-active patients with and without autoantibody staining (Figure S2).

Taking a step further, we tried to de-activate/denature the antibodies and to check whether the effect of serum on enteric neurons was changed before and after the de-activation. Sera were heated at 72 °C for 10 and 30 minutes. As is shown in figure 3.19 staining was eliminated with longer heating time.

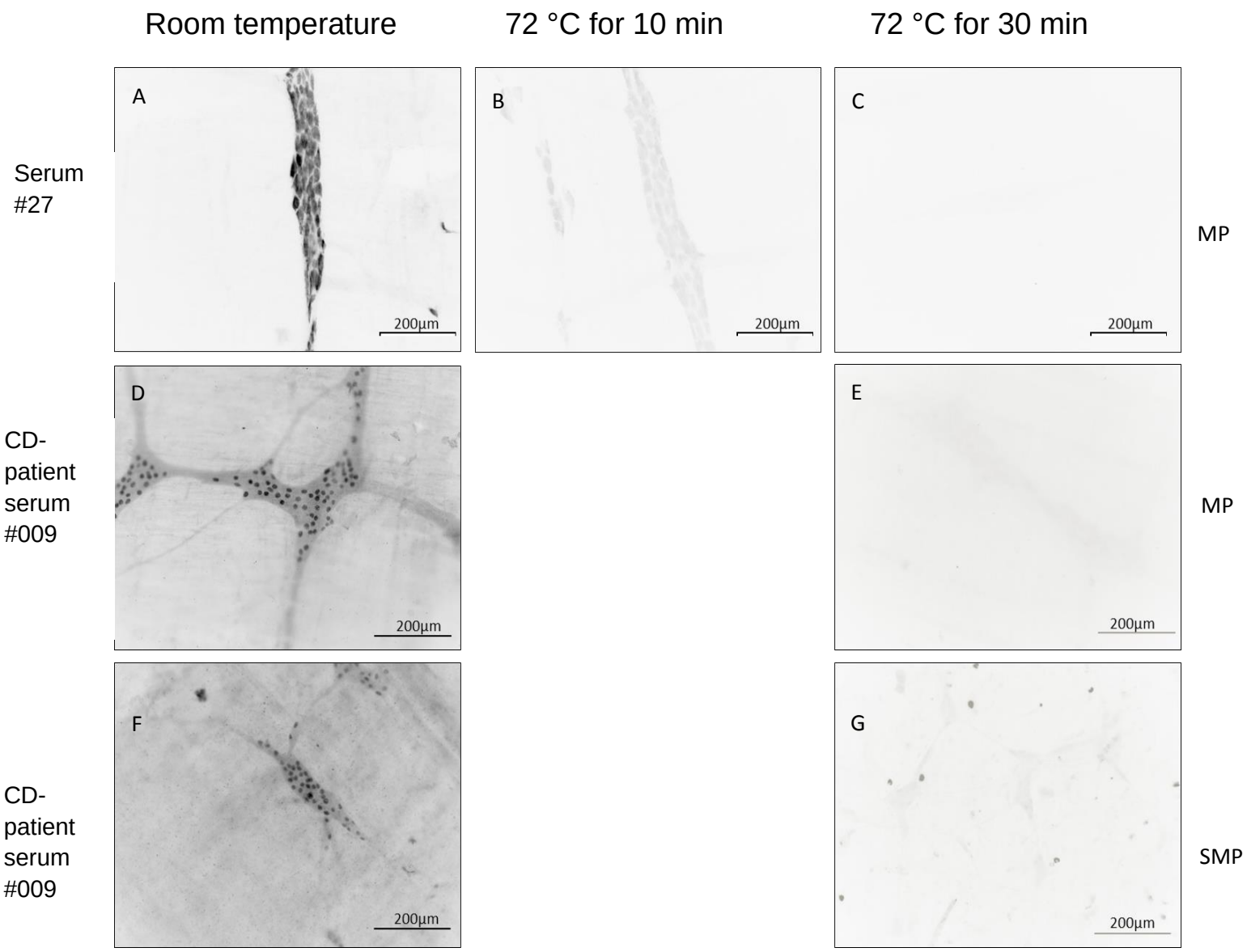


Figure 3.19: Denaturation of IgG antibodies

Staining of guinea pig tissue by the serum of a patient with chronic intestinal pseudo-obstruction of non-paraneoplastic origin (patient #27) and by the serum of a CD patient (patient #009). (A) Serum from patient #27 labelled specifically myenteric neuronal bodies. (B) Patient #27 serum was heated at 72 °C for 10 min resulting in a lesser staining of guinea pig myenteric neurons. (C) Increasing the incubation time to 30 min alleviated the staining. (D, F) Serum of patient #009 stained myenteric and submucous neurons, respectively. (E, G) Heat-treated samples did not stain any neurons neither in the myenteric (MP) nor in the submucous plexus (SMP).

We tested three different CD-active sera previously showing an autoantibody staining. Two sera showed a decrease in neuronal response after the de-activation of the IgG, while one serum (#006) caused an increased response (Figure 3.20).

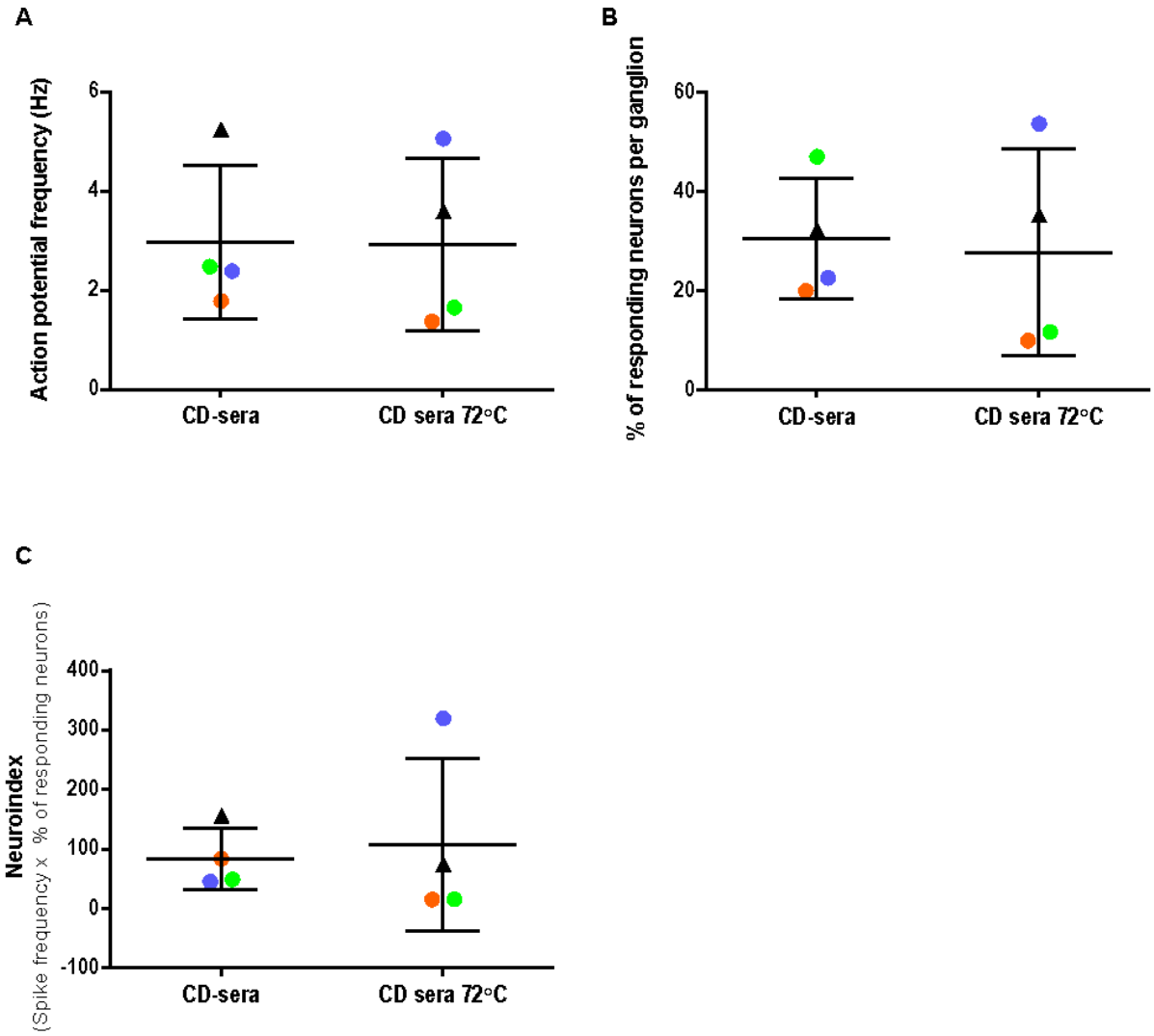


Figure 3.20: Neuronal responses after deactivation of IgG antibodies

(A) The serum of patient #023 and #026, but not #006(●) caused lower action potential discharge after de-activation of IgG. (B) Also, a lower number of neurons were activated by serum #023 and #026. (C) As a result, the neuroindex was lower for the two sera. Serum from sample #27 (▲) was used as a control as it was tested in previous studies from our group for the role of autoantibodies residing in it.

3.11.3 Co-Immunoprecipitation with magnetic beads

Immunoprecipitation has been widely used for the isolation of proteins out of a solution using the principle of antibody/antigen interaction. Magnetic beads have a defined diameter and are non-porous allowing fast and effective isolation of the proteins. While we isolated the IgG/IgM antibodies out of the serum, when the isolated fraction was applied into the enteric neurons it interfered with the ANEPPS staining. Therefore, we did not proceed with this method, as it was incompatible with our neuroimaging methods.

3.12 Distance between ganglia and blood vessels

Enteric neurons are in proximity to the blood vessels. Hence, we checked whether the distance between them could affect the evoked neuronal responses.

We performed a correlation of the distance with the spike discharge, the proportion of responding neurons and the neuroindex. No significant correlation was shown. We additionally performed a correlation of the distance with the neuronal response based on the type of serum spritzed (CD-active or CD-remission sera) on the ganglia. Again no correlation was noted (Figure 3.21)

The correlation of ganglia-blood vessel-distance to the neuroindex was r^2= 0.014 and p=0.54 for CD-active. For CD remission it was r^2= 0.006 and p=0.70 (Figure 3.21A). Correlation of ganglia-blood vessel-distance to the action potential frequency was r^2= 0.11 and p= 0.08 for CD-active. Values for CD-remission were r^2= 0.009 and p= 0.64 (Figure 3.21B). The correlation to the percentage of responding neurons was r^2= 0.03 for both groups with a p= 0.38 for CD-active and p= 0.36 for CD-remission (Figure 3.21C).

Moreover, we analysed ganglia with no visible blood vessel in the bright field picture to exclude that the assigned distance of 600µm influenced the correlation.

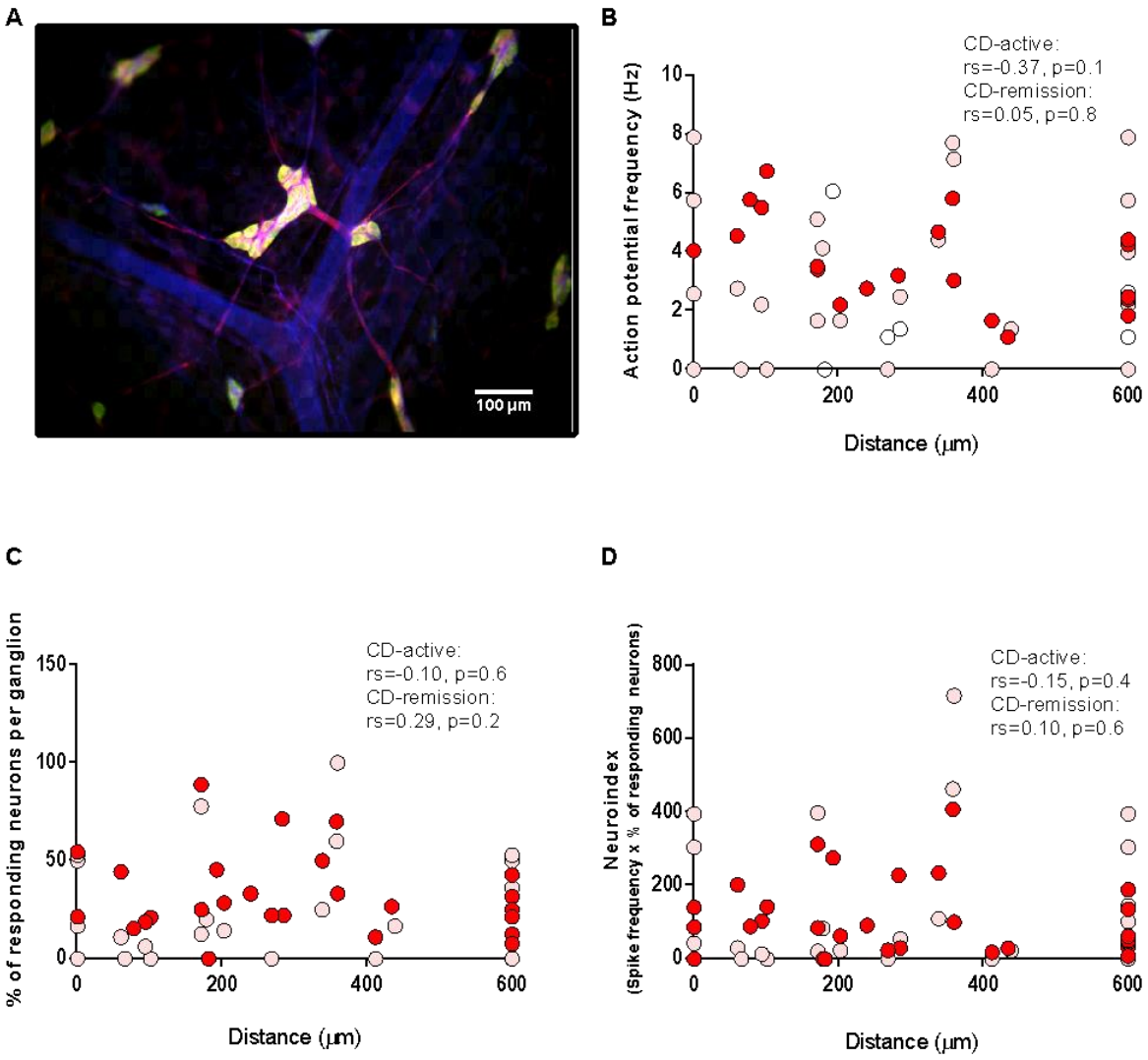


Figure 3.21: Correlation of distance between blood vessels and ganglia to evoked responses after serum application

(A) A ganglion is shown in pink, individual neurons in yellow and blood vessel in blue. (B) Correlation of action potential discharge with the distance of ganglia to the blood vessel. (C) Correlation between the percentage of responding neurons and distance. (D) Correlation of the neuroindex to the distance. Responses of ganglia stimulated with serum from CD-active patients are shown in red and with CD remission in pink. The Spearman correlation analysis was performed. None of the parameters tested revealed any link between the neuronal responses and the proximity of neurons to blood vessels.

3.13 Neurochemical coding of responding neurons

After neuroimaging experiments tissues were fixed and stained for ChAT and VIP. Six ganglia were traced back in four tissues in order to determine the neurochemical coding of the responding neurons. The overlay of two different ganglia and each staining are shown in figure 3.22. Staining was analysed for the neurons responding to serum on neuroimaging experiments (Table 6).

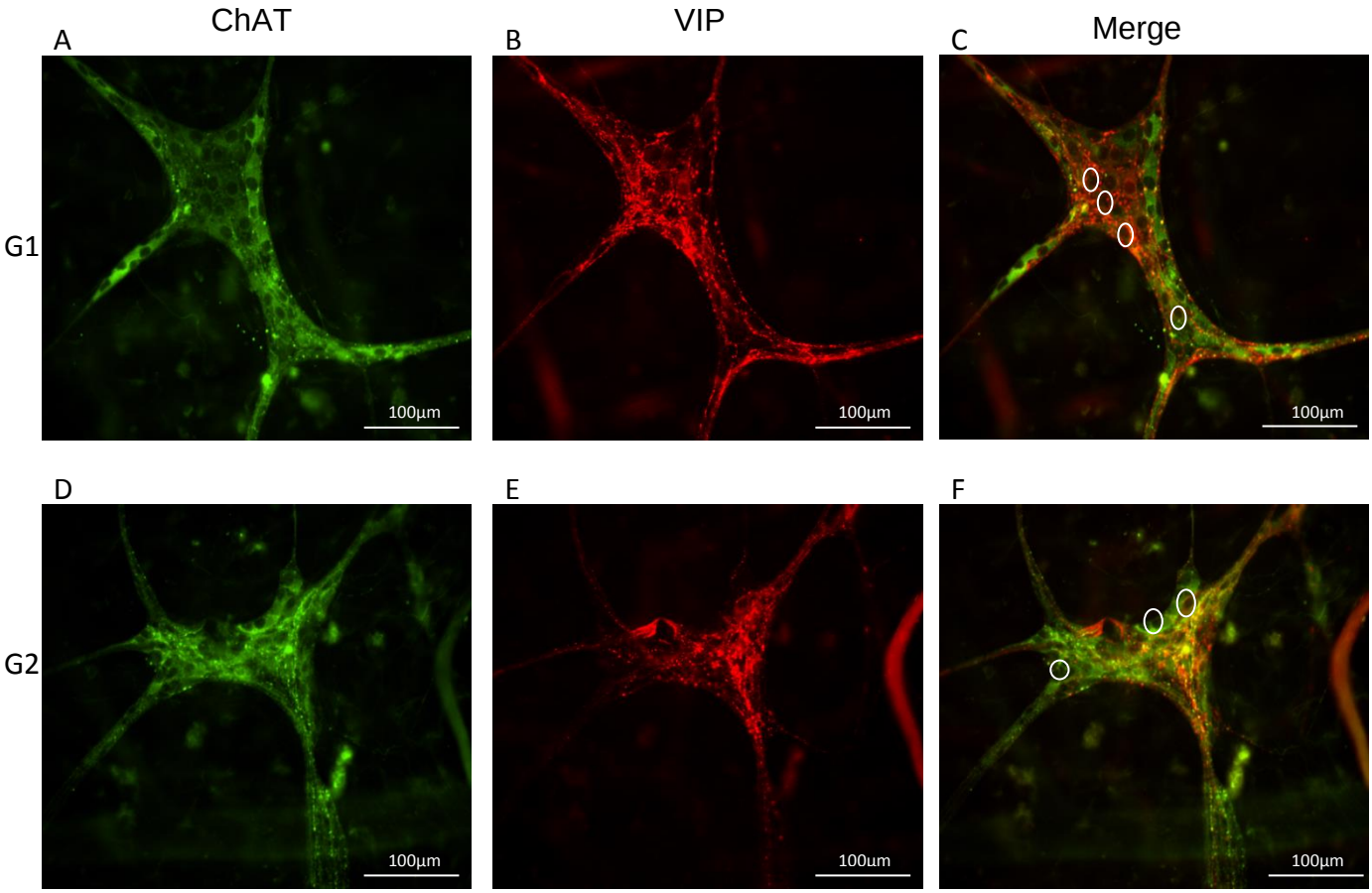


Figure 3.22: Neurochemical coding of neurons responding to patient serum

Tissues were stained for Choline Acetyl Transferase (ChAT) and Vasoactive Intestinal Peptide (VIP). Previously stimulated ganglia and neurons responding to serum from CD patients were identified and are presented here with white circles. (A, D) show staining for ChAT only and (B, E) show the staining for VIP only. The overlay of the ChAT/VIP is shown in (C, F). Images were captured with an x40 objective. G1 and G2 refer to two distinct ganglia.

Approximately equal numbers of neurons responding to serum application were either ChAT-positive or VIP-positive. Out of the 22 neurons responding to serum from CD-active patients 10 showed staining for ChAT and 12 for VIP. For the neurons stimulated with serum from CD-remission patients 8 out of 14 were ChAT-positive and the rest were VIP-positive.

Moreover, no significant difference in the response frequency was observed between ChAT and VIP positive neurons, neither after stimulation with serum from CD-active (p-value= 0.56), nor with serum from CD-remission patients (p-value= 0.53) (Table 6).

Table 6: Summary of the neurochemical coding of responding neurons depending on stimulation with either active or remission CD serum

CD-active 4/5/22; CD-remission: 4/5/14; Wilcoxon test was performed to compare the response frequency between ChAT- and VIP-positive neurons (p =0.56 for CD-active; p=0.52 for CD-remission)

Neurochemical coding	Type of serum					
	CD-active			CD-remission		
	Number of responding neurons	Mean frequency [Hz]	p-value	Number of responding neurons	Mean frequency [Hz]	p-value
ChAT	10	4.65	0.56	8	3.91	0.53
VIP	12	4.44		6	5.66	

3.14 TNF-α effect on cultured enteric neurons

To study the influence of TNF-α on neuronal maturation we cultured enteric neurons with different TNF-α concentrations and for different periods and assess axonal complexity. Neurons were fixed and stained with the neuronal markers Tuj1 and HuC/D to quantify the numbers of neurons. Axonal complexity was calculated using Sholl analysis (Figure 2.4). This analysis indirectly measures the axonal length and axonal branching.

The axonal complexity was increasing linearly to the distance from the ganglion centre for all the TNF treatments (Figure 3.23).

The biggest sprouting was noted at 180μm from the ganglion centre and it was chosen for the further analysis of time and concentration-dependent effects. Analysis of neurons cultured with 10ng/ml and 50ng/ml of TNF-α showed a statistically significant reduction in axonal complexity at 180μm from the ganglion centre compared to neurons cultured with lower TNF-α concentration (Figure 3.24; p =0.02, two-way ANOVA). Neuronal sprouting was independent of incubation time with TNF-α (p=0.5, two-way ANOVA).

Using the HuD staining we calculated the number of neurons per frame. We analysed 12 frames for each TNF-α treatment condition. There was no difference in the number of neurons in any of the conditions tested (Figure 3.24B; p =0.62 for different incubation times; p =0.05 for different TNF-α concentration; two-way ANOVA).

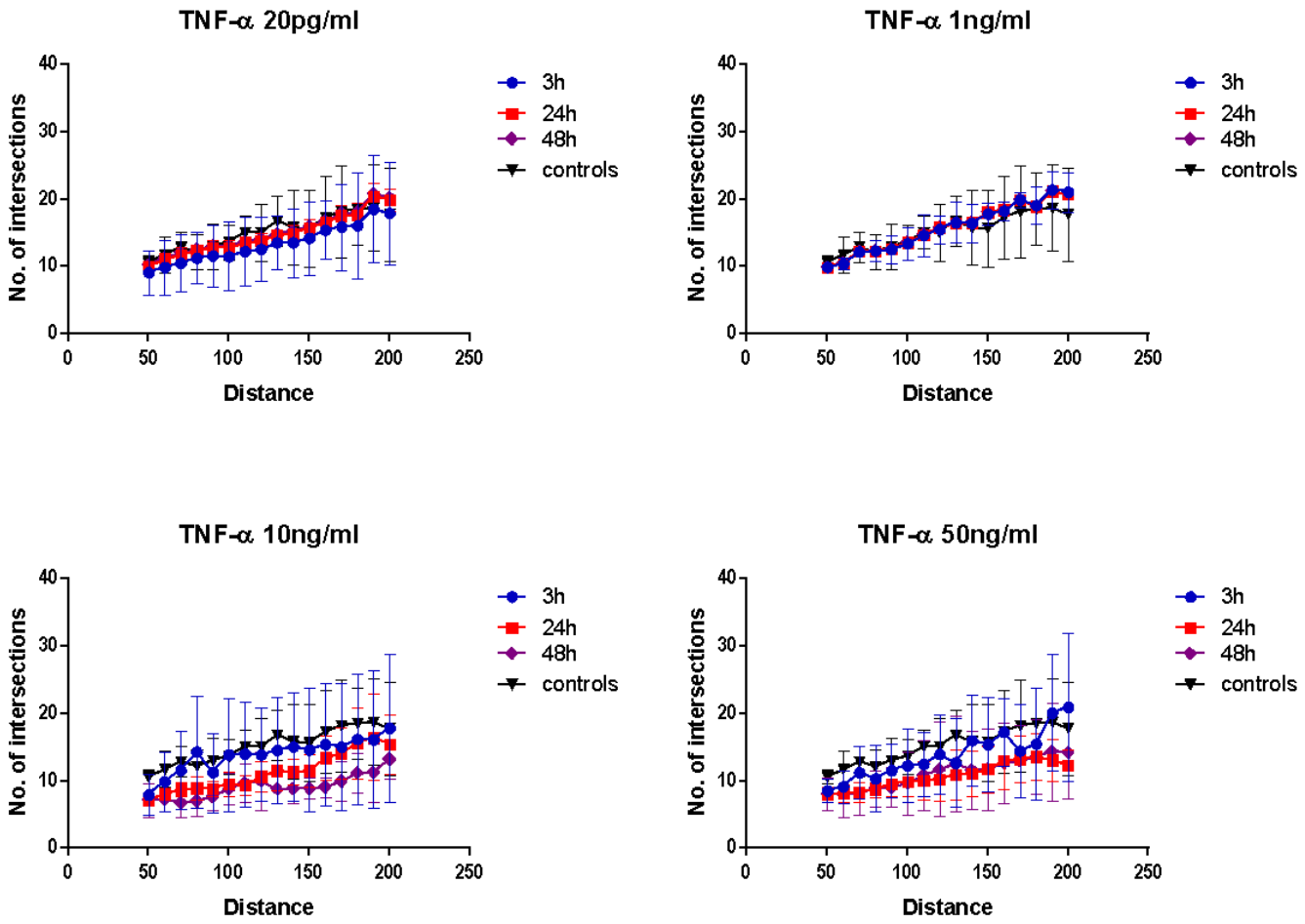


Figure 3.23: Neuronal sprouting correlation with the distance from the ganglion centre

A linear increase in the number of intersections with the increased distance from the ganglion centre was noted in all of the tested concentrations of TNF-α. Cell cultures were incubated with 20pg/ml, 1ng/ml, 10ng/ml and 50ng/ml TNF-α. Incubation with TNF-α for 3h is shown in blue, for 24h in red and 48h in purple. Black refers to cultures not treated with TNF-α.

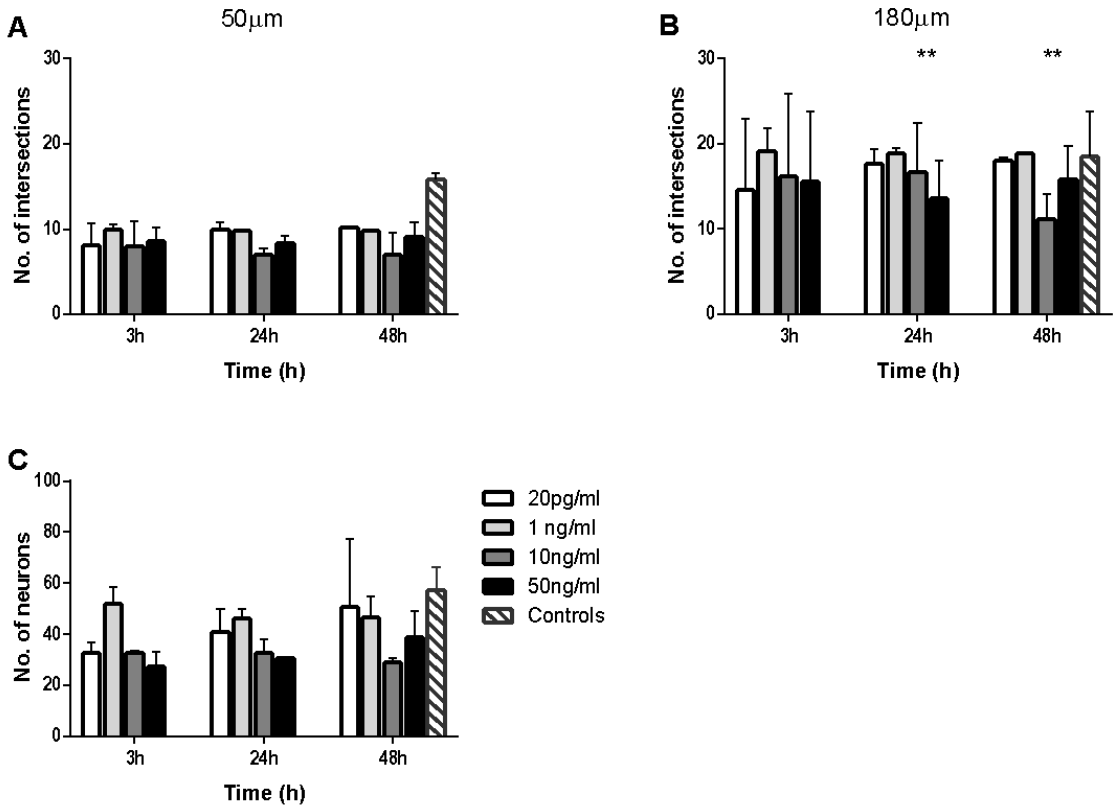


Figure 3.24: Neuronal sprouting and number of neurons correlation with concentration and exposure time to TNF-α

(A) For neurons cultured with 10ng/ml and 50ng/ml TNF-α there was a tendency for lower sprouting at 50μm from ganglion centre. (B) The number of intersections at 180μm for neurons cultured with 10ng/ml and 50ng/ml TNF-α was lower than the ones cultured with 1ng/ml TNF-α (two-way ANOVA; p=0.02). (C) There was no difference in the number of neurons based on neither the exposure time nor the concentration of TNF-α (two-way ANOVA; p=0.63, p=0.05).

4. Discussion

The present study demonstrated for the first time that sera from CD patients activate enteric neurons to a greater extent than sera from controls. Furthermore, activation of enteric neurons was independent of the disease stage. Sera from patients with active and inactive CD evoked similar strong action potential discharge in GP SMP neurons. Inhibiting TNF-α with a specific antibody (Adalimumab), pointed to its role in ongoing neuronal activation. In parallel, these functional experiments suggested the importance of other cytokines residing in the serum, as neuronal activation was not completely blocked by the TNF-α antibody.

Furthermore, the study pointed to differences in neuronal activation between the two main IBD types. Although sera from UC patients activated guinea pig enteric neurons, the effect was not different to the activation caused by sera from healthy controls. The evoked action potential discharge and the number of neurons responding to the serum of UC patients were smaller compared to the ones from CD patients.

Our results indicate that altered signalling may be important in the pathophysiology of CD patients.

4.1 Sera from CD patients excite enteric neurons

Our study was performed in guinea pig submucosal neurons. Immune cells like mast cells and lymphocytes are primarily located in the mucosal and submucosal regions. Furthermore, previous work from our group suggested that the functional neuroimmune interactions occur mainly in the submucous plexus area, where immunocytes and nerve processes are in close contact rather in the myenteric and muscle layers (Buhner et al., 2012). Our results support the relevance of altered signalling between immune cells, epithelial cells and nerves in Crohn’s disease patients.

ENS controls motor functions, local blood flow, absorption, secretion and moderates immune and endocrine functions. Structural and functional abnormalities of ENS were associated with CD. Perianal complications such as strictures, fistulas, and abscesses are common in CD patients (Safar and Sands, 2007). Structural aberrations such as hypertrophy, hyperplasia and damages were observed in the nerve fibres, the neuronal cell bodies and the enteric glial cells. Irregular nerve fibre hypertrophy can occur in all

plexuses in the ileum and colon. Such hypertrophy is linked to an increased inflammatory cell infiltration. Neuronal hyperplasia is a feature of CD with a three-fold increase in the number of cells of the ileal myenteric plexus as measured in 24 CD patients (Davis et al., 1955). Hyperplasia was also reported in the submucous plexus of CD patients. Moreover, the degeneration of neuronal cells was described in areas with inflammation (Geboes and Collins, 1998).

Changes in the neurochemical coding upon inflammation were found. Immunohistochemistry in whole-mount preparations from inflamed segments from CD patients' ileum showed an increased number of VIP, NOS and neuropeptitde Y-immune-reactive cell bodies in the myenteric plexus (Belai et al., 1997). An analogous study in the submucous plexus revealed an increase in the VIP/- population in the non-inflamed rectum of CD patients compared to controls, indicating that inflammation at one site can prompt widespread alterations in the ENS (Schneider et al, 2001). In contrast, inflamed areas in the colon showed a lower concentration of SP and VIP neuropeptides (Sjölund et al., 1983).

It is plausible that structural changes in the innervation of the GI tract due to inflammation could induce functional alterations. Changes in enteric neurons are likely to play a role in the differential regulation of motility, secretion or immune responses (Lomax et al., 2005).

The present study showed that factors from sera samples of CD patients excited guinea pig submucosal neurons stronger than sera from healthy controls. Previous studies have revealed that supernatants from mucosal biopsy samples and PBMC and from IBS patients have direct excitatory actions in the ENS (Buhner et al., 2009; Hughes et al., 2013). Since IBS and CD are both characterized by inflammation, inflammatory mediators may perturb gastrointestinal responses (Barbara et al., 2002). Nevertheless, it remains speculative how ENS activation is connected to dysfunction.

Intriguingly, sera from CD patients in active and remission stage caused a similar excitatory effect on submucosal neurons, suggesting that the neuronal activity is independent of the state of inflammation. Remission is characterised by a decrease or disappearance of the symptoms (like diarrhoea, pain and fatigue) and upon an endoscopy, the mucosal membranes appear healed. Nonetheless, alterations in the gut function are

observed even after the regression of inflammation. Patients with IBD-remission often experience abdominal pain and distension symptoms reminiscent of IBS symptoms (Piche et al., 2010). IBS-like symptoms were reported in 35.4% of CD patients (Vivinus-Nébot et al., 2014). Although these symptoms may be suggestive of a coincident occurrence of IBS, studies identifying high levels of faecal calprotectin, a colonic low-grade inflammation marker, support that symptoms are due to a persistent low-grade non-detectable inflammation (Keohane et al., 2010). Furthermore, IBD patients experiencing IBS-like symptoms had persistent epithelial barrier defects, such as increased paracellular permeability and alterations to TJs. Especially for CD patients in remission an increased mucosa permeability compared to controls was observed (Buhner et al., 2006). Increased paracellular permeability was associated with the severity of the IBS symptoms. Furthermore, remission IBD patients with IBS-like symptoms had a significantly higher mRNA and protein TNF-α expression compared with those without. TNF-α can alter the TJ permeability via cytoskeletal contraction and thus, alter the intestinal permeability. In contrast, IBS patients expressed TNF-α at levels similar to healthy controls (Vivinus-Nébot et al., 2014). Inflammation-induced changes persist even after long periods of remission and this might explain the neuronal activation seen in our experiments.

The observed neuronal activation did not correlate with the clinical characteristics (CRP levels, CDAI score) confirming that neuronal activation is independent of the inflammation state. Similarly to our results, neuron excitation by IBS supernatants appeared unrelated to clinical symptoms. Although, D-IBS and C-IBS patients have distinct symptoms their supernatants evoked similar excitatory actions on human submucosal neurons. Additionally, the same mediators were identified in both patient sera and were responsible for the neuronal responses (Buhner et al., 2009). D-IBS and C-IBS supernatants augmented spike discharge in visceral afferents to the same degree (Barbara et al., 2007; Cenac et al., 2007). Accordingly, CD-remission patients may have the same mediators in their serum as CD-active patients. This would explain why IBD patients often experience relapses.

4.2 TNF-α depolarizes enteric neurons

A role of TNF-α in gut-related diseases has been indicated in many studies. An enhanced released of TNF-α has been measured in mucosal biopsies from IBD patient's tissue

(Dionne et al., 2000; McCormack et al., 2001). Also, mice deficient in TNF-α do not develop an experimentally induced chronic colitis (Neurath et al., 1997). Although IBS is not considered an inflammatory disease, immunological alterations are reported enhancing the hypothesis of low-grade immune activation. PBMC supernatants of IBS-D patients sensitized the mouse colonic afferent nerve endings highlighting the importance of interactions between the immune and the nervous systems. The evoked mechanical hypersensitivity was predominantly due to the increased concentration of TNF-α (Hughes et al., 2013).

Since TNF-α has been widely described as one of the major pro-inflammatory cytokines involved in the pathogenesis of CD, we attempted to assess its effect on enteric neurons. Our study clearly demonstrated that incubation of sera with anti-TNF-α antibody reduced the activation of enteric neurons in an active and remission stage of the disease. Although remission patients were already under treatment with adalimumab, further incubation with it resulted in reduced neuronal activation in a lower proportion of enteric neurons.

Enhanced neuronal excitability has been observed in animal models of inflammation infected with parasites (Palmer et al., 1998). In a TNBS-induced colitis animal model excitability of AH myenteric neurons was enhanced, after-hyperpolarisation was smaller, they were more prone to receive fast excitatory synaptic input and showed enhanced spontaneous activity (Linden et al., 2003).

TNF-α was shown to inhibit the release of noradrenaline from longitudinal muscle/ myenteric plexus preparation. Its effect was suppressed by cyclo-oxygenase inhibitors such as indomethacin or piroxicam (Hurst and Collins, 1994). TNF-α hyperpolarized cultured myenteric neurons via cyclooxygenase metabolites and protein tyrosine phosphorylation (Rehn et al., 2004).

On the contrary, our results showed an immediate excitation of guinea pig colonic neurons upon TNF-α application. Our experiments were performed in freshly dissected intact preparations of submucous plexus while the experiments showing hyperpolarization were performed on myenteric-cultured neurons. Another difference may be due to the different resting potential of neurons between the two studies. Resting potential affects the permeability of the voltage-gated channels and therefore the depolarization or

hyperpolarization of the membrane upon stimulation. Additionally, the incubation time used was different. In our study, we measured the response to an acute application of TNF-α while in the previous study neurons were pre-treated with TNF-α for 20 hours (Rehn et al., 2004).

Our results are in agreement with studies in inflammation models of TNBS colitis showing an excitation of enteric neurons. Neurons have their processes in the lamina propria, where the inflammation is centred and where the inflammatory mediators and trophic factors are. Enhanced excitability of those neurons supports a neuro-immune interaction (Linden et al., 2003). As a result, the intrinsic motor reflexes may be disrupted in the inflamed colon leading to the dysmotility observed in inflammatory diseases (Linden et al., 2003).

4.3 Cytokines in the serum of CD patients

Our experiments with incubation of serum with Adalimumab suggested the role of TNF-α on the evoked neuronal activation. However, other cytokines residing in the serum are important, as the neuronal activation was not completely blocked by the TNF-α antibody. For this reason, we performed a cytokine analysis in the serum of CD patients.

All cytokines tested were identified in sera from both active and remission patients. All mediators were slightly higher in the active disease than those in the remission. Nevertheless, only IL-1β was significantly higher in CD-patients compared to remission ones. IL-1β was shown to be elevated also in mucosal biopsies of CD-patients (Sher et al., 1995). Along with TNF-α, they have a secretory effect of human distal colon mediated by the release of PGE_2 (Bode et al., 1998). IL-1β excites directly the somal membranes of guinea pig entering neurons in both plexuses. In parallel, it suppresses the release of norepinephrine at sympathetic synapses on submucous neurons and cholinergic transmission on myenteric neurons (Kelles et al., 2000; Xia et al., 1999). In agreement with our study, work of Martinez-Borra (2002) showed a decrease in circulating levels of IL-1β upon treatment with infliximab. IL-1β was detectable in the pre-treated sera of all patients at a concentration of 6-45 pg/ml and reduced to 4-35 pg/ml at week 6 after treatment. We detected much lower levels ranging from 0.01-2.60 pg/ml for active patients and 0.06-0.49 pg/ml for patients treated with Adalimumab. Differences in the cytokines

level among the studies may attribute to different techniques used. Paradoxically, multiplex immunoassay yield lower levels or even non-detectable levels of circulating cytokines rather than sandwich-ELISA. This may be due to the lower incubation time with the sera (Martínez-Borra et al., 2002; Smids et al., 2017).

Levels of INF-γ in the serum of the patients tested in our study were higher compared to healthy controls and in agreement with the existing literature proposing its role in the pathogenesis of IBD by its production from intestinal LPMC (Fais et al., 1991; Sasaki et al., 1992). We did not observe any differences in the levels between active and remissive patients. In contrast to our results, other studies reported higher INF-γ levels in the serum of active CD patients compared to those in inactive CD (Sasaki et al., 1992). The levels of INF-γ are correlated with the area involved in inflammation. A larger area of inflamed mucosa is associated with higher serum levels of INF-γ supporting the higher levels observed during an active phase compare to the remission phase (Ito et al., 2006; Sasaki et al., 1992). (Unfortunately, in our study we could not test for these parameters and compensate for them).

4.4 Sera from UC patient cause minimal neuronal activation

UC is a common disease under the umbrella term of IBD. Despite the common features between UC and CD, the two diseases can differ to genetic predisposition, risk factors and clinical, endoscopic and histological characteristics. In this study, we showed that the serum of those patients contains substances that have different activation potential.

Application of sera from UC patients evoked neuronal responses similar to the ones evoked by control sera. In contrast, sera from CD patients caused higher neuronal responses compared to controls. Comparison of CD and UC evoked responses revealed a tendency to lower spike frequency upon application of sera from UC patients.

Despite the low number of UC sera used in the study, we could speculate that the lower frequency of generated action potentials may be due to different cytokines residing in the serum.

UC and CD have overlapping genetic profiles. Nonetheless, they are characterised by different repertoire of gut infiltrating T-cells. Also, the numbers of activated CD4+ T-cells were decreased in the peripheral blood (Kontiainen et al., 1996). Cytokines driving UC

were identified as having Th2 “like” characteristics in contrast to Th1/Th17 characteristics of cytokines mediate CD. Th1 cells produce IFN-γ and IL-2 while Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Romagnani, 2000). UC is characterised as Th2 “like” disease as IL-4 is not increased (Strober and Fuss, 2011). Although IL-6 is considered a Th2 cytokine, in our study it was significantly elevated in CD active patients compared to the UC ones. In agreement, IL-6 was proposed as a putative mediator involved in the regulation of VIP expression upon CD. IL-6 mRNA expression was upregulated in supernatants from non-infected areas for CD, but not for UC (Soufflet et al., 2018). The discrepancy can be explained by the ability of IL-6 to induce differentiation of Th17 cells from naïve CD4 T-cells (Diehl and Rincón, 2002; Yang et al., 2007). Th17 cells are associated with Crohn’s disease as they were detected in the peripheral blood and the gut of the patients (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007). Furthermore, IL-6 was associated with excitation of secretomotor neurons (Xia et al., 1999). Elevated levels of IL-6 in the serum of the CD patients compared to the UC may well correlate with their ability to evoke a neuronal response in our study.

Cytokines analysis in our study showed increased levels of IL-8 in the sera of UC patients. This finding is in agreement with previous studies showing elevated levels of this cytokine, in the mucosa and the circulation in UC patients compared to controls and CD patients (Izzo et al., 1993; Jones et al., 1993; Mahida et al., 1992). The high IL-8 concentration supports its role as a potent chemoattractant and activator for neutrophils; thus, mediating neutrophil infiltration of the gut wall, a crucial mechanism of UC (Mitsuyama et al., 1994; Raab et al., 1993; Rodríguez-Perlvárez et al., 2012). Whether IL-8 could have a direct effect on enteric neuronal activation remains unclear.

Enteric neurons are close to mast cells. This anatomical vicinity has a functional relevance as is the basis for neuroimmune interactions in the lamina propria (Buhner and Schemann, 2012). Mast cells respond to neurotransmitters and release proteases, which in turn diffuse in a paracrine fashion to enhance the excitability of ENS neurons (Wang et al., 2009). IL-8 is one of the cytokines released by human mast cells in order to induce neutrophil chemotaxis on contact with activated T-cells (Salamon et al., 2005). Besides IL-8, mast cells are releasing a spectrum of proinflammatory mediators. A cocktail of mast cell mediators had an excitatory effect on guinea pig and human enteric ganglia (Schemann et al., 2005). More precisely, the mast cell to enteric nerve signalling is

mediated through the release of histamine, serotonin and tryptase, which can activate submucosal enteric neurons (Breunig et al., 2007; Buhner et al., 2009; Mueller et al., 2011). Although these mediators were identified in supernatants, which respond to a local effect, the high level of IL-8 in the serum may be suggestive of their systemic effects.

4.5 Non-IBD sera

4.5.1 Sera from patients with functional symptoms

Anti-enteric neuronal antibodies are frequent in IBD patients and their occurrence suggests the immune-mediated nature of the disease (Mitsuyama et al., 2016). Sera from two patients with strong functional symptoms, but no sign of inflammation also showed staining of the enteric plexuses. Therefore, we wanted to test the effect of those sera on neuronal excitability.

Serum from a patient (AK) diagnosed with slow transit constipation showed staining of myenteric neurons. Application of this serum did not evoke any spike discharge in either of the plexuses.

MF serum refers to a patient diagnosed with abdominal adhesions. Abdominal adhesions can interfere with intestinal motility and transit processes (Tabibian et al., 2017). The patient further suffered from a number of food allergies and intolerances. The serum stained both the submucous and the myenteric plexuses. It excited enteric neurons with a higher frequency compared to the serum of IBD patients. In contrast, the number of neurons responding upon MF application was lower than IBD and comparable to controls.

Mediators released from mucosal biopsies of IBS patients were shown to activate human submucosal neurons. Proteases, histamine, and serotonin were the mediators associated with neuronal activation (Buhner et al., 2009). While some mediators have local effects and can be detected primarily in the tissue others may be detected at the systemic level (Korolkova et al., 2015). Based on this idea, and since MF is a patient with a functional disorder, we tested whether the neuroactive components contained in the serum of MF are similar to the ones found in IBS patients. We used a pharmacologic approach targeting the receptors for serotonin and histamine. Our study did not point to the involvement of these mediators on the excitatory response of guinea pig neurons. We further tested the effect of TNF-α, as its concentration in the serum of MF was similar to the one observed

in IBD patients. Although a tendency to lower neuronal responses after incubation with anti-TNF-α antibody was observed, the effect was not significantly different from the application of serum without the antibody. We speculate that TNF-α might affect evoked activation. However, other mediators residing in the serum have a more pronounced effect. Indeed, cytokine analysis of this serum revealed high levels of IFN-γ.

IFN-γ is a pro-inflammatory cytokine with a role in innate immunity. It is produced primarily by NK cells and NK T-cells, as well as in adaptive immunity through CD8 and CD4 Th1 effector T-cells (Schoenborn and Wilson, 2007). The increased levels of IFN-γ may point to ongoing inflammation. The presence of abdominal adhesions adds to an ongoing inflammation as they may form secondary to inflammatory conditions of the abdomen (Tabibian et al., 2017).

4.5.2 Sera from patients with acute inflammation

IBD is a chronic inflammatory condition of the intestine. We, therefore, compared the effects of sera from IBD patients with sera from patients with an acute inflammatory condition.

We screened sera from two patients admitted for an appendectomy. Application of those sera did not evoke any neuronal response. Nonetheless, the samples were taken post-operatively and patients were under antibiotics. This could have possibly masked any neuronal response.

In the serum of those patients, we measured high concentrations of IL-8 and TNF-α and a low concentration of IL-22. The other cytokines we tested for were either below the detection range or not present in the serum. In contrast, in the serum of IBD patients were additionally detected INF-γ, IL-10, IL-12, IL-1, IL-6, IL-12p70 and IL-1β. Hence, the combination of mediators might be responsible for the generation of a neuronal response rather than single cytokines.

Furthermore, it has to be taken into account that 40% of all appendices being removed show no signs of inflammation. The increase in neurotransmitters in the absence of acute inflammation of the appendix suggested a different pathological entity, neuroimmune appendicitis/ neurogenic appendicopathy (Di Sebastiano et al., 1999; Franke et al., 2002). Symptoms and laboratory findings of neurogenic appendicopathy mimic acute

appendicitis thus, making it difficult to differentiate the two diseases pre-operatively (Tatekawa et al., 2011).

Immunohistochemistry of acute appendicitis post-operatively revealed lower amounts of SP-immunoreactive and VIP-immunoreactive nerves in the mucosal layer of the appendix in contrast to non-acute appendices. Non-acute appendices showed a close contact of nerve fibres to the marginal layer of lymphatic follicles pointing to neuro-immune interaction for the pathogenesis of pain (Di Sebastiano et al., 1999).

Serum in our study was collected from patients admitted to the clinic for appendectomy. Since is difficult to delineate between acute appendicitis and neurogenic appendicopathy pre-operatively, we could not define the acute condition of the sera used in the study.

4.6 Anti-enteric neuronal antibodies

Current literature supports a dysregulated immune system in IBD. The excessive or insufficient production of cytokines is responsible for pathological conditions (Van der Meide and Schellekens, 1996). In parallel, the dysregulated innate immunity favours the production of autoantibodies and microbial antibodies (Mitsuyama et al., 2016). Autoantibodies from patients with paraneoplastic GI syndromes had an excitatory effect on enteric neurons and visceral afferents proposing a link between nerve activation and symptom generation in these patients (Li et al., 2016).

Hence, we screened for the presence of anti-enteric neuronal antibodies in the serum of patients with IBD and controls. Autoantibodies were present in 42.3% CD patients, 25% of UC patients and 26% of controls. Work from Lütt et al. (2018) showed staining in 17% of CD patients and 12% of UC patients. The differences may attribute to the low number of patients, as well as to the animal model used in this study. We performed experiments in guinea pig tissue while their experiments were done on rat tissues (Lütt et al., 2018). A more elegant approach would have been to test for autoantibodies in human tissue. Using animal models is a more convenient approach, but differences in the amino acid sequences might result in reduced specificity to the target. The most promising strategy would be the use of monoclonal recombinant antibodies against the autoantibodies. As an example can serve the study of Kreye et al. (2016) where they suggest that binding of NR1 monoclonal antibodies from patients with the anti-NMDA receptor encephalitis on the

respective receptor can cause morphological and electrophysiological changes in neurons.

Since IBD is a group of multifactorial conditions, we could also argue that the expression of autoantibodies in only a subset of patients may point to a different cause of the disease. Gene mutations or polymorphisms can result in the production of a different antibody and subsequently, to an altered immune response (Mitsuyama et al., 2016). Accordingly, future studies correlating genetic predisposition or microbiota patterns with the expression of autoantibodies would be of interest.

In the present study, a high number of control sera also revealed autoantibody staining. Although this is striking, the work of other groups also showed staining in a high number of controls (Lütt et al., 2018; Wood et al., 2012). The explanation proposed by Chongsrisawat suggested that the presence of those antibodies might have been caused by residues after a previous viral infection (Chongsrisawat, 2012).

Therefore, whether the presence of the anti-enteric neuronal antibodies is directly linked to gastrointestinal symptoms or it is just an epiphenomenon is currently unclear. In our study, the application of sera positively stained for autoantibodies evoked similar neuronal responses compared to the application of sera without any antibody staining. Interestingly, the four CD-active sera showing neuronal staining belong to smokers (3 current smokers, 1 former smoker). Smoking behaviour can modify the association between SNPs and CD. Many of these SNPs are located near or within genes that might be involved in or interfere with the adaptive immune response or the mucosal barrier function (Yadav et al., 2017). Autoantibody production and smoking were also interrelated in other autoimmune disorders. A significant association was found between smoking and the presence of antibodies to cyclic citrullinated peptides in Rheumatoid Arthritis (Lee et al., 2009). Also, a link of smoking with dsDNA autoantibody production was shown in patients with systemic lupus erythematosus (Freemer et al. 2006).

In an attempt to determine the role of serological antibodies in the pathogenesis of Crohn's disease, we try to thermally deactivate the antibodies. Natural antibodies are composed of 6 to 70 immunoglobulin fold domains. While a single immunoglobulin fold domain can be refolded after heat denaturation, pairs of such domains cannot (Akazawa-Ogawa et al.,

2017). Each domain unfolds at different temperature. Initially, the CH1 domain, in the Fab fragment of the immunoglobulin was considered to be the less thermostable and to unfold first (Roterman et al., 1994; Vermeer and Norde, 2000). Later, it was shown that Fc is not a single unit but rather consists of two domains, CH2 and CH3 that denatured independently. CH2 is the least stable cooperative structural unit of immunoglobulin (Feige et al., 2004). Therefore, alterations of the domains due to heat will prevent the binding of the antibody to its antigen and hence, its biological activity (Mainer et al., 1997). Each immunoglobulin denatures at a different temperature varying from 56-90°C with a thermostability order of IgG >IgA>IgM>IgE (Binaghi and Demeulemester, 1983; Mainer et al., 1997). At 72 °C, IgG, IgA, and IgM in bovine milk are denatured by more than 75% after incubating for 25, 5, and 1.5 minutes, respectively. For our experiments, we used initially a thermal deactivation at 72 °C for 10 minutes but IgG staining was still evident in tissue preparations. Therefore, we proceeded with 72 °C for 30 minutes where no staining was evident.

Application of sera undergoing thermal treatment shows a tendency to lower excitatory effects on guinea pig submucous neurons compared to untreated sera. A tendency for weaker activation in a smaller proportion of neurons was also noted for 2 out of 3 patient sera tested. In contrast, application of serum from patient #006 showed an increased neuronal response in a higher number of neurons. The patient did not show any particular clinical characteristics that would explain its response.

Besides the immunoglobulins, numerous other mediators reside in the serum. While proteins precipitate in cell extracts when heated, serum proteins are not eliminated. Thus, in serum, heat-denatured proteins are still present, but at 72° in a (partially) unfolded state. However, to what extent cytokine receptors recognize only 3D structures or also linear motifs are unknown. Work from the group of J. Wood (1999) showed that heating the solution containing IL-1β to boiling did not alter the depolarization action of the cytokine on guinea pig enteric neurons, arguing that other regions of the molecule are recognised by the receptor. On the other hand, the protein chains are in a hydrophobic pocket, as seen in the crystalline structure. Therefore they might be resistant to the boiling temperature at the altitude of Columbus, OH, which is probably no greater than 100^{o}C (Xia et al., 1999). One could even speculate that some of the unfolded proteins re-fold as molecules can return spontaneously to their native state (Anfinsen, 1973).

To specify that this effect of heating was truly due to the antibodies, we attempted to isolate the IgA/IgG from the patient sera and test whether they can elicit activation of enteric neurons. We could not proceed with functional readouts after isolation of IgA/IgG from the samples since the chemical solutions used were interfering with the neuronal staining.

4.7 Parameters deriving from complete blood cell counts

Complete blood cell count is a common laboratory test used to assess the overall health. An abnormal increase or decrease of cell counts can designate an underlying medical condition. In our study, all values fell in the physiological range.

There was a slight increase in the count of leucocytes and platelets of CD patients compared to the control samples. Platelets play a role in blood homeostasis by discriminating between intact and injured endothelium. Increased platelet count was correlated with an exacerbation of clinical activity in IBD patients (Morowitz et al., 1968). A platelet count >450×10^9/L is a common characteristic of IBD patients in an active stage (Talstad et al., 1973). Platelet count has been considered as a measure for the distinction of IBD from infective diarrhoea (Harries et al., 1982). Moreover, platelet aggregates were found *in vivo* circulating in IBD, but this was not the case for other chronic inflammatory conditions (Collins et al., 1994)). Besides their traditional role in homeostasis, recent studies pointed to a role of platelets as potent pro-inflammatory cells. Upon exposure to soluble factors, platelets are activated and release biologically active molecules stored in their granules. Hence, they can activate both immune and nonimmune cells, which mediate inflammation and lead to further platelet activation (Klinger, 1997; Page, 1989; Thomas and Storey, 2015). The secreted mediators modify leucocyte and endothelial responses. Furthermore, P-selectin up-regulation mediates the formation of aggregates between platelets and leucocytes (Nash et al., 1996). They further form bridges between leucocytes and endothelium that subsequently result in leucocyte transmigration. This migration was largely mediated by platelet P-selectin, thus amplifying the local immune response (Thomas and Storey, 2015). An additional role of platelets and leucocytes in mucosal inflammation has been postulated. At low shear rates, leucocytes utilize endothelial P-selectin to bind to venular endothelium. Thus, they can attract platelets which can further interact with endothelium (Danese et al., 2004; Russell et al., 2003). Therefore platelet activation can lead to activation of leucocytes and contribute to inflammation locally and then systemically supporting the elevated platelets and leucocytes levels observed in CD active patients in our study.

4.8 TNF-α affects neuronal complexity

Application of TNF-α on submucosal enteric neurons evoked neuronal responses. This result suggested that TNF-α may affect the function of enteric neurons and thus, contribute to the development of symptoms. Therefore, we examined its effect on intestinal neuroplasticity. More precisely, we investigated how it affected neuron numbers and fibre outgrowth using an enteric neuron-glia co-culture method that yields a highly enriched neuronal population (Le Berre-Scoul et al., 2017).

Incubation of enteric neurons with TNF-α at concentrations of 10ng/ml and 50ng/ml decreased axonal complexity without affecting neuron number suggesting that TNF-α regulates the patterning of neuronal processes, independently of its effect on neuronal differentiation and survival.

Previous studies showed that TNF-α exerted both neurotrophic and neurotoxic effects on neurons (Bessis et al., 2005). Our study is in agreement with previous findings where TNF-α had an inhibitory effect on neurite growth and branching of cultured hippocampal neurons, whereas neuronal survival was not altered (Neumann et al., 2002). Also, in a rat ENS co-culture model, TNF-α increased the numbers of neurites two-fold compared to controls through the activation of the NF-kB signalling pathway (Gougeon et al., 2013).

Studies regarding the role of inflammation in axonal growth are also inconclusive. Electron microscopy studies supported that axonal necrosis was a characteristic feature of Crohn's disease (Dvorak and Silen, 1985; Steinhoff et al., 1988). Axonal necrosis/degeneration was observed in inflamed and non-inflamed areas, thus excluding local inflammation as the primary cause (Dvorak and Silen, 1985). Later studies suggested that axonal damage is not specific to CD patients but rather a feature of IBD (Brewer et al., 1990). Indeed axonal damage was also described in a Dinitrobenzene sulphonic acid (DNBS)-induced colitis mouse model (Sanovic et al., 1999).

TNF-α addition to enteric neurons did not affect neuron survival in our study which was in agreement with Gougeon et al. (2013) where neither TNF-α nor IL1β had an effect. In contrast, DNBS-induced colitis model caused a significant neuronal loss in the inflamed region, an increase in smooth muscle cell number, and damage to surviving axons. The number of myenteric ganglia was not affected while there was a significant decrease in

the number of ganglia in the submucosal plexus (Sanovic et al., 1999). In contrast, in Dextran sulfate sodium colitis and Citrobacter rodentium colitis mice models led to increased colonic neurons supporting enteric neurogenesis. Sox-2 and PLP-1 expressing cells which represent enteric glia or neural progenitors gave rise to enteric neurons in the colitis models (Belkind-Gerson et al., 2017).

4.9 Distance between ganglia and blood vessels

Neurons and blood vessels are in close proximity. 3D neurohistology experiments revealed close anatomical associations between the ENS and the vasculature. They exposed capillaries specific to the villi, the crypts and the myenteric plexus (Fu et al., 2013). Although early studies supported that notion of avascular ganglia, later studies showed a small class of submucous cholinergic neurons (12%) project to the mucosa and the local blood vessels (Costa et al., 2000; Kiernan, 1996). Capillaries in sensory and sympathetic ganglia are fully permeable to macromolecules. Ganglia of ENS lack perineum, so they are exposed to extracellular fluid with an exchange of plasma proteins between blood and extracellular fluid to occur (Kiernan, 1996). Substances can diffuse from blood vessels to ganglia and vice versa via the concept of extra-synaptic communication. This slow, widespread communication is based on a volume transmission between the source and the target (Zoli et al., 1999).

Thus, we were interested if factors released by blood vessels could influence the response of adjacent ganglia. We determined the distance between ganglia and blood vessels and compared it with the response to the serum. We measured the distance of 0-600 µm between ganglion and blood vessel. There was no difference in the recorded evoked response in ganglia close to blood vessel or ganglia with a distance of 600 µm away from it. Non-synaptic volume transmission from source to target can occur in a distance of µm to mm (Zoli et al., 1999). In our experiments, the distance was measured only in the range of µm, which might not be sufficient to reveal any differences in remote neurotransmission via blood.

Visualization of ganglia and blood vessels was performed with a fluorescence microscope and that was a limitation of our study. Using a confocal microscope would allow capturing 3D images. This might reveal blood vessels under or on top of the ganglia and would allow a better estimation of the vicinity among ganglia and blood vessels by identifying the correct layer of the ganglia. Furthermore, discriminating between veins or arteries might have pointed to a distinct group of neurons with different neurochemical coding. Studies in vasoconstricting neurons innervating the ear of the guinea pig showed different neurochemical groups of neurons between innervated veins and arteries (Gibbins and Morris, 1990; Gibbins et al., 2003).

4.10 Neurochemical coding of neurons responding to CD-serum

Neurochemical coding of enteric cell bodies can be used to describe neuronal function. Consequently, we investigated transmitter patterns in the colon submucosal neurons that responded to the serum of CD-patients. Four preparations were double-labelled for ChAT and VIP as they are the vast majority of the submucosal neurons. ChAT-positive neurons represented 45% and VIP- positive neurons 54% of the responding neurons. Our results are in agreement with a previous study showing 47±1% ChAT-positive neurons and 53±1% VIP-positive neurons in the guinea pig proximal colon (Neunlist et al., 1998).

Immunohistochemistry in whole-mount preparations from inflamed segments from CD patients' ileum showed an increased number of VIP, NOS and neuropeptide Y-immune-reactive cell bodies in the myenteric plexus (Belai et al., 1997). An analogous study in the submucous plexus revealed an increase in the VIP/- population in the non-inflamed rectum of CD patients compared to controls (Schneider et al., 2001). In contrast, inflamed areas in the colon showed a lower concentration of SP and VIP neuropeptides (Sjölund et al., 1983). These findings indicated adaptive changes of enteric neurons to inflammation. Thus, we examine whether the acute application of CD serum would activate a distinct neuronal population. Both ChAT and VIP neurons responded to CD serum at a similar degree. In addition, there was no difference in the response between the application of active and remission sera.

In contrast to this study where tissue from guinea pig intestine was used, previous studies showing a change in the neurochemical coding were performed in human gut segments. In human submucosal plexus, there is a co-localization of ChAT and VIP while in the guinea pig plexus there are two distinct populations (Neunlist et al., 1998; Schneider et al., 2001).

Nevertheless, the neurochemical coding in this study should be interpreted with caution as the quality of the tissue might be compromised due to neuroimaging experiments performed before the fixation of the tissue. While ChAT staining is specific and argues in favour of a non-affected plexus, neurons stained for VIP appeared to be damaged.

4.11 Methodological considerations

In the study, sera from human samples were applied to guinea pig enteric neurons. One could argue that using human enteric neurons would be a better approach. Guinea pigs have been extensively used in gastroenterology studies, especially in studies of the enteric nervous system. Work from our group showed that guinea pig neurons, except individual cases such as in the PAR mediated action (Mueller et al., 2011), act very similarly to enteric neurons. Evoked response to supernatants was similar in guinea pig and human enteric neurons (Buhner et al., 2014). Also, their intestinal permeability is closer to humans compared to the other lab rodents (Belai et al., 1997; Delahunty and Hollander, 1987). Exposure of animals to environmental cues can be controlled, while this is not possible with human tissue. Using human tissue would have added another variation parameter to our study as cross-reaction of human tissue with the serum might occur.

Our study was performed in guinea pig submucosal neurons since previous work from our group suggested that the functional neuro-immune interactions occur primarily in the submucous plexus area, where immunocytes and nerve processes are in close contact in contrast to the myenteric and muscle layers (Buhner et al., 2012).

Studies regarding circulating cytokines in IBD show high variability. Whether cytokines detected at an end-organ level can directly translate to cytokines in the bloodstream is unknown. Existing studies classify patients based on their disease index and not due to the localization of inflammation. Since CD can affect different segments along the gastrointestinal tract it would be plausible that inflammation localized in different segments may account for differences in the levels of circulating cytokines (Korolkova et al., 2015).

Also, cytokines elevated at a tissue level may have relatively short half-lives and appear in the bloodstream only in advanced pathological conditions (Korolkova et al., 2015).

Low cytokines level in the bloodstream may be due to the increased binding to the soluble receptors. The notion comes from the ineffectiveness of Etanercept. Etanercept is an engineered sTNF-R2 that neutralizes soluble TNF-α. In contrast, both antibodies (Infliximab and Adalimumab) showing an effect can bind both forms of TNF-α (Smids et al., 2017).

IBD patients also receive a wide range of medications. Treatment received at the time of blood sampling may greatly influence the cytokines levels. Immunosuppressive drugs act on the production and/or on the action of cytokines. Infliximab, a common medication of IBD patients influences the levels of inflammatory markers in Rheumatoid Arthritis patients (Korczowska et al., 2013). In accordance, cytokines concentration in the serum of the CD-remission patients under Adalimumab treatment might be compromised.

The observed heterogeneity may also attribute to the serum storage conditions. Serum samples stored at ≤−80°C and <−130°C for up to 90 days remain unaltered. In contrast, storage at 4°C and −20°C induced substantial decreases in cytokine concentration (Zander et al., 2014). In our study, all samples were stored at ≤−80°C. Moreover, concentration tends to decrease over time. In general, cytokine levels decrease approximately 10–20% each year after two years of storage. Eventually, a five-fold change in cytokine levels can occur after five years as a result of cross-reactivity between protein epitopes (Butterfield et al., 2011). Also, cytokines levels are affected by repetitive freeze/thaw cycles (Keustermans et al., 2013). Some of the samples used in this study were stored for more than five years at ≤−80°C. We tried to avoid frequent freeze/thaw cycles by aliquoting the sample into 5µl aliquots upon arrival. We measured cytokines levels in serum samples with an interval of 6 months and we did not observe any significant difference in their levels.

Another limitation is the type of assay used to determine cytokine concentrations. Besides the immuno-PCR which yields high levels of TNF-α, conventional ELISA and electrochemiluminescence or microsphere immunoassays failed to detect or detected lower concentrations of respective cytokines (Korolkova et al., 2015).

4.12 Future Perspectives

Our study demonstrated that sera from CD patients could activate enteric neurons independently of the disease stage. Our results pointed to a role of TNF-α in neuronal activation. They further suggested that other mediators residing in the serum are responsible for the observed responses. Future experiments could focus on the identification of these mediators and the synergistic effect among them to provoke excitation of enteric neurons.

Additionally, a thorough analysis of the mediators residing in the serum of the IBD patients would be of interest as we already detected differences in the response upon application of CD-sera and UC-sera.

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Appendix

Table S1: Crohn's Disease Patient Characteristics (summary)

Median age (at diagnosis in years)	25
Subjects:	
Male	12
Female	18
Median CDAI score at diagnosis	297± 79.50
Smoking status at diagnosis	
• Never	10, (53%)
• Current	5, (26%)
• Stop	4, (21%)
Disease location at diagnosis (Montreal)	
• Ileal L1	1, (5%)
• Colonic L2	10, (53%)
• Ileocolonic L3	8, (42%)
• Upper GI	2, (11%) (also in L2 or L4)
• Perianal	8, (42%)

Disease behaviour at diagnosis (Montreal)

- Inflammatory B1 — 16, (84%)
- Stricturing+ B2 — 2, (11%)
- Penetrating B3 — 1, (5%)
- Perianal p — 5, (26%)

Body Mass Index (BMI)

- Underweight = <18.5 — 5, (20,8%)
- Normal weight = 18.5–24.9 — 2, (8,3%)
- Overweight = 25–29.9 — 13, (54,2%)
- Obese=<30 — 4, (16,7%)

Treatment (all patients included)

- No medication — 4, (13%)
- Anti-inflammatory — 8, (27%)
- Immunosuppressant — 15 (50%)
- Anti-TNF-α — 6 (20%)
- Analgetics — 3 (10%)
- Spasmolytics — 1 (3%)
- Vitamins — 2 (7%)
- Diazepam — 1 (3%)
- Other — 2 (7%)

Table S2: Medication Sorting

Immunosuppressive	***Anti-inflammatory***	***Antibody***	***Vitamins***	***Antibiotics***
Azitothymidine	Mesalazine	Hepatitis B	Calcium 50mg	Bactum Fort
Prednisone	Esomeprazol		Magnesium B6	Metronidazol
Budesonide	Tramdaol		Magnesium 2	
Azathioprine	Nifinam		Phosphorus	
Mercaptopurine			Depot Eisen	
Methylprednisolon			Toco	
			Eisen	
			Vitamin B12	
Analgesics	***Spasmolytic***	***Anti-depressive***	***Diazepam***	***Other***
Paracetamol	Trimebudin	Venlafaxin	Clorazepam	Allopuriol
Macrogol			Diazepam	Ciate Tripotanig
				Loperamid
				Lévonorgestrel, Ethinylestradiol
				Metoclopramid
				Phloroglucinol dihydrate

Table S3: Crohn's Disease samples

HB=hemaglobulin, HCT=hematocrit, GB= leucocytes, CRP= C-reactive protein, BMI=Body mass index, S0= before administration of adalimumab, S14= after administration of adalimumab, perf. Date= perfusion date, CDAI. Crohn's disease activity index

n°patient	*Age at diagnosis*	*Gender*		*S0*	*HB (g/dl)*	*HCT (%)*	*GB (cells/mm³)*	*Platelets (cells/mm³)*	*CRP (nmol/L)*	*S14*	*HB (g/dl)*	*HCT (%)*	*GB (cells/mm³)*	*Platelets (cells/mm³)*	*CRP (nmol/L)*	*BMI*
002		M	**perf. date**	**10/6/2009**						**1/11/2010**						
	29		CDAI	368	14.9	44	7.13	307	24	77	16.4	48.4	6.3	271	6	24.0
006		F	**perf. date**	**11/23/2009**						**3/1/2010**						
	29		CDAI	320	11.7	35.4	20.9	459	4	277	12.4	37.7	8	462	4	21.6
007		F	**perf. date**	**11/27/2009**						**19/02/2010 (S12)**						
	22		CDAI	304	8.9	27.4	5.7	667	79	256	9.7	30.3	8.1	505	91	15.9
009		F	**perf. date**	**1/26/2010**						**5/4/2010**						
	22		CDAI	197	14.8	42.8	18.9	391	ND	34	13.3	39.7	4.5	432	4	26.0
011		M	**perf. date**	**3/4/2010**						**28/05/2010 (S12)**						
	24		CDAI	154	13.9	43	7.95	416	17	21	15.2	47.3	7.4	328	3.3	21.3
014		F	**perf. date**	**3/31/2010**						**7/2/2010**						
	24		CDAI	297	13.2	37.7	9	276	284.2	0	13.6	43	7.03	226	2.7	21.9
015		F	**perf. date**	**4/12/2010**						**8/2/2010**						
	10		CDAI	325	9.2	31.3	5.31	546	84.2	119	9.8	32.8	6.13	409	12.5	17.2
017		M	**perf. date**	**5/5/2010**						**8/9/2010**						
	20		CDAI	214	13.7	41.4	18.52	558	3	82	13.3	39.8	5.8	282	2.4	24.7
018		M	**perf. date**	**6/2/2010**						**9/20/2010**						
	36		CDAI	226	12.8	38.1	12.58	585	38	133	14	42.6	7.55	241	0	23.9

019		F	**perf. date**	**9/30/2010**						**1/10/2011**							
	39		CDAI	235	8.9	29.9	14.3	684	ND	32	12.6	36.9	9.3	367	5	21.5
020		M	**perf. date**	**11/10/2010**						**2/14/2011**							
	21		CDAI	273	11.2	35.5	6.55	520	94.6	39	15.1	47.5	6.8	289	7	21.1
021		M	**perf. date**	**1/25/2011**						**5/5/2011**							
	22		CDAI	454	12.4	36.8	7.97	463	113.2	0	16.1	47.5	4.68	274	1.2	22.0
022		F	**perf. date**	**2/15/2011**						**5/23/2011**							
	24		CDAI	503	13.1	40.2	12.6	421	139.1	20	13.6	42.3	6.8	317	5.1	25.7
023		M	**perf. date**	**2/21/2011**						**5/30/2011**							
	27		CDAI	288	12.7	38.1	8.16	324	16.9	132	14.1	41.3	6.9	358	5.6	18.8
024		F	**perf. date**	**3/16/2011**						**NA**							
	50		CDAI	429	8.6	27.5	12.47	470	40.1							26.8
026		F	**perf. date**	**4/19/2011**						**7/13/2011**							
	21		CDAI	619	12.8	39.3	9.1	354	14	661	13.1	39.5	6.9	260	8	22.0
6249		M	**perf. date**	**9/30/2008**	**S0 Infliximab**												
	14		CDAI	183	11.7	36	6	287	33.2								
8080		M	**perf. date**	**5/20/2010**	**Quiescent infliximab 10 mg/kg**												
	23		CDAI	30	10.7	37.5	6.69	303	4.1								
8778		F	**date (colonoscopy)**	**4/11/2011**	**pentasa 3 g**												
	50		CDAI	106	12.8	37.6	6.28	253	4.2								
81009		F	**date (colonoscopy)**	**5/25/2010**	**no treatment**												
	49		CDAI	250	13.7	39.2	12.84	304	6.1								

82010		F	**date (colonoscopy)**	**6/22/2010**	**no treatment**				
	43		CDAI	31	14.2	41.9	5.58	270	< 3
87009		F	**perf. date**	**3/2/2011**	**2e injection adalimumab**				
	3		CDAI	0	ND	ND	ND	ND	ND
91040		F	**date (colonoscopy)**	**11/9/2011**	**adalimumab 80 mg/week**				
	4		CDAI	acute flare	ND	ND	ND	ND	ND
96089		M	**date (colonoscopy)**	**6/22/2012**	**pentasa + entocort**				
	50		CDAI	acute flare	16.8	48.6	19.85	249	15.6
103077		F	**date (colonoscopy)**	**2/20/2013**	**no treatment**				
	27		CDAI	acute flare	ND	ND	ND	ND	ND
103084		M	**date (colonoscopy)**	**2/22/2013**	**adalimumab 40 mg/week**				
	42		CDAI	acute flare	13.8	40	3.90	297	ND
93012		M	**perf. date**	**2/13/2012**	**adalimumab 80 mg/week**				
	20		CDAI	219	10	31.1	2.52	213	234.5

Table S4: Ulcerative Colitis samples
HB=hemaglobulin, HCT=hematocrit, GB=leucocytes, CRP= C-reactive protein

n°patient	*Age at diagnosis*	*Gender*	*Lichtigher score*	*HB (g/dl)*	*HCT (%)*	*GB (cells/mm^3)*	*Platelets (cells/mm^3)*	*CRP (nmol/L)*
64/26	**46**	**F**	**10/21**	**12.4**	**39.3**	**10900**	**338000**	**ND**
59/32			**3/21**	**14.3**	**44.3**	**5700**	**263000**	**ND**
76/68	**48**	**M**	**16/21**	**13**	**37.8**	**5400**	**336000**	**negative**
79/030			**1/21**	**13.6**	**39.9**	**4450**	**264000**	**ND**
53/23	**27**	**F**	**11/21**	**10.1**	**29.5**	**14700**	**458000**	**ND**
64/24			**3/21**	**11.8**	**35.1**	**12400**	**304000**	**ND**
29/61	**63**	**M**	**10/21**	**11.9**	**35.8**	**5200**	**261000**	**ND**
4 70			**4/21**	**12.8**	**37.8**	**6900**	**203000**	**negative**

Table S5: Control samples

n° control	*Age at blood withdrawal*	Gender
105079	37	M
107077	46	M
112050	26	F
113086	47	M
114078	49	M
116014	25	M
117062	24	F
117064	30	F
117065	35	M
119089	32	F
120019	46	M
120074	60	M
120093	72	M
124027	34	M
HSC 002	51	F
ML	24	F
Vb234	36	F
Vb236	27	F
Vb238	39	F
Vb240	23	F
Vb242	27	M
Vb61	67	F
Vb68	39	M
Vb72	62	M
Vb73	60	M

Table S6: CDAI questionnaire

Initials of Patient ___ /___	Nº inclusion ___
Visit for selection/ inclusion	

IL-15- Crohn's disease
Selection/Inclusion Date: (__/__/__)

Verification of inclusion/selection criteria		
Criteria of selection/inclusion	Yes	No
Over 18 years old	☐	☐
Read the information and signed an informed consent	☐	☐
Adequate contraception for women of childbearing age	☐	☐
Diagnosis of Crohn's disease based on clinical, endoscopic and histological criteria	☐	☐
Patient on active stage (HBI > 4)	☐	☐
Indication to treatment with anti-TNF	☐	☐
No previous treatment with anti-TNF	☐	☐

Verification of non-inclusion criteria		
Criteria of non- inclusion	Yes	No
Already received anti-TNF treatment	☐	☐
Exclusively anal disease	☐	☐
Indication for a surgical treatment	☐	☐
History of colorectal dysplasia	☐	☐
History of cancer within 5 years of inclusion	☐	☐
Infection HIV, HBV active	☐	☐
Bacterial infection, viral or parasites active and untreated	☐	☐
Latent tuberculosis	☐	☐
IDR > 5mm	☐	☐
Heart failure grade III /IV	☐	☐
PNN < 1500 mm^3 or platelets < 100 000/mm^3	☐	☐
Unexplained increase in transaminases or alkaline phosphatases > 3N	☐	☐
Pregnant or breastfeeding patient	☐	☐

Initials of Patient ___ /___	Nº inclusion ___
Visit for selection/ inclusion	

Selection/Inclusion Date: (__/__/__)

Date of birth: __/__/__ Sex: M F

Height: ___ cm Weight: ___ kg

1. **Smoking:**
   Current smoker: Yes No
   If yes: since __/__/__
   If no, former smoker: Date of quitting: __/__/__
   Cumulative smoking in packs/ years: ___

2. **Date of diagnosis of Crohn's disease**
   (Date of the first finding of morphological abnormalities of the intestine)
   __/__/__

3. **Topography of Crohn's disease during follow-up**
   Check the corresponding box:

Localisation	L1, terminal Ileum	
	L2, colon	
	L3, ileocolon	
	L4, upper GI	
	p, perineal disease	

*L4 and p are not exclusive and can be associated with other locations

4. **Type of disease at inclusion time**

   Check the corresponding box:

Type	B1, non-penetrating – non-stricturing	
	B2, stricturing	
	B3,penetrating	
	p, perineal disease	

Initials of Patient ___ /___ Nº inclusion ___

Visit for selection/ inclusion

Treatment at the beginning of inclusion

Non-commercial	**Dosage**	**Start date**	**Indication**	**Specification**

Harvey Bradshaw Index (HBI)

1. **Well being**
   (good = 0; well = 1; moderate = 2; poor = 3; very poor = 4) ____
2. **Abdominal pain**
   (absent = 0; mild = 1; moderate = 2; severe =3) ____
3. **Number of liquid stools per day** ____
4. **Abdominal mass**
   (absent = 0; doubtful = 1; certain = 2; certain with defence=3)
   ____
5. **Complications**
   (Count one point for each of the following items arthralgia; uveitis; erythraemia; aphids; pyoderma gangrenosum; anal fissure; fistula; abscess) ____

Total ____

The variables 1, 2 and 3 are related to the day before the examination
An HBI > 4 signifies active disease.

Initials of Patient ___ /___	Nº inclusion ___
Visit S__	

Date: (__/__/__)

CDAI score

1. Number of liquid stools per week	**_ _ _ x 2 = _ _ _**
2. Abdominal pain (absent = 0; mild = 1; moderate = 2; severe =3)	**_ _ x 5 =_ _ _**
3. Well being (good = 0; well = 1; moderate = 2; poor = 3; very poor = 4)	**_ _ x 7 =_ _ _**
4. Other elements related to the disease: Count 1 point for each category of elements present and highlight the present element: • Arthralgia, arthritis • Iritis, uveitis • Erythraemia; aphids; pyoderma gangrenosum, mouth ulcer • Anal fissure; fistula; abscess anal or rectal • Other fistula • Fever > 38 ºC in the last week	**_ _ x 20 =_ _ _**
5. Taking antidiarrhealls (no = 0; yes = 1)	**_ _ x 30 =_ _ _**
6. Abdominal mass (absent = 0; doubtful = 2; certain = 5)	**_ _ x 10 =_ _ _**
7. Haematocrit: Haematocrit[1]= _ _% Add or subtract according to the sign: Male: 47 – Hte = _ _ Female : 42 – Hte = _ _	**_ _ X6 = _ _**
8. Theoretical weight [1,2]___ - Actual weight[1]___ x 100 / Theoretical weight [1,2]___	**_ _x1= _ _**

Total **CDAI** = _____

1. The numbers with comma will be rounded:
   to the next digit if the number after the decimal point is **≥ 5**
   to the lower digit if the number after the decimal point is < **5**
2. See table of theoretical weights in annex. In case of overweight the added value is 10.

Biological results

	value	unit	Abnormal(1)/ Normal (0)	Significance of anomaly 1-3	Interpretation 1-3
Hg		**g/dl**			
Ht		**%**			
Leucocytes		**cells/mm^3** (cells/µl)			
Platelets		**cells/mm^3** (cells/µl)			
CRP		**mg/L**			
IL-15		**pM**			
Sil-15Ra		**pM**			
Infliximab		**pM**			

Current treatments

Non-commercial	Dosage	Start date	Indication	Specification

Table S7: Simple Endoscopic Score for Crohn's Disease

Severity	0	1	2	3
Ulcerations	none	aphtoid <0,5cm	0,5 – 2cm	>2cm

Ulcerated surface	none	<10%	10 – 30%	>30%
Inflamed surface	none	<50%	50 – 75%	>75%
Stenosis	none	single, passable	multiple, passable	not passable

Table S8: Lichtiger Clinical Index for Ulcerative Colitis

Variable	**0**	**1**	**2**	**3**	**4**
Diarrhea (number of daily stools)	0-2	3-4	5-6	7-9	≥ 10
Nocturnal diarrhea	No	Yes			
Visible blood in stool (% of movements)	0	<50	≥ 50	100	
Fecal incontinence	No	Yes			
Abdominal pain or cramping	None	Mild	Moderate	Severe	
General well-being	Perfect	Very good	Good	Average	Poor
Abdominal tenderness	No	Mild and localized	Mild to moderate and diffuse	Severe or rebound	
Need for anti-diarrheal drugs	No	Yes			

Table S9: Ulcerative Colitis Endoscopic Index of Severity

Severity	**1**	**2**	**3**	**4**

Vessels	normal	partial loss	total loss	
Bleeding	none	mucosal	luminal bleeding (mild)	luminal bleeding (severe)
Erosions/ulcerations	none	erosion	superficial ulcerations	deep ulcerations

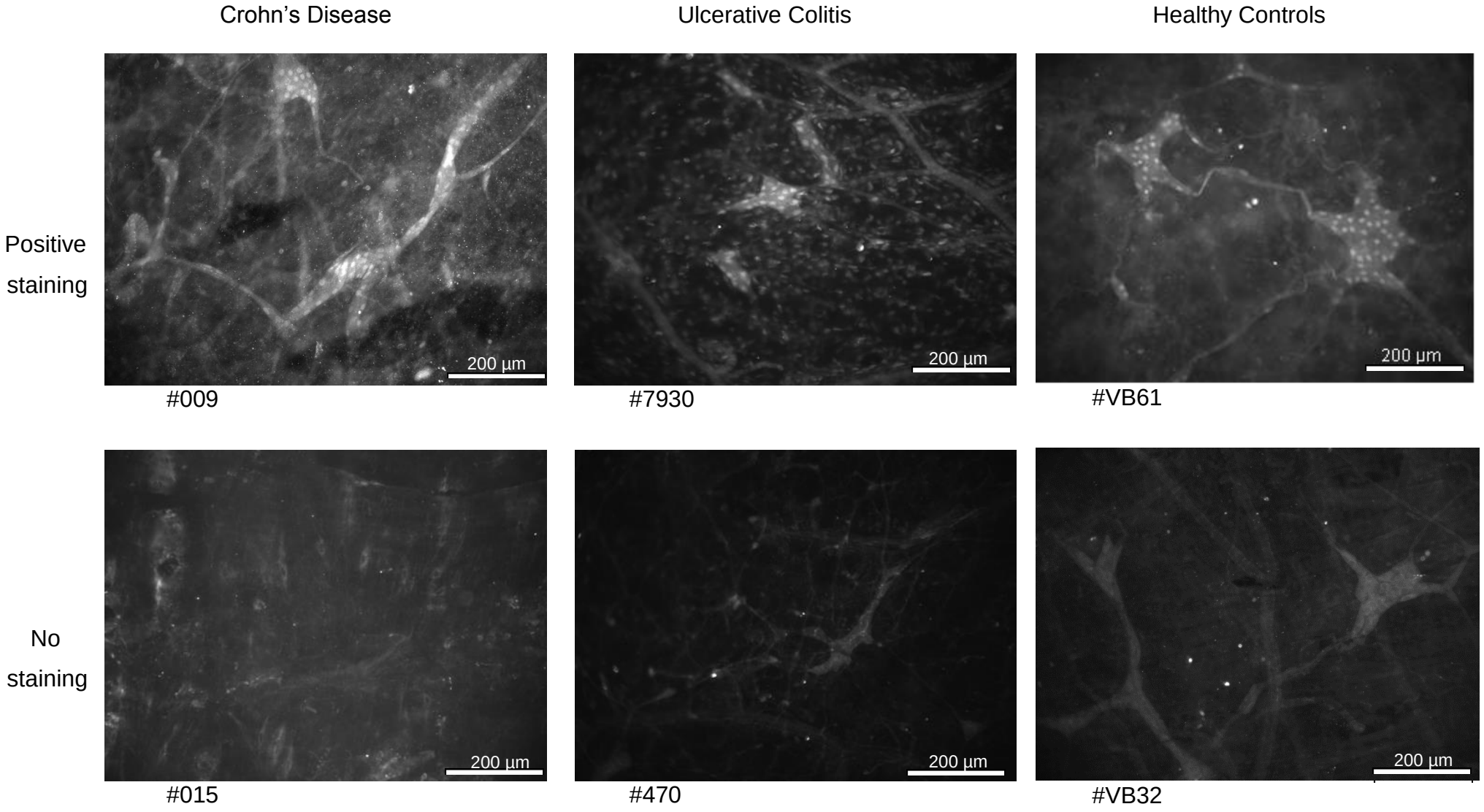

Figure S1: Immunostaining of serum autoantibodies in the guinea pig submucous plexus neurons
The upper panel shows immunostaining of autoantibodies residing in the subject sera in the guinea pig submucous plexus neurons. The autoantibodies labelled the cell bodies of submucous neurons. The bottom panel refers to sera without autoantibodies as ganglia were not stained.

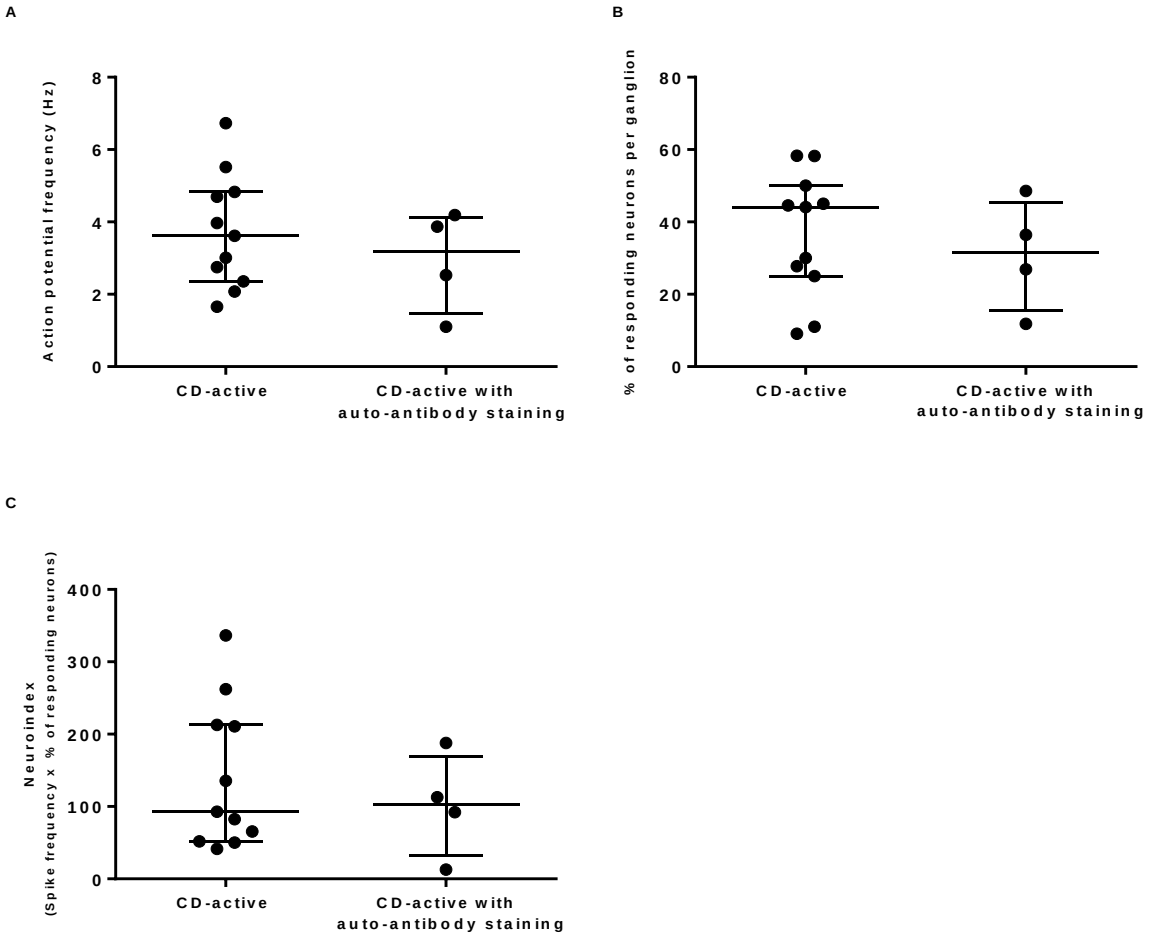

Figure S2: Sera showing autoantibody staining evoke similar neuronal responses to sera without any staining

(A) The evoked neuronal response was similar between sera with and without anti-enteric staining. (B) The percentage of the responding neuron and the neroindex (C) was also comparable between the two groups. CD-active n=13; CD-active with autoantibody staining n=4; Wilcoxon test; Median values with interquartile range.

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Curriculum Vitae

Working experience

09/2017- today	Clinical Trial Administrator, PSI-CRO, Germany

Education

11/2014- today	**PhD Technical University of Munich, Germany** *Thesis: The effect of serum from Crohn's Disease patients on enteric neurons*
09/2013-10/2014	**MSc Molecular and Cellular Basis of Human Diseases, University of Sheffield, United Kingdom** *Thesis: Generation of a neuronal cell line from murine Hippocampus*
09/2009-06/2013	**BSc Biological Sciences, University of Cyprus, Cyprus** *Thesis: The effect of E7 oncoprotein on the DNA damage response upon externally induced double strand breaks*

Sworn Statement

(Eidesstattliche Erklärung)

Ich erkläre an Eides statt, dass ich die bei der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Effect of sera from IBD patients on guinea pig enteric neurons

am Lehrstuhl für Humanbiologie unter der Anleitung und Betreuung durch Prof. Dr. Michael Schemann ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 6 und 7 Satz 2 angegebenen Hilfsmittel benutzt habe.

Ich habe keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht, oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.

Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt.

Die vollständige Dissertation wurde noch nicht veröffentlicht.

Ich habe den angestrebten Doktorgrad noch nicht erworben und bin nicht in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

Die öffentlich zugängliche Promotionsordnung der TUM ist mir bekannt, insbesondere habe ich die Bedeutung von § 28 (Nichtigkeit der Promotion) und § 29 (Entzug des Doktorgrades) zur Kenntnis genommen. Ich bin mir der Konsequenzen einer falschen Eidesstattlichen Erklärung bewusst.

Mit der Aufnahme meiner personenbezogenen Daten in die Alumni-Datei der TUM bin ich einverstanden.

Freising, den 07.11.2019 _____

Maria Lazarou