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Neural Actions of Proteases and Probiotics

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ZUSAMMENFASSUNG

In der Behandlung von Reizdarm (IBS) und chronisch entzündlichen Darmentzündungen (CED), wie Colitis ulcerosa und Pouchitis haben sich Probiotika förderlich erwiesen, indem sie zur Symptomlinderung und Verlängerung der Remissionsphase beitragen. Ferner wurde gezeigt, dass Probiotika die intestinale Barrierefunktion und Sekretion modulieren können. Das enterische Nervensystem (ENS) wird als möglicher Angriffspunkt für Probiotika und probiotische Mediatoren (z.B. Proteasen oder Proteaseinhibitoren) diskutiert, weshalb die neuronale Wirkung von Probiotika untersucht wurde. Eine Fütterungsstudie mit dem Probiotikagemisch VSL#3 in $TNF^{\Delta ARE/WT}$ Mäusen wurde hierzu durchgeführt. $TNF^{\Delta ARE/WT}$ Mäusen waren durch eine Beeinträchtigung der nerval- und Epithelzell- vermittelten Sekretionsleitung des Colons gekennzeichnet. Mit VSL#3 war es jedoch nicht möglich die beeinträchtige Sekretionsleistung zu normalisieren. Lösliche Mediatoren aus Überständen der probiotischen Stämme *Lactobacillus (L.) paracasei* NCC2461 und *Bifidobacterium (B.). longum* NCC3001 induzierten keine spezifischen neuronalen Effekte. Vielmehr zeigte sich, dass es nicht möglich war unspezifische Effekte des Wachstumsmediums zu kontrollieren, welche durch bestimmte Inhaltsstoffe, pH-Wert oder Osmolarität bedingt wurden.

Aufgrund der negativen Ergebnisse mit den löslichen probiotischen Mediatoren, wurde die neuronale Wirkung einer bakteriellen Protease untersucht. Gelatinase (GelE) ist eine Metalloprotease, die von dem kommensalen Bakterium *Enterococcus faecalis* OG1RF sezerniert wird und im genetisch prädisponierten Wirt eine Entzündung induziert. Es konnte gezeigt werden, dass GelE Neurone des Meerschweinchens erregte. Die Vorbehandlung mit GelE reduzierte die PAR2-AP induzierte neuronale Erregung, was möglicherweise auf eine Desensibilisierung des PAR2 Rezeptors zurückzuführen ist. Dies ist der erste wissenschaftliche Beleg, dass bakterielle Proteasen enterische Neurone erregen können. Es scheint, dass GelE PAR2 exprimierende Signalwege beeinflusst, was jedoch weiterer Untersuchungen bedarf.

Proteasen werden von verschiedenen Zelltypen, wie beispielsweise Epithel-, Immun-, Blutzellen oder der Mikrobiota sezerniert. Sie spielen eine zentrale Rolle in der Signalweiterleitung zu enterischen Neuronen und in der Integration von Informationen aus dem enterischen Nervensystem (ENS), dem Blut oder dem intestinalen Lumen. Von

besonderem Interesse sind Thrombin, Mastzell Tryptase und die von pathogenen, kommensalen und probiotischen Bakterien sezernierten Proteasen. Die Effekte der Proteasen werden durch Protease-aktivierte Rezeptoren (PARs) vermittelt. Die vier bekannten PARs - PAR1, PAR2, PAR3, PAR4 - sind G-Protein gekoppelte Rezeptoren. Sie werden durch Entfernung des N-terminalen Liganden mittels Proteasen aktiviert. Synthetisch hergestellte Peptide, bekannt als PAR-aktivierende Peptide (PAR-APs), aktivieren spezifisch PAR1, PAR2 und PAR4. PARs werden im Gastrointestinaltrakt von enterischen Neuronen, Glia-, Epithel- und Glattmuskelzellen exprimiert. Funktionelle als auch entzündliche Darmerkrankungen sind durch eine erhöhte Menge an Proteasen gekennzeichnet. Viele Untersuchungen an Tiermodellen konnten zeigen, dass neuronale PARs und hierbei vor allem PAR2 an der Entstehung von Hyperalgesie und Entzündung beteiligt sind. Die neuronale Wirkung von Proteasen im humanen ENS ist jedoch unbekannt. Folglich war es ein Ziel der vorliegenden Arbeit den Effekt der unterschiedlichen PAR-APs auf den humanen submukösen Plexus und den des Meerschweinchens zu untersuchen. PAR1-AP führte zur Aktivierung humaner submuköser Neurone und zum Anstieg der intrazellulären Calcium Konzentration $[Ca]_i$ in der Mehrheit der submukösen Neuronen und Gliazellen. PAR2 und 4-AP hingegen bedingten nur eine minimale Aktivierung in einer signifikant geringeren Anzahl an Neuronen. Im submukösen Plexus des Meerschweinchens induzierte PAR2-AP eine Aktivierung von Neuronen und Gliazellen, während PAR1-AP nur eine signifikant geringere Menge an Neuronen aktivierte. Der PAR1 Antagonist SCH79797 reduzierte die $[Ca]_i$ Antwort der humanen submukösen Neurone und Gliazellen dosisabhängig. Im Meerschweinchen wurde die PAR2-AP Antwort durch den PAR2 Antagonisten LIGK-NH_2 reduziert. In Übereinstimmung mit der PAR-AP Wirkung im humanen ENS induzierte PAR1-AP, nicht jedoch PAR2-AP, eine nerval vermittelte Sekretion, welche spezifisch durch SCH79797 inhibiert werden konnte. PAR4-AP besaß keine prosekretorische Wirkung, was mit seiner schwachen Wirkung im ENS einhergeht. Des Weiteren bedingte PAR2-AP einen Anstieg der $[Ca]_i$ in CD68-positiven Makrophagen in der humanen Submukosa. Thrombin bedingte eine spezifische Aktivierung von PAR1 in Neuronen und Gliazellen des humanen submukösen Plexus, während Tryptase nur eine schwache neuronale Erregung induzierte. Insgesamt konnten die Untersuchungen zeigen, dass PAR1 humane Neurone und Gliazellen aktiviert und PAR2 und PAR4 eher eine untergeordnete Rolle im humanen ENS spielen.

Serotonin, Histamin und Proteasen wurden als die neuronal erregenden Mediatoren in Überständen mukosaler Biopsien von IBS Patienten identifiziert. SCH79797 reduzierte die

durch IBS Überstände induzierte neuronale Erregung. Dieses Ergebnis unterstreicht die Bedeutung von PAR1. Zukünftige Studien sollten somit die Wirkung neuronaler PARs, und im speziellen PAR1, im humanen Darm untersuchen und PAR1 bei der Entwicklung von Medikamenten für Darmkrankheiten mit einem erhöhten Proteasespiegel berücksichtigen.

ABSTRACT

Probiotics have been shown to be effective in the treatment of irritable bowel syndrome (IBS) and inflammatory bowel diseases (IBD), like ulcerative colitis and pouchitis by inducing symptom relief and prolonging remission phase. Furthermore probiotics have been reported to improve mucosal barrier function and to modulate mucosal secretion. Certain studies suggested the enteric nervous system (ENS) as possible target for probiotics or released probiotic mediators (e.g. proteases, protease inhibitors) by inducing their beneficial effects. This has triggered further research, looking into the neural action of probiotics and soluble factors released by probiotics. A VSL#3 feeding study in a mouse model of experimental ileitis revealed that $TNF^{\Delta ARE/WT}$ mice suffered from impaired mucosal secretion as both epithelial as well as nerve mediated secretion was much weaker than in tissues from WT animals. VSL#3 did not reverse the impaired secretion and had also no effect on transepithelial resistance. Pure, concentrated and reconstituted lyophilised supernatants were obtained from the probiotic strain *Lactobacillus (L.) paracasei* NCC2461 and *Bifidobacterium (B.) longum* NCC3001. Neither of these supernatants evoked any specific effects on enteric neurons. Under *in vitro* conditions it eventually turned out to be impossible to control for unspecific actions, as the control medium itself exerted effects. These were either caused by ingredients which are obligatory for the bacterial growth, the pH value or the osmolarity.

Since our *in vitro* experiments about the neural actions of soluble mediators released by probiotics revealed negative results, we decided to test the neural action of a bacterial derived protease. Gelatinase (GelE) is a metalloprotease released by the commensal bacterium *Enterococcus faecalis* OG1RF, which has been shown to induce inflammation in a genetic susceptible host. GelE induced spike discharge in guinea-pig submucous neurons. The protease-activated receptor2-activating peptide (PAR2-AP) induced neural excitation was significantly reduced after pre-treatment with GelE, very likely due to PAR2 desensitization. This is the first report describing neural excitation by a bacterial protease. It appears that GelE actions involve PAR2 expressing pathways which, however, await further confirmation.

Proteases, which are released from various cell types including epithelial cells, immune cells, blood cells or microbiota are central players that send signals to enteric neurons, which then

integrate the information arising from the enteric nervous system (ENS), blood or intestinal lumen. Proteases of interest are the blood derived thrombin, the mast cell tryptase and proteases released from pathogenic, commensal or probiotic bacteria. The effects of proteases are mediated through protease-activated receptors (PARs). The four known PARs - PAR1, PAR2, PAR3 and PAR4 - are G-protein coupled tethered ligand receptors, which are activated by proteolytic cleavage of the N-terminal ligand. Synthetic peptides, known as PAR-activating peptides (PAR-APs), mimic the action of the PAR-specific ligands and selectively activate PAR1, PAR2 or PAR4. PARs are expressed in the gastrointestinal tract by enteric neurons, glia, epithelial cells and smooth muscle cells. Excessive release of proteases has been reported in functional and inflammatory bowel diseases. Several animal studies detected an involvement of neural PARs and particular PAR2 in inducing hyperalgesia and inflammation. Neural actions of proteases in the human ENS are unknown; therefore the main aims were to demonstrate the effects of PAR-APs in the human and guinea-pig submucous plexus. PAR1-AP evoked a prominent spike discharge and $[Ca]_i$ transients in the majority of human submucous neurons and glia, whereas PAR2 and 4-APs induced significant weaker responses in a minor population. In the guinea-pig submucous plexus it was PAR2-AP, which induced neural and glia activation, whereas the PAR1-AP exhibited a much lower neural activation. The PAR1 antagonist SCH79797 dose dependently inhibited the PAR1-AP evoked neural and glia responses. In the guinea-pig the PAR2 antagonist LIGK-NH_2 significantly reduced the neural and glia responses. Both results supported the specific actions of PAR1- and PAR2-APs. In agreement with their actions in the ENS the PAR1-AP, but not PAR2-AP, induced a nerve mediated secretion in human mucosa/submucosa preparations, which was inhibited by SCH79797. The PAR4-AP had no pro-secretory action which corresponds to the weak effect in the ENS. In the human, but not in the guinea-pig submucous layer, PAR2-AP evoked $[Ca]_i$ signals in CD68-positive macrophages. The endogenous protease thrombin specifically induced a strong PAR1 activation of human submucous neurons and glia, whereas tryptase caused only a weak neural excitation. In summary the results demonstrate for the first time that PAR1 rather than PAR2 or PAR4 activated nerves and glia in the human submucous plexus.

Mucosal biopsy supernatants from IBS patients have been shown to excite enteric neurons. Serotonin, histamine and proteases have been identified as the main excitatory mediators. By using the PAR1 antagonist SCH79797 a significant reduction of the IBS supernatant induced excitation was detected, which supported the role of PAR1 in mediating the neural action of

proteases, released by the IBS mucosal biopsies. These findings further emphasize the need to focus on PAR1 for future studies on neural PARs mediated actions in the human intestine and to consider PAR1 as a drug target in gut diseases associated with increased protease levels such as IBS.

1. Introduction

Research in the field of neurogastroenterology began about a century ago after the first description of enteric neurons organised in ganglionated plexuses within the wall of the intestine by Auerbach (1862) and Meissner (1857) and the first functional discovery of the enteric nervous system by Bayliss and Starling (1899). Bayliss and Starling isolated a loop of dog intestine and by increasing the pressure within the loop they could cause contractions, which pushed the intestinal contents forward. They called this phenomenon 'the law of intestine', which is today known as the peristaltic reflex. After the disconnection of all nerve fibres between the gut and the central nervous system (CNS), the peristaltic reflex was unaffected. This observation led them to the conclusion that an autonomic nervous system must exist within the gut wall. The anatomical and functional resemblance of the enteric nervous system regarding neurons, glia and neurotransmitters with the central nervous system created the expression 'second brain' (Gershon 1998). Since the early discovery of the enteric nervous system (ENS) many research areas, like the one regarding gastrointestinal motility, secretion or neuro-immune interaction under physiological and pathophysiological conditions emerged. Alterations of the ENS are associated with gastrointestinal inflammatory diseases, like inflammatory bowel disease (IBD) and functional diseases, such as irritable bowel syndrome (IBS) (Lomax et al. 2005; Mawe et al. 2004; Ohman and Simren 2010). IBS, which affects 10-15% of the population in developed countries (Johanson 2004; Camillileri 2005) and in general, mostly women (Gloro et al. 2005) is characterized by continuous or remittent abdominal discomfort/pain, bloating, and abdominal distension, associated with altered bowel function, like diarrhea, constipation or altered diarrhea and constipation.

The human enteric nervous system contains about 100 million neurons and exhibits many different types of neurons, which are differently distributed and have functional region and species differences (Timmermans et al. 1990). Neurons of the enteric nervous system have been classified according to their morphological, neurochemical, electrophysiological or functional properties. With regard to morphology they have been classified into Dogiel type I, II or III neurons (Furness 2000) and electrophysiologically into AH- and S-type neurons. The functional classification distinguishes sensory neurons, interneurons, motoneurons and intestinofugal neurons. In general the enteric neurons are organised in ganglionated plexuses. The two major ganglionated plexuses are the Auerbach's or myenteric plexus and the Meissner's or submucous plexus. The myenteric plexus is located between the longitudinal

and circular muscle layers from the oesophagus to the rectum. The submucous plexus is situated between the mucosa and the circular muscle layer. In the human, the submucous plexus consists of three layers: an inner plexus located at the serosal side of the muscularis mucosae (Meissner's plexus) and an outer plexus (Schabadasch's) adjacent to the circular muscle coat and a third intermediated plexus between Meissner's and Schabadasch's plexus (Furness JB 2000; Timmersmanns et al. 1992; Costa et al. 2000; Furness 2006a). The ganglia of the submucous plexus in small mammals, like guinea-pigs, most closely resemble those of the inner submucous plexus of larger species, like humans (Furness 2006a). The submucous plexus is only found in the small and large intestine. Although scattered ganglia are found in the submucous layer in the oesophagus and stomach, these do not form a ganglionated plexus comparable to that of the intestine (Furness 2006a). The neuronal density of the myenteric plexus is higher compared to the one of the submucous plexus (Wood et al. 1999). Additionally, the intraganglionic fibre tracts of the submucous plexus are finer and ganglia are smaller than those of the myenteric plexus (Timmermanns et al. 2001). In addition to neurons, enteric glia are part of the enteric ganglia. The majority of enteric glia is found in the myenteric and submucous plexus ganglia (Wedel et al. 1999; Mestres et al. 1992). Glia cells are also present in the interconnecting nerve strands of the ganglionated plexuses (Gershon and Rothmann 1991; Gabella 1981) and in the mesentery accompanying the extrinsic nerves to the gut (Bjorklund et al. 1984). In the human submucous plexus the glia to neuron ratio is 1.3 to 1.9 and in the guinea-pig submucous plexus 0.8 to 1.0 (Hoff et al. 2008). Enteric glia, similar to their CNS counterparts, are a predominant cell type in the ENS and express glial fibrillary acidic protein (Jessen and Mirsky 1980) and the S-100 Ca^{2+} binding protein (Ferri et al. 1982; Hanani and Reichenbach 1994), which are both typical markers of CNS astrocytes (Furness 2006b). Furthermore enteric glia share many morphological, molecular and electrophysiological (Hanani et al. 2000) features with CNS astrocytes. Enteric glia are involved in the regulation of physiological functions such as neurotransmission, motility, and epithelial barrier as well as in pathophysiological conditions such as intestinal inflammation (Bassotti et al. 2006; Neunlist et al. 2007; Savidge et al. 2007). Regarding neuro-glia interaction, it has been reported that enteric glia respond to a variety of neuroligands, like ATP, serotonin, histamine and PAR-agonists with an increase of intracellular Ca^{2+} $[Ca]_i$ transients (Garrido et al. 2002; Kimball and Mulholland 1996).

The anatomical proximity in the intestine serves as the basis for a bi-directional interaction between neurons and glia with immune cell-, blood- and lumen-derived factors. The neuro-

immune interaction has been shown to play an important role in the physiology and pathophysiology of the gut. Several immune cells, like polymorphonuclear leucocytes, lymphocytes, macrophages and mast cells are present in the intestinal mucosa, lamina propria and smooth muscle, where they are in close proximity to enteric neurons, vagal nerve fibers and spinal sensory nerves (Stead et al. 1987; Gottwald et al. 1995; Wiliams et al. 1997; Stead et al. 1989, Barbara et al. 2004). After antigen recognition, mast cells release a variety of mast cell mediators, such as histamine, mast cell tryptase, prostaglandins, leukotrienes, pro-inflammatory cytokines (IL-1β, IL-6) and serotonin (5-HT), which act on various cells including the enteric neurons (Wood 1993; Liu et al. 2003). Many electrophysiological studies have provided evidence that a single inflammatory mediator can elicit a direct excitatory action on enteric neurons (Frieling et al. 1997; Mawe et al. 2004; Sharkey and Mawe 2002). Of importance are histamine (Bruenig et al. 2007) and tryptase (Reed et al. 2003; Gao et al. 2002), which are major inflammatory mediators released by mast cell degranulation. Additionally, Schemann et al. (2005) showed that a mediator cocktail released from IgE stimulated human intestinal mast cells excited human submucous neurons. These degranulated mediators (e.g. bradykinin, 5-HT (Bueno et al. 1997)) have been further shown to activate vagal and spinal afferent neurons to induce hyperalgesia/pain (Coelho et al. 1998), to sensitize silent nociceptors (Jiang et al. 2000; Kirkup et al. 2003) and to induce motility disorders (Collins 1996).

Proteases, which are released in the gut from various cell types, such as epithelial cells, immune cells (e.g. mast cells), blood cells or microbiota, are central players in these neuro-immune / neuro-blood and neuro-lumen interactions. Proteases of interest are for example the blood derived thrombin, the mast cell derived tryptase, the neutrophil derived cathepsin G or luminal proteases from pathogenic or commensal bacteria. Excessive concentrations of proteases in the gut or in the stool have been observed in inflammatory states as well as in IBS patients. There is compelling evidence that proteases function, besides their role in digestive degradation and wound healing, as signalling molecules and many of their effects are mediated through activation of protease-activated receptors (PARs) (Vergnolle 2005; Hollenberg 2010). The four known PARs - PAR1, PAR2, PAR3 and PAR4 (Vu et al.1991; Nystedt et al. 1995; Ishihara et al. 1997; Kahn et al. 1998; Xu et al. 1998) are G-protein coupled receptors which are characterised by a unique mechanism of activation. Serine proteases, such as thrombin, trypsin or mast cell tryptase, cleave an extracellular domain of the receptor, which frees a tethered ligand that binds intramoleculary and activates the

receptor (Hollenberg and Compton 2002). PAR1, PAR3 and PAR4 are predominantly activated by thrombin. Mast cell tryptase and trysin activate PAR2. PAR4 is activated by thrombin and trypsin. Cathepsin G, released by neutrophiles, activates PAR4. Short synthetic peptides, known as PAR activating peptides (PAR-APs), correspond to the tethered ligand released upon cleavage of the PARs and specifically activate PAR1, PAR2 or PAR4 thereby mimicking the action of the rather non-selective endogenous PAR activators thrombin, trypsin or tryptase. So far, no PAR3-AP has been described (Reed et al. 2003; Covera et al. 1999; Linden et al. 2001; Gao et al. 2002). Endogenous activators of PARs as well as PAR-APs show strong tachyphylaxis due to cleavage, internalization and lysosomal degradation of the receptor. Once activated, PARs induce different G-protein coupled intracellular signalling pathways. PARs stimulate, amongst others, a PLC- and IP_3- dependent increase of intracellular calcium (Macfarlane and Plevin 2003).

PAR1, 2 and 4 are expressed in the gastrointestinal tract by different cell types, such as endothelial cells (Vergnolle 2005), epithelial cells (Buresi et al. 2001; Cenac et al. 2002; Mall et al. 2002), smooth muscle cells (Kawabata et al. 2004; Al-Ani et al. 1995), enteric neurons (Covera et al. 1999; Reed et al. 2003; Gao et al. 2002; Linden et al. 2001), extrinsic afferent neurons (Kirkup et al. 2003) and enteric glia (Garrido et al. 2002). PARs are also expressed by intestinal immune cells including dendritic cells, mast cells, macrophages and T cells (Colognato et al. 2003; Li et al. 2008; Mari et al. 1996), these immune cells are also a rich source of proteases. PARs are not only expressed in the intestine, but also in other organs, such as the brain, kidney, skin, pancreas, liver, bladder, lung and cardiovascular system, where they are important in physiology and pathophysiology, like arthritis, cancer, pancreatitis and neurodegenerative disorders (Multiple Sclerosis, Alzheimer's disease). Several PAR1 antagonists (e.g. E5555 atopaxar; Goto et al. 2010) have been developed as antiplatelet drugs and for the treatment of atherothrombotic, inflammatory, proliferative and neurodegenartive diseases (Cirino and Severino 2010). However, these drugs have exerted certain limitations including low efficacy or bioavailability. SCH530348 for instance is the first oral thrombin receptor antagonist selective for PAR1 in phase III of a clinical trial to proof its efficacy as an anti-thrombotic agent in humans (Hildemann and Bode 2009).

Enteric neurons and glia express PARs (Reed et al. 2003; Covera et al. 1999; Linden et al. 2001; Gao et al. 2002; Garrido et al. 2002). Proteases, like thrombin, trypsin and mast cell tryptase excite enteric neurons and increase intracellular calcium levels particularly through

activation of PAR1 and PAR2 (Reed et al. 2003; Covera et al. 1999; Linden et al. 2001; Gao et al. 2002). Most of these studies focused on the effects of PAR activators in rodent myenteric neurons where 40-80% responded to PAR-APs. Such systemic studies have not been performed in the submucous plexus, except for one study demonstrating an excitatory action of mast cell tryptase and PAR2-AP (Reed et al. 2003). The effects of PAR1 and PAR4-APs in the guinea-pig submucous plexus are not known.

PAR2 has received much attention recently as visceral and somatic nociception, inflammation as well as intestinal permeability is attenuated in PAR2 knock out mice (Cenac et al. 2002; Cenac et al. 2007; Vergnolle et al. 2001). Additionally, it has been shown that PAR2-AP induced intestinal barrier breakdown, long lasting visceral hyperalgesia, allodynia (Coelho et al. 2002) or neurogenic inflammation (Steinhoff et al. 2000; Nguyen et al. 2003; Cenac et al. 2002; Cenac et al. 2004). Likewise, supernatants of colonic biopsy samples of IBS patients release mediators that directly sensitize murine sensory neurons and generate visceral and somatic hypersensitivity through the activation of PAR2 (Cenac et al. 2007). Also fecal supernatants of diarrhea-predominant irritable bowel syndrome (D-IBS) patients, which exhibit increased serine protease contents, induce allodynia and increase paracellular permeability via PAR2 (Gesce et al. 2008). These data from animal models support an important role of PAR2 as pro-inflammatory and pro-algesic mediator. Despite the strong focus on PAR2, also PAR1 and PAR4 are extensively studied *in vitro* and *in vivo*. PAR1 and PAR4 agonists, identical to PAR2 agonists, evoke depolarizing responses in guinea pig myenteric neurons (Gao et al. 2002), increase intestinal permeability (Chin et al. 2003) and induce inflammation (Dabek et al. 2009; Dabek et al. 2010). In contrast to PAR2, sub-inflammatory doses of PAR1 and 4 exert an analgesic effect by suppressing somatic and visceral hyperalgesia and pain (Asfaha et al. 2002; Asfaha et al. 2007; Vergnolle et al. 2003; Coelho and Bunnett 2003; Auge et al. 2009).

It has been previously reported that activation of PAR1 and PAR2 affects intestinal chloride secretion (Cuffe et al. 2002; Mall et al. 2002; Green et al. 2000) in a nerve-dependent as well as nerve-independent manner (Coelho et al. 2002) suggesting a role for these receptors in intestinal secretion. *In vitro* studies have shown that differences in the secretion mechanisms are regional and species dependent. In the rat jejunum (Vergnolle et al. 1998), in the mouse colon (Cuffe et al. 2002) as well as in human rectal mucosal biopsies (Mall et al. 2002) the basolateral PAR2 stimulation induce a nerve independent Cl^--secretion, whereas in the pig

ileum opiod sensitive enteric neurons mediate the ion transport (Green et al. 2000). In a human duodenal epithelial cell line (SCBN) (Buresi et al. 2001) the basolateral application of PAR1 agonists induce Cl^--secretion, whereas in human colon (Mall et al. 2002) and in rat jejunum (Vergnolle et al. 1998) the PAR1 agonist thrombin exerts no pro-secretory action. In the mouse caecum it has been shown that PAR1 and PAR2 stimulate Cl^--secretion, but only the PAR1 induced secretion is nerve-dependent (Ikehara et al. 2010). PAR4, which is expressed in the small and large intestine (Xu et al. 1998) and in the epithelium (Ferazzini et al. 2003), did not induce any ion transport in human rectal mucosal biopsies (Mall et al. 2002).

Proteases and PARs appear to be particularly relevant in the pathology of inflammatory and functional bowel diseases. Excessive concentrations of proteases have been found in the stool of patients with ulcerative colitis (UC) or IBS (Roka et al. 2007; Annahazi et al. 2009; Bustos et al. 1998; Cenac et al. 2007). Additionally, an increased expression of PAR2 in UC patients has been detected (Kim et al. 2003). Moreover, mast cells in mucosal biopsies from IBS patients release more tryptase than those from control patients (Cenac et al. 2007; Barbara et al. 2004). The functional relevance of enhanced protease levels has been demonstrated by studies which investigated the neural actions of mucosal biopsy supernatants from IBS patients. These supernatants activated human submucous neurons (Buhner et al. 2009) and rat visceral nociceptive neurons (Barbara et al. 2007). In both studies proteases predominantly contributed to the supernatant evoked nerve excitation, this was revealed by inhibition of protease activity in the supernatants. The above findings strongly suggest a role of PAR signalling in the human enteric nervous system and additionally suggest proteases and PARs as relevant factors in the pathophysiology of functional and inflammatory bowel disorders.

The functional bowel disorder IBS is characterized by continuous or remittent abdominal discomfort/pain, bloating, and abdominal distension, associated with altered bowel function, like diarrhea, constipation or altered diarrhea and constipation. Based on the bowel symptoms different IBS subtypes get defined with a predominance of constipation (C-IBS), diarrhea (D-IBS) or with alternating constipation and diarrhea (A-IBS). After an episode of gastrointestinal infection (e.g. bacterial gastroenteritis) 18% of these patients develop post-infectious IBS (PI-IBS), which is a pathophysiologically defined subtype of IBS (Spiller 2003; Collins et al. 2001). Additionally, IBS-like symptoms can be developed by patients in remission from IBD (Simren et al. 2002; Quingley 2005a). However, the organic basis for

IBS is still unknown, but recent studies provided some mechanisms involved in IBS pathophysiology. There are multiple factors contributing to IBS pathophysiology like motility disorders, visceral hypersensitivity, low-grade inflammation or immune activation, altered intestinal permeability, altered intestinal microbiota, abnormal CNS modulation and psychological factors (Gloro et al. 2005; Lee et al. 2010; Mayer and Collins 2002). Many studies reported visceral hypersensitivity in some IBS patients, which is characterized by decreased pain thresholds (allodynia) and increased nociceptive responses (hyperalgesia) to balloon distension in the rectum of IBS patients (Mertz et al. 1995; Bradette et al. 1994; Naliboff et al. 1997; Bouin et al. 2002). The underlying causes for this hypersensitivity are however still unknown, but low grade inflammation has been proposed in the pathogenesis of hypersensitivity (Collins et al. 2001; Bueno and Fioramonti 2002). Microscopic inflammation has recently also been suggested to be associated with the development of IBS symptoms in some patients (Tornblom et al. 2002; Barbara et al. 2007; Bercik et al. 2005; Collins et al. 2001; Spiller 2004; Andrews and Shaffer 2004; Chadwk et al. 2002; Gwee et al. 2003). Colonic biopsy specimens from IBS patients has been shown to exhibit increased mast cell numbers (O'Sullivan et al. 2000; Park et al. 2003) and increased tryptase content due to mast cell degranulation (Barbara et al. 2004). Moreover, the quantitative and qualitative composition of the fecal microbiota in IBS patients varies from healthy controls, which emphasizes the importance of an altered intestinal microbiota in the pathogenesis of IBS (Tana et al. 2009; Kerckhoffs et al. 2009; Kassinen et al. 2007). Furthermore to the changed intestinal microbial composition an increased amount of fecal serine-proteases has been detected in D-IBS patients (Roka et al. 2007; Gesce et al. 2008).

With regard to the afore mentioned pathophysiological factors and the fact that PARs are present in most cells that are potentially actors in the generation of IBS symptoms, it is important to consider luminal and endogen proteases and their receptors in IBS, especially as potential future therapeutic targets. In animal studies PAR2 seem to play an important role in IBS symptom generation, since PAR2 agonists are able to induce visceral hyperalgesia (Coelho et al. 2002), increase intestinal permeability (Cenac et al. 2004), induce intestinal inflammation (Cenac et al. 2003), facilitate intestinal transport (Kawabata et al. 2001) and induce intestinal chloride secretion (Cuffe et al. 2002), effects which are prevented by PAR2 antagonism or PAR2 knock out. The clinical relevance of these phenomena is still unclear and requires further studies in human intestine and patients. Regarding the altered microbiota in IBS patients (Madden and Hunter 2002; Malinen et al. 2005; Pimentel et al. 2000; Si et al.

2004; Balsari et al. 1982; King et al. 1998; King et al. 2002; Nobaek et al. 2000), probiotics also seem to be a promising therapeutic option inducing symptom relief in IBS patients. Accordingly, certain clinical trials and animal studies with single-organism or probiotic mixtures have been conducted.

Probiotics, which are defined as live organisms that, when ingested in adequate amounts, exert a health benefit on the host (Fuller R, 1991; Schrezenmeir J and de Vrese M, 2001) (according to the WHO/FAO definition in 2001) contain as the most common representatives *Lactobacilli*, *Bifidobacteria*, *Streptococci*, non-pathogenic *Escherichia coli* and non-pathogenic yeasts, such as *Saccharomyces boulardii*. Recent studies not only use live bacteria, but also dead organisms, bacterial DNA or supernatants of bacterial cultures to study probiotic action.

To date there have been a few clinical trials which showed that treatment with various probiotic bacteria, such as VSL#3 (Kim et al. 2003; Kim et al. 2006), *Lactobacillus plantarum* (Niedzielin et al. 2001; Sen et al. 2002) and *Bifidobacterium infantis* 35624 (O'Mahony et al. 2005; Whorwell et al. 2006) improved IBS symptoms, including pain/discomfort, distension/bloating and defecation. This has been further supported by various animal models, where different probiotics, including *Lactobacillus reuteri*, *Lactobacillus farciminis* and *Lactobacillus paracasei* NCC 2461 have been shown to reverse hypersensitivity (Verdu et al. 2006; Kamiya et al. 2006; Ait-Belgnaoui et al. 2006) and exert anti-inflammatory (Verdu et al. 2006), barrier-enhancing (Ait-Belgnaoui et al. 2006) and neuromodulatory actions. Further studies have indicated that probiotics have the potential to counteract the subtle changes in the mucosal immune system and low grade inflammation (Rodriguez and Ruigomez 1999; Collins et al. 2001) observed in IBS patients. For example, peripheral blood mononuclear cells from IBS patients revealed an abnormally low IL-10/IL-12 ratio (O'Mahony et al. 2005), which was reversed by the probiotic bacterium *Bifidobacterium infantis* 35624 (O'Mahony et al. 2005; Quingley 2005b) and VSL#3 (O'Mahony et al. 2005). The results of these human and animal studies suggest that probiotic treatment is a promising therapy for IBS. There are still remaining questions concerning their mode of action whether probiotics interact directly with target cells, whether probiotics release active compounds, such as bacterial proteases and what the target cells of probiotics are?

There are a few studies investigating the influence of probiotics on the enteric nervous system as a possible target. They showed that probiotic supplemented diet (*Saccharomyces boulardii* (Kamm et al. 2004), *Pedicoccus acidilactici* (Di Giancamillo et al. 2010)) changed the neurochemical code of enteric neurons, which seems to support the idea that the enteric nervous system is a possible target for probiotic bacteria and yeasts. But these studies did not reveal the underlying molecular mechanisms. A new study by Kunze et al. (2009) provided direct evidence that the probiotic *Lactobacillus reuteri* interacted with colonic enteric neurons. After 9 days ingestion, *Lactobacillus reuteri* increased the excitability of myenteric AH/Dogiel type II neurons by targeting an ion channel (IKCa), which finally contributed to motility changes of rat colon (Wang 2010). Besides this first report of a neuron-probiotic interaction, it remains to be elucidated whether specific probiotics or their soluble mediators (e.g. proteases) directly target enteric neurons and exert their positive effects via this way.

The dysregulated bacteria-host interaction in the development and progression of IBD make probiotics an interesting therapeutic approach. IBD is a chronic, spontaneously relapsing, immunologically mediated disorder of the gastrointestinal tract. IBD includes two major pathologies, Crohn's Disease (CD) and ulterative colitis (UC), which differ in pathogenesis, underlying inflammation profiles, symptoms and treatment strategies. CD is identified by transmural, granulomatous inflammation, which can spread throughout the whole gastrointestinal tract, but can be mainly found in the terminal ileum. UC exhibits a mucosal inflammation, which is restricted to the colon and generally begins in the rectum and spreads proximally, depending on the severity of the disease (Papadakis and Targan 2000). The aetiology of IBD is still unknown, but genetic (e.g. NOD2/CARD15 polymorphism (Clavel and Haller 2007; Hugo et al. 2001)), environmental (e.g. intestinal microflora, smoking) and immunological factors (e.g. altered cytokine release) are thought to play an important role in the disease development (Danese et al. 2004; Hanauer 2006; Bouma and Strober 2003). There is increasing evidence that IBD develops in genetically predisposed subjects due to dysregulated immune response towards the intestinal commensal microflora (Strober et al. 2007). It seems that IBD patients lost the normal immune tolerance to commensal bacteria (Duchmann et al. 1995). Restoration of a balanced gastrointestinal microbiota and following reduction of the intestinal inflammation are major aims in the treatment of IBD. Therefore probiotics as modulators of the gastrointestinal flora have come into focus as potential therapeutic agents.

Probiotic therapy is focused on induction and maintenance of remission in UC, CD or pouchitis (postoperative inflammation of ileal pouch). Several studies have highlighted the potential of different probiotic strains, such as *Escherichia coli* (*E.coli*) Nissle 1917 (Kruis et al. 2004; Kruis et al. 1997; Rembacken et al. 1999), *Lactobacillus* GG (Zocco MA et al, 2006) and VSL#3 (Venturi et al. 1999; Tursi et al. 2004) to induce and maintain the remission of UC. Additionally, VSL#3 showed to be effective in treating chronic relapsing or refractory pouchitis and to avoid occurrence of pouchitis after proctocolectomie (Gionchetti et al. 2003; Pronio et al. 2008; Gionchetti et al. 2000; Mimura et al. 2004; Venturi et al. 1999). In contrast to these promising results probiotic intervention (*E.coli* Nissle 1917 (Malchow 1997), *Saccharomyces boulardii* (Guslandi et al. 2000) was found to be ineffective in CD patients. Although probiotic efficacy in the prevention and treatment of several IBD indications has been reported in clinical studies, the active components/structures, the molecular mechanisms or the primary probiotic target (e.g. cell types, like neurons, epithelial cells and region, like inflamed vs uninflamed) remain to be clarified.

Proteases, either from bacterial or endogenous (e.g. inflammatory cells) origin, play an important role in the pathogenesis of chronic intestinal inflammation. Gelatinase (GelE) a secreted metalloprotease from *Enterococcus faecalis* OG1RF, a commensal lactic acid bacterium of the human gastrointestinal tract (Tannock 2002), induces colitis in germfree IL-$10^{-/-}$ mice by targeting barrier regulating proteins of tight and adherence junctions and thus impairing the epithelial barrier function (Steck et al. 2009; Steck et al. 2011), whereas GelE exerts no barrier braking effects in WT mice. The barrier impairment leads to an enhanced translocation of antigens which triggers an inflammatory response. This barrier breakdown also facilitates the penetration of bacterial proteases into the subepithelial area and enables proteases access to the submucous ganglia. This result supports the notion that bacterial proteases target intestinal epithelial cells to induce inflammation in a genetically susceptible host. Proteases from endogenous sources, such as immune cells, have been shown to activate enteric neurons via PARs and to mediate inflammatory conditions. However, the potential of luminal bacterial proteases from pathogenic, commensal or probiotic origin to stimulate enteric neurons has not yet been studied. However, the bacterial protease gingipain released from the pathogenic bacteria *Porphyromonas gingivalis* has been shown to cleave and activate PAR2 on human neutrophils (Lourbakos et al. 1998). This raises the question of whether the commensal protease GelE can target enteric neurons and if this effect is mediated via PARs.

All functions described to be altered by proteases and/or probiotics are regulated by the ENS and it is likely that the ENS acts as an interface for their mode of actions. Hence, the aim of our study was to investigate the effect of probiotics and proteases on the enteric nervous system from mouse, guinea-pig or human.

There are many clinical and experimental studies supporting a positive effect of probiotics in IBS and IBD, but there are just a few studies examining the enteric nervous system as a possible target for probiotics. Since probiotics may release proteases and/or protease inhibitors, the neural actions of soluble factors released by probiotic bacteria was of interest.

(1) Therefore, one aim of the project was to determine whether soluble factors released by probiotic bacteria (*Lactobacillus (L.) paracasei* NCC 2461, *Bifidobacterium (B.) longum* NCC 3001) are able to directly activate guinea-pig enteric neurons and to elucidate the respective molecular mechanism.

$TNF^{\Delta ARE/WT}$ mice are an experimental model for Crohn's-like intestinal bowel disease. These mice develop an ileal inflammation due to overproduction of the pro-inflammatory cytokine TNFα mediated by the deletion of the TNF AU-rich elements (ARE) (Kontoyiannist et al. 1999), whereas the colon stays macroscopically uninflamed. Since probiotics have been shown to influence mucosal barrier function and to modulate intestinal secretion, $TNF^{\Delta ARE/WT}$ and WT mice were fed with the probiotic mixture VSL#3 (*L. acidophilus, L. bulgaricus, L. casei, L. plantarum, Streptococcus thermophilus, B. breve, B. infantis, B. longum*) or placebo.

(2) The first aim of these experiments was to study the neural and epithelial mediated secretory potential and the transepithelial resistance of the colon of $TNF^{\Delta ARE/WT}$ mice compared to WT mice without probiotic intervention.

(3) A further aim was to determine whether probiotic feeding of VSL#3 affects colonic secretion and permeability.

Endogenous proteases, like thrombin, trypsin, mast cell tryptase and bacterial proteases are discussed in the pathophysiology of functional and inflammatory bowel disorders. Endogenous proteases have been shown to activate enteric neurons via PAR receptors, but the potential of bacterial proteases to excite enteric neurons has not yet been studied. GelE, a protease released by the commensal bacterium *Enterococcus faecalis* OG1RF, which has been shown to induce inflammation in a genetic susceptible host, was used

(4) to study the effect of a bacterial protease on submucous neurons of human and guinea-pig submucous plexus and to elucidate the signalling mechanism.

Findings in animal experiments strongly suggest a role of PAR signalling in the human enteric nervous system. While functional consequences of activation of neural PARs have been reported in several animal models there are no data on the effect of PAR activation in the human enteric nervous system. Therefore the major aim was to demonstrate the effects of PAR-APs in the human submucous plexus:

(5) by investigating if PAR1, PAR2, or PAR4-APs modulate nerve or glia cell activity and

(6) by studying the effects of PAR1, PAR2 and PAR4-APs on mucosal ion secretion in human intestine.

Complementary results from guinea-pig submucous plexus will be reported for two reasons. Firstly, there are no systematic studies on effects of PAR1, PAR2 and PAR4-APs in guinea-pig submucous neurons and, secondly, to emphasize with this comparative study the human specific modes of actions of PAR-APs.

(7) Furthermore the neural action of the endogenous proteases thrombin and tryptase and

(8) the expression of PAR1 and PAR2 using immunohistochemistry will be examined.

Supernatants from mucosal biopsy specimens of IBS patients, which contain histamine, serotonin and serine proteases as main mediators, have been shown to excite enteric neurons (Buhner et al. 2009). The mediator cocktail of C-IBS and D-IBS biopsies evoked the same neuronal excitation and the serotonin, histamine and tryptase levels in the supernatants correlated with the supernatant induced magnitude of spike discharge.

(9) The aim was to identify the underlying PAR receptor mediating the protease induced neuronal action by the IBS supernatant. Since PAR1 has been shown to be the most important receptor in the human enteric nervous system, the IBS supernatants were applied in the presence of the PAR1 antagonist SCH79797.

2. Material and Methods

2.1 Tissue samples and tissue preparations

Human samples

Human tissue samples of large and small intestine were obtained from 145 patients (71 female, 74 male, 64.1 ± 1.2 years (mean ± SEM)) undergoing surgery at the Medical Clinics in Freising, Rechts der Isar in Munich and Großhadern in Munich. Samples were taken from macroscopically unaffected, non-damaged areas. Patients receiving surgery had been previously diagnosed with carcinoma (99), diverticulitis (15), polyps (5), fistula (3), Morbus Crohn (3), chronic pancreatitis (4), ileus (3), stenosis (4), stoma (5), perforation (2), rectum prolapse (1), endometriosis (1). Recordings of nerve and glia activity or mucosal secretion were performed on 60 preparations from the small intestine and 85 preparations from the large intestine. Procedures were approved by the ethics committee of the Technische Universität München (1748/07 and 2595/09) and of the Ludwig-Maximilians-Universität (tissue samples were obtained and experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR, with the informed patient's consent). After removal specimens were placed in ice cold oxygenated sterile Krebs solution and immediately transferred to the institute. Segments were dissected in oxygenated (95% O_2 and 5% CO_2, Carbogen (*Westfalen Gas AG, Münster, Germany*), pH = 7.4) ice cold Krebs solution containing (in mM): 117 NaCl, 4.7 KCl, 1.2 $MgCl_2.6H_20$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2.2H_20$, 11 glucose, to obtain mucosa/submucosa preparations containing the submucous plexus for Ussing Chamber experiments and the inner submucous plexus for the neuro imaging experiments.

Guinea-pig samples

Tissue samples were obtained from 98 male guinea-pigs (Dunkin Hartley, Harlan Winkelmann, Borchen, Germany). The animals were kept under standardised conditions at the institute at least one week before the start of the experiments (isolation board, *Uni-Protect, Ehret, Emmendingen*). Animals received standard food (*Altromin Spezialfutter GmbH & Co. KG, Lage, Germany*) and drinking water *ad libitum* and were kept at 20-24°C room temperature, 60% humidity and a day night cycle of 14:10 hrs. The weight of the guinea-pigs at the time point of killing was 353.6 ± 6.3g (mean ± SEM). The animals were killed by cervical dislocation and exsanguination in accordance with the german ethical

guidelines for animal experiments. Colonic segments were removed for the imaging experiments. Segments were dissected in ice cold oxygenated Krebs solution. The mucosa, the circular and longitudinal muscle and the myenteric plexus were removed in order to obtain the submucous plexus for the neuro imaging experiments with the use of microscissors (*FST #14058-11, Fine Science Tools*) and forceps (*Dumostar #5, Dumont, Switzerland*).

Mice samples

The probiotic feeding experiments were performed in collaboration with the Chair of Biofunctionality, ZIEL-Research Centre for Nutrition and Food Science, Technische Universität München, Germany (with kind support by G. Hörmannsperger). They conducted the feeding studies and provided us with the proximal and distal colon of the mice. Conventionally raised $TNF^{\Delta ARE/WT}$ mice on C57BL/6 background and wildtype (WT) C57B/6 mice were fed $1.3x10^9$ cfu of VSL#3 bacteria in 13.2% (w/v) gelatine, 20% (w/v) glucose in water every weekday from day one after birth for 18 weeks in a pre-weaning experimental setup with additional feeding of the mother mice (as soon as plaque was detected until the end of the weaning period). The offspring were fed with liquid VSL#3-suspension that was prepared freshly every day for the first two weeks after birth. The gelatine was prepared freshly every third day. Placebo fed mice were used as controls. The mice were kept under standard conditions with 23-25°C room temperature, 40-60% humidity and a 12 hrs day/night rhythm. Previous experiments performed in the laboratory showed that $TNF^{\Delta ARE/WT}$ mice develop chronic inflammation in the distal ileum 18 weeks after birth (Kontoyiannis et al. 1999). $TNF^{\Delta ARE/WT}$ mice were used as a model for experimental ileitis. Mice were killed at the age of 18 weeks and the proximal and distal colon was removed. Colonic segments were dissected in ice cold oxygenated Krebs solution. The circular and longitudinal muscle and the myenteric plexus were removed in order to obtain mucosa/submucosa preparations of the distal and proximal colon for the Ussing Chamber experiments. The placebo fed WT mice had a mean weight of 28.6g at the time point of killing (n = 8), the VSL#3 fed WT mice of 25.4g (n = 5), the placebo fed $TNF^{\Delta ARE/WT}$ mice of 22.9g (n = 8) and the VSL#3 fed $TNF^{\Delta ARE/WT}$ mice of 24.5g (n = 9). The weight did not differ significantly between the different treatment groups and genetic background.

2.2 Imaging activity of enteric neurons and glia using voltage- and calcium-sensitive dyes

2.2.1 Principle of the neuro imaging technique

The neuro imaging technique (mulit-site optical recording technique, MSORT) used to study neural and glia activation by using voltage- and calcium-sensitive dyes has been previously described in detail (Schemann et al. 2002; Michel et al. 2005; Schemann et al. 2005; Neunlist et al. 1999). The neuro imaging technique allows direct and simultaneous recordings of neuronal activity from all neurons of a given ganglion with a high spatial and temporal resolution (Schemann et al. 2002). Calcium imaging is a more general reporter of cell activation allowing glia activation and neuronal activation unrelated to action potential discharge to be assessed.

The activity of enteric nerves can be monitored by recording their membrane potential changes or changes in intracellular Ca^{2+} $[Ca]_i$ level. The activity of enteric glia can be detected by changes in $[Ca]_i$. Activated nerve cells fire action potentials, which are detected by the voltage-sensitive dye (VSD) Di-8-ANEPPS (1-(3-sulfanato-propyl-4-[β-[2-(di-*n*-octylamino)-6-naphthyl]vinyl] pyridinium betaine; *Molecular Probes, Eugene, OR, USA*). The VSD incorporates into the plasma membrane of enteric neurons and changes its dye absorption and emission spectra with the membrane potential (Fromherz and Lambacher, 1991). Neurons and glia respond to certain stimuli with an increase of the $[Ca]_i$ level, via Ca^{2+} influx through voltage gated calcium channels or through release from intracellular calcium stores. The calcium-sensitive dye (CSD) Fluo-4 AM (*Invitrogen, Darmstadt, Germany*) binds free cytosolic Ca^{2+}, whereby the fluorescent intensity increases on Ca^{2+} binding. These fluorescent changes are recorded by CCD chip technology.The optical data generated by these techniques are then transformed into electrical data.

<u>Advantages and disadvantages of the neuro imaging technique (Schemann et al. 2002)</u>

The main advantage of the neuro imaging technique is the possibility to record simultaneously, and with high spatial and temporal resolution, neuronal responses of all cells of a given ganglion (Neunlist et al. 1999). This possibility allows following network activity. This method is non-invasive providing a means to study neurons, which would ordinarily be too small to be impaled by microelectrodes for intracellular recordings without changing their physiological properties.

However, there are also limitations to this neuro imaging technique. The signal to noise ratio does not allow small changes in membrane potential to be detected (Neunlist et al. 1999). Using a local microejection technique to apply the VSD through a micropipette directly into the ganglion, improves the signal to noise ratio. A further improvement can be the use of CSDs instead of VSDs, because of their higher signal to noise ratio. Due to the phototoxicity and bleaching of the dye the recordings are limited to a certain timeframe. Using the VSD Di-8-ANEPPS only short recording periods of up to 4s are possible. The time-course of intracellular calcium signals is generally slower and therefore the recording frequency (40 Hz vs 1 kHz) can be reduced and a longer recording timeframe (up to 20s) is possible. However, as with the VSD phototoxicity and bleaching need to be taken into account. Furthermore another difficulty using Di-8-ANEPPS is that only action potential discharge, but not subthreshold depolarisation can be detected. An additional feature of the calcium imaging is the possibility to study enteric glia.

2.2.2 Experimental procedure of the neuro imaging technique

The submucous plexus preparations of the guinea-pig and human intestine were pinned onto a Sylgard ring (5 x 10mm; *Dow Corning, Midland, MI*) and placed into a recording chamber with a 42-mm diameter glass bottom (130- to 170µm thickness; *Sauer, Reutlingen, Germany*). For the calcium imaging experiments the tissue was stained before transferring into the recording chamber. The recording chamber was continuously perfused with 37°C warm Krebs solution, containing (in mM): 117 NaCl, 4.7 KCl, 1.2 $MgCl_2.6H_20$, 1.2 NaH_2PO_4, 20 $NaHCO_3$, 2.5 $CaCl_2.2H_20$, 11 glucose and was carbonated with 95% O_2 and 5 % CO_2 (pH = 7.4). The perfusing Krebs solution was circulated at a speed of 20mL/min from a reservoir (1L) to the chamber. The plastic tubes (*Tygon R3607*) connecting the pump with the chamber had a diameter of 2.79mm. The temperature (37°C) and pH (7.4) were kept stable during the experiments.

Staining method with the voltage-sensitive dye Di-8-ANEPPS

To study membrane potential changes individual ganglia were stained with the fluorescent VSD Di-8-ANEPPS by intraganglionic dye application through a microejection pipette (Figure 1). The glass pipettes (GB100F-10; *Science products, Hofheim, Germany*) were pulled with a Flaming/Brown micropipette puller (*Sutter instrument Co., Novato, CA, USA*).

The glass pipettes were loaded with 20µM Di-8-ANEPPS dissolved in DMSO (final concentration, 0.125%), Pluronic F-127 (final concentration, 0.014%) and Krebs solution. The filled pipettes were gently positioned onto a submucous ganglion and pressure ejection pulses were used to apply the dye into the ganglion. The pressure ejections were performed with a pressure ejection system (*PDES-2L, npi electronic GmBH, Tamm, Germany; A310 accupulser, World precision instruments, Inc. Sarasota, FL, USA*). The ejection pulses lasted for 200-800ms with a pressure of 0.5bar. The staining was followed by a 5-10min incubation time to allow the dye to incorporate into the plasma membrane before starting the experiments. Neunlist et al. (1999) have shown that the dye does not change the electrophysiological properties of the nerve cells.

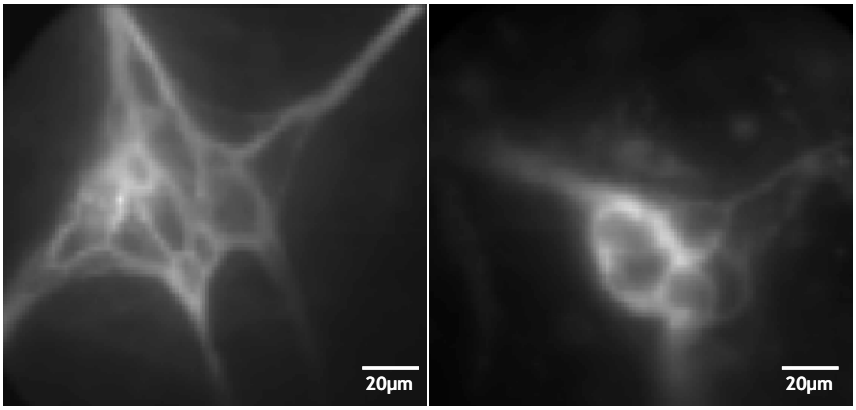


Figure 1: Di-8-ANEPPS staining of a guinea-pig and human submucous ganglion: The individual cells can be identified since the dye incorporates into the plasma membrane of the enteric neurons, revealing the outline of the neurons of guinea pig (left) and human (right) ganglia.

Staining method with the calcium-sensitive dye Fluo-4 AM

To study changes of $[Ca]_i$ the submucous plexus of human and guinea-pig was pinned onto a sylgard ring and covered with 1,3ml of a 30µM Fluo-4 AM solution, dissolved in Krebs solution, in a tissue culture dish (35/10mm, *cellstar, greiner bio-one, Frickenhausen, Germany*). The dye was loaded for 2hrs at room temperature in the dark and was continuously bubbled with carbogen. Afterwards the preparation was transferred into 100mL oxygenated Krebs solution at room temperature, in the dark for an additional 1hrs to cleave the AM from the Fluo-4 AM (Wunderlich et al. 2008). The ring was then transferred into the recording chamber perfused with oxygenated Krebs solution at 37°C and left for an equilibration period of 20min before the experimental protocol was started (Figure 2).

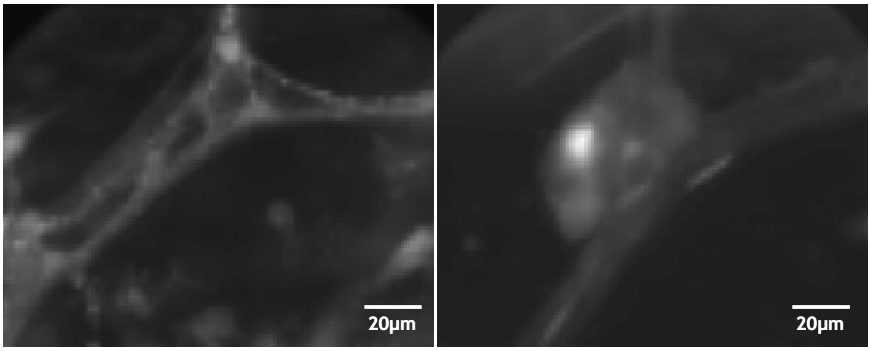
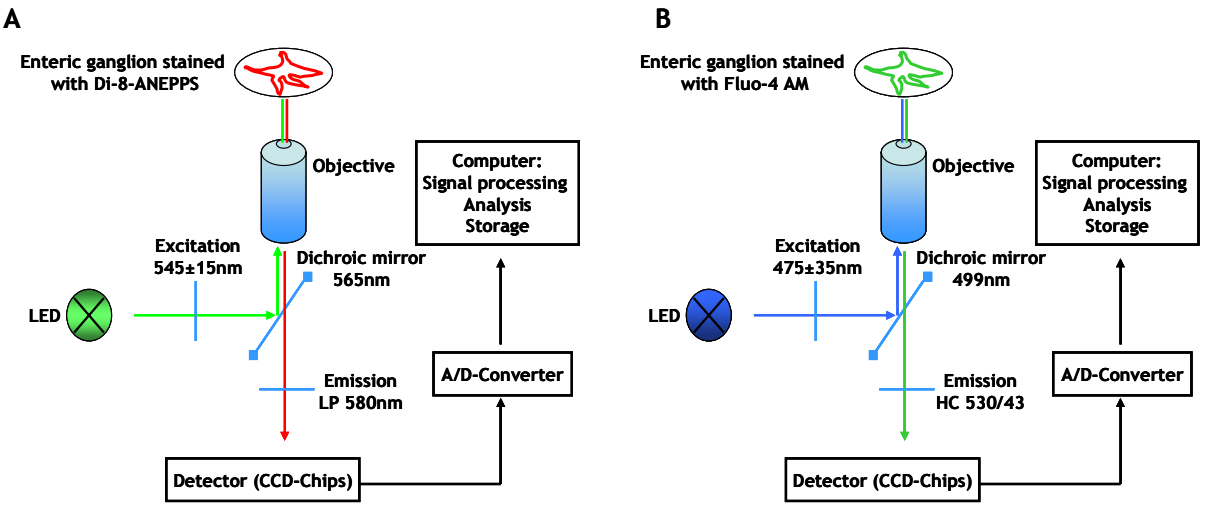


Figure 2: Fluo-4 AM staining of a guinea-pig (left) and human (right) submucous ganglion.

Optical recording method:

The experiments were done on an inverted microscope (Zeiss Axio Observer.A1 microscope; *München, Germany*) with the NeuroCCD SM system from RedShirtImaging (*Decatur, USA*). The NeuorCCD SM system consists of a fast cooled charge-coupled device (CCD) camera, a computer and the Neuroplex software for data acquistion and analysis. To detect Di-8-ANEPPS signals a modified Cy3 filterset was used (545±15nm excitation interference filter, 565nm dichroic mirror and 580nm emission, *Ahf Anaysentechnik, Tübingen*, Germany). For the Fluo-4 AM a FITC filterset was used (excitation: HD475/35, dichroic: BS499 and emission: HC 530/43; BrightLine FITC HC Basic BP-Filterset, *Ahf Anaysentechnik, Tübingen, Germany*). A blue LED (3W blue and 3W royal blue, *Philips Lumiled, Conrad, München, Germany*) or a green LED (LE T S2W and LE T A2A, *Osram, München, Germany*) was used to excite Fluo-4 AM or Di-8-ANEPPS, respectively (Figure 3). Visualization of the ganglia and signal recordings were done with an x100 objective (UPLANAPO, NA=1.35, *Olympus, Hamburg, Germany*) resulting in a spatial resolution of 2.0µm^2 per pixel. The fluorescence images were acquired and processed by the Neuroplex 9.1.0 software (*RedShirtImaging, Decatur, GA, USA*). The imaging set-up allows the relative changes in the fluorescence (ΔF/F) to be measured, which for the voltage-sensitive dye is linearly related to changes in the membrane potential (Neunlist et al. 1999). The fluorescence changes were detected with a CCD camera made of 80 pixels x 80 pixels (*RedShirtImaging, Decatur, GA, USA*). Optical signals were processed with a computer. Frame rates for VSD and calcium imaging were 1kHz and 40Hz, respectively. The overlay of signals and ganglion images allowed the analysis of the response of individual cells (Michel et al. 2005).



Adapted from Schemann et al. *Am J Physiol Gastrointest Liver Physiol* 2002; 282: 919-925

Figure 3: Illustration of the neuro imaging technique for experiments with the voltage-sensitive dye Di-8-ANEPSS (A) and the calcium-sensitive dye Fluo-4 AM (B): To detect Di-8-ANEPSS signals a modified Cy3 filterset was used. For the Fluo-4 AM a FITC filterset was used. A blue LED or a green LED was used to excite Fluo-4 AM or Di-8-ANPEPPS, respectively. The fluorescence changes were detected with a CCD camera. The optical signals were processed with a computer.

Electrical stimulation of intraganglionic fiber tracts

Intraganglionic fiber tracts were stimulated with 25µm Teflon-coated platinum electrode (*Science Products, Hofheim, Germany*), connected to a constant-voltage stimulator, to evoke fast excitatory postsynaptic potential (fEPSPs). The stimulus parameters were defined as suprathreshold with pulse duration of 600µs and amplitudes from 1 to 8V (*Grass SD9 Stimulator, Quincy Mass, USA*).

Testing viability of the neuronal network

To ensure the viability of the studied neuronal network the intraganglionic fiber tracts were either electrically stimulated to induce fEPSPs, 100µM nicotine or 50mM KCl were microejected onto the ganglion to evoke action potential discharge or $[Ca]_i$ transients. This was done at the beginning and at the end of each individual experimental protocol.

Application protocols

Accordingly to previously published calibration curves, any substances applied by pressure pulse ejection will be diluted by approximately 1:10 once it reaches the ganglion (Breunig et al. 2007).

In preliminary experiments different application protocols were tried to study PAR-APs action in guinea-pig colon and in human intestine: (1) 800ms pressure pulse ejection of 100µM or 1mM PAR-AP and than 3 consecutive acquisitions of 1.8s with different time intervals (3s, 5s) in between; (2) 3 times 800ms pressure-pulse ejection of 1mM PAR-AP and than acquisitions for 2.5s after 20s, 30s, 50s, 60, 90s or 120s; (3) long term incubation of 100µM PAR-AP and recording after 2min, 4min and 12min of incubation; (4) local perfusion of 3000nl, 6000nl or 9900nl PAR-AP (100µM) and then 3 consecutive acquisitions 0min, 1min and 2min after the local perfusion; (5) 2s microejection of 100µM PAR-AP and recording for 4s (see chapter 3.3.2 and 3.3.3).

Based on these preliminary experiments to validate the action of PAR-APs two different protocols for VSD recordings in the guinea-pig and human preparations were established. The PAR-APs, PAR-reversed peptides (PAR-RPs) (100µM) were either microejected or locally perfused over the ganglion. In the human submucous plexus, the PAR-APs and -RPs were microejected for 2s (0.5bar) onto the ganglion and the response was recorded for 4s. This protocol revealed reliable responses in the human submucous plexus but not in the guinea-pig submucous plexus. In guinea-pig preparations reliable responses were recorded only when 3000nl of the PAR-APs and -RPs were locally perfused for 30s at a rate of 100nl/s over the ganglion followed by a recording period of 1.8s. The local perfusion was coordinated by a digital volume controlled injector (*UltraMicroPump II, World Precision Instruments Inc., Sarasota, FL, USA*) and a microprocessor-based controller, Micro-4 (*Micro Syringe Pump Controller, World Precision Instruments Inc., Sarasota, FL, USA*). The syringe used was a 1000µl plastic syringe (*Dispomed, Geinhausen, Germany*) filled with the agonist solution and inserted into the volume controlled injector. The syringe was connected to a rigid plastic tube (10cm in length with 1mm diameter; *KronLab, Sinsheim, Germany*), which connected the syringe to the glass pipette placed over the ganglion. The whole system was bubble-free filled with the agonist solution. With the Micro-4 pump controller it was possible to perfuse a defined volume (e.g. 3000nl) of the agonist solution at a certain rate (e.g. 100nl/s) over the ganglion.

Since VSD imaging reliably reveals action potential discharge but is of limited use as a general reporter of cell activation, calcium imaging in human and guinea-pig submucous plexus was also performed. For these experiments an 800ms puff application of PAR-APs was found to be an optimal protocol evoking reliable responses. Therefore the PAR-APs and

-RPs (100µM) were 800ms microejected over the ganglion (0.5 bar). Also the endogenous proteases thrombin (100nM) and tryptase (100nM) were applied for 800ms.

The probiotic supernatants (MRS- or MT5 supernatants: pure, concentrated and resuspension of lyophilised supernatants) and the yeast extract solution were microejected (400ms - 800ms) onto the ganglion (0.5 bar) and the response was recorded 1.8s to 2.5s (using VSD Di-8-ANEPPS).

GelE (100nM) and control were perfused locally for 30s over the ganglion (3000nl at a rate of 100nl/s) in accordance with the guinea-pig PAR-AP experiments and the responses were recorded immediately after the application period for 1.8s (using Di-8-ANEPPS). For calcium imaging experiments GelE (100nM) and control were microejected (800ms) onto the ganglion (0.5 bar).

IBS supernatants were microejected onto the ganglion for 800ms and the response was recorded for 1.8s by using VSD recordings.

Reproducibility test

To test the reproducibility of the PAR-APs induced responses, the PAR-APs were applied at 10min intervals. Responses to PAR-APs were not reproducible in accordance with already published data (Reed et al. 2003), which shows a desensitization of the receptor. Due to the PAR-AP induced tachyphylaxis, the pharmacology was performed on different ganglia.

To test the reproducibility of the IBS supernatants, the supernatants were applied at 20min intervals. Responses to IBS supernatants were reproducible similar to the findings by Buhner et al. (2009). Therefore the pharmacology was performed on the same ganglion.

Pharmacology

For pharmacological studies the following substances were added to the Krebs solution perfusing the tissue: the PAR1 specific antagonist SCH79797 (*Tocris Bioscience, Bristol, UK*) at concentration of 100nM, 1µM, 10µM, the PAR2 antagonist LIGK-NH_2 (100µM; *Cambridge research biochemials, UK*; *kindly supplied by D. Bulmer*) and the neural blocker TTX (0.5µM; *Tocris Bioscience, Bristol, UK*). To study possible presynaptic actions the PAR1-AP was applied in the presence of a low Ca^{2+}/high Mg^{2+} Krebs solution, containing (in

mM): 98 NaCl, 4.7 KCl, 16 $MgCl_2.6H_20$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 0.25 $CaCl_2.H_20$, 11 glucose which blocks synaptic input (Mazzouli and Schemann 2009). The antagonists were perfused for approximately 20min before starting the pharmacological studies.

Due to the PAR-AP induced tachyphylaxis, the pharmacology was performed on different ganglia. In a given tissue the PAR-AP response was evoked in one ganglion and the blocking effects were recorded in a different ganglion in the same preparation. The thrombin experiments were performed in the same way.

For the IBS supernatants, the pharmacology was performed on the same ganglia. After 20min perfusion of the PAR1 antagonist (10µM SCH79797, added to the superfusing Krebs solution) the IBS supernatant was applied.

The serine protease inhibitor FUT-175 (Nafamostat mesylate) (*Merck KGaA, Darmstadt, Germany* and *Sigma-Aldrich, Schelldorf, Germany*) was added to the Fluo-4 AM dye solution and the tissue perfusing Krebs solution. In the presence of the FUT-175 (50µg/ml) calcium imaging experiments with the PAR2-AP were performed. PAR2-AP was applied for 800ms and the response was recorded for 20s. The results were compared to PAR2-AP induced $[Ca]_i$ responses without FUT-175 treatment.

2.2.3 Data analysis and statistics of the neuro imaging experiments

The analysis of the neuro imaging experiments was done with Neuroplex 9.1.0 software (*RedShirtImaging, Decatur, GA, USA*). The statistics and graphics were done with Sigma Plot 9.0 for Windows (*Systat Software Inc., Erkrath, Germany* and Igor Pro for Windows (*Version 6.03, WaveMetrics, Lake Oswego, USA*).

For the fast imaging studies, the number of dye-labelled neurons and glia in the field of view were counted and the number of responding neurons or glia per ganglion as well as the frequency of action potentials or the maximal change in $[Ca]_i$ fluorescence ($\Delta F/F$) were analysed (Figure 4). The Di-8-ANEPPS staining clearly reveals the outline of enteric neurons (Michel et al. 2005) (Figure 1). This allowed counting of all neurons in a given ganglion and therefore the percentage of responding neurons could be calculated. Fluo-4 AM staining does

not readily reveal outlines of all ganglion cells (Figure 2). Therefore the neurons and glia which showed an increased $[Ca]_i$ response to PAR-APs were counted without analysing the relative proportion of responding cells in a given ganglion. Nevertheless an estimation of the relative proportion of neurons and glia which respond to PAR-APs with $[Ca]_i$ signals based on previously published numbers of neurons or glia per ganglion in guinea-pig and human submucous plexus were provided (Hoff et al. 2008). The action potential frequency [Hz] was determined by dividing the number of action potentials by the recording time in s. The maximum $[Ca]_i$ increase was determined by the difference between baseline before application and the maximum $[Ca]_i$ ΔF/F change. Figure 4 shows an example for action potentials or $[Ca]_i$ transients in human submucous neurons to PAR1-AP.

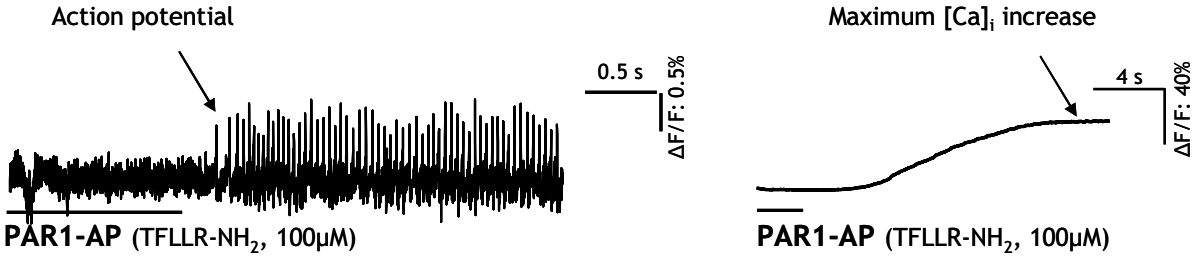


Figure 4: Representative response of a human submucous neuron to PAR1-AP application: The left trace shows a representative response to 2s microejetion (indicated by the horizontal bar) of PAR1-AP (TFLLR-NH_2). Each peak corresponds to one action potential. The right traces shows a representative neuronal $[Ca]_i$ signal after PAR1-AP (TFLLR-NH_2) application (800ms, indicated by the horizontal bar) in a human submucous neuron.

The statistical analysis was performed with the responding cells. The figures for VSD experiments indicate the total number of neurons (T/G/N is tissue/ganglia/neurons), whereas the figures for the CSD experiments indicate the responding neurons or glia (T/G/N is tissue/ganglia/neurons or T/G/G is tissue/ganglia/glia).

All data are expressed as median with the 25^{th} and 75^{th} percentiles given in brackets. To detect significant differences various statistical tests were used, depending on the distribution of data and the study design. Normally distributed data was analysed by using parametric tests, like the Students' *t*-Test, the paired *t*-Test or the One Way Analysis of Variance. Not normally distributed data was analyzed by the Mann-Whitney Rank Sum Test, the Wilcoxon Signed Rank Test or the Kruskal-Wallis One Way Analysis of Variance of Ranks. The multiple comparisons were done with Dunn's Method and Tukey Test. Differences were considered significant when P was < 0.05.

2.3 Ussing Chamber

2.3.1 Principle of the Ussing Chamber technique

The Ussing Chamber was developed by Hans Ussing in 1951 to study the electrophysiological behaviour of epithelial ion transport mechanisms, like that seen in the gastrointestinal tract. This technique considers two important characteristics of epithelia: polarity and permeability. The Ussing Chamber is a two chamber system, which connects two chambers separated by an epithelial tissue (Figure 5). The mucosa/submucosa preparation of the intestine (proximal and distal colon of the mouse; small and large intestine of the human) was used as epithelial tissue, which is polar with a mucosal and serosal side.

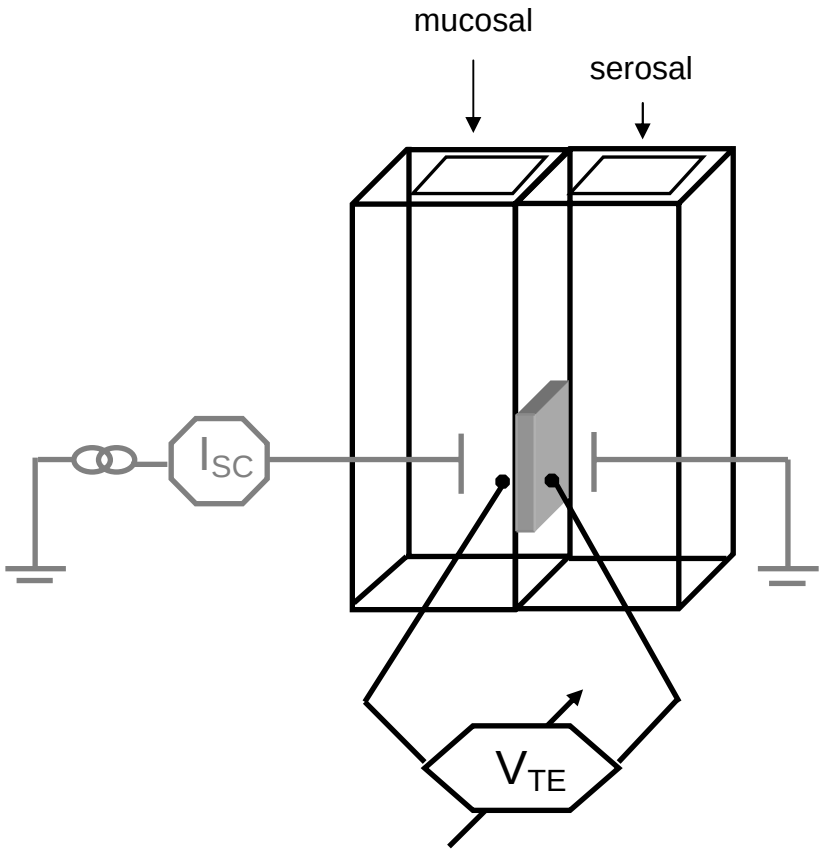


Figure 5: Illustration of the Ussing Chamber set up: The mucosa/submucosa preparation (grey) is fixed onto a slider and placed in between two Krebs solution filled chambers (5mL volume). The black electrodes measure the transepithelial potential (V_{TE}) and the grey electrodes apply the short-circuit-current (I_{SC}).

The vectorial transport of ions over the epithelial tissue creates a potential over the tissue, the so called transepithelial potential (V_{TE}). The short-circuit-current technique was used, in which a certain short-circuit-current (I_{SC}) is applied to clamp the transepithelial potential to

0mV (voltage-clamp). The current, which is therefore necessary, is identical with the current generated by active ion transport. Thus by measuring the short-circuit-current the net secretion or net absorption of the epithelium can be determined indirectly. The short-circuit-current is defined as follow:

$$I_{SC} = V_{TE} / R$$

I_{SC}: short-circuit-current [µA]

V_{TE}: transepithelial potential [V]

R: electrical resistance [Ω]

The transport of positive charge from mucosal to serosal side or of negative charge from serosa to the lumen induces a positive short-circuit-current. This corresponds to a cation net resorption and anion net secretion, respectively.

The tightness/permeability of the epithelium and integrity of the tissue can be determined by measuring the transepithelial resistance. The electrical resistance is defined as follow:

$$R = \rho * L / A$$

R: electrical resistance [Ω]

ρ: material specific electrical resistance constant [$\Omega*m^2/m$]

A: cross section area of the flow through tissue [m^2]

L: length or size of the flow through tissue [m]

The electrical resistance is experimentally determined indirectly using the Ohm's law:

$$R = \Delta U / \Delta I$$

R: electrical resistance [Ω]

ΔU: change of the electrical potential over the tissue [V]

ΔI: change of the electrical current over the tissue [A]

The corresponding values for potential and current can be read off the voltage current clamp/pulse generator (*Physiologic Instruments, San Diego, CA, United States*). The current corresponds to the value necessary to clamp the potential to 0mV. The potential difference over the tissue is determined by suppressing the application of the short-circuit-current.

2.3.2 Experimental procedure of the Ussing Chamber technique

Before the start of the experiments the Ussing chambers (*Easy Mount Chambers, Physiologic Instruments, San Diego, Ca, Unites States*) were calibrated. To calibrate the system a calibration block was put between the two chambers. The calibration block allows the fluid exchange (Krebs solution) between the two chambers. Two potential electrodes were placed close to the tissue to measure the transepithelial potential and two current electrodes, which apply the short-circuit-current, were placed distant to the tissue (*Easy Mount Electrode Set P2020, Physiologic Instruments, San Diego, CA, United States*). These Ag/AgCl electrodes were connected to the Krebs solution via a 3M KCl-filled agar bridge and to the AD-converter (*PowerLab, ADInstruments, Spechbach, Germany*) via the voltage clamp generator (*VCC 600L, Physiologic Instruments, San Diego, CA, United States*). The Software Chart (*Version 5, Chart Software: AD Instruments, Spechbach, Germany*) detected the short-circuit-currents during the experiment. After inserting the calibration blocks the chambers were filled with Krebs solution, which get warmed to 37°C and continuously bubbled by carbogen, and then calibrated to 0mV.

The Ag/AgCl electrodes were produced by filling the electrodes with 30-40µL of a 3-4% KCl-agar (*Merck, Darmstadt, Germany*). After hardening of the KCl-agar the electrode tips were bubble free filled with 3M KCl solution. They were stored in a 3M KCl solution at 4°C.

To test the effects of the PAR1, 2 and 4-APs on ion secretion in mucosa/submucosa preparations of human intestine or to test the effect of probiotic feeding on nerve- and epithelial dependent secretion in mucosa/submucsoa preparation of murine intestine, the tissue specimens were mounted into the Ussing chambers. The exposed tissue area was 0.5cm^2 (human) and 0.25cm^2 (mouse). Mucosal and serosal sides were bathed separately in 5mL Krebs solution, continuously bubbled with 95% O_2 and 5% CO_2 (carbogen) and maintained at 37°C. The transepithelial potential difference was measured by a pair of Ag/AgCl electrodes, connected to a voltage clamp apparatus that compensated for the solution resistance between the electrodes. Ion secretion was measured as short-circuit-current (I_{SC}) and expressed in µA/cm^2.

After an equilibration period of 1hrs (human) / 30min (mice) to adjust the short-circuit-current to a stable base value the experimental protocols were started.

PAR-AP study

For the PAR-AP experiments, first of all the electrical resistance was determined, as described in 2.3.1. Then the tissue was electrically stimulated by silver electrodes placed on either side of the tissue and connected to a constant voltage stimulator (*Grass SD-9; Grass-Telefactor*). The neural stimulation of the human tissue was achieved by delivering a train of pulses with supramaximal stimulus parameters (pulse amplitude, 20V; pulse frequency, 10Hz; pulse duration, 1ms; train duration, 10s). After 30min the PAR-APs (10µM), PAR-RPs (10µM), the antagonists (100nM, 1µM SCH79797, 100µM LIGK-NH_2, 100µM KGIL-NH_2) or the blocker (0.5µM TTX) were added to the serosal side of the preparation. 20min after the antagonist or blocker application the specific PAR-APs were added. The second application of PAR-APs was also carried out 20min after the first application. After 30min the transepithelial resistance was again determined and the tissue was electrically stimulated.

Probiotic feeding study

In the probiotic feeding experiments the transepithelial resistance of the murine tissue was determined three times in 30min intervals. Shortly after the determination of the resistance, the tissue was electrically stimulated (supramaximal stimulus parameters: pulse amplitude, 6V; pulse frequency, 10Hz; pulse duration, 0.5ms; train duration, 10s) to study the nerve mediated secretion. 30min after the last electrical field stimulation 1µM forskolin was added to the serosal side of the mucosa/submucosa preparation to study the epithelial mediated secretion.

2.3.3 Data analysis and statistics of the Ussing Chamber experiments

The graphical analysis of the Ussing Chamber experiments was done with the programs Chart for Windows (*Version 5, Chart Software, AD Instruments, Spechbach, Germany*), Igor Pro for Windows (*Version 6.03, WaveMetrics, Lake Oswego, United States*) and Sigma Plot 9.0 for Windows (*Systat Software Inc., Erkrath, Germany*).

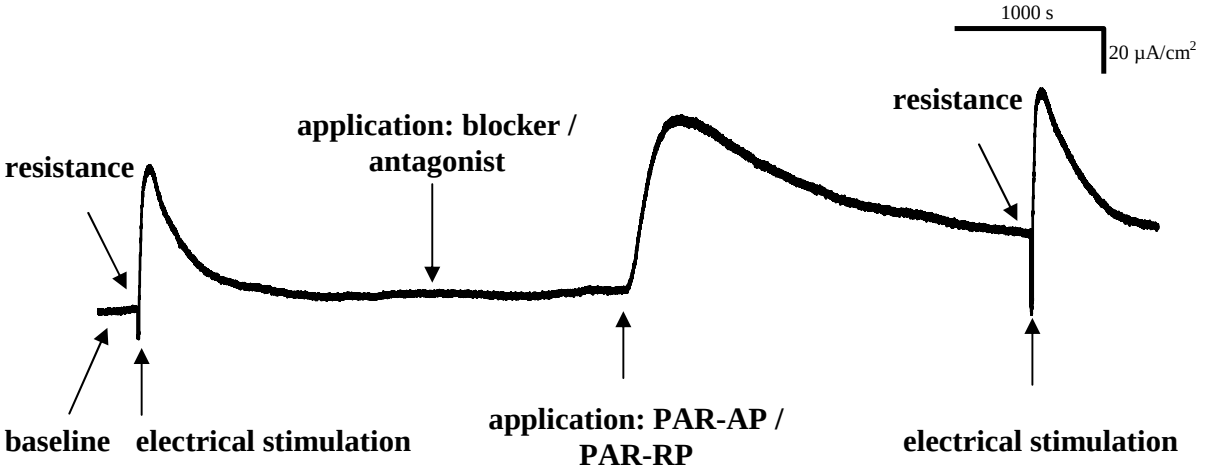


Figure 6: Illustration of the experimental procedure and definition of the analysed parameters of the PAR-AP study: transepithelial resistance, electrical stimulation, blocker (TTX), antagonists (SCH79797, LIGK-NH_2, KGIL-NH_2) and PAR-APs, -RPs.

Figure 6 illustrates the experimental procedure and a representative experiment with PAR-APs, how it was recorded by the Chart for Windows program (*Version 5, Chart Software, AD Instruments, Spechbach, Germany*). The transepithelial resistance [Ω*cm^2], the electrical field stimulation induced secretion [$\mu A/cm^2$] and the PAR-APs, -RPs induced secretion [$\mu A/cm^2$] were determined. The absolute delta I_{SC}-value was defined as difference between the baseline before application/stimulation and the maximum peak.

The statistics are based on the number of patients. Each agonist, blocker or antagonist were tested in separate tissue preparations from the same patient.

Figure 7 illustrates the experimental procedure and a representative experiment for the murine feeding experiments. The transepithelial resistance [Ω*cm^2], the electrical field stimulation induced secretion [$\mu A/cm^2$] and the forskolin induced secretion [$\mu A/cm^2$] were determined. The absolute delta I_{SC}-value was defined as difference between the baseline before application/stimulation and the maximum peak.

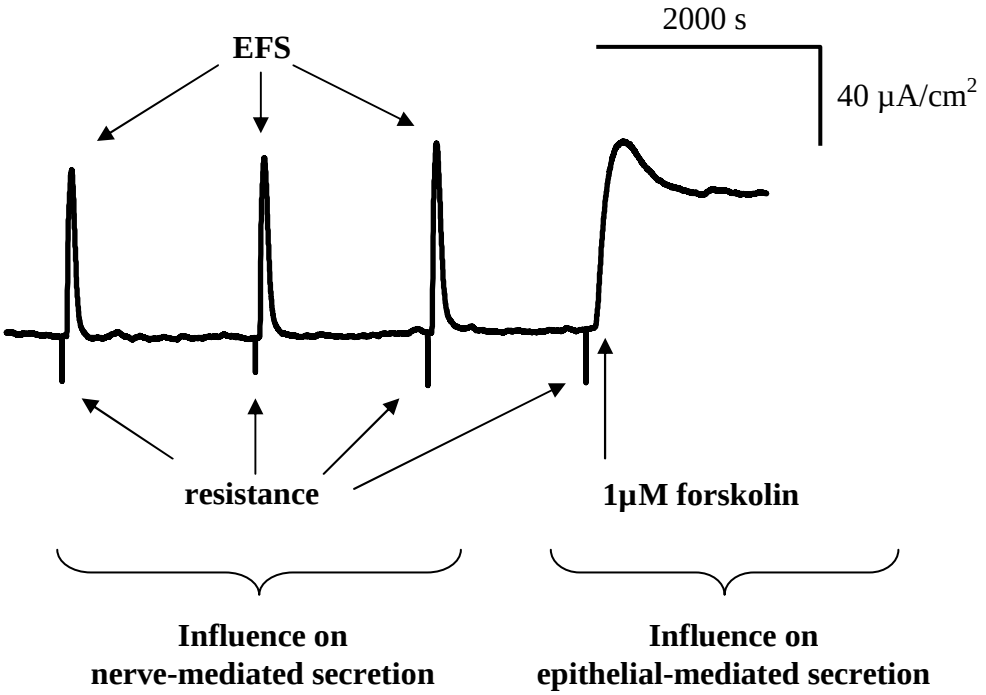


Figure 7: Illustration of the experimental procedure and definition of the analysed parameters of the probiotic feeding study: transepithelial resistance, electrical field stimulation (EFS), forskolin (1µM).

The statistics are based on the number of mice. For every region (proximal, distal) two tissues were used to do a repeat determination. The mean of the two obtained values was used to do the statistical testing. The mean of the transepithelial resistance and of the electrical field stimulation induced secretion was determined, because they did not differ significantly between the different time points. Statistical analysis was performed with the mean values of the transepithelial resistance and electrical field stimulation induced secretion.

The statistical analyses were performed with Sigma Plot 9.0 (*Systat Software Inc., Erkrath, Germany*). All data are expressed as median with the 25^{th} and 75^{th} percentiles given in brackets. To detect significant differences various statistical tests were used, depending on the distribution of data and the study design. Normally distributed data was analysed by using the Students' *t*-Test, the paired *t*-Test or the One Way Analysis of Variance and the One Way Repeated Measures Analysis of Variance. Not normally distributed data was analysed by the Mann-Whitney Rank Sum Test, the Wilcoxon Signed Rank Test or the Kruskal-Wallis One Way Analysis of Variance on Ranks and the Friedman Repeated Measures Analysis of Variance on Ranks. The multiple comparisons against the control were done with the Bonferroni *t*-Test, the Student-Newman-Keuls Method, or the Dunn's Method and the Tukey Test. Differences were considered significant when P was <0.05.

2.4 Immunohistochemistry

In order to study the expression of PAR1 and PAR2 in guinea-pig and human intestine (Table 1) and to selectively label enteric neurons, glia or macrophages staining procedures as previously described (Hoff et al. 2008; Schemann et al. 2010), were performed. Fresh human and guinea-pig preparations were fixed overnight at room temperature in formaldehyde (4%) or formaldehyde plus 0.2% picric acid, respectively. For some preparations alternatively an acetone fixation (10min) was performed (Table 1). After a washing step (3 x 10 min) in PBS buffer, the preparations were incubated for 1hr 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) or PBS/NaN_3/HS) to block unspecific binding. The tissues then were incubated for 40 hrs (human) / 12-16hrs (guinea-pig) at room temperature in solution containing the primary antibodies. As primary antibodies mouse anti-PAR2 (SAM-11 (1:200 and 1:1000), *sc-13504, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA),* mouse anti-Thrombin R (ATAP-2; 1:100, 1:500, 1:1000), *sc-13503, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA*), goat anti-Thrombin R (C-18; 1:100, 1:500, 1:1000, 1:2000, *sc-8202, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA*), goat anti-Thrombin R (N-19; 1:100, 1:500, *sc-8203, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA*), mouse anti-CD68 (1:200; *AbD Serotec, Düsseldorf, Germany*), mouse anti-Sox8/9/10 (1:10; *M. Wenger, Universität Erlangen-Nürnberg, Germany*), sheep anti-PGP 9.5 (1:10000; *PH164; The Binding Site, Birmingham, UK*), rabbit anti-neuron-specific enolase (NSE) (1:2000; *Polysciences, Epplheim, Germany*) were used. After washing in PBS (3 x 10min), samples were exposed to the species-specific secondary antibodies (Cy2-, Cy3-, Cy5-labeleled anti-mouse, anti-sheep, anti-goat and anti-rabbit secondary antibodies from *Dianova, Hamburg, Germany*) for 3.5hrs (human) / 1.5hrs (guinea-pig). After washing (3x 10min in PBS) the human preparations were 1hr incubated in a 1mM $CuSO_4$ - 50mM CH_3COONH_4 buffer. After a final wash (3 x 10min), preparations were mounted on poly-L-lysine coated slides and cover slipped with a solution of PBS (pH= 7.0) / NaN_3 (0.1%) / glycerol (80%). For the inspection of the tissue, an Olympus microscope (*BX61 WI; Olympus, Japan*) with appropriate filter blocks was used. The microscope was equipped with an SIS Fview II charge-coupled device (CCD) camera and an analySIS 3.1 software (*Soft Imaging System GmbH, Münster, Germany*) for image acquisition, processing and editing. If an antigen-retrieval was performed the tissue was fixed on a sylgard slice (1x1cm) and was 40min heated in citratbuffer (pH 6.0) at 95°C. After this step the tissue was 3 times washed in PBS buffer and then the blocking with PBS/NaN_3/HS and the normal staining steps were performed.

Table 1: Immunohistochemical stainings of whole-mount preparations of human and guinea-pig submucous plexus (SMP) and myenteric plexus (MP) with primary antibodies against PAR1 and PAR2

	Primary antibody	Concentration	Membrane permealizer Triton X-100	Antigen retrieval	Aceton fixation	Tissues
PAR1	ATAP-2 (mouse anti-Thrombin R)	1:100 1:500 1:1000	+ / -	+	-	human SMP guinea-pig SMP
PAR1	C-18 (goat anti-Thrombin R)	1:100 1:500 1:1000 1:2000	+	+	+	human SMP guinea-pig SMP
PAR1	N-19 (goat anti-Thrombin R)	1:100 1:500	+ / -	-	-	human SMP guinea-pig SMP
PAR2	SAM 11 (mouse anti-PAR2)	1:200 1:1000	+ / -	-	-	human SMP, MP guinea-pig SMP, MP

In order to selectively label enteric neurons, enteric glia or macrophages preparations were incubated with their specific antibodies (neurons: sheep anti-PGP 9.5 (1:10000; *PH164; The Binding Site, Birmingham, UK*), rabbit anti-neuron-specific enolase (NSE) (1:2000; *Polysciences, Epplheim, Germany*); glia: mouse anti-Sox8/9/10 (1:10; *M. Wenger, Universität Erlangen-Nürnberg, Germany*); macrophages: mouse anti-CD68 (1:200; *AbD Serotec, Düsseldorf, Germany*). The responding cell type was identified by superimposing the image of the imaging experiment with the immunohistochemical image. Labelling for neurons and glia was not always performed as in many cases the cell morphology during $[Ca]_i$ imaging clearly distinguished neurons from glia.

2.5 Drugs, Supernatants and Solutions

PAR-APs and PAR-RPs

As PAR-APs amino acid sequences specific for human (first sequence in parenthesis) or rodent (second sequence) receptors were used: PAR1-AP (TFLLR-NH_2, SFFLR-NH_2), PAR2-AP (SLIGKV-NH_2, SLIGRL-NH_2) and PAR4-AP (GYPGQV-NH_2, GYPGKF-NH_2). As controls the reversed peptides (RPs) were used: PAR1-RP (RLLFT-NH_2, RLFFS-NH_2), PAR2-RP (VKGILS-NH_2, LRGILS-NH_2), PAR4-RP (VQGPYG-NH_2, FKGPYG-NH_2) (*all from D.McMaster, Peptide Synthesis Core Facility, Department of Medical Biochemistry, Health Science Centre, University of Calgary, Alberta, Canada*). PAR-APs and PAR-RPs

were prepared as 10mM Stock solutions in deionised water and stored at -20°C. PAR-APs and -RPs were freshly dissolved in oxygenated Krebs solution (pH 7.4) in a concentration of 100µM and either microejected onto the ganglion or locally perfused over the ganglion. In the Ussing Chamber experiments the PAR-APs and -RPs at a concentration of 10µM were serosally applied to the mucosa/submucosa preparations.

SCH79797 (PAR1 antagonist)

SCH79797 dihydrochloride (*Tocris Bioscience, Bristol, UK*), N^3-Cyclopropyl-7-[[4-(1-methylethyl)phenyl]methyl]-7*H*-pyrrolo[3,2-*f*]quinazoline-1,3-diamine dihydrochloride, is a potent and selective non-peptide PAR1 antagonist (Ahn et al. 2000). This PAR1 antagonist was dissolved in 100% dimethyl sulphoxide (DMSO) and the 1mM and 10mM stock solutions were stored at 4°C. DMSO (content: 0.1%) had no effect on basal activity of enteric neurons (nicotine response in absence and presence of DMSO was not significantly different: 10.5 %ΔF/F [3.7/27.3%ΔF/F]; T/G/N: 13/31/175 vs 8.5%ΔF/F [4.1/15.8%ΔF/F]; T/G/N: 13/35/180; P = 0.285) or on basal secretion in Ussing Chamber experiments. Neither the PAR1 antagonist nor the solvent DMSO had adverse effects on the intestinal tissues; because the transepithelial resistance (100nM: 58.1 Ωcm^2 [42.8/97.9 Ωcm^2] vs 55.7 Ωcm^2 [39.3/103.3 Ωcm^2]; N: 13; P = 0.233; 1µM: 86.6 Ωcm^2 [38.6/126.2 Ωcm^2] vs 63.4 Ωcm^2 [35.6/106.4 Ωcm^2]; N=12; P = 0.214) as well as the electrical field stimulation induced secretion (100nM: 11.5 µA/cm^2 [6.4/25.7 µA/cm^2] vs 15.7 µA/cm^2 [6.0/21.3 µA/cm^2]; N: 13; P = 0.922; 1µM: 13.5 µA/cm^2 [7.6/34.3 µA/cm^2] vs 16.6 µA/cm^2 [12.8/29.0 µA/cm^2]; N: 13; P = 0.685) were not changed. The PAR1 antagonist was used in different concentrations (100nM, 1µM, 10µM dissolved in Krebs solution), by continuously perfusing the tissue with the PAR1 antagonist (neuro imaging experiment). In the Ussing Chamber experiments the PAR1 antagonist (100nM, 1µM) was added serosally to the mucosa/submucosa preparations 20min before application of the PAR1-AP.

LIGK (PAR2 antagonist) and KGIL (reversed sequence of the PAR2 antagonist)

The peptide antagonist LIGK-NH_2 (ENMD-1005) is a truncated version of the PAR2-AP which blocks the PAR2 receptor. LIGK-NH_2 and its reversed sequence KGIL-NH_2 (*Cambridge research biochemials, UK*) were dissolved in PBS and 100mM stock solutions were stored at -80°C. For the neuro imaging experiments LIGK-NH_2 was dissolved in Krebs solution in a concentration of 100µM and continuously perfused. In the Ussing Chamber

LIGK-NH_2 or KGIL-NH_2 (100µM) were added serosally 20min before the application of the PAR2-AP.

Thrombin

The human plasma derived thrombin (citrate free, *Merck KGaA, Darmstadt, Germany*) was stored at -80°C in a concentration of 2,14mg/ml. Thrombin was freshly dissolved in oxygenated Krebs solution in a concentration of 100nM and microejected (800ms) onto the human submucous ganglion.

Tryptase

The human lung derived tryptase (*Merck KGaA, Darmstadt, Germany*) was stored at -20°C in a concentration of 1mg/ml. Tryptase was freshly dissolved in oxygenated Krebs solution in a concentration of 100nM. After pH adjustement to 7.4 by oxygenation tryptase was microejected for 800ms onto the human submucous plexus.

FUT-175

The serine protease inhibitor FUT-175 (Nafamostat mesylate) (*Merck KGaA, Darmstadt, Germany* and *Sigma-Aldrich, Schelldorf, Germany*) was dissolved in deionised water to get a stock solution of 5mg/ml, which was stored at 4°C. In a final concentration of 50µg/ml FUT-175 was added to the Fluo-4 AM dye solution and the tissue perfusing Krebs solution. In the presence of the FUT-175 (50µg/ml) calcium imaging experiments with the PAR2-AP were performed. PAR2-AP was applied for 800ms and the response was recorded for 20s.

GelE and Control

The experiments with the bacterial protease (GelE) were done in collaboration with the Chair of Biofunctionality, ZIEL-Research Centre for Nutrition and Food Science, Technische Universität München, Germany (with kind support by N. Steck). They purified and concentrated the GelE and produced the control.

The *Enterococcus faecalis* OG1RF protease GelE (32kDa) was purified by using fast protein liquid chromatography (anionexchanger, *GE Healthcare, München, Germany*) and concentrated by using Amicon® Ultra-15 tubes (exclusion size 10kDa, *Millipore, Schwalbach/Ts, Germany*). The control, which displays the concentrated buffer solution, contained 20mM L-histidine and 125mM NaCl (pH 6.0). The GelE sample was obtained and had a protein content of 1284µg/ml determined by Bradford protein assay, which correspond

to a 40.1µM stock solution. The activity of GelE was determined by an azocasein assay. To get a final concentration of 100nM or 312nM GelE, GelE and similarly the control were diluted in oxygenated Krebs solution. The pH was adjusted to 7.4. The GelE and control solution were locally perfused (3000nl at a rate of 100nl/s) over the ganglion (VSD experiments) or 800ms microejected onto the ganglion (calcium imaging experiments).

Probiotic supernatants (conditioned media)

The experiments with the probiotic supernatants were done in collaboration with the Chair of Biofunctionality, ZIEL-Research Centre for Nutrition and Food Science, Technische Universität München, Germany (with kind support by T. Clavel). They produced the probiotic MRS- and MT5-supernatants (pure, concentrated and lyophilised).

Pure control MRS-supernatant (1:10) and pure L. paracasei MRS-supernatant (1:10)

The MRS (*Fluka Analytical, Sigma Aldrich, Buchs, Switzerland*) contained 2 g/L hydrogen phosphate, 20 g/L glucose, 0.2 g/L magnesiumsulfate heptahydrate, 0.05 g/L manganous sulfate tetrahydrate, 8 g/L meat extract, 10 g/L peptone, 5 g/L sodium acetate trihydrate, 2 g/L triammonium citrate, 4 g/L yeast extract (pH 6.2 ± 0.2). Bacteria (*L. paracasei* NCC 2461) were revived from cryo-aliquots (100µl) in 10ml MRS and subcultured once in MRS for 24h. Filter-sterilized MRS medium (30ml) was inoculated with 500µl preculture. Supernatants were obtained by centrifuging bacterial cells (4,300 x g, 5min, RT). Negative controls (control MRS-supernatant) were media without bacteria. After 48h at 37°C, growth was monitored by plating and OD measurement at 600nm. Pure supernatants, like the control MRS-supernatant and *L. paracasei* MRS-supernatant, were stored at -20°C. The pure MRS-supernatants were 1:10 diluted in Krebs solution. The pH was adjusted to 7.4 and the osmolarity was 0.29Osmol. The pure MRS-supernatants were 400ms microejected onto the ganglion.

Pure control MT5-supernatant (1:1 in high osmolarity Krebs) and pure L. paracasei or B. longum MT5-supernatant (1:1 in high osmolarity Krebs)

Bacteria (*L. paracasei* NCC 2461, *B. longum* NCC 3001) were revived from cryo-aliquots (100µl) in 10ml MRS and subcultured once in MRS for 24h. Filter-sterilized MT5 medium (30ml) was inoculated with 500µl preculture. The MT5 contained (per liter distilled H_20): 4.2g $NaHCO_3$, 1g yeast extract, 720mg $Na_2HPO_4 \cdot 2H_20$, 500mg cystein, 400mg KH_2PO_4, 300mg NaCl, 124mg $MgSO_4 \cdot 7H_2O$, 20mM glucose (3.6g), 10µM $FeCl_3$ (pH=7.7). Supernatants were obtained by centrifuging bacterial cells (4,300 x g, 5min, RT). Negative

controls (MT5-control supernatant) were media without bacteria. After 48h at 37°C, growth was monitored by plating and OD measurement at 600nm. Pure supernatants, like the control MT5-supernatant, *L. paracasei* MT5-supernatant and *B. longum* MT5-supernatant, were stored at -20°C. The pure MT5-supernatants were 1:1 diluted in high-osmolarity Krebs solution (Krebs solution + 50mM Mannitol + 150mM NaCl). The pH was adjusted to 7.4 and the osmolarity was 0.27Osmol. The pure MT5-supernatants were 400ms microejected onto the ganglion.

Concentrated control MT5-supernatant and concentrated L. paracasei MT5-supernatant

Bacteria (*L. paracasei* NCC 2461) were revived from cryo-aliquots (100µl) in 10ml MRS and subcultured once in MRS for 24h. Filter-sterilized MT5 medium (30ml) was inoculated with 500µl preculture. After 48h at 37°C, growth was monitored by plating and OD measurement at 600nm. Samples were centrifugated (4,300 x g, 5min, RT) and supernatants (25ml) were concentrated using Amicon® Ultra-15 tubes (*UFC901024, Millipore, Schwalbach/Ts, Germany*; 3,000 x g, 25min, RT). Concentration factors were calculated by measuring retentate volumes. Concentrated supernatants, like the concentrated control MT5-supernatant and *L. paracasei* MT5-supernatant, were stored at -20°C. The concentrated MT5-supernatants were depending on their concentration factor 1:1 diluted in Krebs solution (e.g. 10µl concentrated control MT5- supernatant (x65) in 10µl Krebs solution; 10µl concentrated *L. paracasei* MT5-supernatant (x70) in 10.7µl Krebs solution). The pH was adjusted to 7.4 and the osmolarity was 0.29Osmol. The concentrated supernatants were 400ms microejected onto the ganglion.

Lyophilised control MT5-supernatant and lyophilised L. paracasei MT5-supernatant

To prepare lyophilized materials, cultures in MT5 (30ml) were frozen at -80°C and dried by lyophilization for 48h (*Alpha 1-4 LDplus, Christ*). Lyophilized supernatants, like the lyophilized control MT5-supernatant and *L. paracasei* MT5-supernatant, were stored at RT in an excicator. 17.6mg of the *L. paracasei* MT5-supernatant were dissolved in 400µl Krebs solution and accordingly also the control MT5-supernatant (400µl: minimal volume to get it in solution). The pH was adjusted to 7.4. The osmolarty of the control MT5-supernatant was 0.7Osmol and of the *L. paracasei* MT5-supernatant was 1.0Osmol. The resuspension of lyophilised supernatants were 800ms mircoejected onto the ganglion.

Yeast extract

The yeast extract, which was used for the MT5-medium, was dissolved in distilled H_20 in the same concentration as it exists in the concentrated control MT5-supernatant and 1:1 diluted in Krebs solution. The pH was adjusted to 7.4 and the osmolarity was 0.35Osmol. The yeast extract was 800ms microejected onto the ganglion.

IBS supernatants

Patients underwent left colonoscopy after cleaning of the distal colon with a 500mL water enema performed the evening before and the morning of the procedure. Four mucosal biopsy samples, taken from the proximal descending colon, were used to obtain an incubation supernatant. The incubation of the mucosal biopsy samples to obtain mucosal mediators spontaneously released from colonic biopsies was performed as previously published (Barbara et al. 2007). Briefly, upon removal, biopsies were rapidly immersed in hard plastic tubes containing 1mL of Hank's solution (*Sigma-Aldrich, Schelldorf, Germany*) continuously oxygenated (95%CO_2, 5%O_2) at 37°C. After 25min incubation, the bathing solution was removed. All samples were centrifuged at 200g for 10min, and 150µL of supernatant aliquoted (5µL) and stored at -80°C (Buhner et al. 2009). The IBS supernatants were defrosted and 1:1 diluted in Krebs solution and than 800ms microejected (0.5bar) onto the ganglion (Table 2).

Table2: Characteristics of the study participants

Sample	Age	Sex
MZ-9	52	male
D-IBS 24	68	female
D-IBS 134	32	male
D-IBS 137	35	male

TTX

The neural blocker tetrodotoxin (TTX; *Biozol Diagnostica, Eching, Germany*) was dissolved in distilled H_20 and stored as a 1mM stock solution at -20°C. In the Ussing Chamber TTX (0.5µM) was added serosally 20min before the application of the PAR1, 2-AP, respectively. In the neuro imaging experiment TTX was added (0.5µM) to the superfusion Krebs solution.

Forskolin

Forskolin (*Sigma-Aldrich, Schelldorf, Germany*), was disolved in 100% DMSO and stored as 10mM stock solution at -20°C. 10µM forskolin was serosally applied to the mucosa/submucosa preparations. The bicyclic diterpenoide forskolin (7β-Acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one) stimulates the adenylatcyclase, which induces the formation of cAMP and following activates the cAMP-dependent Cl^--secretion (de Souza et al. 1983; Seamon et al. 1981).

Preparation Krebs solution

The preparation Krebs solution (pH 7.4, 4°C) was used to do the tissue preparation and to keep the tissue over night at 4°C. The Krebs solution contained (per liter dH_20): 1.2mM $MgCl_2*6H_2O$, 2.5mM $CaCl_2*2H_2O$, 1.2mM NaH_2PO_4, 117mM NaCl, 25mM $NaHCO_3$, 11mM $C_6H_{12}O_6$, 4.7mM KCl (*Sigma Aldrich, Schnelldorf, Germany*).

Experimental Krebs solution

The experimental Krebs solution (pH 7.3-7.4, RT) was used for the neuro imaging and Ussing Chamber experiments. The Krebs solution contained (per liter dH_20): 1.2mM $MgCl_2*6H_20$, 2,5mM $CaCl_2*2H_2O$, 1.2mM NaH_2PO_4, 117mM NaCl, 20mM $NaHCO_3$, 11mM $C_6H_{12}O_6$, 4.7mM KCl (*Sigma Aldrich, Schnelldorf, Germany*).

Low Ca^{2+}/high Mg^{2+} Krebs solution

The low Ca^{2+}/high Mg^{2+} Krebs solution (pH 7.4, 37°C) contained (per liter dH_20): 16mM $MgCl_2*6H_20$, 0.25mM $CaCl_2*2H_2O$, 1.2mM NaH_2PO_4, 98mM NaCl, 25mM $NaHCO_3$, 11mM $C_6H_{12}O_6$, 4.7mM KCl (*Sigma Aldrich, Schnelldorf, Germany*).

Solutions in distilled H_20

The 10mM Stock solution of PAR2-AP (SLIGRL-NH_2) and PAR2-RP (LRGILS-NH_2) was accidentally dissolved in distilled H_20 to get the final concentrations of 1µM, 5µM, 10µM, 100µM. The PAR2-AP and PAR2-RP (800ms) were then microejected onto submucous ganglia of the guinea-pig. For recording the neuronal responses different acquisition protocols were used: (1) 4 times 1.8s acquisition with 3s interval in between, (2) 4 times 1.8s acquisition with 4s interval in between, (3) 3 times 1.8s acquisition with 5s interval in between, (4) 3 times 1.8s acquisition with 8s interval in between, (5) 3 times 1.8s acquisition with 10s interval in between. The comparison of the action potential discharge between

different time points, concentrations of PAR2-AP or between PAR2-AP and -RP did not provide any differences. Additionally, the application of pure distilled H_2O induced the same neural responses. Based on these results the crucial message of these experiments is that the distilled H_20 induced the action potential discharge in submucous neurons and not the PAR2-AP or PAR2-RP. Table 3 shows consequently the spike discharge in response to distilled H_20 at different time points after the application and the proportion of responding neurons to distilled H_20. Hence, it needs to taken in consideration to minimize the amount of distilled H_2O in solutions and to additionally ensure a physiological pH (7.4) and osmolarity (0.29Osmol) of the solution to avoid unspecific nerve activation.

Table 3: Excitation of guinea-pig submucous neurons by distilled H_2O: The table shows the spike discharge in response to 800ms microejection of PAR2-AP (SLIGRL-NH_2) or PAR2-RP (LRGILS-NH_2) dissolved in distilled H_2O, recorded for different time points (3 acquisitions of 1.8s with 3s interval in between).

1. application		AP-frequence [Hz] (3 acquisitions of 1800ms with 3s interval) (median [25/75th percentile]				proportion of responding neurons [%]	T/G/N
		0-1800ms	4800-6600ms	9600-11400ms	14400-16200ms		
1µM	SLIGRL (in dH_20)	2.5 [1.4/3.6]	1.9 [0.6/2.5]	0.6 [0.3/3.3]	1.1 [0.0/3.6]	6.5%	2/7/124
5µM	SLIGRL (in dH_20)	0.0 [0.0/1.7]	3.3 [0.6/5.6]	3.1 [1.1/6.7]	2.2 [0.6/5.0]	26.9%	2/11/201
10µM	SLIGRL (in dH_20)	1.1 [0.0/2.2]	2.2 [0.7/4.4]	2.2 [0.6/3.9]	1.7 [0.0/3.3]	48.2%	6/12/192
100µM	SLIGRL (in dH_20)	0.6 [0.0/1.7]	3.3 [1.1/6.7]	3.3 [1.1/7.2]	2.2 [0.6/5.0]	31.0%	10/26/406
10µM	LRGILS (in dH_20)	1.1 [0.0/1.7]	2.8 [2.8/4.4]	2.2 [0.6/3.9]	2.2 [0.0/4.4]	11.8%	2/9/161
100µM	LRGILS (in dH_20)	0.6 [0.0/2.2]	2.2 [1.1/5.6]	4.2 [1.1/6.1]	3.3 [1.7/6.1]	38.0%	2/5/100

3. Results

3.1 Neural actions of probiotics

The rational for this part of the project is that probiotic bacteria target the enteric nervous system to modulate epithelial functions in the gut as well as visceral sensation. Recently published data suggest that the neural action profile of probiotic bacteria may contribute to their efficacy in symptom relief in patients with functional and inflammatory bowel diseases (see introduction). The aim of the work was to identify such neural action.

3.1.1 Effect of probiotic conditioned media (supernatants) on enteric neurons

Supernatants (pure, concentrated or reconstituted lyophilised supernatant) obtained from the probiotic strain *L. paracasei* NCC 2461 and *B. longum* NCC 3001 were used to study the effect of soluble factors released into the medium. Direct effects of *L. paracasei* NCC2461 and *B. longum* NCC 3001 supernatants were studied with the neuro imaging technique. Over 600 submucous neurons (51 ganglia from 14 guinea pigs) were examined and the immediate effects of *L. paracasei* NCC 2461 and *B. longum* NCC 3001 supernatants by direct microejection onto the ganglion were analysed. Representative traces are shown in Figure 8 to illustrate responses of individual enteric neurons to the application of the reconstituted lyophilised probiotic supernatant and control supernatant (medium alone).

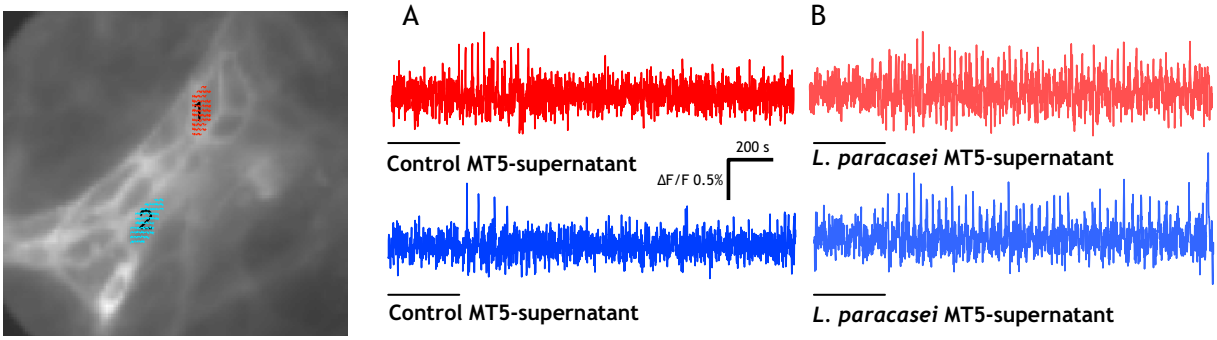


Figure 8: Resuspension of lyophilised control MT5-supernatant (medium alone) and lyophilised *L. paracasei* MT5-supernatant excited enteric neurons: The picture on the left side illustrates a Di-8-ANEPPS stained guinea-pig ganglion. Individual neurons in the ganglion of the submucous plexus can clearly be seen by the bright outline of the outer membrane. The response of two representative neurons

(marked in red and blue) to a 400ms microejection of control MT5-supernatant (A) and *L. paracasei* MT5-supernatant (B) are shown on the right side. Neurons showed an immediate onset of spike discharge after the application of control and probiotic supernatant.

In the first step, *L. paracasei* NCC 2461 was cultivated in MRS-medium to obtain supernatants. For the neuro imaging experiments the pure control and probiotic supernatants were diluted 1:10 in Krebs solution to get a physiological pH of 7.4 and a physiological osmolarity of 0.29Osmol. The control MRS-supernatant and the *L. paracasei* MRS-supernatant excited the same proportion of submucous neurons (15.4% [0.0/25.0%] vs 12.7% [0.0/34.8%]) and induced a similar action potential frequency (2.1Hz [1.8/4.7Hz] vs 2.4Hz [1.2/3.8Hz] (Figure 9). In conclusion, using *L. paracasei* NCC 2461 cultured in the MRS-medium it was not possible to detect any specific effects of the probiotic conditioned medium on enteric neurons.

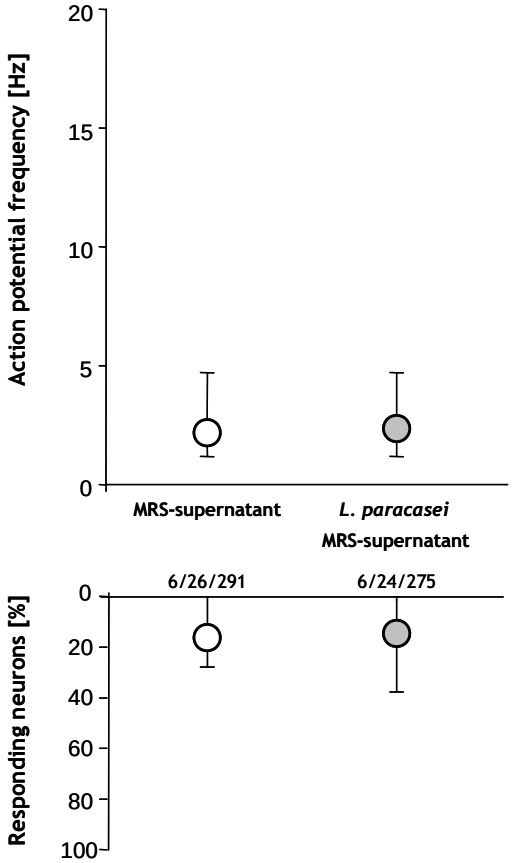


Figure 9: Control MRS-supernatant and *L. paracasei* MRS-supernatant (pure) excited guinea-pig submucous neurons: The control (white) and the probiotic (grey) supernatants excited the same proportion of enteric neurons and induced a similar spike discharge (Mann-Whitney Rank Sum Test: *P* = 0.961 and *P* = 0.854). Numbers indicate numbers of tissues/ganglia/neurons studied.

Due to the unspecific nerve-activating effects of the commercially available MRS-medium a new medium was designed. This new medium, MT5 ("Medium Tom 5" by T. Clavel) contained yeast extract, glucose, L-cysteine and salts still providing appropriate conditions for the growth of the probiotic strains. To compensate for the low osmolarity of the MT5-medium the control and probiotic supernatants were diluted 1:1 with a Krebs solution enriched with 50mM Mannitol and 150mM NaCl, however the pure supernatants remained still hypo-osmotic (control MT5-supernatant: 0.27Osmol, *L. paracasei* MT5-supernatant: 0.26Osmol, *B. longum* MT5-supernatant: 0.26Osmol). The pH was adjusted to 7.4. Pure control and probiotic supernatants excited enteric neurons with a comparable number of neurons responding with a similar magnitude (control MT5-supernatant: 1.8Hz [1.8/4.7Hz] vs *L. paracasei* MT5-supernatant: 1.8Hz [0.9/5.3Hz] vs *B. longum* MT5-supernatant: 2.9Hz [2.4/3.5Hz]) (Figure 10). These results again showed a non-specific effect of the probiotic conditioned medium on enteric neurons. However, experiments have shown that changes in osmolarity have profound effects on neuronal spiking suggesting that the low osmolarity of the supernatants was probably responsible for these non-specific neuronal responses.

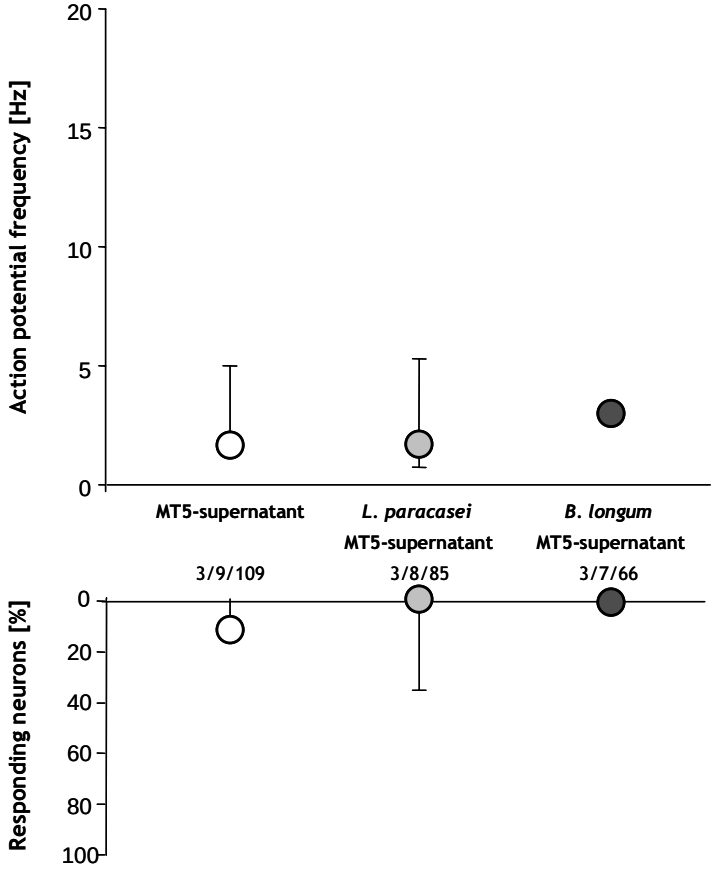


Figure 10: Control MT5-supernatant, *L. paracasei* and *B. longum* MT5-supernatant (pure) excited guinea-pig enteric neurons: The control (white) and the *L. paracasei* (light grey) and *B. longum* (dark grey) supernatants excited submucous neurons to a similar extent (Kruskal-Wallis One Way Analysis of

Variance of Ranks: proportion of responding neurons: *P* = 0.231 and spike discharge: *P* = 0.779). Numbers indicate numbers of tissues/ganglia/neurons studied.

In the next step concentrated control MT5-supernatant and concentrated *L. paracasei* MT5-supernatant were dissolved in Krebs solution. This supernatant solution had a physiological pH (7.4) and osmolarity (0.29Osmol). After microejection both the control and probiotic supernatants excited a similar proportion of enteric neurons (10.0% [0.0/24.25] vs 0.0% [0.0/10.7%]) and induced an almost identical action potential discharge (1.8Hz [1.8/3.5Hz] vs 2.1Hz [0.9/4.4Hz]) (Figure 11). These data show that even concentrated supernatants were ineffective at eliciting direct neuronal responses. The non-specific effects of the control supernatant were still observed suggesting that the pH and osmolarity effects seen before can be excluded and therefore some of the ingredients in the MT5-medium were suspected to be responsible for the nerve activation. In order to investigate this further a yeast extract solution with physiological pH and osmolarity in the same concentration as the yeast extract in the concentrated control MT5-supernatant was prepared. After microejection of this yeast extract solution 58.8% [11.1/91.7%] of the neurons responded with an action potential frequency of 2.5Hz [1.3/4.6Hz]. Based on these results, the yeast extract seemed to be one of the factors inducing nerve activation by the bacterial growth medium.

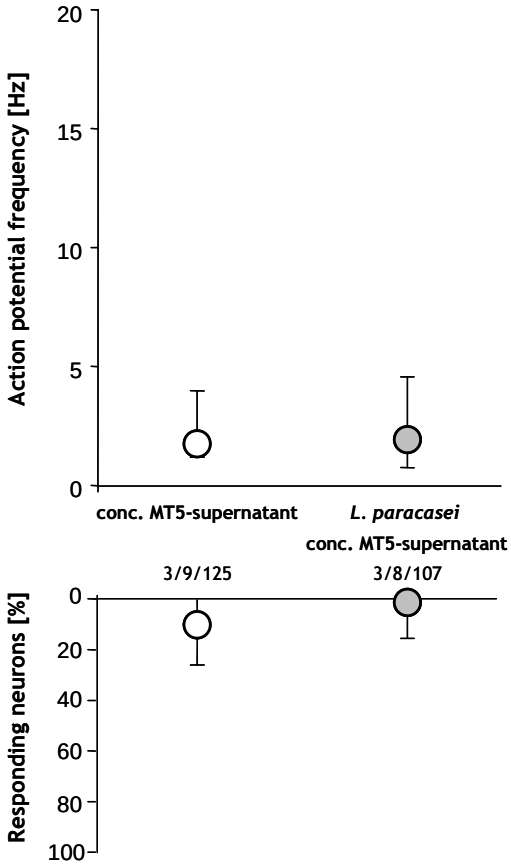


Figure 11: Concentrated control MT5-supernatant and concentrated *L. paracasei* MT5-supernatant excited guinea-pig enteric neurons: The control (white) and the probiotic (grey) supernatants excited a similar percentage of neurons with a comparable action potential frequency (Mann-Whitney Rank Sum Test: *P* = 0.358 and *P* = 0.802). Numbers indicate numbers of tissues/ganglia/neurons studied.

Finally, the effect of lyophilised control MT5-supernatant and lyophilised *L. paracasei* MT5-supernatant, dissolved in Krebs solution, on neuronal activation was studied. The control and probiotic solution had a physiological pH, but were hyper-osmotic (control MT5-supernatant: 0.7Osmol vs *L. paracasei* MT5-supernatant: 1.0Osmol). Compared to the control MT5-supernatant, the *L. paracasei* MT5-supernatant excited a higher proportion of enteric neurons (70.8% [58.3/81.8%] vs 90.0% [74.0/100.0%]) with a significant higher action potential frequency (3.1Hz [1.3/6.5Hz] vs 11.9Hz [7.5/17.5Hz]) (Figure 12). However, this increased spike frequency is very likely caused by the high osmolarity. Unfortunately, it was not possible to adjust the osmolarity to near physiological values as this would have involved removing all sodium from the Krebs solution. This would have interfered with the spiking behaviour of enteric neurons as action potential discharge is dependent on sodium influx through fast sodium channels.

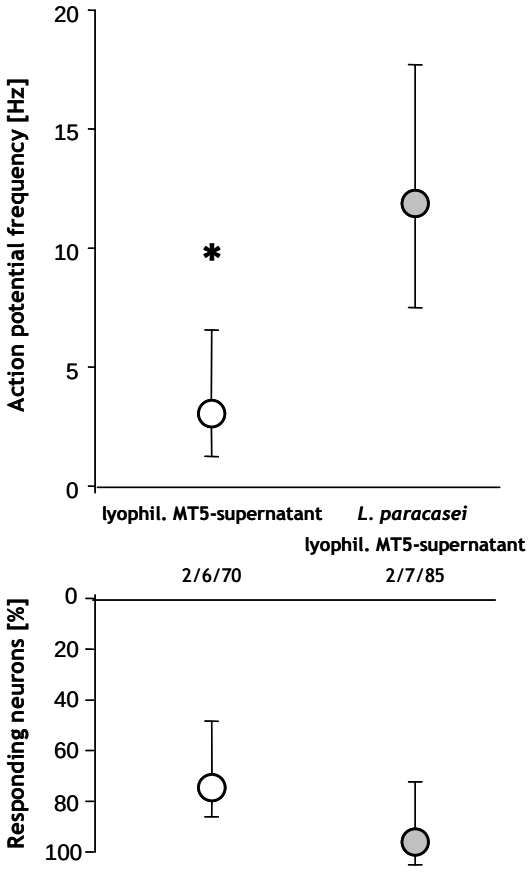


Figure 12: Reconstituted lyophilised control MT5-supernatant and lyophilised *L. paracasei* MT5-supernatant excited guinea-pig enteric neurons: The probiotic (grey) supernatant excited a higher proportion of neurons with a significantly higher action potential frequency compared to the control (white) (Mann-Whitney Rank Sum Test: $P = 0.073$ and $P < 0.001$). Numbers indicate numbers of tissues/ganglia/neurons studied.

At least under our *in vitro* experimental conditions, it must be concluded that soluble factors in *L. paracasei* NCC 2461 conditioned media had no direct effect on enteric neurons. Whenever spike discharge after application of *L. paracasei* NCC 2461 supernatants was observed, the effect was mimicked by the control medium. It turned out to be impossible to avoid these non-specific medium effects due to pH, osmolarity or medium ingredients which forced us to stop this project.

3.1.2 Effect of probiotic feeding on secretory behaviour of WT and $TNF^{\Delta ARE/WT}$ mice

In the $TNF^{\Delta ARE/WT}$ mouse model of IBD, the effect of probiotic feeding of the probiotic mixture VSL#3 on secretory behaviour in proximal and distal colon was investigated. Two stimuli were applied in order to assess whether probiotic treatment would affect the secretory activity of the intestinal tissues. The first stimulus was electrical field stimulation (EFS) with 6V, which leads to submaximal activation of nerve-mediated secretion. The resulting secretory response reflects the net effect of a physiological neurotransmitter cocktail. The second stimulus was serosal application of the cAMP activator forskolin, which leads to secretion that is primarly mediated by direct actions on the epithelial cell causing secretion through activation of the CFTR chloride channel. The two stimuli were applied in the same tissue. Animals were divided into four groups: $TNF^{\Delta ARE/WT}$ mice treated with placebo (n = 8), or with VSL#3 (n = 9) and WT mice treated with placebo (n = 8) or with VSL#3 (n = 5).

Figure 13 shows that the probiotic feeding with the probiotic mixture VSL#3 had no effect on the EFS induced secretion, neither in the proximal nor in the distal colon of WT and $TNF^{\Delta ARE/WT}$ mice. Instead it has been detected, that in the proximal colon of $TNF^{\Delta ARE/WT}$ mice the EFS evoked response was significantly smaller compared to the WT mice in both placebo and VSL#3 fed animals. In the distal colon only the EFS evoked response of VSL#3 treated $TNF^{\Delta ARE/WT}$ mice was significantly lower compared with WT mice.

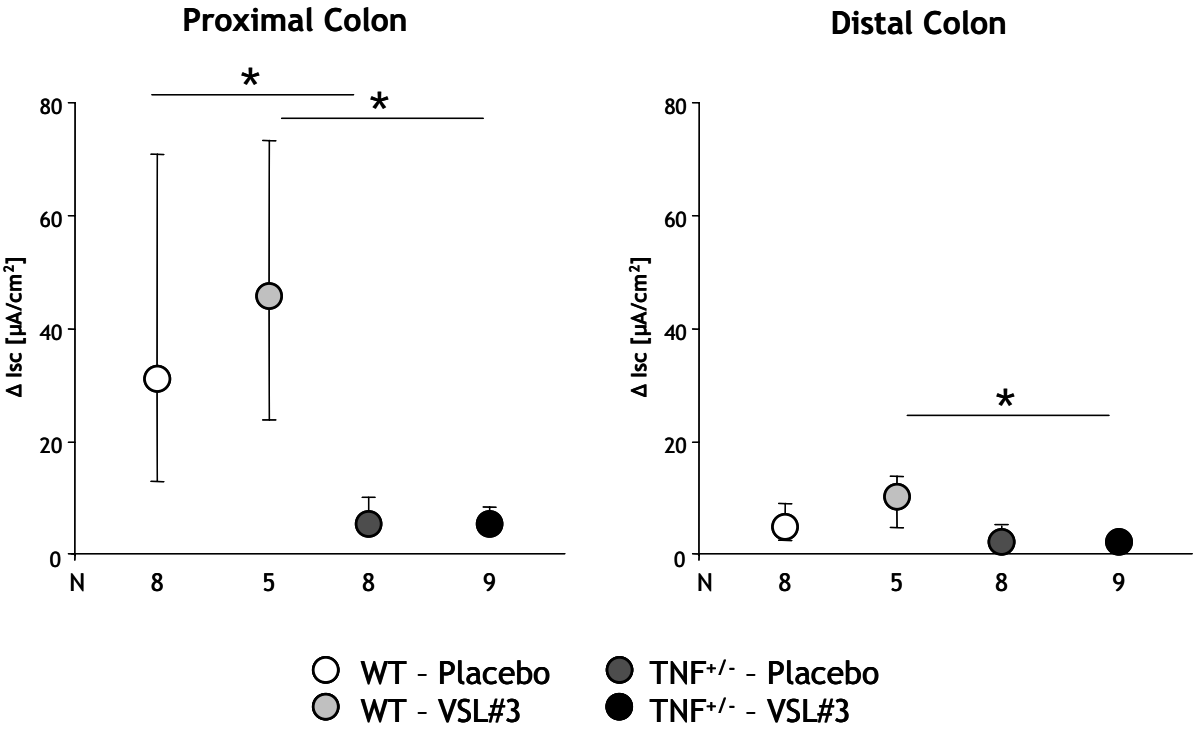


Figure 13: Effect of probiotic feeding (VSL#3) on nerve evoked secretion in proximal (left) and distal colon (right): The graph shows the response to EFS evoked secretion in WT mice treated with placebo (white) or with VSL#3 (light grey) and TNF$^{\Delta ARE/WT}$ mice treated with placebo (dark grey) or with VSL#3 (black). In the proximal colon, the EFS evoked response was significantly smaller in the TNF$^{\Delta ARE/WT}$ mice compared to the WT mice in both placebo and VSL#3 fed animal (Kruskal-Wallis One Way Analysis of Variance on Ranks, Dunn's Method, $P < 0.001$). In the distal colon the EFS evoked response of the VSL#3 treated TNF$^{\Delta ARE/WT}$ mice was significantly smaller compared to the WT mice (One Way Analysis of Variance, Tukey Test, $P = 0.011$). VSL#3 treatment had no effect on the EFS induced secretion, neither in the proximal nor in the distal colon. Symbols mark significant difference.

Similar results were also seen with epithelial mediated secretion. VSL#3 treatment had no effect on forskolin induced secretion, neither in the proximal nor in the distal colon of WT and TNF$^{\Delta ARE/WT}$ mice. Forskolin induced secretion was significantly smaller in the proximal colon of TNF$^{\Delta ARE/WT}$ mice compared to WT mice. However, in the distal colon there were no significant differences in terms of forskolin induced responses (Figure 14).

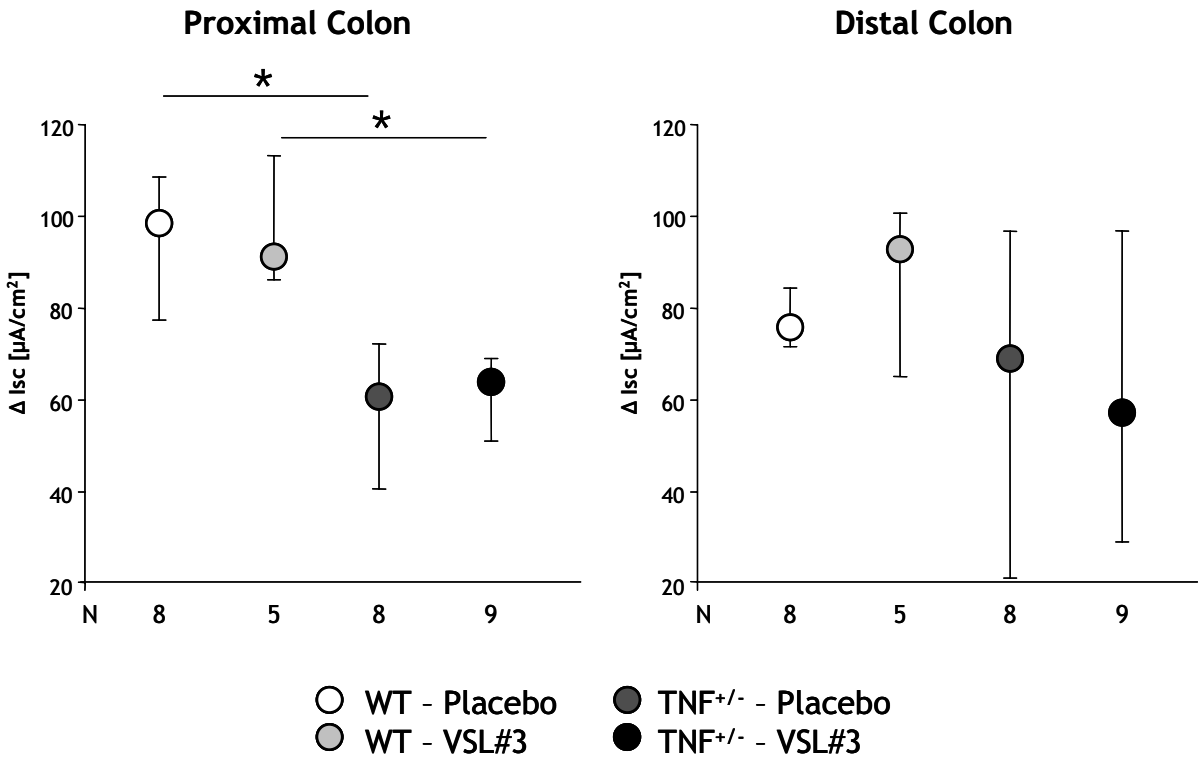


Figure 14: Effect of probiotic feeding (VSL#3) on forskolin activated secretion in proximal (left) and distal colon (right): The graph shows the responses to forskolin induced secretion in WT mice treated with placebo (white) or with VSL#3 (light grey) and TNF$^{\Delta ARE/WT}$ mice treated with placebo (dark grey) or with VSL#3 (black). In the proximal colon the forskolin induced secretion was significantly smaller in TNF$^{\Delta ARE/WT}$ mice compared to WT mice in both placebo and VSL#3 treated tissues (One Way Analysis of Variance, Tukey Test, *P* < 0.001). Whereas in the distal colon the forskolin induced secretion was not significantly different (Kruskal-Wallis One Way Analyis of Variance on Ranks, *P* = 0.5). The VSL#3 treatment had no effect on the forskolin induced secretion. Symbols mark significant difference.

The transepithelial resistance was not significantly changed by VSL#3 treatment in the WT or in the TNF$^{\Delta ARE/WT}$ mice. Furthermore the transepithelial resistance was not significantly different between WT and TNF$^{\Delta ARE/WT}$ mice (Figure 15).

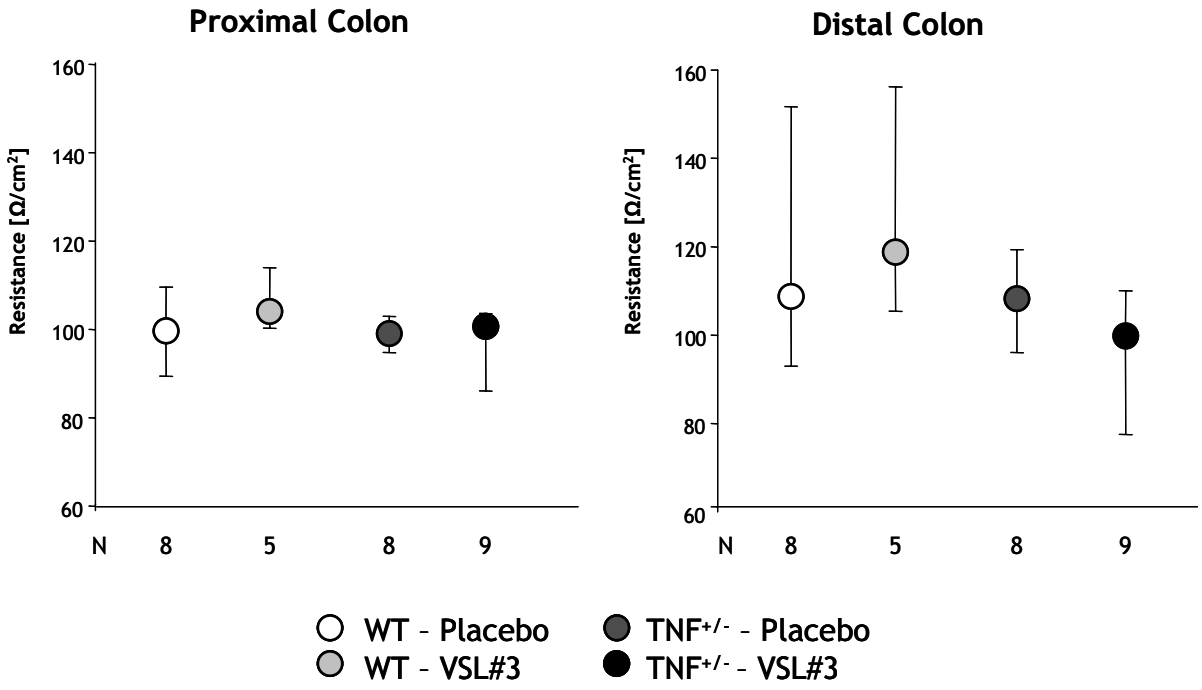


Figure 15: Effect of probiotic feeding (VSL#3) on transepithelial resistance in proximal (left) and distal colon (right): The graph shows transepithelial resistance in WT mice treated with placebo (white) or with VSL#3 (light grey) and TNF$^{\Delta ARE/WT}$ mice treated with placebo (dark grey) or with VSL#3 (black). TNF$^{\Delta ARE/WT}$ mice and WT mice showed the same transepithelial resistance and VSL#3 treatment did not change the transepithelial resistance in the proximal (One Way Analysis of Variance, *P* = 0.361) or the distal colon (One Way Analysis of Variance, *P* = 0.191). Symbols mark significant difference.

These data suggest that the VSL#3 treatment had no significant effects on the integrity of the colonic mucosa and its secretory activity. Furthermore these results show that TNF$^{\Delta ARE/WT}$ mice exerted an impaired mucosal secretion due to significantly smaller nerve and epithelially mediated pro-secretory responses compared to WT mice. However, the VSL#3 treatment did not reverse the impaired secretion observed in TNF$^{\Delta ARE/WT}$ mice.

3.2 Neural action of the bacterial protease gelatinase from *Enterococcus faecalis* (GelE)

3.2.1 The bacterial protease GelE evoked spike discharge in guinea-pig colonic submucous neurons

Since our *in vitro* experiments to study the neural actions of soluble mediators released by probiotics revealed negative results, we decided to test the neural action of the bacterial derived protease GelE. After the purification of GelE from *Enterococcus faecalis* OG1RF (by N. Steck, Biofunctionality, ZIEL-Research Centre of Nutrition and Food Science, Technische Universität München, Germany) the effect of GelE on enteric neurons of the guinea-pig colon was tested. A local perfusion protocol was used, by which individual ganglia were exposed to 3000nl (100nl/s) of GelE in a concentration of 100nM or 312nM. These concentrations were chosen on the basis of N. Steck results, where 2-10µg/ml of GelE was effective to decrease the tranepithelial electrical resistance (TEER). As a control the concentrated buffer solution was applied. 100nM GelE and 312nM GelE caused spike discharge in previously quiescent neurons and increased spike discharge in spontaneously active neurons. However, the 100nM GelE induced spike discharge was significantly higher compared to the 312nM GelE induced response. The proportion of activated neurons did not differ between the two GelE concentrations (100nM GelE: 20.0% [7.1/42.9%] vs 312nM GelE: 18.2% [7.5/25.4%]), whereby around 80% of the remaining neurons showed no response. GelE evoked spike discharge was significantly higher than basal (no application; data not shown) and control conditions, with the control buffer solution exerting negligible nerve activity (Figure 16)

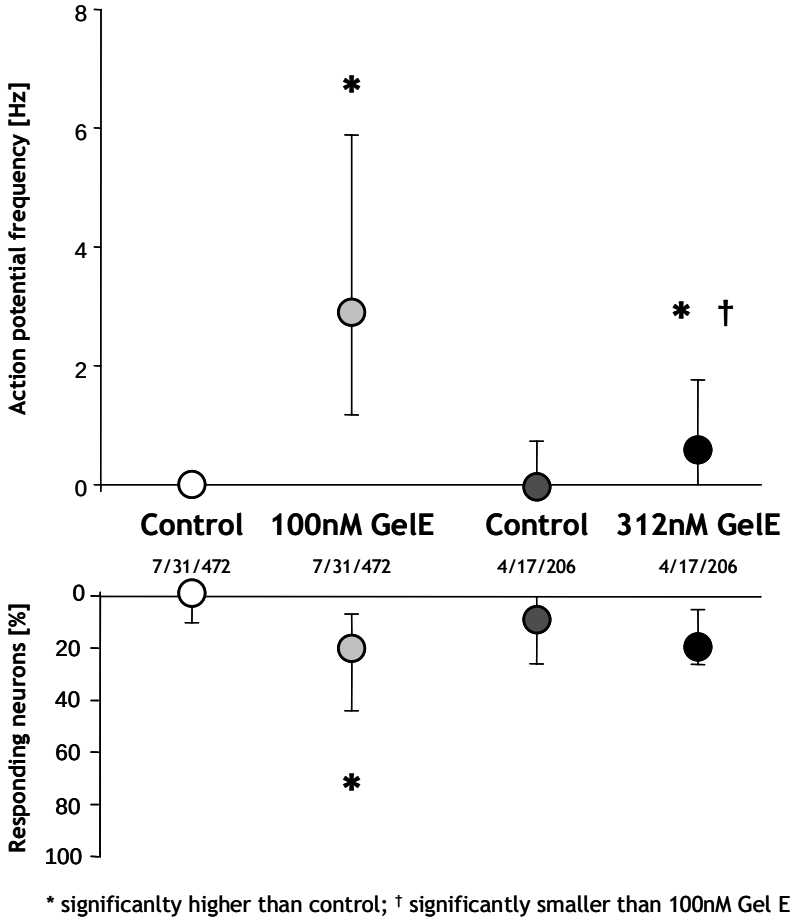


Figure 16: Effect of the bacterial protease GelE on guinea-pig submucous neurons: The application of GelE (100nM) for 30s induced an action potential spike discharge, which was significantly higher than the 312nM GelE induced response. The control buffer solution alone had no effect. Symbols mark significant differences as explained in the figure. Numbers indicate numbers of tissues/ganglia/neurons studied.

To elucidate the signalling mechanism of GelE, in the next set of experiments PAR2-AP (100µM) was applied 10min after the GelE (100nM) application. The PAR2-AP was applied for 30s as described in chapter 3.3.2. PAR2-AP caused a significant increase in neuronal activity; however following GelE treatment the response to PAR2-AP was significantly reduced. Furthermore the PAR2-AP induced spike discharge after GelE treatment was significantly lower than the GelE spike discharge per se (Figure 17). These data created the hypothesis that GelE may mediate is neuronal action via PAR2.

PAR2-AP induced a marked $[Ca]_i$ increase in enteric neurons and glia (see chapter 3.3.4.2), therefore the effect of GelE on $[Ca]_i$ transients using calcium imaging experiments were investigated**.** The experimental protocol was based on the 800ms microejection protocol used for the PAR studies (see chapter 3.3.2.). Figure 18 shows that neither GelE nor the control

buffer changed the $[Ca]_i$ in the submucous neurons. In addition no glia activation by GelE or control buffer could be detected.

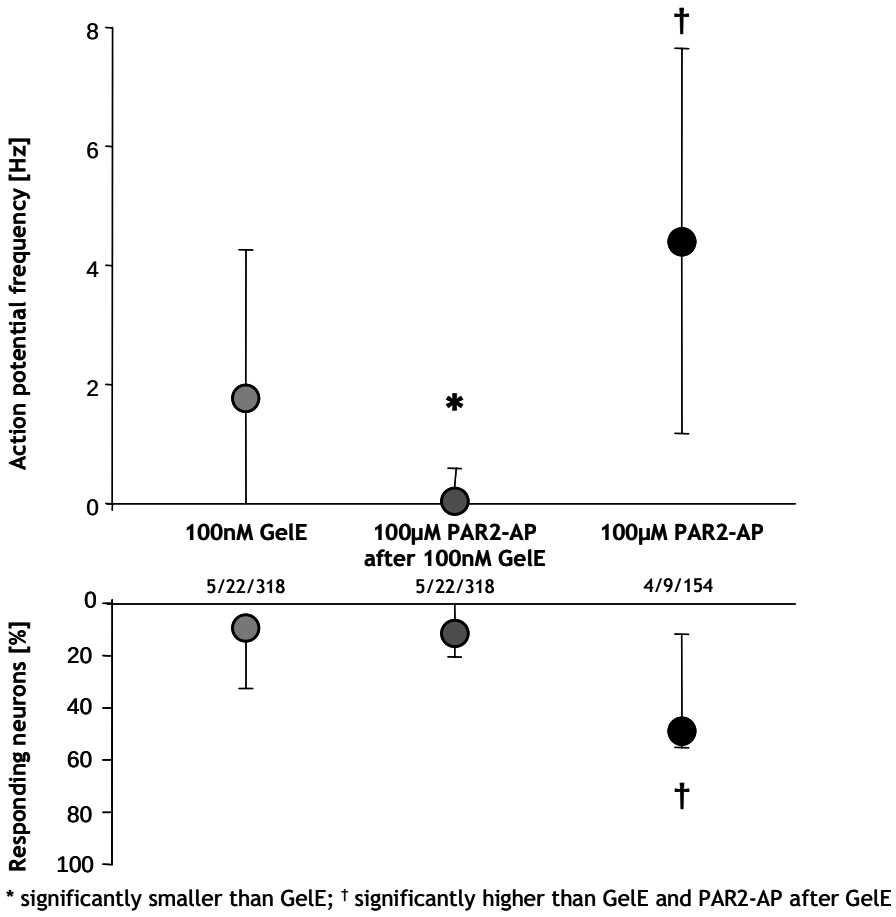


Figure 17: Reduced action of PAR2-AP after the application of the bacterial protease GelE on guinea-pig submucous neurons: The application of the PAR2-AP after 100nM GelE induced a significant lower spike discharge compared to PAR2-AP without preceding GelE treatment. Symbols mark significant differences as explained in the figure. Numbers indicate numbers of tissues/ganglia/neurons studied.

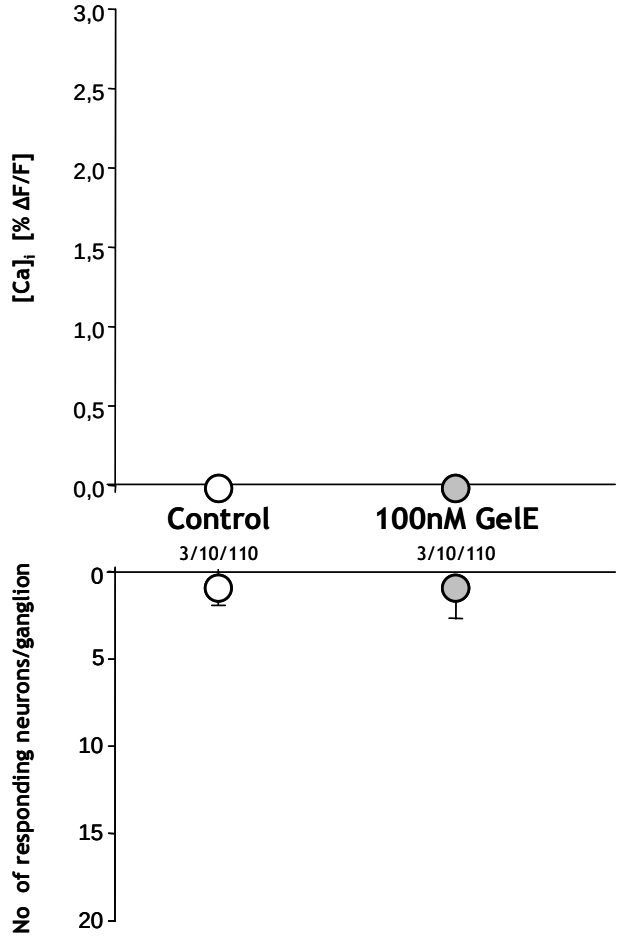


Figure 18: No effect of the bacterial protease GelE on $[Ca]_i$ in guinea-pig submucous neurons: Neither GelE (100nM) nor the control buffer exhibited any neuronal activating potential. Numbers indicate numbers of tissues/ganglia/neurons studied.

In the next step, functional studies were performed in order to assess whether the neural action of GelE caused increased secretion. Serosal application of 100nM GelE to mucosa/submucosa preparations of the guinea-pig colon did not induce any measurable changes of the I_{sc} (data not shown).

The above results suggest that GelE induced spike discharge in guinea-pig submucous neurons, but did not evoke any $[Ca]_i$ transients or epithelial ion secretion. After GelE treatment the PAR2-AP mediated neural action was significantly reduced, which supports the hypothesis that GelE interferes with the PAR2 response. However, the lack of $[Ca]_i$ increase in response to GelE argues against a direct action of GelE via the PAR2 Ca^{2+} pathway. Further studies are needed to address this issue by applying GelE in the presence of a PAR2 antagonist.

3.2.2 The bacterial protease GelE evoked no $[Ca]_i$ in human submucous neurons and glia

Neither GelE nor the control buffer could induce any $[Ca]_i$ response (Figure 19) in human submucous neurons and glia which is comparable to the data obtained in guinea-pig submucous plexus. It remains to be shown whether GelE would evoke any action potential discharge which would require VSD imaging in human submucous neurons. In human mucosa/submucosa preparation GelE did not change the basal I_{sc}, which exclude any pro-secretory potential (n = 6).

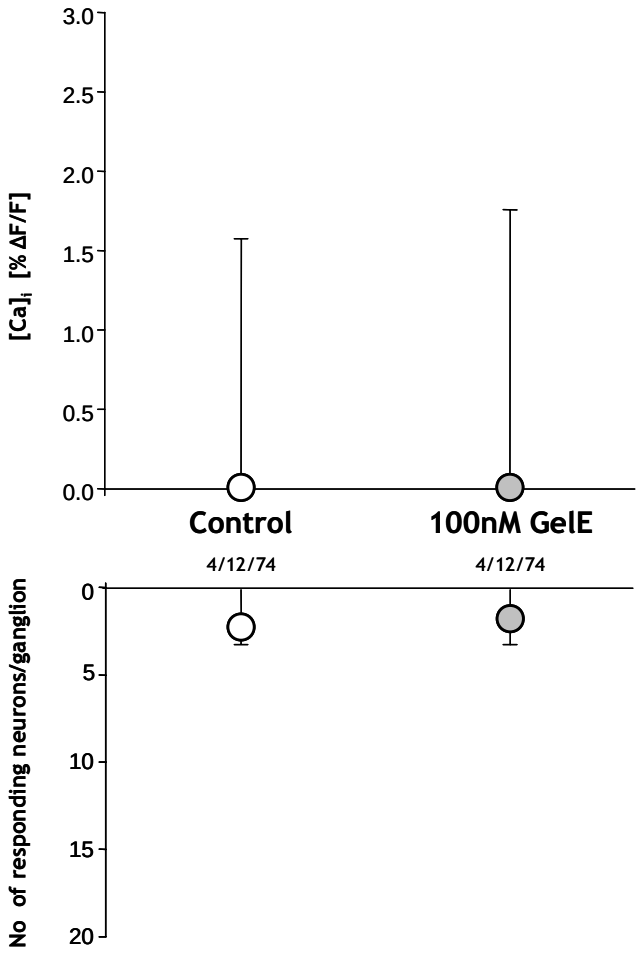


Figure 19: Bacterial protease GelE had no effect on $[Ca]_i$ in human submucous neurons: Neither GelE (100nM) nor the control buffer exhibited any neuronal activating potential. Numbers indicate numbers of tissues/ganglia/neurons studied.

3.3 Neural actions of Protease-Activated Receptors (PARs)

The rational for this part of the project is that proteases are targeting enteric neurons and are involved in the pathophysiology of IBD and IBS as it has been shown in animal studies (see introduction). We therefore wanted to study if PARs, which are activated by proteases, are expressed by human and guinea-pig submucous neurons and if their activation modulates neuronal activity.

3.3.1 Lack of PAR1 and PAR2 immunohistochemistry in human and guinea-pig submucous neurons and glia

Immunohistochemical stainings were performed to localise PAR1 and PAR2 in human and guinea-pig enteric neurons and glia. PAR2 was studied in human and guinea-pig submucous and myenteric plexus preparations by using the primary antibody SAM-11. Using Triton X-100 to permeabilise the membrane a non-specific PAR2 positive staining was observed in all guinea-pig submucous and myenteric neurons as well as in all human submucous neurons. In the human myenteric plexus no PAR2 positive cells could be detected. Since SAM-11 targets the extracellular N-terminus of the PAR2 receptor the stainings were repeated without Triton X-100. However, no specific staining was observed.

Since the PAR1 antibody ATAP-2 (mouse anti-Thrombin R) did not induce any PAR1 positive staining the goat anti-Thrombin R C-18 was used, which in contrast to the previous one targets the C-terminus of the PAR protein. However, no positive staining was observed in guinea-pig submucous plexus or in human submucous plexus (Figure 20), independent of the staining protocol used (see chapter 2.4 and Table 1). Similarly, the PAR1 antibody goat anti-Thrombin R N-19, which targets the N-terminus of the PAR1 protein, did not reveal stainings in guinea-pig or human submucous plexus.

In summary, none of the antibodies revealed any specific staining in the human or guinea-pig enteric nervous system.

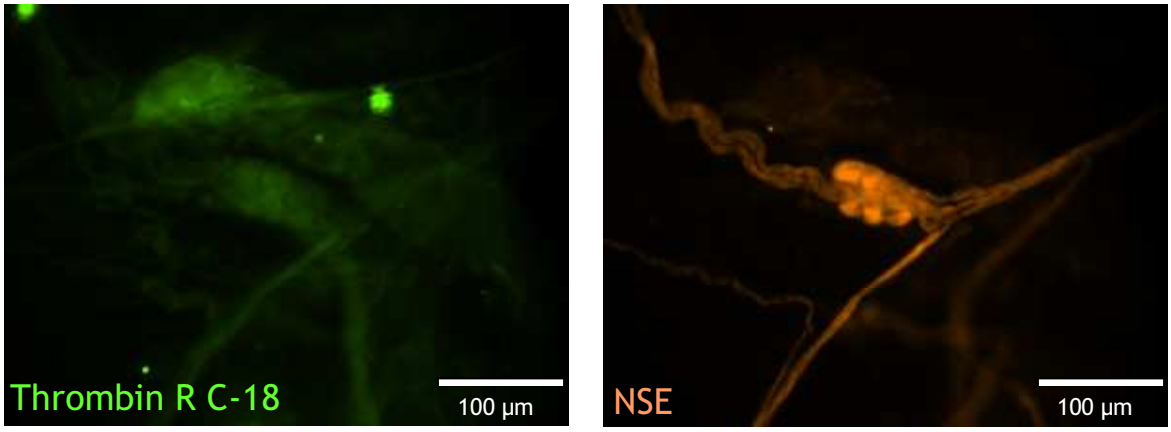


Figure 20: Lack of PAR1 positive staining by the PAR1 antibody goat anti-Thrombin R C-18 (1:500/Cy2) in whole-mount preparation of human submucous plexus. The pan-neuronal marker rabbit NSE (1:2000/Cy5) exhibited a cytoplasmatic staining pattern and labelled also nerve fibres.

3.3.2 Preliminary experiments to establish a protocol for voltage- and calcium-sensitive dye recordings to validate PAR-activating peptides (APs) action in guinea-pig submucous plexus preparations

Based on already published data on the action of PAR-APs on guinea-pig submucous and myenteric neurons (see introduction), one aim was to establish a protocol for VSD recordings in guinea-pig submucous plexus preparations to validate PAR-APs action. Linden et al. (2001) microejected the PAR2-AP (SLIGRL-NH_2) and trypsin for 900ms onto the myenteric neurons of the guinea-pig ileum and detected a depolarisation. Reed et al. (2003) and Gao et al. (2002) added the PAR agonists to the superfusion solution and obtained after a brief application of several minutes a depolarisation of submucous and myenteric neurons. Since Reed et al. (2003) showed that PAR2-AP excited guinea-pig ileal submucous neurons, our first aim was to reproduce this in guinea-pig colonic submucous neurons.

Initially, 100µM PAR2-AP was microejected for 800ms for up to 2s onto submucous ganglia of the guinea-pig colon. But PAR2-AP did not induce any spike discharge (Figure 21).

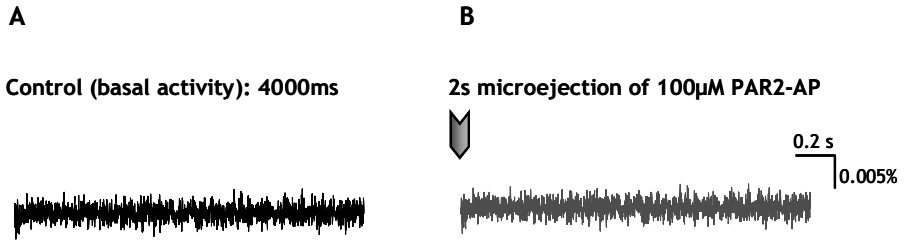


Figure 21: No immediate response after the microejection of PAR2-AP (100µM) onto submucous ganglia of guinea-pig colon: (A) Basal activity of submucous neurons. (B) The PAR2-AP (100µM) did not induce any spike discharge after 2s microejection.

Consequently, the concentration of PAR2-AP was increased to 1mM and the recording protocol was changed. Multiple acquisitions with varying time intervals in between, starting from 1s up to 12s were performed. But PAR2-AP did again not evoke any action potential discharge (Figure 22). These two experimental approaches led to the conclusion that PAR2-AP did not induce any immediate spike discharge and that longer exposures are needed.

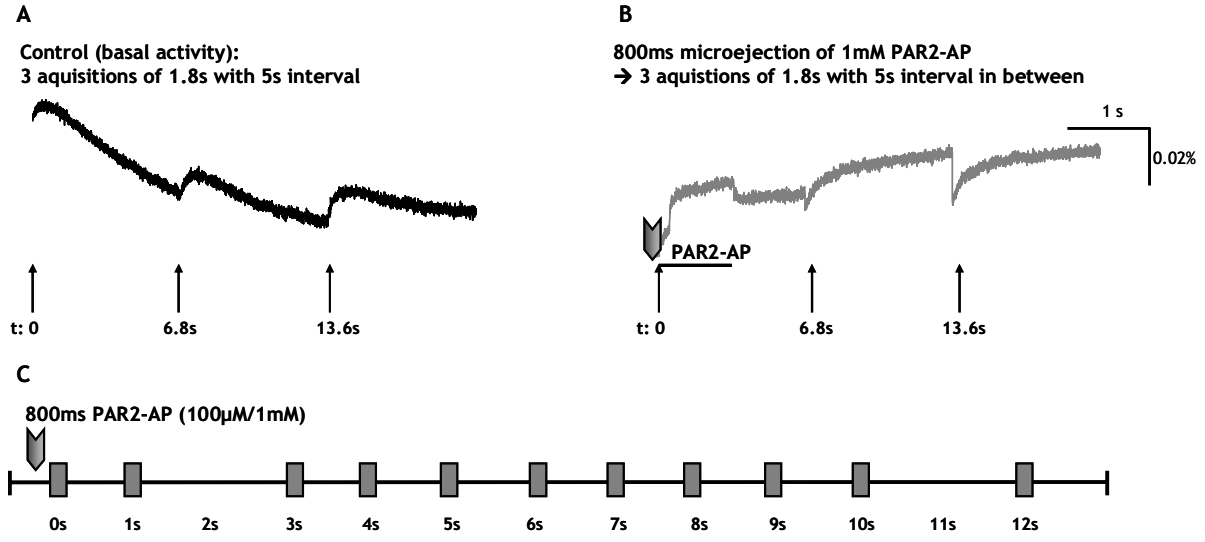


Figure 22: No immediate response after the microejection of PAR2-AP (1mM) onto submucous ganglia of guinea-pig colon: (A) Basal activity of submucous neurons. (B) The PAR2-AP (1mM) did not induce any spike discharge neither 0ms, 6.8s nor 13.6s after the microejection (800ms). (C) Time scale indicates the different interval periods used to record the neuronal response.

PAR2-AP (1mM) was applied 4 times for 800ms onto the ganglion and the response up to 120s after the application was recorded. However, no neuronal activation was observed using this approach (Figure 23). The next approach was to increase the responsiveness of enteric neurons by increasing the potassium concentration in the Krebs solution superfusing the tissue, but PAR2-AP still had no effect.

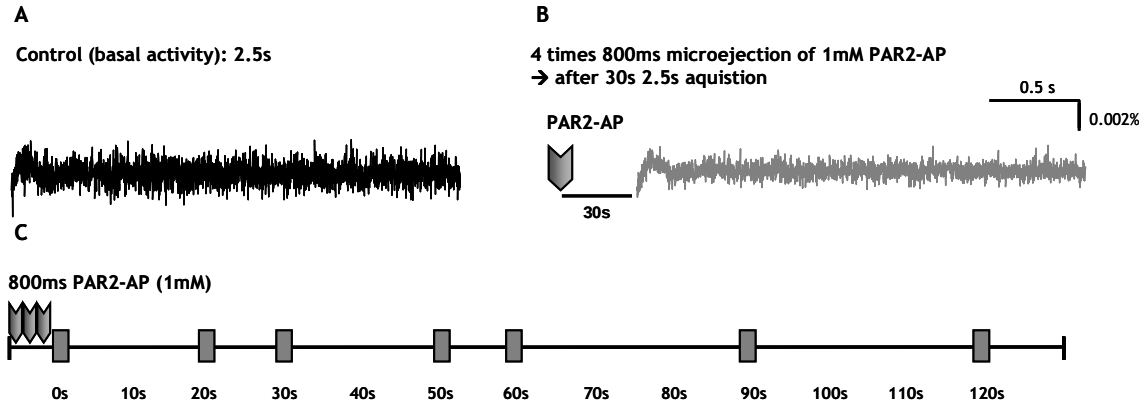


Figure 23: No long-term response after the microejection of PAR2-AP onto submucous ganglia of guinea-pig colon: (A) Basal activity of submucous neurons. (B) The PAR2-AP (1mM) did not induce any late onset spike discharge 30s after the microejection (800ms). (C) Time scale indicates the time between application and start of recording.

Since the microejection approach was unsuccessful in causing any immediate or long-term spike discharges, PAR2-AP (100µM) was added to the superfusing Krebs solution to study late onset spike discharge after long-term incubation. As Figure 24 shows, after 2min of long-term incubation of PAR2-AP the submucous neurons started firing action potentials which continued for up to 12min. The above results suggest that PAR2-AP induces a slowly developing late onset spike discharge.

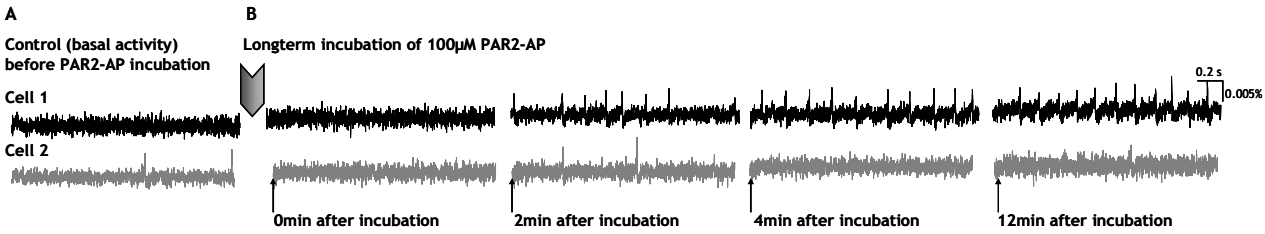


Figure 24: Spike discharge after the long-term incubation of colonic guinea-pig submucous ganglia with PAR2-AP: (A) Basal activity of submucous neurons before incubation. (B) PAR2-AP (100µM) induced spike discharge after 2min of long-term incubation.

Based on the physiology of the PAR receptors a desensitization of the receptors to a second application of the agonist occurs (Reed et al. 2003; Gao et al. 2002). This tachyphylaxis made long-term incubation of the entire tissue quite unattractive, because it would allow studying just one ganglion per tissue. To overcome this problem a local perfusion protocol was established. Three different protocols were evaluated: (1) 3000nl (100nl/s) local perfusion with recording 0, 1 and 2min after perfusion; (2) 6000nl (100nl/s) local perfusion with recording 0, 1 and 2min after perfusion; (3) 9900nl (100nl/s) local perfusion with recording

0, 1 and 2min after perfusion (Figure 25). 3000nl, 6000nl and 9900nl local perfusion of 100µM PAR2-AP induced action potential firing in guinea-pig submucous neurons 0, 1, and 2min after the perfusion.

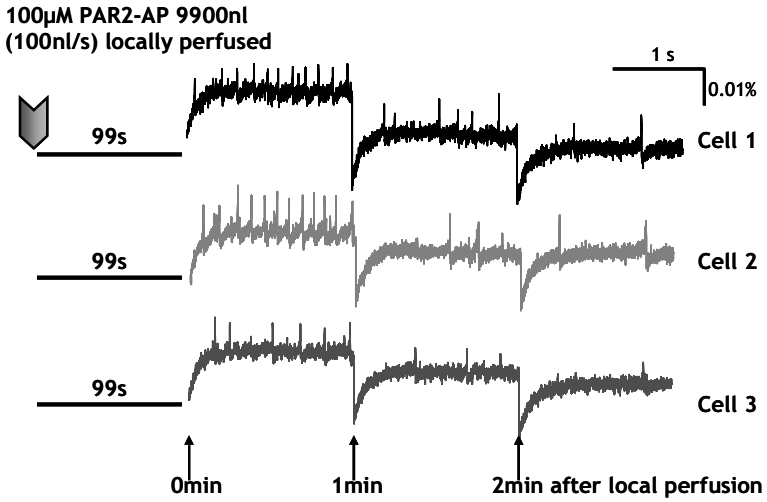


Figure 25: Spike discharge after local perfusion of PAR2-AP onto colonic guinea-pig submucous ganglia: Local perfusion of 9900nl PAR2-AP (100µM) for 99s at a rate of 100nl/s induced action potentials 0, 1 and 2min after the perfusion.

Only the following protocol revealed reliable responses in the guinea-pig submucous plexus and was therefore used as standard protocol for studying the effect of PAR-AP and -RP on guinea-pig colonic submucous neurons: 3000nl of PAR-APs or PAR-RPs (100µM) were locally perfused for 30s over the ganglion at a rate of 100nl/s followed by a recording period of 1.8s (Figure 26).

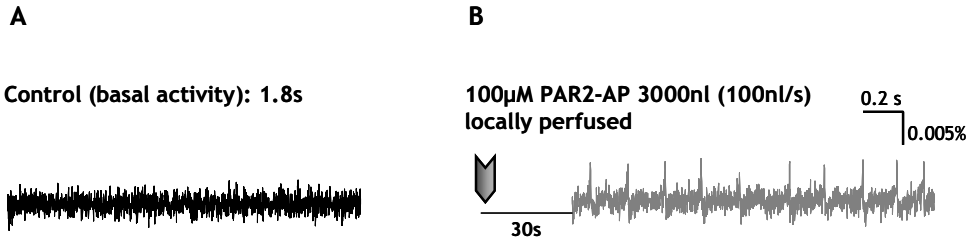


Figure 26: Spike discharge response after 30s local perfusion of PAR2-AP onto colonic guinea-pig submucous ganglia: (A) Basal activity of submucous neurons. (B) Local perfusion of 3000nl PAR2-AP (100µM) for 30s at a rate of 100nl/s induced action potentials.

Since VSD imaging reliably reveals action potential discharge but is of limited use to resolve subthreshold membrane depolarisations, in addition calcium imaging in guinea-pig submucous plexus were performed. Since PAR2-AP induced only late onset spike discharge after local perfusion, calcium imaging ensured additionally the detection of early onset

subthreshold responses by PAR-APs or responses that remain subthreshold throughout. For these experiments it turned out that a 800ms puff application of PAR-APs evoked reliable responses. This was detected by using a 20s recording period.

3.3.3 Preliminary experiments to establish a protocol for voltage- and calcium-sensitive dye recordings to validate PAR-APs action in human submucous plexus preparations

The human submucous neurons responded quite differently to PAR-APs which required a further modification of the application method. Neither microejection nor local perfusion of the PAR2-AP could induce any reliable neuronal activation (Table 4). Therefore the effect of another PAR-AP, PAR1-AP was investigated. PAR1-AP evoked action potential discharge when microejected for 2s onto the ganglion. Regarding the timing of the response the immediate response of human submucous neurons differed from the late onset response in guinea-pig neurons. Consequently, the local perfusion protocol (3000nl at a rate of 100nl/s) was also applied in the human tissue. However, the 2s microejection included in a 4s recording period evoked a stronger and more reliable neuronal activation and was therefore used as standard protocol for experiments in the human submucous plexus (Table 4).

Table 4: Comparison of the action potential frequency [Hz] and the proportion of responding neurons [%] between 2s microejection and 3000nl (100nl/s) local perfusion of PAR1, 2 and 4-APs in human submucous plexus preparations.

Median [25th/75th percentile]	Action potential frequency [Hz]		Statistics	Proportion of responding neurons [%]		Statistics	Tissues/Ganglia/Neurons	
	2s micro-ejection	3000nl local perfusion		2s micro-ejection	3000nl local perfusion		2s micro-ejection	3000nl local perfusion
PAR1 TFLLR-NH_2	2.2 [1.0/3.8]	1.5 [0.6/3.2]	p=0.204 Mann-Whitney Rank Sum Test	71.4 [50.0/83.7]	42.2 [0.0/61.9] *	p=0.021 t-Test	11/19/108	5/12/79
PAR2 SLIGKV-NH_2	0.8 [0.3/1.3]	1.8 [0.6/2.8] *	p=0.015 Mann-Whitney Rank Sum Test	6.3 [0.0/27.1]	0.0 [0.0/20.0]	p=0.713 Mann-Whitney Rank Sum Test	3/8/59	13/44/359
PAR4 GYPGQV-NH_2	0.8 [0.3/2.4]	0.6 [0.0/1.8]	p=0.632 Mann-Whitney Rank Sum Test	33.3 [8.3/56.3]	6.1 [0.0/29.2]	p=0.096 t-Test	4/12/89	4/12/75

As with the guinea-pig preparations calcium imaging experiments were also performed in human submucous plexus. For these experiments PAR-APs and -RPs were microejected for 800ms onto the ganglia and the responses were recorded for 20s.

3.3.4 Neural actions of PAR-APs in guinea-pig colonic submucous plexus

3.3.4.1 PAR-APs evoked spike discharge in guinea-pig colonic submucous neurons

Application of PAR2-AP onto guinea-pig submucous neurons induced a median action potential frequency of 4.4Hz [1.2/7.6Hz] in 47.1% of the submucous neurons (on average 8 neurons per ganglion). The PAR2-AP mediated a significantly higher spike discharge compared to the PAR1-AP (1.2Hz [0.0/2.4Hz]) and PAR4-AP (0.6Hz [0.6/1.8Hz]) (Figure 27). As expected, a second application of PAR-APs, 10min after the first one, led to a significantly weaker response in a much smaller proportion of submucous neurons due to receptor desensitization and/or internalisation. The weak PAR4-AP induced spike discharge was smaller, yet not significant, after the second application (Figure 27).

To test the specificity of the PAR-APs the effect of the reversed sequences (PAR-RPs) was studied. None of the PAR-RPs had effect on nerve activity in the guinea-pig submucous plexus (Table 5).

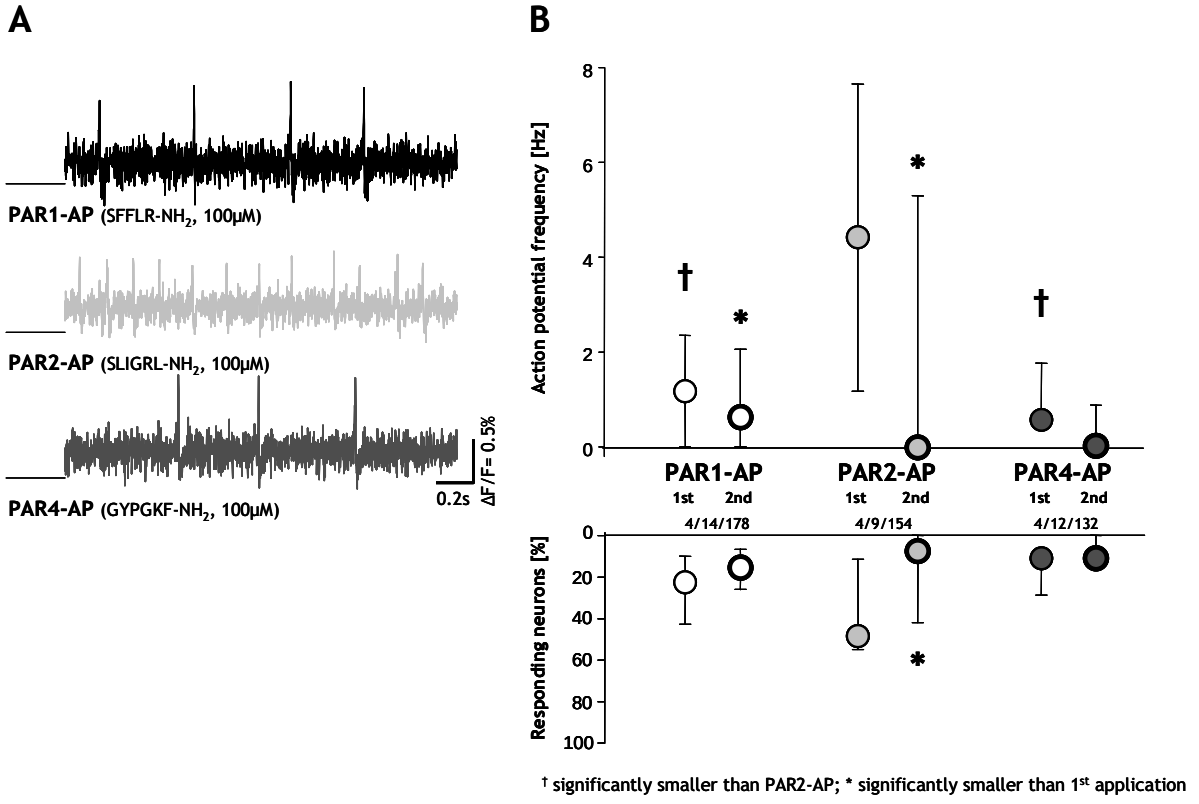


Figure 27: Neural action of PAR-APs in guinea-pig submucous neurons recorded with Di-8-ANEPPS imaging: (A) The traces show representative responses to PAR1, 2 and 4-APs after 30s of application (indicated by the horizontal bars). Each peak corresponds to one action potential. (B) PAR2-AP (100µM) induced the highest neural activation, followed by PAR1-AP (100µM) and PAR4-AP (100µM). Due to

receptor tachyphylaxis the second application was significantly smaller after PAR1 and 2-AP. The second application of PAR4-AP was smaller, but this effect was not significant. Symbols mark significant differences as explained in the figure. Numbers below PAR-APs indicate numbers of tissues/ganglia/neurons studied.

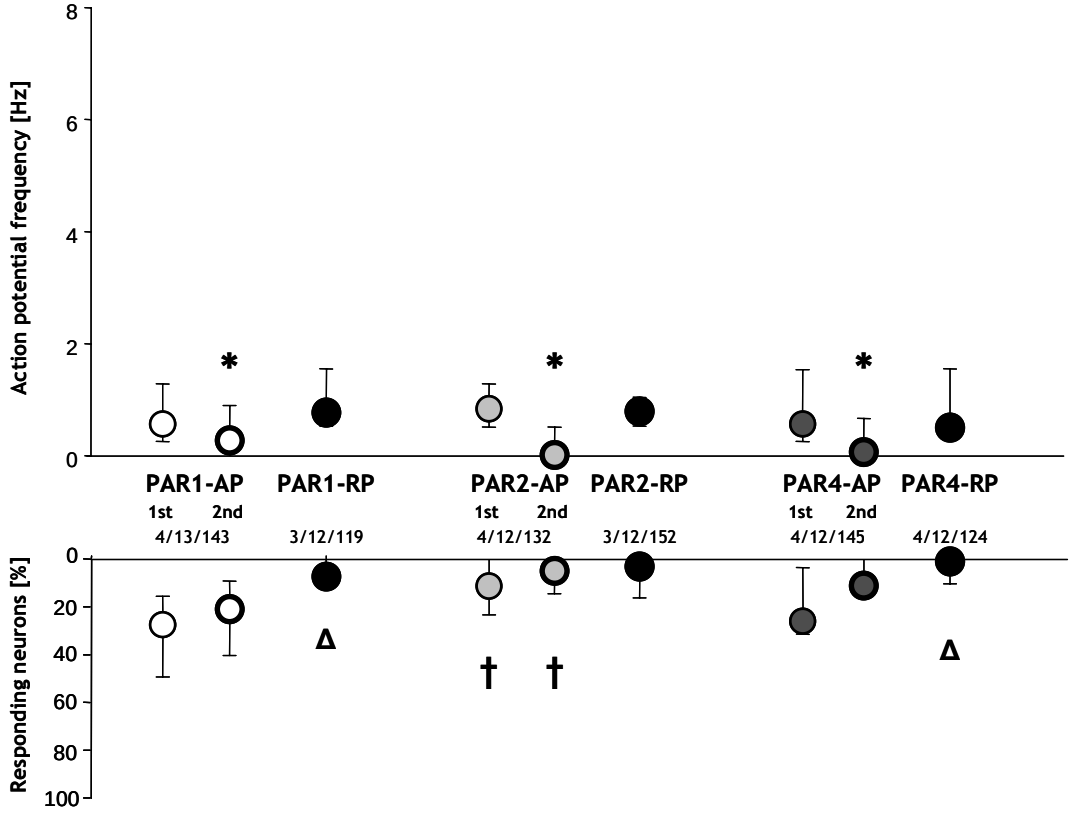
Table 5: Neural action of PAR-APs and -RPs in guinea-pig submucous neurons recorded with Di-8-ANEPPS imaging: The PAR-RPs had no significant nerve activating action. Asterisks mark significant differences ($p<0.005$).

	PAR1-AP	PAR1-RP	PAR2-AP	PAR2-RP	PAR4-AP	PAR4-RP
Action potential frequency [Hz]	1.2 [0.0/2.4]	1.8 [1.2/4.2]	4.4 [1.2/7.6]	1.2 [0.7/2.2]*	0.6 [0.6/1.8]	1.8 [1.8/2.9]*
Statistics	p=0.191 Mann-Whitney Rank Sum Test		p=0.022 Mann-Whitney Rank Sum Test		p=0.002 Mann-Whitney Rank Sum Test	
Proportion of responding neurons [%]	21.5 [10.0 /41.2]	0.0 [0.0/7.5]*	47.1 [13.2/52.7]	10.5 [4.5/12.8]*	10.6 [6.5/12.8]	4.2 [0.0/24.0]
Statistics	p=0.004 Mann-Whitney Rank Sum Test		p=0.020 Mann-Whitney Rank Sum Test		p=0.386 Mann-Whitney Rank Sum Test	
Tissues/Ganglia/Neurons	4/14/178	3/11/148	4/9/154	4/7/128	4/12/132	4/12/121

The above results suggest that it was possible to induce neuronal excitation by using PAR-APs and support the importance of PAR2 in the guinea-pig intestine as it has been previously shown by Reed et al. (2003), Covera et al. (1999), Linden et al. (2001) and Gao et al. (2002). Furthermore, the above results suggest that PAR2 is the most important PAR receptor in guinea-pig submucous plexus, followed by PAR1 and PAR4. In addition, further evidence was provided for the specificity and described tachyphylaxis of the receptor.

Due to the different timings of the PAR-APs induced responses in guinea-pig and human submucous neurons different experimental protocols (3000nl local perfusion vs 2s microejection) were established. However, for consistency the 2s microejection protocol was also systematically performed in the guinea-pig submucous plexus. Figure 28 shows that PAR1, PAR2 and PAR4-APs induced a weak neuronal response which was significantly smaller after the second application, but this reduction was not significantly different between the PAR-APs. With the 2s application the PAR-APs and PAR-RPs evoked similar action potential frequencies. However, the proportion of responding neurons was significantly smaller after the application of PAR1-RP and PAR4-RP. The spike frequency evoked by the

PAR-APs was rather low (PAR-1 AP: 0.5Hz [0.3/1.3Hz] vs PAR-2 AP: 0.8Hz [0.5/1.3Hz] vs PAR-4 AP: 0.5Hz [0.3/1.5Hz]) and did not differ from the frequency of PAR-RPs and between the PAR-APs. These results confirm the rational to use the local perfusion protocol for studying neuronal action of PARs in guinea-pig submucous plexus.



† significant difference between PAR-APs; * significanlty smaller than 1st application; Δ significant difference between PAR-AP and PAR-RP

Figure 28: Neural action of guinea-pig submucous neurons after 2s microejection of PAR-APs and -RPs recorded with Di-8-ANEPPS imaging: PAR1, PAR2 and PAR4-APs induced a similar weak neural excitation, which was significantly smaller after the second application, but was not significantly different from the neuronal action of the PAR-RPs. Symbols marks significant differences as explained in the figure. Numbers below PAR-APs indicate numbers of tissues/ganglia/neurons studied.

3.3.4.2 PAR-APs evoked $[Ca]_i$ signals in guinea-pig colonic submucous neurons and glia

Since the local perfusion protocol allowed only the detection of late onset responses in the guinea-pig and revealed only excitation associated with action potential discharge calcium imaging was used as a more general reporter of cell activation. During the experiment it was noted that PAR-APs evoked $[Ca]_i$ mobilisation not only in neurons but also in glia. In the guinea-pig submucous plexus we only studied $[Ca]_i$ transients after PAR1-AP and PAR2-AP because of the almost negligible effect of the PAR4-AP on spike discharge.

The PAR2-AP induced a large $[Ca]_i$ increase in guinea-pig enteric neurons and glia cells. The $[Ca]_i$ raise to a second application of the PAR2-AP, 10min after the first one, was significantly smaller in the enteric neurons and glia cells (Figure 29).

PAR1-AP induced a $[Ca]_i$ increase in enteric neurons, but not in enteric glia. The PAR1-AP induced $[Ca]_i$ response was significantly lower compared to the one evoked by PAR2-AP (Figure 30).

Based on previously published quantitative evaluation of the average number of neurons and glia in guinea-pig submucous ganglia (Hoff et al. 2008) it was possible to calculate the average number of responding glia and neurons. This analysis revealed that significantly more neurons responded to PAR-AP application with $[Ca]_i$ signals than with spike discharge. In the guinea-pig submucous plexus the PAR2-AP evoked a $[Ca]_i$ increase in 11.0 [9.0/12.3] neurons per ganglion, which correspond to 67% of the neurons, while the spike discharge was recorded in 47.1% of the neurons (8.0 [1.8/9.3] neurons per ganglion) ($P = 0.035$). PAR1-AP evoked $[Ca]_i$ transients in 12.0 [9.3/16.8] neurons per ganglion, which correspond to 73% of the neurons, while the spike discharge was recorded in 21.5% of the neurons (3.0 [1.0/5.0] neurons per ganglion) ($P < 0.001$). 52% of the enteric glia responded to PAR2-AP in the guinea-pig submucous plexus, whereas none of the glia cells responded to PAR1-AP.
The efficacy of the different PAR-APs to evoke neural $[Ca]_i$ confirmed the result obtained with Di-8-ANEPPS. PAR2-AP rather than PAR1-AP activated guinea-pig submucous neurons.

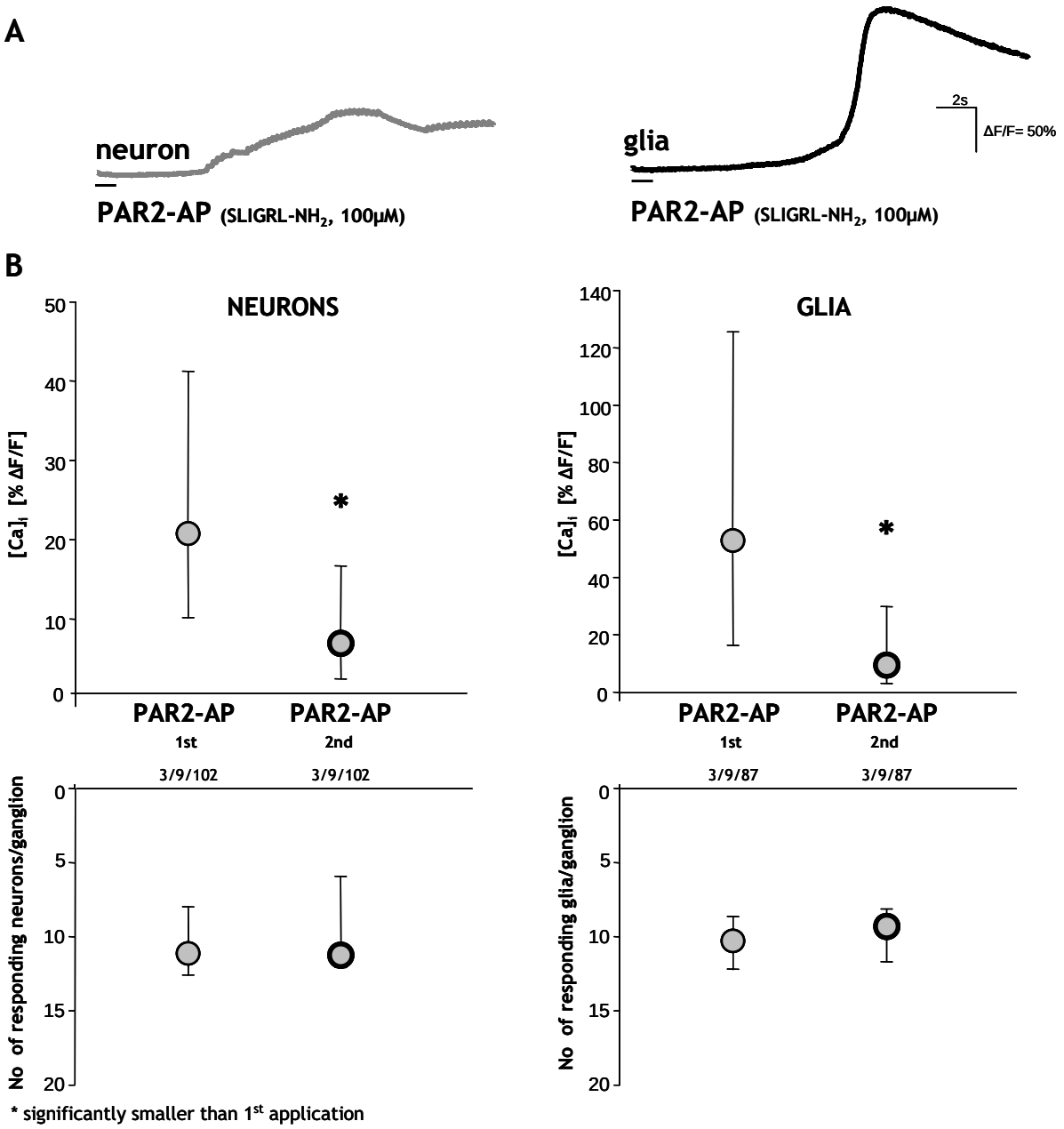


Figure 29: Effect of PAR2-AP on $[Ca]_i$ in guinea-pig submucous neurons and glia: (A) The traces show representative $[Ca]_i$ signals after spritz application of 100µM PAR2-AP (800ms; indicated by horizontal bars below the traces) in guinea-pig submucous neurons and glia. (B) PAR2-AP induced a $[Ca]_i$ raise in both enteric neurons and glia cells. The response to a second application was significantly smaller. Symbols mark significant differences as explained in the figure. Numbers below PAR-APs indicate number of tissues/ganglia/neurons or tissues/ganglia/glia studied.

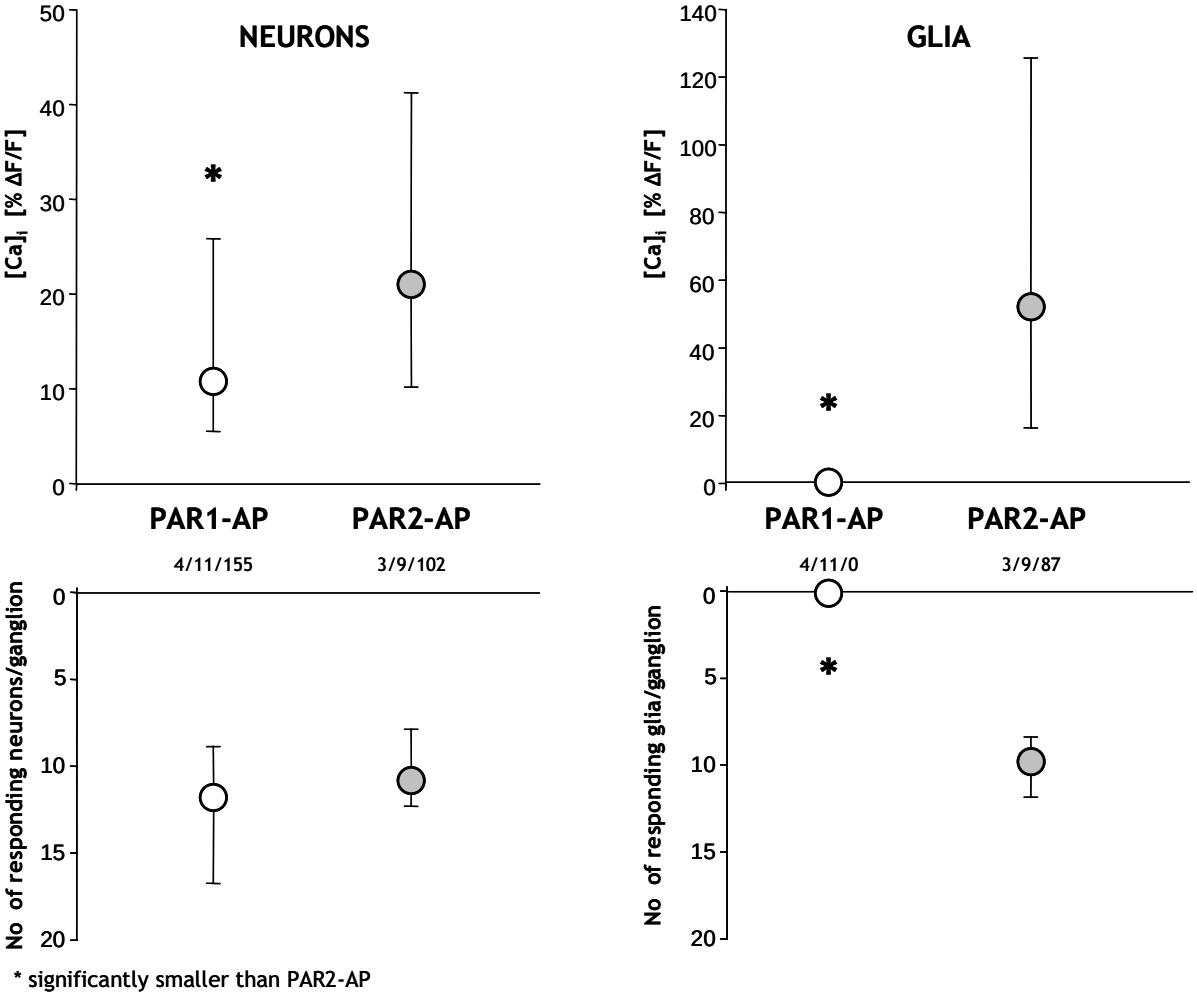


Figure 30: Effect of PAR1-AP and PAR2-AP on $[Ca]_i$ in guinea-pig submucous neurons and glia: PAR2-AP induced a $[Ca]_i$ raise in both enteric neurons and glia cells, whereas PAR1-AP increased $[Ca]_i$ only in enteric neurons. This PAR1-AP induced neuronal response was significantly lower compared to the PAR2-AP induced response. Symbols mark significant differences as explained in the figure. Numbers below PAR-APs indicate numbers of tissues/ganglia/neurons or tissues/ganglia/glia studied.

3.3.4.3 PAR2 antagonist reduced PAR2-AP induced $[Ca]_i$ increase in guinea-pig colonic submucous neurons and glia

The above results suggest a prominent role of PAR2 in neurons and glia of guinea-pig submucous plexus. To test the specificity of the PAR2-AP evoked responses the PAR2 antagonist LIGK-NH_2 was used. Calcium imaging was performed for these experiments because it revealed more responsive cells and allowed to study PAR2 antagonism in neurons and glia. The PAR2 antagonist significantly reduced the PAR2-AP induced $[Ca]_i$ increase in enteric neurons and glia cells (neurons: PAR2-AP: 20.9%ΔF/F [12.1/30.5%ΔF/F] vs PAR2-

AP in LIGK-NH_2: 7.4%ΔF/F [3.7/17.0%ΔF/F]; $P < 0.001$; glia: PAR2-AP: 66.9%ΔF/F [37.6/93.7%ΔF/F] vs PAR2-AP in LIGK-NH_2: 20.2%ΔF/F [10.0/40.0%ΔF/F]; $P = 0.002$; Figure 31), and in addition significantly reduced the number of responding glia per ganglion. This suggests that LIGK-NH_2 was able to block PAR2-AP mediated $[Ca]_i$ raise in guinea-pig enteric neurons and glia cells.

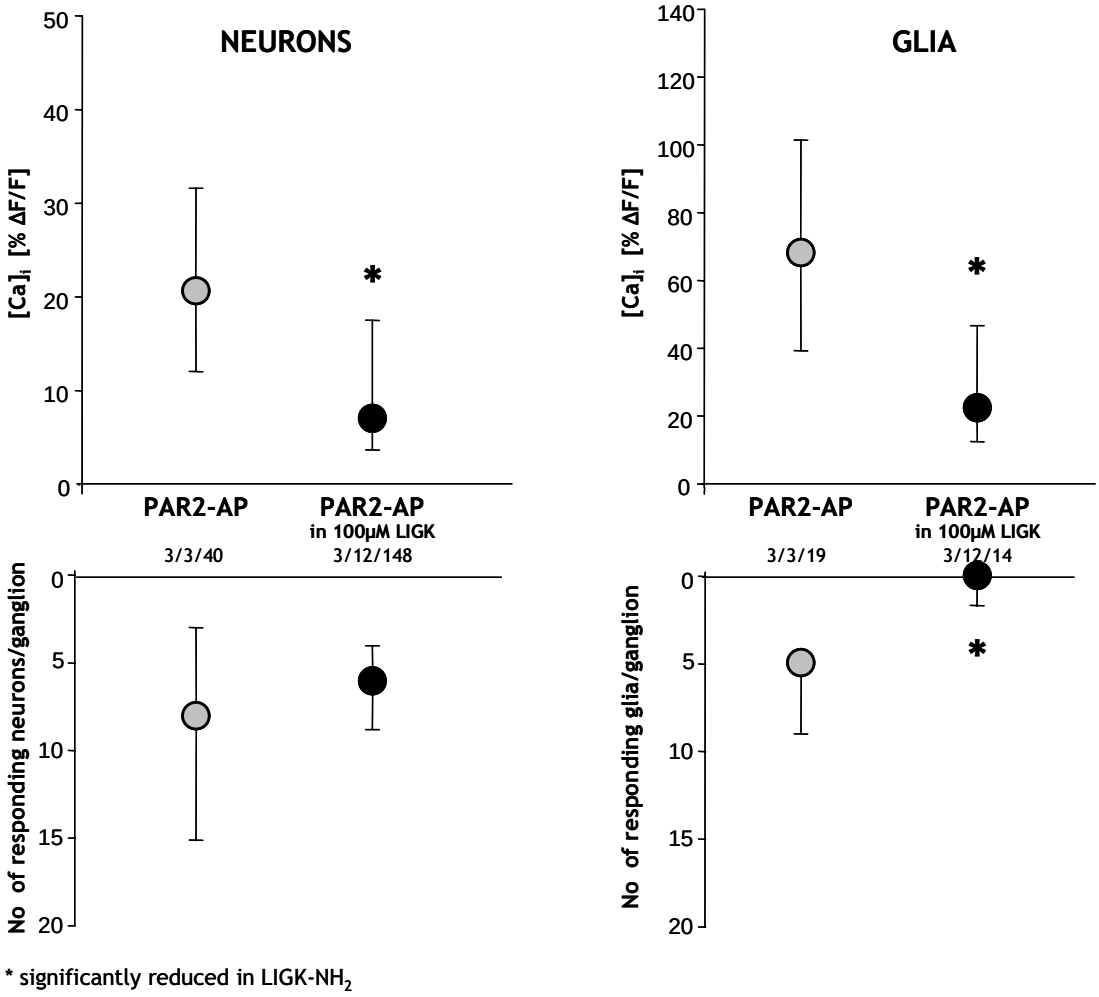


Figure 31: PAR2-AP induced neuronal and glia activation was reduced by the PAR2 antagonist LIGK-NH_2: The PAR2-AP induced $[Ca]_i$ raise was significantly reduced in the presence of the PAR2 antagonist LIGK-NH2 (100µM) in both enteric neurons and glia cells. The number of responding glia was significantly reduced, whereas the number of responding neurons was unchanged. Symbols mark significant differences as explained in the figure. Numbers below PAR-APs indicate numbers of tissues/ganglia/neurons or tissues/ganglia/glia studied.

3.3.4.4 PAR1 antagonist reduced PAR1-AP induced $[Ca]_i$ increase in guinea-pig colonic submucous neurons

To study the specificity of the PAR1-AP induced $[Ca]_i$ increase the PAR1 antagonist SCH79797 (1µM) was used. $[Ca]_i$ imaging was performed for these experiments because it revealed more responsive cells and allowed to compare the data to human experiments. The PAR1 antagonist significantly reduced the PAR1-AP induced $[Ca]_i$ raise in the enteric neurons as well as the number of responding neurons per ganglion (Figure 32). These data further support the involvement of PAR1 in enteric neurons as well as the specificity of the PAR1-AP.

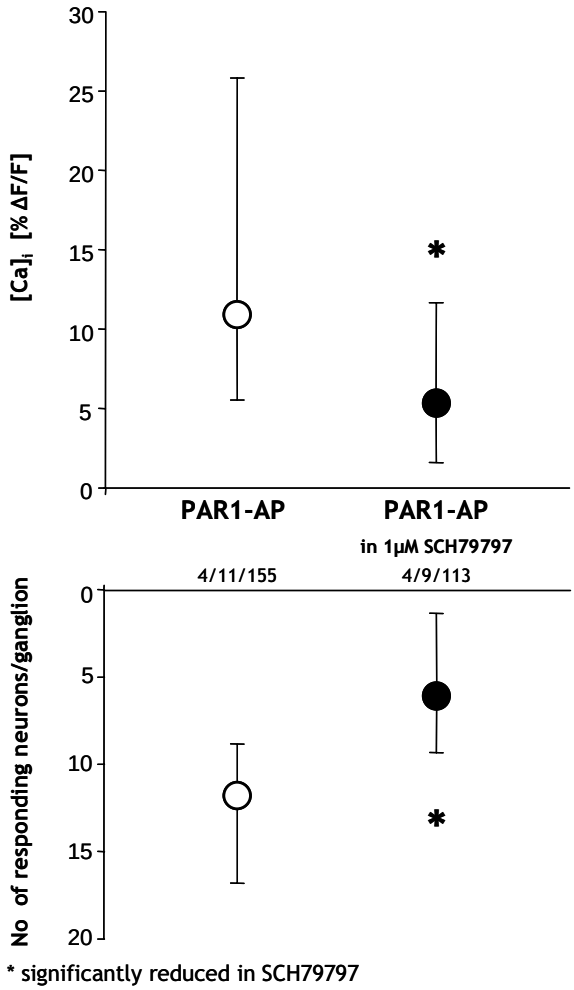


Figure 32: PAR1-AP induced neuronal activation was reduced by the PAR1 antagonist SCH79797: The PAR1-AP induced $[Ca]_i$ raise in guinea-pig enteric neurons and the number of responding neurons per ganglion were significantly reduced in the presence of the PAR1 antagonist SCH79797 (1µM). Symbols mark significant differences as explained in the figure. Numbers below PAR-APs indicate numbers of tissues/ganglia/neurons studied.

In conclusion, PAR1 and PAR2 specifically activated enteric neurons of the guinea-pig colon, with PAR2 appearing to be dominant in both neurons and glia.

3.3.5 Neural actions of PAR-APs in human submucous plexus

3.3.5.1 PAR-APs evoked spike discharge in human submucous neurons

Microejection of PAR1-AP (100µM) onto human submucous ganglion induced a spike discharge (2.1Hz [0.8/4.2Hz]) in 71.4% of the submucous neurons (on average 3 neurons per ganglion). The PAR1-AP activated a higher proportion of neurons with a significantly higher spike discharge compared to PAR4-AP (0.8Hz [0.3/2.4Hz]; 33.3% responding neurons) and PAR2-AP (0.8Hz [0.3/1.3Hz]; 6.3% responding neurons) (Figure 33). Even longer applications of PAR2-AP for 30s did not increase the number of responding neurons (median 0%) or the spike discharge (1.8Hz [0.6/2.8Hz]; T/G/N: 13/44/359; P = 0.713) (Table 4). Interestingly, longer application of PAR1-AP for 30s resulted in a significantly smaller activation (median 42.2% of the submucous neurons (T/G/N: 5/12/79), (P = 0.021); Table 4).

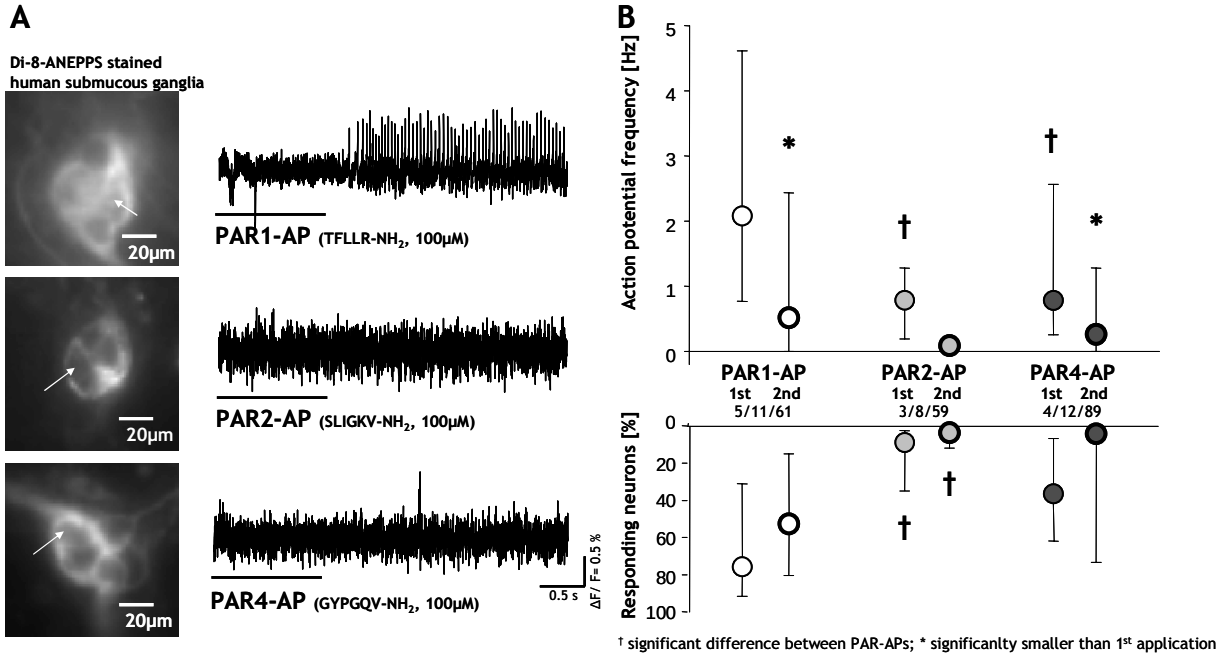


Figure 33: Neural action of human submucous neurons after 2s microejection of PAR-APs recorded with Di-8-ANEPPS imaging: (A) The images show outlines of Di-8-ANEPPS stained neurons of human submucous ganglia. The traces next to the images show representative responses to a 2s spritz application (indicated by the horizontal bars) of PAR1, 2 and 4-APs. The traces are from the neurons marked by white arrows in the images. Each peak corresponds to one action potential. (B) Statistical analysis of the experiments revealed that PAR1-AP induced a strong spike discharge in the majority of human submucous neurons, whereas the PAR2 and PAR4-AP caused significantly weaker nerve activation in a lower proportion of neurons. The response to a second application was significantly smaller due to receptor tachyphylaxis. Symbols mark significant differences as explained in the figure. Numbers below PAR-APs indicate numbers of tissues/ganglia/neurons studied.

As expected the second application of the PAR1-AP and PAR4-AP, 10min after the first one, significantly reduced the action potential frequency, which indicates a clear desensitization of the PAR receptors (Figure 33). Whereas 71.4% of human submucous neurons responded with a spike frequency of 2.1Hz [0.8/4.2Hz] to the first application of PAR1-AP, just 50% of the neurons responded with a significantly lower action potential frequency to the second application (0.5Hz [0.0/2.4Hz]; T/G/N: 5/11/61; $P < 0.001$). To test the specificity of the PAR-APs the effect of the reversed peptides (PAR-RPs) as negative controls were also studied. None of the PAR-RPs had any effect on nerve activity in the human submucous plexus (proportion of responding neurons: PAR1-RP: 0.0% [0.0/20.9%] vs PAR2-RP: 0.0% [0.0/0.0%] vs PAR4-RP: 0.0% [0.0/11.7%]).

These results underline the importance of PAR1 and the functional insignificance of PAR2 in the human submucous plexus. Furthermore, the results show a marked difference to the contribution of PARs in the guinea-pig submucous plexus. While PAR1-AP evoked the strongest neural activation in the human submucous plexus, it was PAR2-AP that had the strongest effect in the guinea-pig submucous plexus (Figure 34).

In conclusion, PAR1 is the most prominent receptor in the human submucous plexus mediating immediate (71.4% responding neurons) and late onset (42.2% responding neurons) neuronal activation (Table 4), whereas PAR2-AP evoked a much smaller activation.

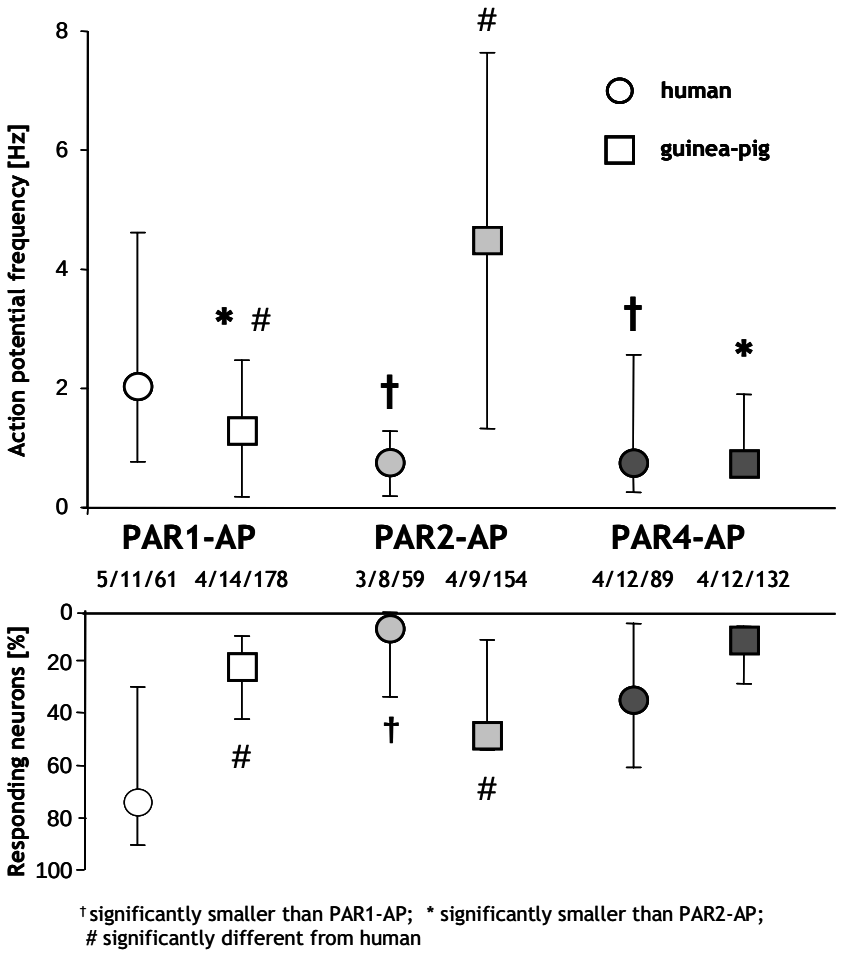
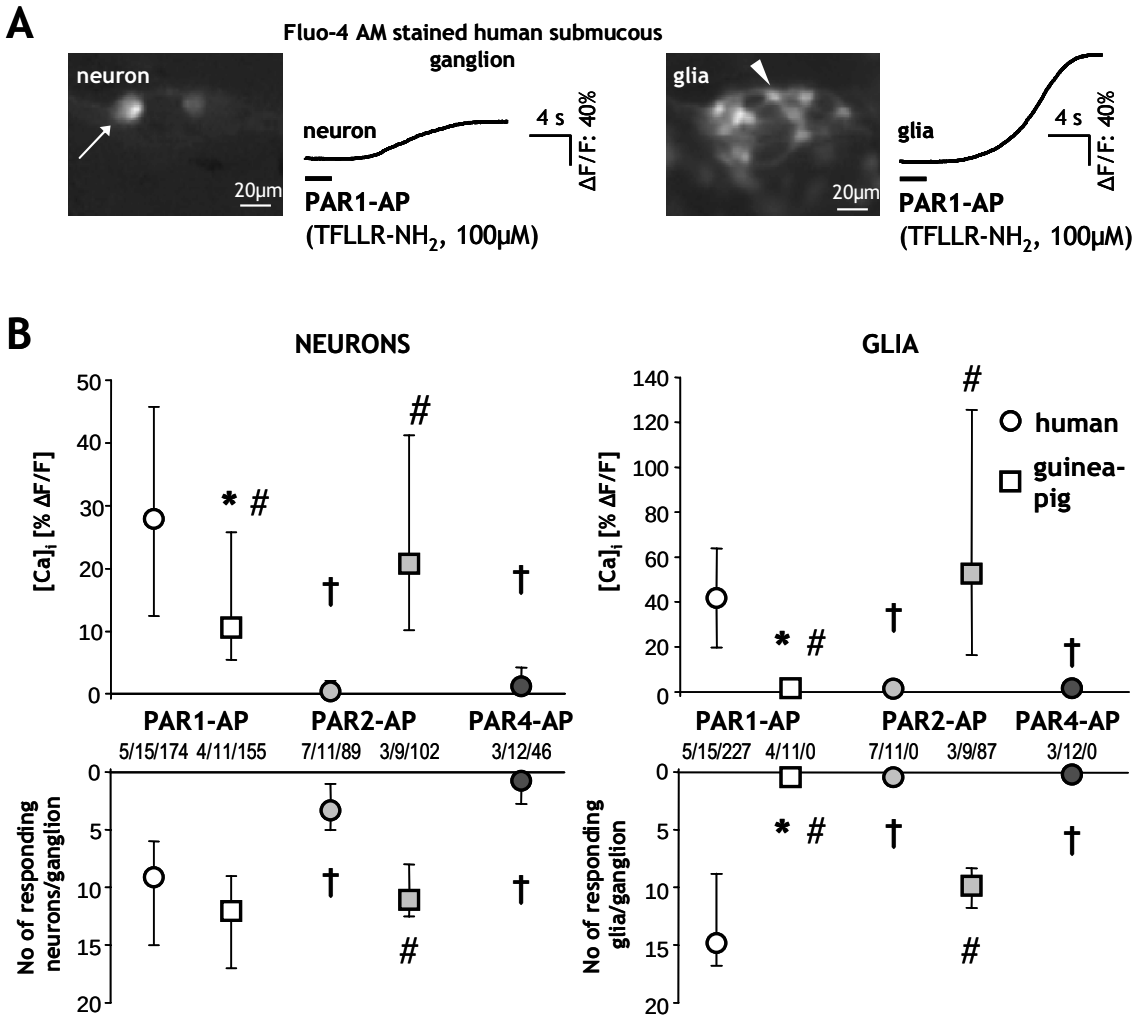


Figure 34: Comparison of the neuronal action of PAR-APs in human and guinea-pig submucous neurons recorded with Di-8-ANEPPS imaging: PAR2-AP induced in the guinea-pig the highest spike discharge, followed by PAR1-AP and PAR4-AP, whereas in the human intestine PAR1-AP evoked the most prominent spike discharge. PAR4-AP and PAR2-AP caused significant weaker responses in a minor population. Symbols mark significant differences as explained in the figure. Numbers below PAR-APs indicate numbers of tissues/ganglia/neurons studied.

3.3.5.2 PAR-APs evoked $[Ca]_i$ signals in human submucous neurons and glia

The above results suggest that human, unlike guinea-pig submucous neurons, respond strongly to PAR1-AP but much less to PAR2-AP or PAR4-AP. Because Di-8-ANEPPS imaging only reveals excitation associated with action potential discharge calcium imaging was also performed as a more general reporter of cell activation. During the experiments we noted that PAR-APs evoked $[Ca]_i$ not only in neurons but also in glia.

PAR1-AP induced a strong $[Ca]_i$ signal in human submucous neurons and glia, whereas the PAR2-AP and PAR4-AP had very minor effects (Figure 35). The PAR1-AP induced increase in $[Ca]_i$ was significantly higher in human than in guinea-pig submucous neurons while the PAR2-AP induced $[Ca]_i$ response was significantly larger in guinea-pig submucous neurons. PAR1-AP, but not PAR2-AP induced $[Ca]_i$ transients in human submucous glia, which is opposite to the results observed in the guinea-pig.



† significantly smaller than PAR1-AP; * significantly smaller than PAR2-AP; # significantly different from human

Figure 35: Effect of PAR-APs on $[Ca]_i$ in human and guinea-pig submucous neurons and glia: (A) The images show a human submucous ganglion at the time where PAR1-AP evoked $[Ca]_i$ was maximal in neurons and glia. One neuron and glia cell marked by arrow and arrowhead, respectively, illustrate the distinctive morphology of the different cell types. The traces show representative $[Ca]_i$ signals after PAR1-AP spritz application (indicated by horizontal bars below the traces) in a submucous neuron and glia. (B) In human submucous plexus PAR1-AP induced an increase of $[Ca]_i$ in enteric neurons and glia. PAR2-AP and PAR4-AP evoked a significantly smaller response in a small number of cells. In contrast, in guinea-pig submucous plexus PAR2-AP induced the strongest $[Ca]_i$ responses in neurons and glia. The PAR1-AP induced $[Ca]_i$ responses in neurons and glia was significantly higher in the human submucous

plexus. Symbols mark significant differences as explained in the figure. Numbers below PAR-APs indicate numbers of tissues/ganglia/neurons or numbers of tissues/ganglia/glia studied.

As expected, the PAR1-RP did not evoke a $[Ca]_i$ signal in human submucous neurons or glia (Figure 36). Tachyphylaxis was also observed for the $[Ca]_i$ transients since a second application of the PAR1-AP evoked smaller $[Ca]_i$ responses in less neurons (1st application: 27.9%ΔF/F [12.5/45.8%ΔF/F] in 9.0 [6.0/14.8] neurons per ganglion; 2nd application: 13.0%ΔF/F [4.3/22.6%ΔF/F] (*P*<0.001) in 9.0 [5.0/13.8] neurons per ganglion; *P*=0.016; T/G/N: 5/15/174) and less glia (1st application: 42.1%ΔF/F [19.7/63.8%ΔF/F] in 15.0 [9.0/17.0] glia per ganglion; 2nd application: 19.9%ΔF/F [8.5/42.6%ΔF/F] (*P*<0.001) in 13.0 [7.5/17.0] glia per ganglion; *P*=0.039; T/G/G: 5/15/227).

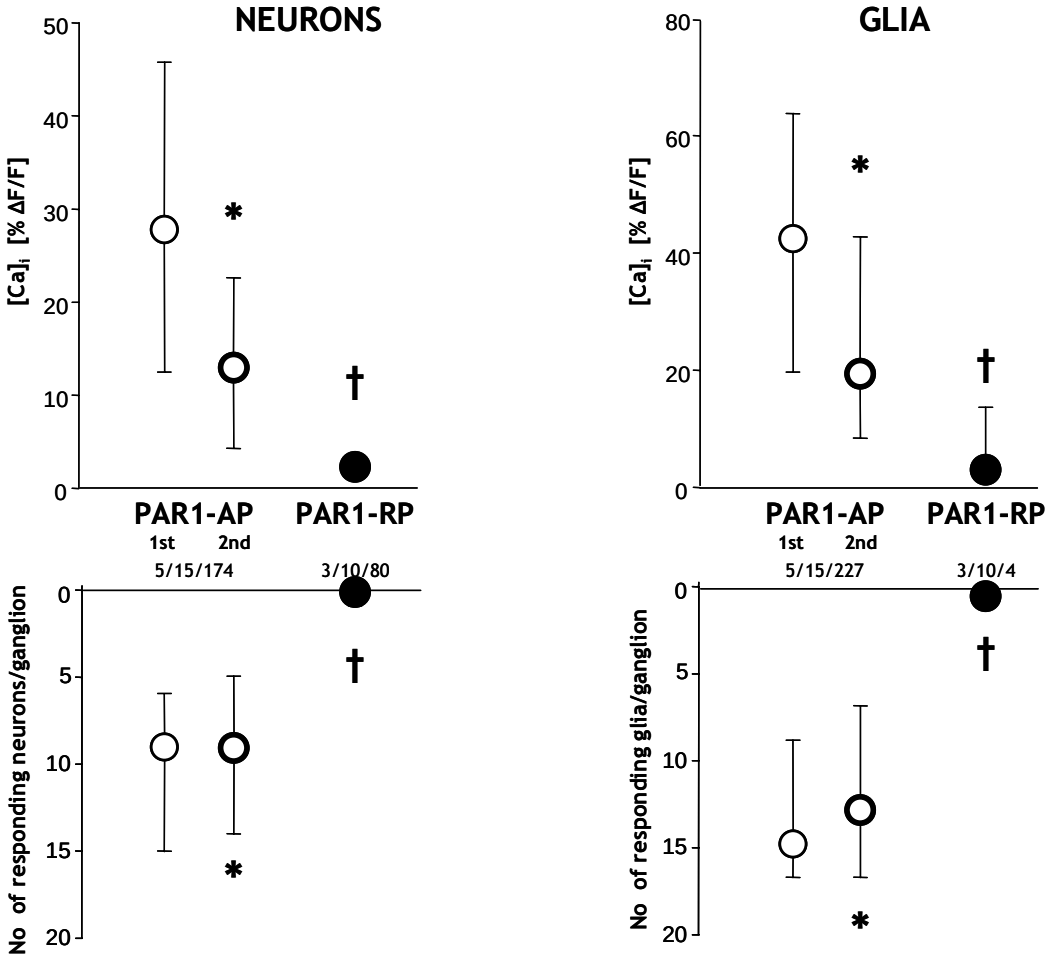


Figure 36: Effect of PAR1-AP and PAR1-RP on $[Ca]_i$ in human submucous neurons and glia: PAR1-AP (100µM) induced a $[Ca]_i$ increase in both enteric neurons and glia, which was significantly smaller after the second application. PAR1-RP (100µM) had no effect on the $[Ca]_i$. Symbols mark significant differences as explained in the figure. Numbers below PAR-APs indicate numbers of tissues/ganglia/neurons or tissues/ganglia/glia studied.

Based on previously published quantitative evaluation of the average number of neurons and glia in human submucous plexus (Hoff et al. 2008), it turned out that also in the human significantly more neurons responded to PAR-AP application with $[Ca]_i$ signals than with spike discharge. In the human submucous plexus the PAR1-AP evoked $[Ca]_i$ increase was observed in 9.0 [6.0/14.8] neurons per ganglion, which corresponds to almost 100% of the neurons, while the spike discharge was observed in 71.4% of the neurons (3.0 [2.0/4.8] neurons per ganglion) ($P < 0.001$). Almost 100% of enteric glia responded to PAR1-AP in the human submucous plexus.

The efficacy of the different PAR-APs to evoke neural $[Ca]_i$ confirmed the results obtained with Di-8-ANEPPS; PAR1-AP rather than PAR2-AP activated human submucous neurons.

Nevertheless, it may be a remote possibility that proteases which are released during surgery and tissue preparation may selectively desensitize PAR2 and thereby compromise PAR2 activation. Therefore, the effect of the PAR2-AP was investigated in the presence of the serine protease inhibitor FUT-175 (50µg/ml). The PAR2-AP induced neuronal $[Ca]_i$ response (PAR2-AP: 0.6%ΔF/F [0.0/2.2%ΔF/F]; T/G/N:7/11/89 vs PAR2-AP in FUT-175: 2.8%ΔF/F [1.3/3.5%ΔF/F]; T/G/N: 4/18/94) as well as the number of responding neurons per ganglion (PAR2-AP: 3.0 [1.3/4.8] vs PAR2-AP in FUT-175: 1.5 [0.0/4.0]) were not changed by FUT-175 treatment (Figure 46). Furthermore, the FUT-175 treatment still did not reveal any responses in glia cells. Consequently, the PAR2-AP response in the presence of FUT-175 was still significantly smaller compared to the PAR1-AP response ($P < 0.001$). This further supports the conclusion, that PAR1-AP rather than PAR2-AP activated human submucous neurons and glia. Furthermore, the FUT-175 treatment also showed that the PAR2-AP did not undergo proteolytic degradation or inactivation while applied onto the ganglia.

In human submucous plexus preparations it has been frequently observed that PAR2-AP, but not PAR1-AP or PAR4-AP evoked $[Ca]_i$ signals in cells outside the submucous plexus. These cells were identified as macrophages by their CD68 immunoreactivity (Figure 37). On average, macrophages showed an increase in $[Ca]_i$ of 17.2%ΔF/F [9.4/35.2%ΔF/F] (8 tissues and 61 regions studied). In the guinea-pig neither PAR1-AP nor PAR2-AP did induce any $[Ca]_i$ response outside the submucous ganglia.

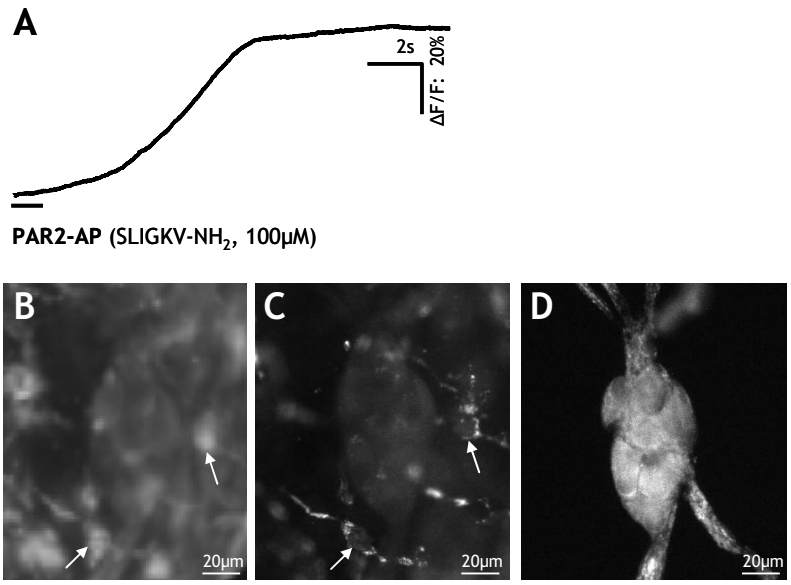


Figure 37: Responses of macrophages to application of PAR2-AP in the human submucous plexus: (A) Representative trace showing the PAR2-AP (application marked by horizontal bar below the trace) induced $[Ca]_i$ increase in a cell that was located outside a ganglion. The images illustrate the origin of the signal. (B) Fluo-4AM stained cells outside a ganglion marked by white arrows. (C) The cells were identified as macrophages by CD68 immunoreactivity (mouse anti-CD68 antibody; 1:200). Arrows mark the same cell as in panel B. (D) A PGP9.5 staining reveals neurons in the ganglion that is faintly stained in panels B and C.

3.3.5.3 PAR1 antagonist reduced PAR1-AP induced $[Ca]_i$ increase in human submucous neurons and glia

These results suggest a prominent role of PAR1 in neurons and glia of human submucous plexus. Therefore the PAR1 antagonist SCH79797 was used in different concentrations (100nM to 10µM) to test the specificity of the PAR1-AP evoked response. Calcium imaging was performed for these experiments because it revealed more responsive cells and allowed to study PAR1 antagonism in neurons and glia.

SCH79797 dose dependently reduced the $[Ca]_i$ increase evoked by PAR1-AP in human submucous neurons and glia. 100nM and 1µM SCH79797 reduced significantly the PAR1-AP induced $[Ca]_i$ increase. The number of responding cells was however unchanged. At a concentration of 10µM SCH79797 completely abolished the response to the PAR1-AP (Figure 38). The PAR1 antagonist consequently reduced specifically and dose-dependently the PAR1 mediated action in both neurons and glia.

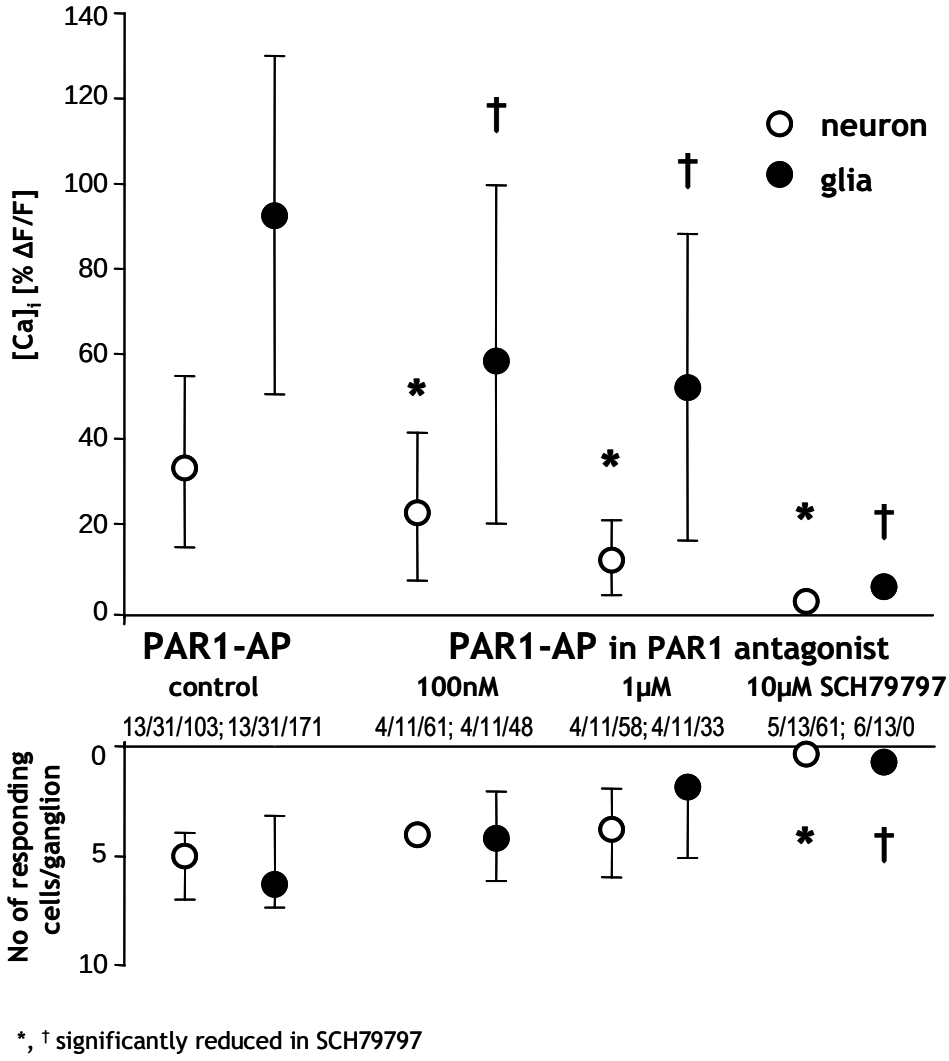


Figure 38: PAR1-AP induced neuronal and glia activation was dose-dependently reduced by the PAR1 antagonist SCH79797: 100nM and 1µM SCH79797 reduced the increase of $[Ca]_i$ to 100µM PAR1-AP significantly in both neurons and glia. 10µM SCH79797 completely abolished the PAR1-AP induced $[Ca]_i$ response. Symbols mark significant differences as explained in the figure. Numbers indicate numbers of tissues/ganglia/neurons or tissues/ganglia/glia studied.

3.3.5.4 PAR1-AP acted directly on human submucous neurons

To study if the PAR1-AP evoked increase in spike discharge is mediated by pre- or postsynaptic activation the PAR1-AP was applied in the presence of a low Ca^{2+}/high Mg^{2+} Krebs solution, which blocks synaptic transmission. The low Ca^{2+}/high Mg^{2+} solution had no effect on the PAR1-AP evoked action potential discharge (2.8Hz [1.2/3.8Hz]; T/G/N: 6/8/47 vs. 1.5Hz [0.8/3.9Hz]; T/G/N: 6/17/105 in low Ca^{2+}/high Mg^{2+} solution; P = 0.267) or the proportion of responding neurons (69.0% [50.0/76.4%] vs. 66.7% [41.5/87.5%]; P = 0.733).

This result strongly suggests that the PAR1-AP induced spike discharge is a result of a direct postsynaptic action rather than a presynaptic facilitation of neurotransmitter release (Figure 39).

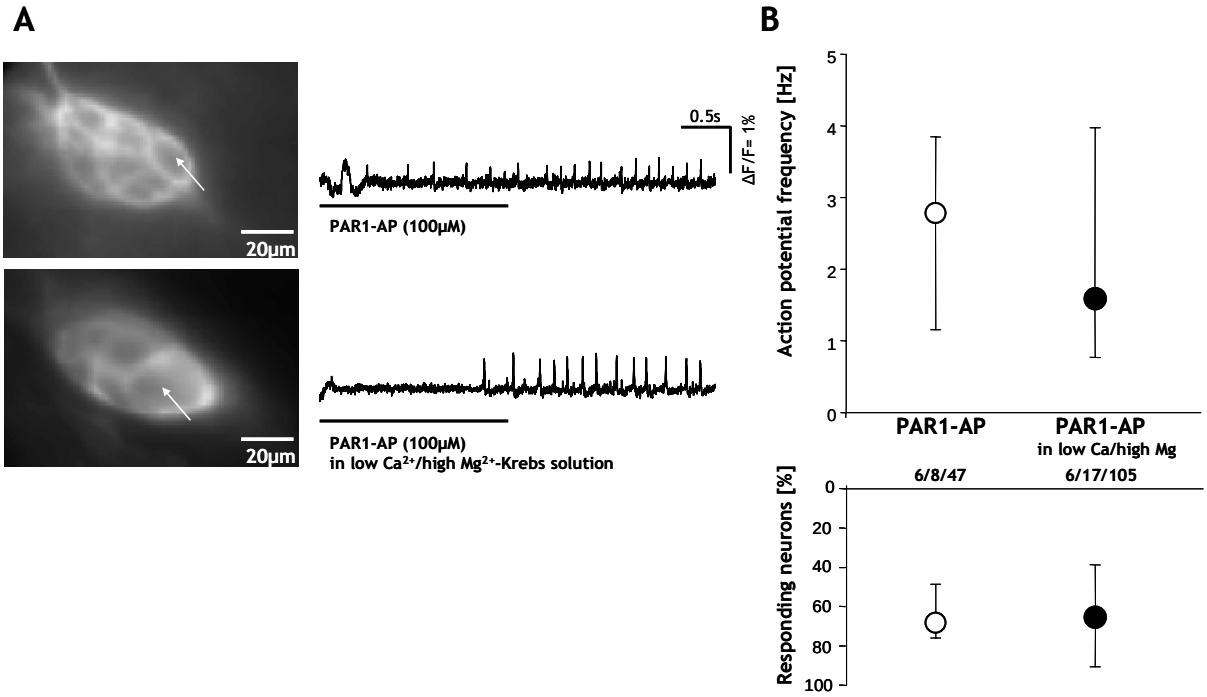


Figure 39: PAR1-AP acted directly on human enteric neurons: (A) The images show the outlines of Di-8-ANEPPS stained neurons in the human submucous ganglia. The traces next to the images show representative responses to a 2s spritz application (indicated by the horizontal bars) of PAR1-AP and PAR1-AP in lowCa^{2+}/high Mg^{2+} solution. Each peak corresponds to one action potential. (B) The PAR1-AP induced action potential discharge and the proportion of responding neurons were not significantly changed in the presence of the low Ca^{2+}/high Mg^{2+} solution. Numbers below PAR-APs indicate numbers of tissues/ganglia/neurons studied.

To study neuron - neuron and neuron - glia communication the PAR1-AP was applied in the presence of the neuronal blocker TTX. The PAR1-AP induced response was significantly reduced in the presence of TTX (0.5µM) in both enteric neurons and glia (Figure 40). This result suggests a functional interaction between neurons and glia. But the reduction of the $[Ca]_i$ signal could be a result of an inhibition of spike discharge. However, a synaptically mediated reduction seems unlikely, because the calcium depleted solution had no effect on the neuronal activity (see results above).

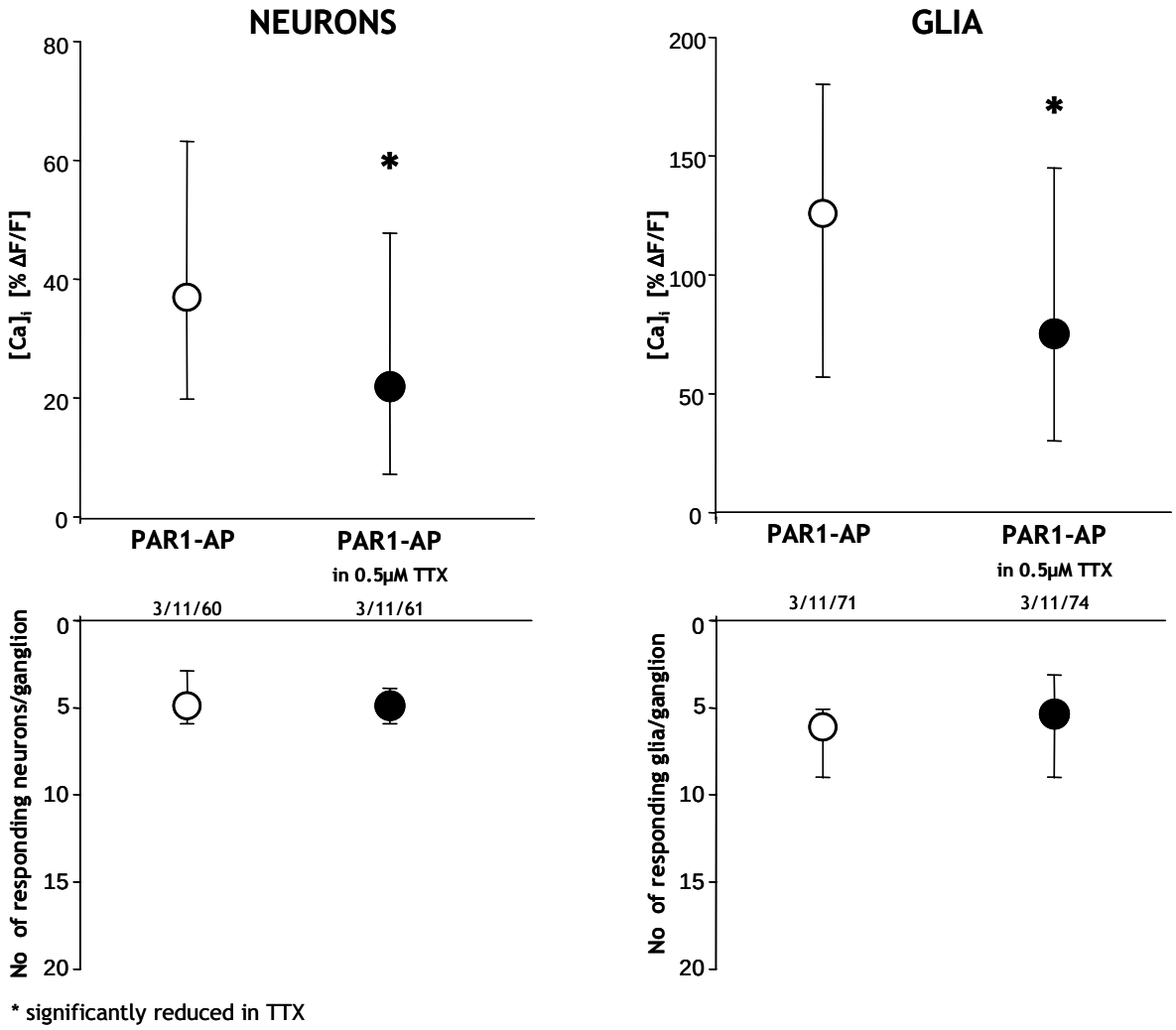


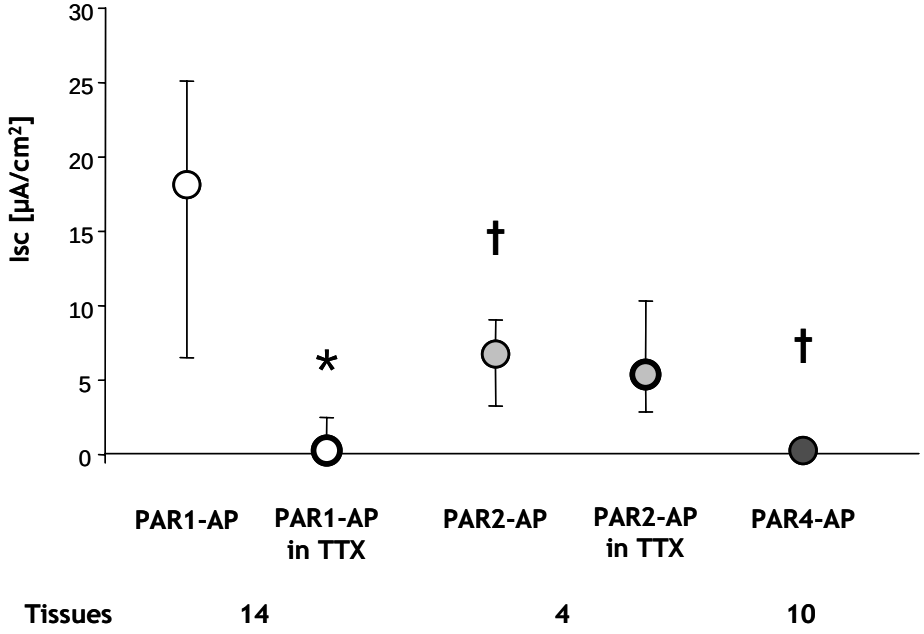
Figure 40: PAR1-AP response in the presence of the neuronal blocker TTX (0.5µM): The neuronal blocker TTX reduced the PAR1-AP induced $[Ca]_i$ increase in human submucous neurons and glia. Symbols mark significant differences as explained in the figure. Numbers below PAR-APs indicate numbers of tissues/ganglia/neurons and tissues/ganglia/glia studied.

3.3.6 Secretory actions of PAR-APs in human intestine

3.3.6.1 PAR1-AP, but not PAR2-AP or PAR4-AP, induced a nerve dependent pro-secretory response in the human intestine

Basolateral application of the PAR1-AP and PAR2-AP led to an increase in I_{sc}, whereupon the PAR1-AP induced a significantly larger response ($P < 0.001$) (Figure 41). Application of PAR4-AP did not induce any measurable change in I_{sc}. The neural blocker TTX (0.5µM) was used to reveal the neural component of the pro-secretory response. The PAR1-AP induced response was abolished in the presence of TTX (18.2µA/cm^2 [6.9/24.6µA/cm^2] vs 0.0µA/cm^2

[0.0/1.1µA/cm^2]; N: 14; $P < 0.001$), which indicates a nerve-dependent pro-secretory action. The PAR2-AP induced response was TTX-resistant ($P = 0.875$), which suggests a pro-secretory action via direct activation of epithelial cells (Figure 41). These results were in accordance with the results of the neuroimaging studies, where PAR1-AP rather than PAR2-AP led to activation of human submucous neurons.

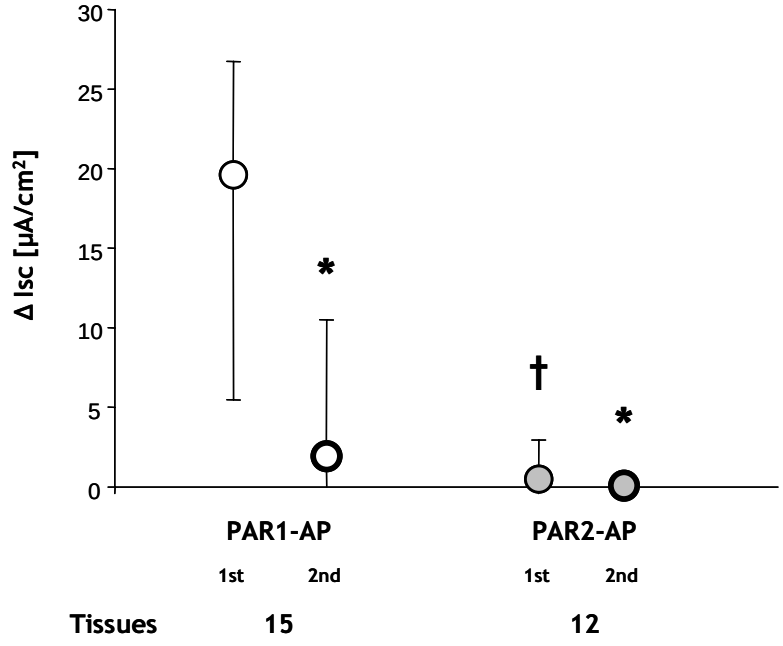


† significantly smaller than PAR1-AP evoked secretion; * significantly reduced in TTX

Figure 41: Effect of PAR-APs on mucosal ion secretion in the human intestine: PAR1-AP (10µM) induced a significantly higher pro-secretory response (ΔI_{sc}) than PAR2-AP (10µM). The PAR4-AP (10µM) did not induce any measurable increase in ΔI_{sc}. The PAR1-AP induced secretion was fully abolished by the nerve-blocker tetrodotoxin (TTX) while the PAR2-AP evoked secretion was TTX insensitive. Symbols mark significant differences as explained in the figure.

The PAR1 and PAR2-AP mediated responses were not region dependent. The PAR1-AP induced pro-secretory response in the small intestine was comparable to the one in the large intestine ($P = 1.0$). TTX significantly reduced the ion secretion in the small and large intestine (small intestine: PAR1-AP: 19.8 µA/cm^2 [8.2/24.5 µA/cm^2] vs PAR1-AP in TTX: 0.0 µA/cm^2 [0.0/0.8 µA/cm^2]; N: 7; $P = 0.001$; large intestine: PAR1-AP: 11.2 µA/cm^2 [7.7/25.3 µA/cm^2] vs PAR1-AP in TTX: 0.0 µA/cm^2 [0.0/2.5 µA/cm^2]; N: 7; $P = 0.004$). Likewise, the PAR2-AP induced secretion was similar in small and large intestine (small intestine: 0.1 µA/cm^2 [0.0/1.0 µA/cm^2]; N: 14 vs large intestine: 2.6 µA/cm^2 [0.1/4.4 µA/cm^2]; N: 15; $P = 0.07$).

The ion secretion to a second application of PAR1 and PAR2-AP 20min after the first one was significantly smaller, which reflected desensitization of the receptors (Figure 42).



† significantly smaller than PAR1-AP evoked secretion; * significantly smaller than 1st application

Figure 42: The PAR1-AP and PAR2-AP induced ion secretion was smaller after the second application: PAR1-AP (10µM) induced a significant higher pro-secretory response than PAR2-AP (10µM). The response to the second application of PAR1 and 2-AP 20min after the first one significantly reduced this pro-secretory response. Symbols mark significant differences as explained in the figure.

As expected, the reverse peptides had no pro-secretory action, which supports the specificity of the PAR-APs (PAR1-RP: 0.0 µA/cm^2; n: 15; PAR2-RP: 0.0 µA/cm^2; n: 8; PAR4-RP: 0.0 µA/cm^2; n: 6).

3.3.6.2 PAR1 antagonist reduced the PAR1-AP induced pro-secretory response in human intestine

To study the specificity of the PAR1-AP induced pro-secretory response the PAR1 antagonist SCH79797 was used in concentrations of 100nM and 1µM. Both concentrations reduced the PAR1-AP induced I_{sc} significantly by 81.5% and 68.8%, respectively (N: 13; $P < 0.001$) (Figure 43). This result supports the specificity of the PAR1-AP mediated pro-secretory response.

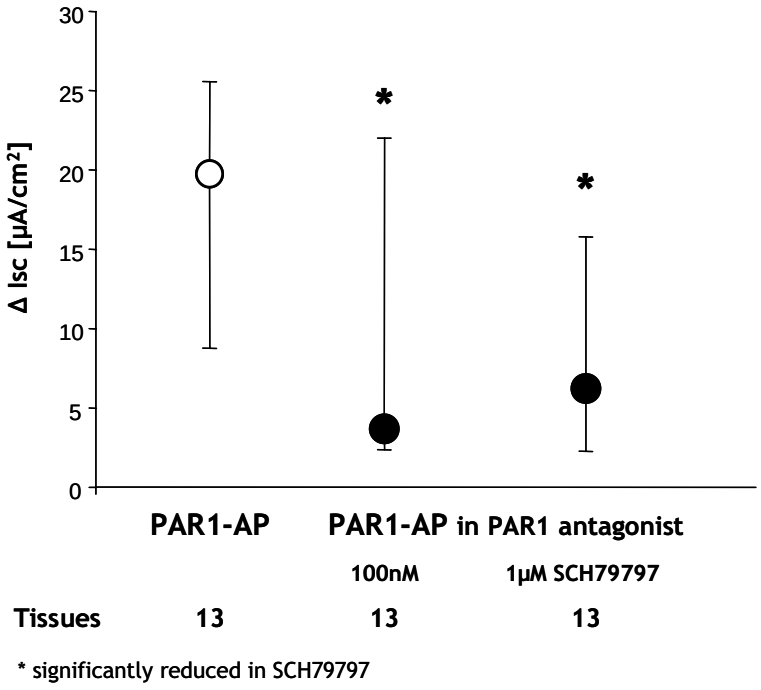


Figure 43: Effect of the PAR1 antagonist SCH79797 on the PAR1-AP induced mucosal ion secretion in the human intestine: The PAR1-AP (10µM) induced pro-secretory response was significantly reduced by 100nM and 1µM SCH79797. Symbols mark significant differences as explained in the figure.

3.3.6.3 PAR2 antagonist did not reduce the PAR2-AP induced pro-secretory response in human intestine

To test the specificity of the PAR2-AP mediated pro-secretory response the PAR2 antagonist LIGK-NH_2 was used. Additionally, the reverse sequence of the PAR2 antagonist KGIL-NH_2 was applied, which should not exhibit any blocking properties. The PAR2-AP induced ion secretion was not significantly changed in the presence of LIGK-NH_2 or KGIL-NH_2 (Figure 44). LIGK-NH_2 and KGIL-NH_2 actually slightly increased the PAR2-AP induced pro-secretory activity (PAR2-AP: 1.7µA/cm^2 [0.3/3.1µA/cm^2] vs PAR2-AP in LIGK-NH_2: 4.7µA/cm^2 [0.5/13.1µA/cm^2] vs PAR-2 AP in KGIL-NH_2: 2.4µA/cm^2 [0.0/4.3µA/cm^2]).

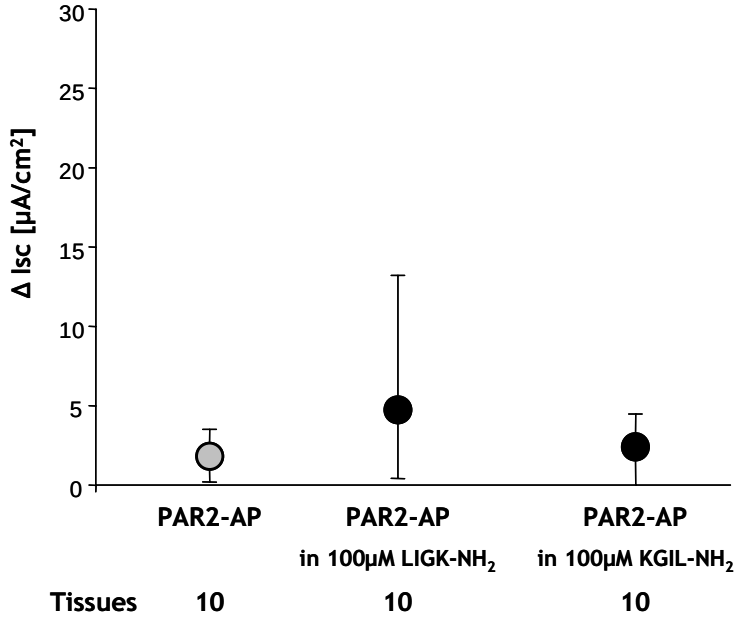


Figure 44: No effect of the PAR2 antagonist LIGK-NH_2 and the reversed sequence of the PAR2 antagonist KGIL-NH_2 on the PAR2-AP induced mucosal ion secretion in the human intestine: The PAR2-AP (10µM) induced pro-secretory response was unaffected by LIGK-NH_2 (100µM) and KGIL-NH_2 (100µM).

In summary the PAR1-AP induced a specific nerve-dependent secretion, whereas PAR2-AP induced an epithelially mediated secretion. The PAR4-AP had no pro-secretory effect. These results were in accordance with the results of the neuroimaging studies, where PAR1-AP rather than PAR2-AP led to activation of human submucous neurons.

3.3.7 Neural actions of the endogenous proteases thrombin and tryptase

The results with the PAR-APs suggest a prominent role of PAR1 and a minor role of PAR2 in neurons and glia of human submucous plexus. Therefore, the neural action of the endogenous proteases thrombin, which has been shown to predominantly activate PAR1, PAR3 and PAR4 and the PAR2 specific activator tryptase were studied. Calcium imaging was performed for these experiments because it allowed studying neurons and glia.

Thrombin (100nM) induced a strong $[Ca]_i$ response in human submucous neurons and glia. The PAR1 antagonist SCH79797 (10µM) significantly reduced the neuronal and completely abolished the glial $[Ca]_i$ response (Figure 45). This result demonstrates the PAR1 specific action of thrombin.

It is noteworthy that thrombin evoked similar $[Ca]_i$ transients comparable to those after PAR1-AP application (Figure 46).

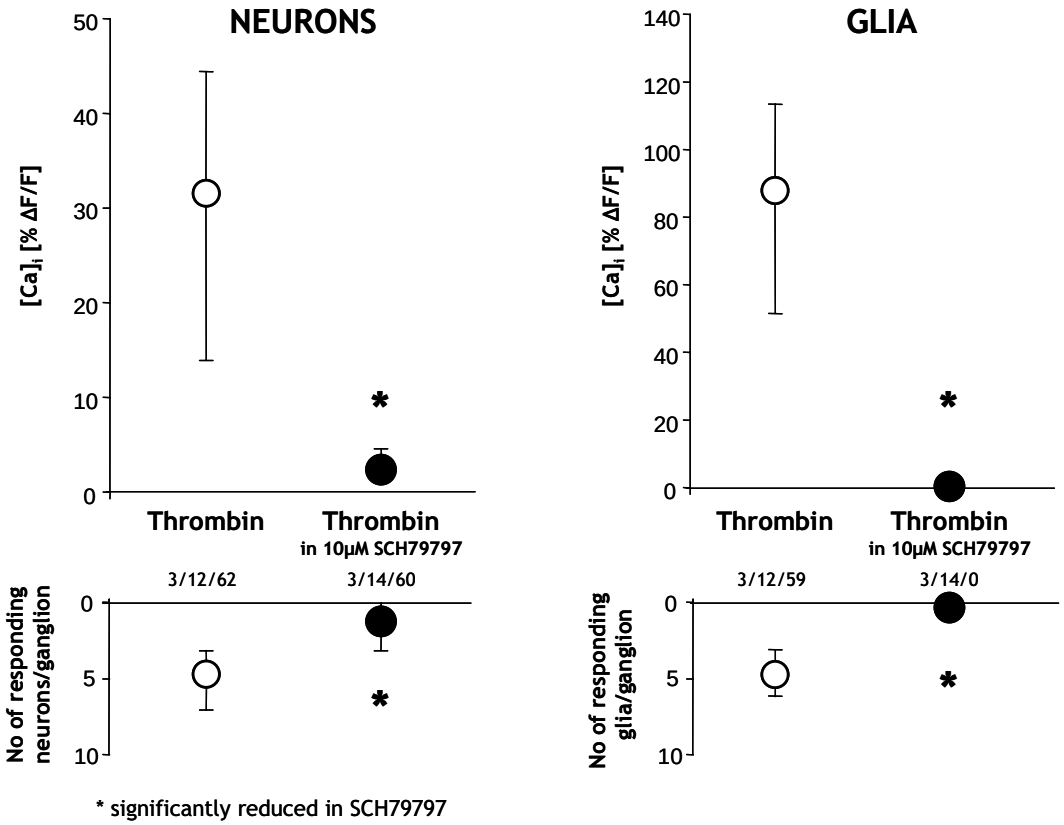


Figure 45: Effect of thrombin on $[Ca]_i$ in human submucous neurons and glia: Thrombin (100nM) induced a $[Ca]_i$ increase in both enteric neurons and glia, which was significantly reduced by the PAR1 antagonist SCH79797 (10µM). Symbols mark significant differences as explained in the figure. Numbers indicate numbers of tissues/ganglia/neurons or tissues/ganglia/glia studied.

Tryptase (100nM) induced a weak neuronal excitation, but no glia activation, thereby resembling the action profile of the PAR2-AP. The neuronal $[Ca]_i$ response to tryptase was significantly smaller compared to the one induced by PAR1-AP or thrombin, but did not differ from the PAR2-AP induced $[Ca]_i$ transients (Figure 46).

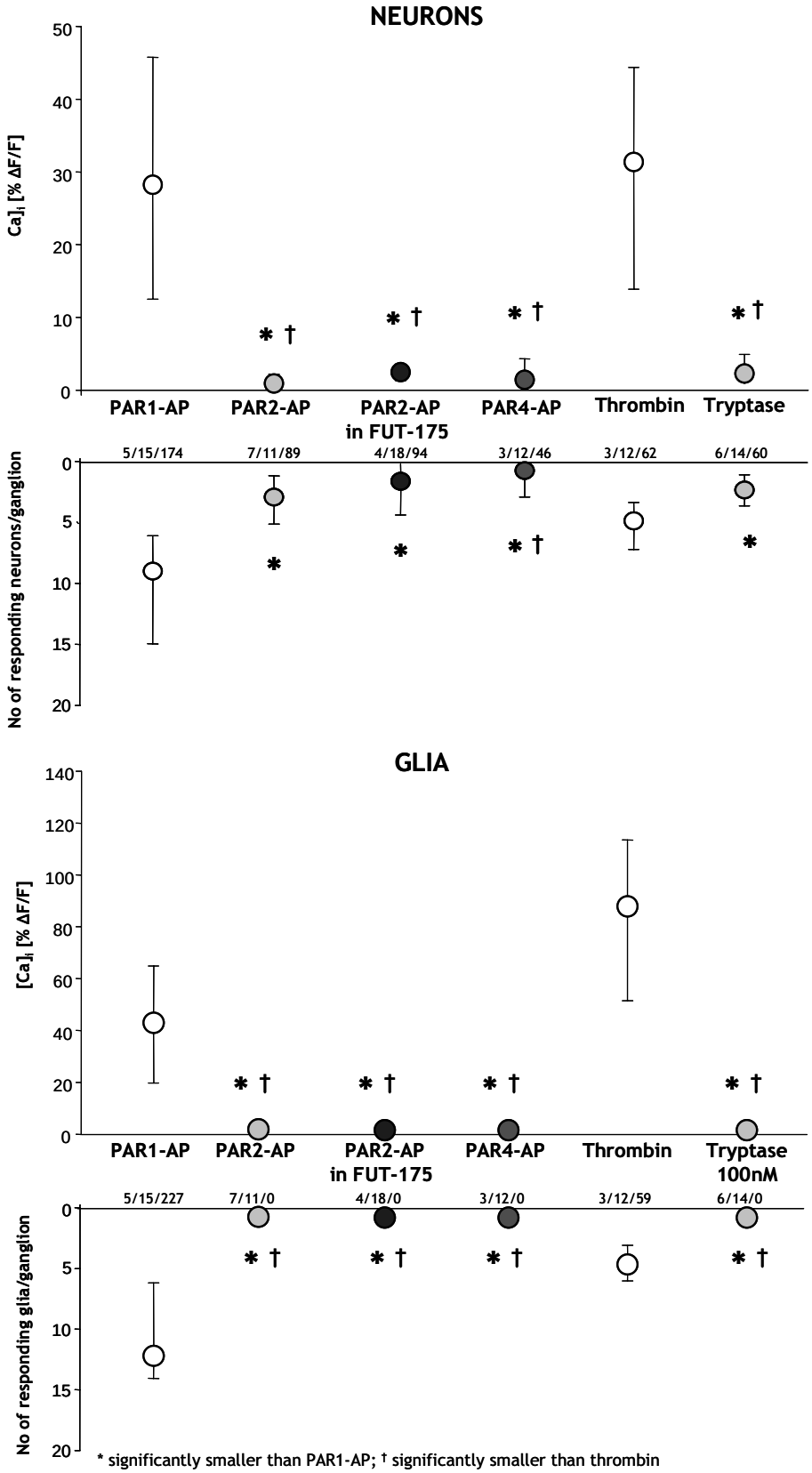


Figure 46: Effect of tryptase on $[Ca]_i$ in human submucous neurons and glia: Tryptase (100nM) induced a weak neuronal excitation, which was significantly smaller compared to the PAR1-AP and thrombin induced $[Ca]_i$ response, but did not differ from the PAR2-AP induced $[Ca]_i$ responses. Trpytase, comparable to PAR2-AP did not induce any glia activation. Symbols mark significant differences as explained in the figure. Numbers indicate numbers of tissues/ganglia/neurons studied.

3.4 Neural actions of IBS supernatants

It has been shown by Buhner et al. (2009) that mediators released from mucosal biopsies of IBS patients activated human submucous neurons. The main mediators found to be involved were histamine, serotonin and proteases. Since PAR1 has been identified as the most prominent PAR receptor in human intestine, one aim was to investigate the effect of PAR1 antagonism on IBS supernatant induced neuronal excitability.

Preliminary experiments with IBS supernatants (MZ-9 from T. Frieling, Helios Clinic Krefeld; IBS-137, IBS-134, IBS-24 from G. Barbara; University Hospital Bologna) showed that the IBS supernatant induced spike discharge was reproducible and was reduced by the PAR1 antagonist SCH79797 (10µM) (Figure 47). Although all of the IBS supernatant induced neuronal responses were reduced only samples MZ-9 and IBS-34 reached a level of significance.

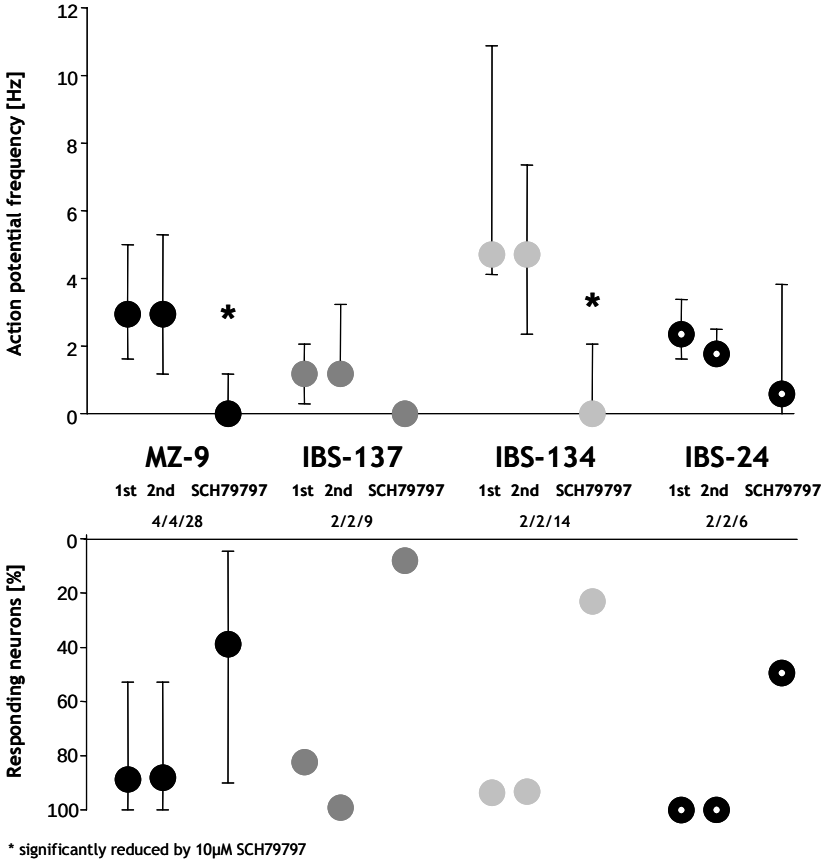


Figure 47: The IBS supernatant induced spike discharge was reduced by the PAR1 antagonist SCH79797 in human colonic submucous neurons: The IBS supernatants induced a clear reproducible spike discharge, which was significantly reduced by the PAR1 antagonist SCH79797 (10µM). Symbols mark significant differences as explained in the figure. Numbers indicate numbers of tissues/ganglia/neurons studied.

4. Discussion

This study primarily aimed to investigate the role of proteases in modulating neuronal activity in the human enteric nervous system. Several experimental strategies were used to achieve this goal. Most importantly, we studied the consequence of direct activation of PARs for the activity level of enteric neurons. There were also several indirect ways to unravel involvement of PARs such as the use of the bacterial protease GelE, feeding of probiotics to study their long term effects on nerve evoked secretion and investigating the neural action of soluble factors released by probiotic bacteria.

The results of this study revealed four main novel findings. Firstly, PAR activation caused neuronal and glia cell activation in human submucous plexus primarily via PAR1 while PAR evoked cell activation in guinea-pig submucous neurons and glia was mainly mediated by PAR2. Secondly, the bacterial protease GelE induced neural excitation of guinea-pig submucous neurons, likely by involving PAR2 expressing pathways. Thirdly, the experiments revealed that $TNF^{\Delta ARE/WT}$ mice suffered from impaired mucosal secretion as the epithelial as well as the nerve mediated secretion was much weaker than in the WT mice. However, the probiotic mixture VSL#3 did not reverse the impaired secretion. Fourthly, soluble factors released by probiotic bacteria had no specific nerve activating actions, mainly because non-specific medium effects were unavoidable.

4.1 Neural actions of probiotics

Despite several attempts to use different isolation techniques and experimental protocols, it was impossible to demonstrate that probiotics or their soluble factors had any specific neural action. The main obstacle, which turned out to be impossible to overcome in *in vitro* experiments, was the effect of the medium itself which had to be used to grow probiotic bacteria. Whenever the pH and/or osmolarity were adjusted to physiological levels neither probiotics nor their soluble factors had any effect on neural activity. However, only an acute application regime was tested in these experiments. This does not rule out that neural effects may occur after chronic application of probiotics for several days. These results demonstrated that data collected from experiments with bacterial conditioned media have to be interpreted

with caution, at least when looking at immediate effects and that proper controls for bacterial conditioned media are mandatory for *in vitro* studies.

There are few studies examining the effect of probiotics on enteric neurons, which all reported on long-term probiotic feeding. They described changes of the chemical code of enteric neurons as a result of probiotic feeding (Kamm et al. 2004; Di Giancamillo 2010). Recently, Kunze et al. (2009) provided evidence that the probiotic *Lactobacillus reuteri* interacted with colonic enteric neurons. After 9 days ingestion, *Lactobacillus reuteri* increased the excitability of myenteric AH/Dogiel type II neurons by targeting an ion channel (IKCa), which finally contributed to motility changes of rat colon (Wang 2010). Bär et al. (2009) showed in an *in vitro* organ bath study the acute action of probiotic supernatants on human colon motility. Cell-free supernatants from *E. coli* Nissle 1917 enhanced colonic contractility by direct stimulation of smooth muscle cells. Similarly to our observations, Bär et al. (2009) reported culture medium effects, which could be attributed to acetic acid and its contractility enhancing effects. Taken these results together is has been shown that long-term exposure to probiotics is able to modify enteric neurons, whereas the experimental settings to study immediate actions of probiotics on enteric neurons need to be optimized and until now did not deliver any evidence for a direct or immediate action of probiotics.

In the present study, even the long term application of probiotics did not reveal any substantive changes in nerve-dependent or -independent mucosal secretion. As a noteworthy and rather incidental result, an impaired secretory activity of the colon of $TNF^{\Delta ARE/WT}$ was discovered. At least under our experimental conditions, VSL#3 feeding of WT and $TNF^{\Delta ARE/WT}$ mice had no significant effects on the integrity of the colonic mucosa or its secretory activity. Furthermore, VSL#3 treatment was not able to reverse the impaired secretion observed in $TNF^{\Delta ARE/WT}$ mice. These results are in accordance with previous studies (unpublished own data and personal communication D. Krueger) with the probiotic bacteria *B. longum* NCC 3001. In these studies, probiotic feeding did also not affect the nerve or forskolin evoked secretory responses. The lack of effect of probiotics on mucosal secretion contradicts results from other studies which reported that probiotics improved mucosal integrity (Ukena et al. 2007; Garcia Vilela et al. 2008; Khailova et al. 2009) and prevented infectious diarrhoea (Schroeder et al. 2006). The lack of effect on impaired secretion in $TNF^{\Delta ARE/WT}$ in the present study may be due to the disability of probiotics to normalize a reduced secretory capacity.

There are several hypotheses which could provide a reason for the secretory dysfunction in the $TNF^{\Delta ARE/WT}$ mice. The animals may suffer from epithelial malfunctions or alterations in the enteric nervous system. The focus has been on the involvement of neurotransmitters inhibiting mucosal secretion, like noradrenaline, somatostatin and adenosine. The secretory deficit in preparations from $TNF^{\Delta ARE/WT}$ mice could be partly reversed by blocking somatostatin actions through SST-2 receptor and by blocking adenosine A_1 receptors (unpublished own data and personal communication D. Krueger). However, there was no full recovery, which suggested that a major part of the secretory dysfunctions may be attributed to alterations in transporter and/or channel functions at the epithelial level. As it has been shown, that the neurochemical code of enteric neurons was altered in inflamed as well as non-inflamed tissue from CD and UC patients (Geboes and Collins 1998; Schneider et al. 2001; Neunlist et al. 2003), the neurochemical code of the colonic regions of the $TNF^{\Delta ARE/WT}$ mice in comparison to the WT mice has been studied. The total number of enteric neurons per ganglion, the ganglionic area and the glia area were not changed in $TNF^{\Delta ARE/WT}$ mice compared to WT mice. But $TNF^{\Delta ARE/WT}$ mice exhibited an increased density of somatostatin-immunoreactive (SOM-IR) nerve fibres in the distal colon and increased area of noradrenergic (Tyrosine Hydroxylase (TH)-IR) nerve fibres in the proximal colon of the submucous plexus (unpublished own data). These results lead to the conclusion that $TNF^{\Delta ARE/WT}$ mice exhibited a functional impairment in mucosal secretion and changes of the chemical code in macroscopically uninflamed colonic regions, which is probably partly mediated by an altered mediator release and altered expression of nerve fibres. Obviously, probiotic feeding with VSL#3 was not able to overcome these alterations. However, one has to consider that our model tried to reveal therapeutic effects of probiotics rather than preventive actions.

The alterations in mucosal secretion in $TNF^{\Delta ARE/WT}$ mice are in accordance with previous reports, where functional and structural changes of the ENS at inflamed and not-inflamed regions in IBD have been shown. In a TNBS-induced ileitis model it has been reported that the ileal inflammation changed the enteric reflex circuits and impaired intestinal secretion in the non-inflamed colon (O'Hara et al. 2007). Similarly, an inflammation induced secretory dysfunction, which involved alterations of the submucous synaptic transmission, has been reported in the uninflamed ileum of a TNBS-colitis model (Hons et al. 2009). In this animal model the forskolin induced secretion was not altered suggesting that impairment of mucosal functions is different between hapten and genetically induced colitis. In addition regional

(proximal vs distal colon) and species (mouse vs guinea-pig) differences have to be considered.

The long-term treatment with the probiotic mixture VSL#3 in the feeding experiment was unable to improve the impaired colonic secretion. This is in contrast to data from IL-$10^{-/-}$ mice, where the long-term treatment with VSL#3 has been shown to normalize the basal secretory potential and to improve the impaired forskolin induced secretion in the inflamed colon (Madsen et al. 2001). Furthermore, short-term application of probiotic bacteria, like *Lactobacillus acidophilus* (Borthakur et al. 2008), *Streptococcus thermophilus* or *Bifidobacterium breve* (Heuvelin et al. 2010) in *in vitro* experiments seemed to be promising in modulating intestinal secretion of human intestinal epithelial cells. Both *Lactobacillus acidophilus* and *Streptococcus thermophilus* have been shown to have protective effects on epithelial dysfunction, such as impaired barrier function and secretion induced by the pro-inflammatory cytokines IFNγ and TNFα (Resta-Lenert and Barrett 2006). In general the ability of probiotics to modulate secretion seems to be promising. There are many potential reasons that VSL#3 had no effect in our study including different underlying pathological mechanisms in the non-inflamed gut region. In addition, it has to be considered, that the $TNF^{\Delta ARE/WT}$ mice is a model for ileitis and exert no inflammation in the colon (Kontoyiannis et al. 1999) while IL-$10^{-/-}$ mice develop colitis. Thus, the efficacy of probiotic treatment may be due to their anti-inflammatory actions which will consequently normalise mucosal function.

There was no significant difference in intestinal permeability between the $TNF^{\Delta ARE/WT}$ and WT mice and no effect of the probiotic mixture was detected. However, a previous study showed that in IL-$10^{-/-}$ mice the permeability of the inflamed colon was impaired, but could be restored by VSL#3 (Madsen et al. 2001), which supports the region-specific action of probiotics, in this case at the site of inflammation.

Increased levels of TNFα have been found in the $TNF^{\Delta ARE/WT}$ mice as well as in the intestinal wall and serum of IBD patients (Breese et al. 1994). Certain studies supported the pro-secretory action of TNFα in porcine ileal mucosa (Kandil et al. 1994) and human distal colon (Schmitz et al. 1996). With regard to the $TNF^{\Delta ARE/WT}$ mice, the impaired secretion seems not to be attributed to the elevated TNFα levels; instead it seems to be induced by functional

changes of the enteric nerve reflex circuits and epithelium, and probably also by receptor desensitization due to the permanently elevated TNFα levels.

Inflammation induced changes of the neurochemical code as well as changes at sites remote of inflammation have been shown by several studies. We also reported alterations of the chemical code by changes in the expression of SOM positive and adrenergic nerve fibres in the non-inflamed colon. Altered expression of SOM and TH positive fibres in the inflamed gut has been also observed by others. Watanabe et al. (1992) showed the reduction of SOM positive submucous neurons in the inflamed colon of CD and UC patients. A loss of nerve fibres in inflamed and non-inflamed gut regions in CD patients has been described by Dvorak et al. (1985). Additionally Straub et al. (2008) detected a loss of TH positive neurons in the inflamed colon of CD patients. The increased area of TH fibres and the increased number of SOM fibres, which we observed in non-affected areas, differ however from these previously observed results in IBD patients. It is well established that intestinal inflammation is associated with functional and structural changes in the ENS. However, the particular alterations depend on the gut region, the inflammatory state and the species.

The lack of effect induced by the probiotic supernatants (*L. paracasei* NCC 2461 and *B. longum* NCC 3001) or by the probiotic feeding (probiotic mixture: VSL#3) agrees with the inability of *B. longum* NCC 3001 or VSL#3 feeding of $TNF^{\Delta ARE/WT}$ mice to reverse the histological scores for ileitis (unpublished data and personal communication T. Clavel; Hörmannsperger et al. 2009). Furthermore *in vitro*, *B. longum* NCC 3001 and *L. paracasei* NCC 2461 had no effect on markers of ER-stress and did not affect TNF induced secretion of the pro-inflammatory chemokine IP-10 (interferon-inducible protein-10) (unpublished data and personal communication T. Clavel). VSL#3, however, was in *in vitro* experiments able to selectively inhibit activation induced secretion of IP-10 in intestinal epithelial cells (Hörmannsperger et al. 2009). However VSL#3 feeding did not exert protective effects on ileal inflammation in $TNF^{\Delta ARE/WT}$ mice and was not able to reduce elevated levels of IP-10 in ileal intestinal epithelial cells. In contrast to $IL\text{-}10^{-/-}$ mice, where the VSL#3 clearly reduced the inflammation in the caecum as well as the level of IP-10 in primary caecal epithelial cells (Hörmannsperger et al. 2009; Hörmannsperger et al. 2010). Thereby, suggesting that the probiotic mixture VSL#3 has region specific probiotic effects in IBD mouse models.

4.2 Neural action of the bacterial protease gelatinase from *Enterococcus faecalis* (GelE)

The purified bacterial metalloprotease GelE from *Enterococcus faecalis* OG1RF activated neurons in the guinea-pig submucous plexus. However, the $[Ca]_i$ recordings did not reveal any neural or glia cell activation by GelE. This suggests a calcium-independent excitation or weak $[Ca]_i$ signals below the detection limit. PAR2-AP after GelE treatment evoked significantly less action potentials. These data suggest that GelE may mediate its neuronal action via PAR2, because it desensitized the receptor and as a result reduced the PAR2-AP induced response. Since GelE did not induce any $[Ca]_i$ response, its PAR2 activating action is, however, unlikely. Only studies with specific PAR2 antagonist allow final conclusions about the activation mechanism of GelE. One alternative mode of action could be that GelE interferes with the extracellular activation of the PAR2 receptor or with intracellular signalling cascades that are shared by PAR2 and GelE. Dulon et al. (2005) have shown that an elastolytic metalloprotease secreted by the lung pathogen *Pseudomonas aeruginosa* disarmed the PAR2 receptor by proteolysis, which silence the receptor causing it to become insensitive to activating proteases. In contrast, a house dust mite cysteine protease allergen has been shown to target PAR2, but inactivate PAR1, in respiratory epithelial cells and to induce the release of pro-inflammatory cytokines (Asokananthan et al. 2002). The data from Steck et al. (2009; 2011) has provided evidence that GelE is affecting intestinal epithelial barrier via targeting of barrier regulating proteins of tight and adherene junctions. GelE has been furthermore shown to only impair the intestinal barrier in genetically susceptible hosts, like IL-$10^{-/-}$ mice, but not in healthy WT mice. Therefore it is important to note, that this metalloprotease derived from a commensal bacterium was able to excite submucous neurons in healthy animals. This is the first report describing a neuron excitation by a bacterial derived metalloprotease. However, the mode of action and functional relevance in the diseased gut remains unknown.

In the human intestine GelE, similar to the guinea-pig submucous plexus did not induce any calcium responses in enteric neurons or glia. But since no VSD experiments have been conducted, it is impossible to draw any conclusion about a possible nerve-activating action of GelE in the human submucous plexus. The basolateral application of GelE did not induce ion transport in human or in the guinea-pig intestine excluding its role in regulating secretory processes. In addition, this result underlines a PAR-independent mode of action of GelE.

4.3 Neural actions of Protease-Activated Receptors (PARs)

This is the first report describing the effects of PAR activation in the human enteric nervous system as well as in guinea-pig submucous plexus. The present study revealed that stimulation of PARs by PAR selective peptides and endogenous proteases activated neurons and glia in the human submucous plexus. The most striking result was that most neurons and glia in human submucous plexus responded to PAR1-AP and thrombin application while activation of PAR2 and PAR4 yielded minor responses in very few neurons. Furthermore this PAR1-AP mediated neural response has been shown to be direct and specific. There was a marked difference between human and guinea-pig submucous neurons and glia in their responses to PAR-APs. In guinea-pig submucous neurons the PAR2-AP evoked the strongest response, followed by weaker responses of PAR1 and PAR4-AP. The difference was more pronounced for glia cell activation through PAR-APs. While human enteric glia responded to PAR1-AP but not to PAR2-AP, it was the other way around in guinea-pig enteric glia. The proportion of PAR2-AP sensitive neurons in the guinea-pig submucous plexus agrees with data reported for the guinea-pig myenteric plexus (Gao et al. 2002). However, the low responsiveness of guinea-pig submucous neurons to PAR1-AP is in contrasts to findings that 87% of myenteric neurons respond to PAR1-AP (Gao et al. 2002). The functional relevance of this plexus specific effectiveness of PAR1-AP remains to be studied. The finding that macrophages in the human, but not in the guinea-pig, submucous plexus preparation respond to PAR2-AP remains descriptive at this stage; functional consequences of PAR activation in tissue resident macrophages in the gut are unknown. But in general it has been already shown that human macrophages express PAR2 and respond to PAR2 agonists with $[Ca]_i$ transients (Colognato et al. 2003).

We mainly focused on the actions of peptides which specifically activate PAR1, PAR2 or PAR4 rather than using non-selective endogenous activators. It has been shown in guinea-pig enteric neurons that PAR1-AP and PAR2-AP mimicked the action of thrombin and trypsin, respectively (Covera et al. 1999; Gao et al. 2002), whereas trypsin is a PAR1 and PAR2 preferring ligand and therefore non-specific (Kwong et al. 2010). Also thrombin has been shown to activate PAR1, PAR3 and PAR4. The following evidences supported specific actions of PAR-APs: we used rodent and human specific PAR-APs, the reversed peptides were without any effects, the effects of the PAR1-AP were blocked by a selective PAR1 antagonist (SCH79797; Ahn et al. 2000) and futhermore the different efficacies of PAR-APs in the human and guinea-pig submucous plexus made cross-reactivity of the PAR-APs

unlikely. But nevertheless, the actions of the endogenous proteases thrombin and tryptase were studied. Thrombin induced a strong PAR1 specific response in human enteric neurons and glia, whereas tryptase caused only a weak neuronal excitation. Finally, the endogenous proteases thrombin and tryptase mimicked the action of the PAR1 and PAR2-APs, respectively and supported the promiment role of PAR1 in the human submucous plexus.

Guinea-pig submucous neurons required much longer exposures to the PAR-APs to elicite spike discharge than human submucous neurons. This may be interpreted as a higher sensitivity of human submucous neurons to PAR-APs. The necessity for long PAR-AP exposure time has been previously observed in guinea-pig myenteric neurons (Covera et al. 1999; Gao et al. 2002). In this study, concentrations of PAR-APs were used that reliably activated guinea-pig myenteric neurons (Covera et al. 1999; Gao et al. 2002). The ejection pipette contained 100µM of the PAR-APs which will be diluted by a factor of ten before they reach the tissue targets (Breunig et al. 2007). At 10µM the PAR1, PAR2 and PAR4-APs caused an almost identical submaximal membrane depolarisation in guinea-pig myenteric neurons (Gao et al. 2002).

The conclusions are based on PAR-APs induced spike discharge in neurons as well as $[Ca]_i$ increase in neurons and glia. As expected, the $[Ca]_i$ recordings revealed responses in a greater number of neurons. This is due to the fact that the membrane potential and calcium-sensitive dyes reported different aspects of cell activation. Di-8-ANEPPS signalling required action potential discharge while the calcium imaging also revealed cell activation not associated with spikes. It is important to note that the recordings of spike discharge and $[Ca]_i$ led to the same conclusion: PAR1 activation produced the greatest response in the human submucous neurons while PAR2 activation mediated the greatest response in the guinea-pig submucous neurons. Using PAR2-AP only weak neural or glial responses could be achieved in human submucous plexus even by varying the application protocol (e.g. microejection vs local perfusion, increased potassium concentration, perfusion of serine protease inhibitor). For several reasons, it is unlikely that recordings of PAR2 activation were per se compromised in human tissue due to excessive release of proteases, in particular from tumors, resulting in PAR2 desensitization. Firstly, release of proteases would be expected to also desensitize PAR1 but this was not the case. Secondly, although most tissues were obtained from cancer patients differences in PAR2 activation in non-cancer tissues were not observed. Moreover, the samples were taken from macroscopically normal non-cancer regions. Thirdly, increases in

$[Ca]_i$ in macrophages and enhanced mucosal secretion in response to PAR2-AP were recorded arguing against PAR2 desensitization. Fourthly, the treatment of tissues with the serine protease inhibitor FUT-175 (50µg/ml) which served to prevent PAR desensitization, did not improve the PAR2-AP induced responses which makes PAR2 receptor desensitization due to tissue handling and surgery or proteolytic degradation and inactivation of the synthetic peptides very unlikely. The failure to detect PAR2 positive immunostaining made it impossible to decide, whether there was low expression of PAR2 receptors or whether the functionality of PAR2 receptors in the human submucous plexus is modified. Compton et al. (2002) showed that cell surface expression and signalling of human PAR2 depend on the glycosylation of the N-terminus. It can be hypothesized that altered glycosylation is responsible for the compromised neural and glial PAR2 action. This, however, would mean that such altered glycosylation only appear in human and not in guinea-pig intestinal preparations.

The observation that PAR1-AP and PAR2-AP activate a signalling cascade leading to an increase of the intracellular calcium level has been previously reported for guinea-pig myenteric neurons (Covera et al. 1999) and glia (Garrido et al. 2002) as well as for epithelial cells (Böhm et al. 1996). In our study PAR2-AP exerted the $[Ca]_i$ transients in guinea-pig submucous neurons and glia, but PAR1-AP generated a $[Ca]_i$ transient only in neurons. Thus there is a plexus specific response to PAR-APs not only in neurons but also in glia.

It has previously been shown that in guinea-pig myenteric neurons and glia cells PAR1 and PAR2 induce the $[Ca]_i$ increase via release of calcium from intracellular stores, which is mediated by a phospholipase C (PLC) dependent mechanism (Garrido et al. 2002; Gao et al. 2002; Covera et al. 1999). However, this intracellular signalling cascade has not been studied in the human and guinea-pig submucous plexus. Therefore a calcium-independent PAR1 response in guinea-pig submucous glia can not be excluded. PAR4-AP, which induced a moderate spike discharge in human submucous neurons, generated a very minor neuronal and no glia $[Ca]_i$ response. This means, that PAR4 could mediate its action via a Ca^{2+}-independent signalling mechanism, as it has been shown for endothelial cell, where PAR4 evoked NO release independent of calcium (Hirano et al. 2007). Augé et al. (2009) has also shown that PAR4-AP did not mobilize calcium in DRG neurons, but inhibited the PAR2-AP induced $[Ca]_i$ increase.

It has been previously shown that guinea-pig myenteric glia showed increased $[Ca]_i$ after PAR1 and PAR2 activation (Garrido et al. 2002). In the brain the PAR1 preferring agonist thrombin evoked glia proliferation and release of cytokines from glia (Wang et al. 2003). Additionally nerve growth factor production (Neveu et al. 1993) or protection form cell death (Perraud et al. 1987) has been shown to be mediated via PAR activation on astrocytes. The functional consequence of PAR-AP mediated $[Ca]_i$ increases in enteric glia of human and guinea-pig submucous plexus and the fact that this is species-specifically signalled through different PARs remains to be studied.

Furthermore it still needs to be clarified whether human enteric glia are directly activated by proteases or indirectly activated by PAR stimulated neurotransmitter release from neurons. The results of the TTX experiments suggested that both routes may be operative in human enteric ganglia. While PAR1-AP mediated $[Ca]_i$ transients were significantly reduced in the presence of TTX in some glia, the $[Ca]_i$ transients in others were TTX-resistant.

The motivation to study PAR-APs effect on epithelial secretion was that this is one of the primary functions of submucous neurons. As one functional consequence in human mucosa/submucosa preparations a PAR1 induced nerve-dependent mucosal secretion was identified whereas the PAR2 evoked secretion was a result of direct epithelial stimulation. This agrees with previous results in mucosal biopsies (Mall et al. 2002). Basolateral PAR2 activation caused increased Cl^--secretion probably by a direct epithelial action as found in our experiments. In the same study (Mall et al. 2002) PAR1 activation had no effect probably because the routine biopsies unlikely contained the submucous plexus which, as shown in the present study, mediated the pro-secretory actions of PAR1-AP. In mucosal biopsy samples PAR4-AP did not induce any secretory response (Mall et al. 2002), as it has been shown for our human mucosa/submucosa preparations. These results are in accordance with the imaging data, where PAR1, but not PAR2 induced nerve activation.

There are many different ways to block PAR1, such as the usage of selective peptidomimetic PAR1 antagonists. It has been shown that peptides and peptidomimetic compounds based on the structure of PAR1 tethered ligand can serve as PAR1 antagonists. But they have certain limitations, such as the lack of specificity, partial agonist activity and low affinity (Bernatowitcz et al. 1996; Rasmussen et al. 1993). There are also non-peptide PAR1 antagonists, such as SCH79797 or SCH530348, which are selective and competitive and

functionally antagonize the thrombin generated tethered ligand (Ahn et al. 2000). SCH530348, is an oral antiplatelet drug under development by Schering-Plough in phase III clinical trails for the treatment and prevention of atherothrombotic events in patients with acute coronary syndrome, previous myocardial infarction, stroke, or existing peripheral arterial disease (Hildemann and Bode 2009).

To block the PAR2 mediated actions also different possibilities exist: small molecule inhibitors, such as ENMD-1068 (N^1-3-methylbutyryl-N^4-6-aminohexanoyl-piperazine), peptide antagonists, such as LIGK-NH_2 (ENMD-1005) or molecular antibodies, such as SAM-11 anti-human monoclonal antibody or B5 anti-rat polyclonal antibody, which target the tethered ligand (Kelso EB et al, 2006). LIGK-NH_2 reduced the PAR2 mediated $[Ca]_i$ response in guinea-pig submucous neurons and glia. However in the present study, LIGK-NH_2 exerted no inhibitory action on the PAR2-AP induced pro-secretory response in human intestine. Instead, LIGK-NH_2 slightly increased the PAR2-AP induced response, which could be interpreted as partial agonistic action of LIGK-NH_2.

PARs play not only a role in the gastrointestinal system, where they exert plexus- and species-specific actions. PARs are also expressed and functional relevant in the vascular, renal, respiratory, musculoskeletal and somatic nervous system. Several animal and human studies emphasize the different regional, species and disease dependent importance of the individual PARs. In human malignant tumors a comparable expression of PAR1 and PAR2 has been determined (Elste and Petersen 2010). In HIV-associated encephalitis an increased expression of PAR1 and an increased level of prothrombin mRNA in the brain has been detected (Boven et al. 2003), whereas in multiple sclerosis an increased level of mast cell tryptase in cerebrospinal liquid and increased importance of PAR2 and has been suggested (Roznieckі et al. 1995).

A few thoughts are at hand as to the relevance of our results for clinical applications. PAR1 antagonists are currently used as antiplatelet drugs and for the treatment of inflammatory, proliferative and neurodegenerative diseases (Cirino and Severino 2010). Those drugs may have effects on gut functions through PAR1 signalling via enteric neurons. Constipation was listed as a frequent adverse event in a clinical trial with a PAR1 antagonist although the incidence was not significantly different from placebo (Goto et al. 2010). The data from the present study may point to new strategies to treat pathological conditions associated with

increased protease levels in the gut and suggest agonists or antagonists of human PAR1 as potential disease modifying therapeutic agents. Interestingly, thrombin positive cells are increased in the colon of diarrhea-predominant IBS patients (Bian et al. 2009) suggesting hyperstimulation of PAR1. It remains to be shown whether the decreased expression of PAR1 in the same patients may be a consequence of and one way to compensate for the increased density of thrombin producing cells (Bian et al. 2009) as our results would suggest that a lower PAR1 expression should result in hypo- rather than hypersecretion. Diarrhea in these patients may also be caused by enhanced PAR2 activation because these patients had higher levels of mast cell tryptase (Bian et al. 2009). Based on our data a PAR2 mediated secretion would result from direct activation of epithelial cells. Excessive thrombin levels could also result from increased vascular permeability which has not yet been studied in IBS. Similar data have been reported for inflammatory bowel diseases where thrombin generation (Saibeni et al. 2010) and vascular permeability enhancing vascular endothelial growth factor is increased (Griga et al. 1998).

Mucosal biopsy supernatants of IBS patients excited human submucous neurons (Buhner et al. 2009). The PAR1 antagonist SCH79797 reduced the IBS supernatant induced spike discharge. These results underline the importance of proteases as one of the main mediators involved in ENS excitation by the supernatants (Buhner et al. 2009). Future studies have to evaluate whether the ability of the PAR1 antagonist to reverse ENS sensitization by IBS supernatants is linked to PAR1 activating factors in the supernatants. One unresolved issue is the discrepancy in the response pattern to IBS supernatants and PAR1-AP. While the latter evoked tachyphylaxis the IBS supernatant-evoked excitation is reproducible. This may be due to the concentration of proteases in the supernatants which might be low enough to not cause PAR desensitization. In addition, other mediators in the IBS supernatants, such as serotonin and histamine, are prominently involved in neural excitation. The interaction between histamine, serotonin and proteases leading to neural excitation are unknown but may affect PAR responsiveness. PAR2 agonists for instance have been shown to cause hyper-responsiveness to histamine in guinea-pig respiratory tract (Barrios et al. 2003). Since different application protocols have been used to study the neural action of PAR1-AP and IBS supernatants, it can be also assumed that this affects the reproducibility. The application of the activating peptide for 800ms did not induce any neural activation, whereas the supernatant clearly evoked spike discharge after 800ms application.

The functional importance of PAR1 in human submucous plexus stands in clear contrast to the prominent role of PAR2 in the guinea-pig intestine as it has been shown by electrophysiological studies as well as in knock out experiments. In summary, one of the main results of this thesis is the dominant contribution of PAR1 in signalling to human enteric neurons and glia. This novel pathway of proteases in the human gut calls for further translational studies which address the potential to target PAR1 in the clinic setting in particular to improve symptoms in gut disorders that are associated with increased levels of PAR1 activating proteases.

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ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
A-IBS	Alternating diarrhea and constipation IBS
ARE	Adenosine uracil rich region
B. longum	*Bifidobacterium longum*
C-IBS	Constipation-predominant IBS
Ca^{2+}	Calcium
$[Ca]_i$	Intracellular calcium concentration
CCD	Charge-coupled device
Cl^-	Chloride
CNS	Central nervous system
CD	Crohn's Disease
CSD	Calcium-sensitive dye
D-IBS	Diarrehea-predominant IBS
E. coli Nissle	*Escherichia coli* Nissle 1917
E. faecalis	*Enterococcus faecalis*
ENS	Enteric nervous system
GelE	Gelatinase from *Enterococcus faecalis*
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IFNγ	Interferon γ
IL	Interleucin
IP_3	Inositol triphosphate
IP-10	Interferon-inducible protein-10
IR	Immunoreactive
I_{SC}	Short-circuit-current
L. paracasei	*Lactobacillus paracasei*
Mg^{2+}	Magnesium
MP	Myenteric plexus
MSORT	Multi-Side Optical Recording Technique
MT5	Medium Tom 5
NSE	Neuron-specific enolase

PARs	Protease-activated receptors
PAR-APs	PAR-activating peptides
PAR-RPs	PAR-reversed peptides
PGP 9.5	Protein gene product 9.5
PI-IBS	Post-infectious IBS
PLC	Phospholipase C
R	Resistance
SMP	Submucous plexus
SOM	Somatostatin
SST-2	Somatostatin 2 receptor
TEER	Transepithelial electrical resistance
TH	Tyrosine hydroxylase
TNBS	Trinitrobenzene sulfonic acid
TNFα	Tumor necrosis factor α
TTX	Tetrodotoxin
TX	Triton X-100
UC	Ulcerative Colitis
VSD	Voltage-sensitive dye
WT	Wild type

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