

# **INTERUNIVERSITY ATTRACTION POLES**

**PHASE V 2002-2006**

**PROJECT 5/20**

**NEURO-ENDOCRINE INTERACTIONS IN THE  
GASTRO-INTESTINAL TRACT UNDER NORMAL  
AND PATHOLOGICAL CONDITIONS**

**PROGRESS REPORT 2003**

# Table of contents

<b>1. Description of the research activities .....</b>	<b>3-21</b>
1. Working package enteric microcircuits .....	3-5
2. Working package inhibitory neurotransmission .....	5-8
3. Working package inflammation .....	8-14
4. Working package VPAC-PAC .....	14-15
5. Working package ghrelin .....	16-19
6. Working package ICC .....	19-21
<b>2. Structure and functioning of the network .....</b>	<b>22</b>
1. Collaborative work performed .....	22
2. Colloquia, seminars, workshops .....	22
3. Formal and informal contacts .....	22
<b>3. Publications .....</b>	<b>23-27</b>
1. Publication list of each IAP partner .....	23-26
2. Joint publications of the IAP-network .....	26-27
<b>Addendum.....</b>	<b>28-30</b>

# 1. Description of the research activities 2003

## 1.1 Morphological, pharmacological and electrophysiological analysis of enteric microcircuits in mammals including man under normal and pathological conditions (working package: enteric microcircuits)

**Age-related changes in the myenteric neurons of the rat esophagus.** (Partner 1 in collaboration with the Department of Medical Physiology and Surgery, UMC Utrecht, The Netherlands)

The data obtained and mentioned in the previous report have now been further collected and published in an extended paper.

### **Optimisation of optical imaging of enteric microcircuits.** (Partner 4)

Our group has successfully developed a method for the optical imaging of neuronal activity. We studied responses to electrical train stimulation (ETS) of interganglionic fibre tracts in entire myenteric ganglia of the guinea-pig small intestine. ETS induced calcium transients in a subset of neurons: 52.2% responded to oral ETS, 65.4% to aboral ETS, and 71.7% to simultaneous oral and aboral ETS. A total of 41.3% of the neurons displayed convergence of oral and aboral ETS-induced responses. Pharmacological analysis showed that responses were due to neuronal conduction and involved N-type calcium channels. Our data reveal complex oral and aboral input to individual myenteric neurons rather than a polarisation in spread of activity.

**Effects of interleukins on enteric neurons in the guinea-pig.** (Partner 1 in collaboration with the Department of Medical Physiology and Surgery, UMC Utrecht, The Netherlands)

Myenteric ICCs are considered to be pacemaker cells for contractile activity in the GI tract.  $Ca^{2+}$  oscillations observed in ICCs are linked to this pacemaker activity. To determine if ICCs are involved in neuro-immune interactions, effects of the pro-inflammatory mediator IL-1 $\beta$  on ICCs were investigated in conventional myenteric plexus preparations of the guinea-pig colon, loaded with Fluo-4-AM. The cytosolic  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) was measured by confocal laser scanning microscopy. All ICCs responded to IL-1 $\beta$  with an immediate fast and transient rise in  $[Ca^{2+}]_i$ . The dose-response relation revealed an  $EC_{50}$  of approximately 50 pM. Continuous perfusion with IL-1 $\beta$  evoked a long-term increase in  $[Ca^{2+}]_i$ . The responses of ICCs to IL-1 $\beta$  were reproducible and reversible and were not affected by blockade of prostaglandin synthase, neural action potential firing, or NOS. Double immunostaining on whole mounts showed the presence of the IL-1 receptor on the cell membrane of myenteric ICCs. In conclusion, these results demonstrate that IL-1 $\beta$  has a marked, direct excitatory effect on ICCs in the myenteric plexus, indicating that these ICCs may be involved in the neuro-immune regulation of GI motility.

**Purinergic receptors in the guinea-pig gastrointestinal tract.** (Partner 1 and partner 5 in collaboration with the Autonomic Neuroscience Institute, Royal Free and University College Medical School, London, United Kingdom and the Department of Biological Structures, Functions and Technology, University of Naples Federico II, Italy)

Purinergic (ATP) neurotransmission is a component of the inhibitory response of the musculature in various regions of the GI tract. So far, seven ionotropic purinergic receptors (P2X<sub>1-7</sub>) have been cloned. As specific antibodies become available, their respective distribution in the GI tract can be elucidated. Here we used high-resolution tricolour confocal

microscopy as previously described, to study the distribution of P2X<sub>7</sub>-ir cells in the muscularis propria of the rat stomach, small intestine and colon. Smooth muscle cells, KIT-ir ICCs and CD34/SK3-ir fibroblast-like cells were P2X<sub>7</sub>-negative, while P2X<sub>7</sub> IR was observed in nerves and S100-ir glial cells. In all regions studied, P2X<sub>7</sub>-ir was also observed in myenteric and submucosal ganglia, where perineuronal nerve endings appeared brightly labelled. Our observations suggest that purinergic signalling could influence the enteric glia through P2X<sub>7</sub> receptors.

Pharmacological studies have provided functional evidence that a P2X receptor, possibly of the P2X<sub>1</sub> subtype, participates in the contractile response of enteric smooth muscle to purines. However, immunohistochemistry failed to detect the P2X<sub>1</sub> receptor in enteric smooth muscle or in enteric neurons. Due to the fact that our knowledge of the expression of the P2X<sub>1</sub> receptor in the GI tract remains obscure, a study was performed to elucidate the distribution of this receptor in the GI tract of the mouse and the guinea-pig. IR was predominantly observed in vascular and enteric smooth muscle in both species. In the guinea-pig, no IR was found in the enteric nervous system, while in the mouse, P2X<sub>1</sub>-positive cholinergic varicosities were detected in the ganglia of both plexuses of the ileum and of the myenteric plexus of the colon. In the guinea-pig, a few oesophageal intramuscular ICCs expressed P2X<sub>1</sub> IR. As such, morphological evidence was provided for postjunctional involvement of the P2X<sub>1</sub> receptor mediating a substantial component of the contractile response of the gut. In the mouse, the P2X<sub>1</sub> receptor was found to be involved in the intrinsic innervation of the small and large intestine.

The P2Y<sub>4</sub> receptor is a member of the P2Y family of G-protein-coupled receptors for extracellular nucleotides. Pharmacological studies showed that the P2Y<sub>4</sub> receptor mediates the UTP- and ATP-induced chloride secretory responses in the small intestine. Therefore, a morphological study was performed to reveal the distribution of this receptor in the GI tract of the mouse and guinea-pig. Preliminary data show that the P2Y<sub>4</sub> receptor is mainly expressed in ICCs in the guinea-pig GI tract, while in the murine GI tract it is predominantly found in glial cells.

**Innervation of motor endplates in the porcine oesophagus.** (Partner 1 and partner 5 in collaboration with the Department of Clinical Physiology, University of Warmia and Mazury, Olsztyn, Poland)

The data obtained and mentioned in the previous report have now been further collected and published in an extended paper.

**Enteric nervous system of the murine oesophagus.** (Partner 1 in collaboration with the Laboratory of Electrobiolgy, University of Antwerp, Belgium)

Morphological studies and intracellular electrophysiological recordings were performed to clarify the possible function of the observed anatomical co-innervation of the motor endplates in the oesophagus by intrinsic nerve fibres, and to characterise the myenteric neurons in the murine oesophagus. Apart from further identification of the neurochemical coding of the murine intrinsic esophageal neurons based on their neurotransmitter/neuromodulator content, immunocytochemical stainings were initiated to map the distribution pattern of distinct potassium channels within the oesophageal wall. Potassium channels are involved in a large number of important cellular events, such as cell growth and neuronal excitability level. Furthermore, studies of potassium currents within the enteric nervous system have demonstrated that different subtypes of potassium channels play a role in the regulation of the resting membrane potential of sensory neurons and in the hyperpolarisation associated with inhibitory postsynaptic potentials. In a first step we focussed on the TASK -family, yielding the presence of TASK 1 and 2 channels in

subpopulations of enteric neurons of the mouse GI tract. In addition, the presence and distribution pattern of another voltage-insensitive and acid-sensitive cation channel, i.e. ASIC3 belonging to the amiloride-sensitive epithelial Na<sup>+</sup> channel/degenerin family, was investigated in control and *Schistosoma*-infected mice. ASIC3 was found to be expressed in the murine oesophagus and ileum, in c-Kit-negative, mostly CD169-positive cells (and thus probably macrophages), but not in nerve fibres or neuronal somata. Preliminary data from inflamed intestine of *S.mansoni*-infected mice did not reveal an apparent upregulation of the ASIC3 channel.

Finally, intracellular recordings of myenteric neurons in the striated muscle of the murine oesophagus have been continued in order to further unravel their electrophysiological properties and correlate these with morphological data.

### **Abbreviations:**

**ATP:** adenosine triphosphate; **[Ca<sup>2+</sup>]<sub>i</sub>:** cytosolic Ca<sup>2+</sup> concentration; **ETS:** electrical train stimulation; **ICC:** interstitial cell of Cajal; **IL-1β:** interleukin-1β; **IR:** immunoreactivity; **ir:** immunoreactive; **GI:** gastrointestinal; **NOS:** nitric oxide synthase; **TASK:** tandem pore acid sensitive K channel; **UTP:** uridine triphosphate.

## **1.2. Inhibitory neurotransmission in the digestive tract (working package: inhibitory neurotransmission)**

### **Serotonergic control of upper stomach motility. (Partner 2)**

The characterisation of the serotonin receptors influencing upper gastric motility was continued. As data on buspirone administered to humans suggest that activation of 5-HT<sub>1A</sub> receptors might lead to proximal stomach relaxation, the influence of the 5-HT<sub>1A</sub> receptor agonist flesinoxan was studied in conscious dogs instrumented with a barostat. Flesinoxan was found to induce a dose-dependent gastric relaxation. The clear-cut gastric relaxant effect of 100 µg.kg<sup>-1</sup> i.v. flesinoxan was dose-dependently reduced by the 5-HT<sub>1A</sub> receptor antagonist WAY100635, indicating that in the dog model, flesinoxan indeed induces gastric relaxation through interaction with 5-HT<sub>1A</sub> receptors. The gastric relaxant effect of flesinoxan was completely prevented by vagotomy, indicating that the vagal pathway is essential. Central activation of the vagal inhibitory nitrergic pathway to the stomach, however, is excluded since the response to flesinoxan was not influenced by the NO synthesis inhibitor N<sup>G</sup>-nitro-L-arginine methyl ester. Nevertheless, flesinoxan might act centrally by activation of other vagal pathways that release purinergic or peptidergic neurotransmitters in the stomach. Alternatively, it might act by inhibition of the vagal stimulatory cholinergic pathway to the stomach, which could occur either at a central or at a peripheral level.

### **Role of NO and VIP. (Partner 1, partner 2 and partner 4)**

Due to the very short half-life of NO, it is important to know the location within the neuron where the molecule is generated, and thus the time to reach its target. nNOS is generally accepted to be a soluble enzyme present in the cytoplasm of the nitrergic neurons. However, in the longitudinal muscle/myenteric plexus preparation of the rat small intestine, we previously found a particulate fraction of nNOS, prompting us to further study the subcellular distribution of nNOS in this preparation. Application of a stronger homogenisation procedure revealed that up to 50 % of immunoreactive nNOS was now sedimentable. By studying the correlation between the distribution of nNOS IR and the IR or enzyme activity of several cell component markers, we attempted to identify the nNOS-binding cellular component. nNOS appeared associated with a single subcellular structure that differed from the plasma membrane, rough and smooth endoplasmic reticulum, mitochondria, lysosomes, VIP-containing particles and synaptobrevin-containing exocytotic particles.

### **Role of SERCA in the relaxant effect of NO. (Partner 2)**

Although NO is established as an ubiquitous inhibitory neurotransmitter in the gastrointestinal tract, the mechanism by which NO elicits relaxation remains largely unresolved. We investigated the possible role of sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) in nitrergic relaxation of rat gastric fundus since this location has been proposed as the site of action of NO in some tissues. Rat gastric fundus tissue has to be contracted to allow observation of nitrergic relaxation. Following contraction by prostaglandin  $\text{F}_{2\alpha}$ , gastric fundus tissues were completely relaxed by nifedipine, indicating that contraction was due to  $\text{Ca}^{2+}$  entry through L-type voltage-operated  $\text{Ca}^{2+}$  channels. However, contraction of gastric fundus tissues to a similar degree by the SERCA inhibitor thapsigargin led to a 20% tone reduction by nifedipine, indicating that contraction was mainly related to  $\text{Ca}^{2+}$  entry through store-operated  $\text{Ca}^{2+}$  channels. In the latter condition, the relaxant responses to exogenous NO and to endogenous NO, liberated by electrical field stimulation of the tissues, were greatly reduced in comparison with tissues contracted with prostaglandin  $\text{F}_{2\alpha}$ . This finding indicates that an active SERCA pump is required for NO to induce relaxation. Since the relaxations induced by exogenous VIP were also partly reduced in tissues contracted with thapsigargin, activation of SERCA does not appear unique for nitrergic relaxation.

### **Interference with gastric motility via prostanoid receptors. (Partner 2)**

Our interest for gastric relaxant mechanisms is related to the possible therapeutic application of selective gastric relaxant procedures in functional disorders such as functional dyspepsia patients with impaired gastric accommodation. The interaction of prostaglandins with gastric motility is largely unexplored. Therefore, we investigated the influence of prostanoids in circularly oriented muscle strips of pig gastric fundus. Tissues were incubated with  $^3\text{H}$ -choline, which is used by the cholinergic neurons to build up  $^3\text{H}$ -acetylcholine. By measuring tritium outflow upon electrical field stimulation, the acetylcholine release induced by electrical stimulation of the cholinergic neurons is directly assessed. When omitting the influence of endogenous prostaglandins by working in the presence of indomethacin,  $\text{PGE}_2 > \text{PGF}_{2\alpha} > \text{PGI}_2$  inhibited tritium release while the TP receptor agonist U-46619 and  $\text{PGD}_2$  had no effect. This indicates that inhibitory EP receptors are present on the cholinergic nerve endings. The  $\text{EP}_2$  receptor agonist butaprost had no effect, while the  $\text{EP}_1$  and  $\text{EP}_3$  receptor agonist sulprostone mimicked the effect of  $\text{PGE}_2$ . As the latter effect was not influenced by AH6809, that is able to antagonize  $\text{EP}_1$  receptors, presynaptic  $\text{EP}_3$  receptors are the subtype of EP receptors present on the cholinergic nerve endings. This suggests that selective activation of  $\text{EP}_3$  receptors might be a mechanism to relax the stomach by counteracting the tonic cholinergic drive to the stomach. However, all prostanoids including  $\text{PGE}_2$  also induced contraction of the tissues by interaction with muscular prostaglandin receptors. Although the muscular effect of  $\text{PGE}_2$  certainly involves interaction with  $\text{EP}_1$  receptors, it is also partly due to interaction with  $\text{EP}_3$  receptors. Stimulation of these contractile receptors would, of course, counteract the gastric relaxing effect of selective  $\text{EP}_3$  receptor agonists via the presynaptic  $\text{EP}_3$  receptors.

### **Role of ATP: (Partner 1)**

The role of ATP in enteric neurotransmission is complex and much debated upon. ATP mediates fast synaptic transmission in the enteric nervous system but may also act as a non-adrenergic non-cholinergic (NANC) neurotransmitter in the gut. The functional evidence reported in favour of ATP is well balanced by evidence against ATP being a NANC neurotransmitter in the gut. We therefore studied the role of ATP as NANC neurotransmitter in circular muscle of the murine jejunum. Jejunal circular muscle strips were mounted in organ baths for isometric tension recording. All experiments were performed on prostaglandin  $\text{F}_{2\alpha}$ -contracted strips and in the presence of atropine, guanethidine and L-nitroarginine (blocks NOS). Electrical stimulation (ES, 1-8 Hz) caused fast twitch relaxations

which were abolished by the purinergic P2 receptor blockers suramin and PPADS and by the SK channel blocker apamin. The purines ATP,  $\alpha\beta$ mATP (P2X receptor agonist) and ADP $\beta$ S (P2Y receptor agonist) relaxed jejunal muscle strips with a potency of ADP $\beta$ S > ATP >  $\alpha\beta$ mATP. TTX did not alter the responses to ATP or ADP $\beta$ S but slightly inhibited that to  $\alpha\beta$ mATP. Desensitisation of P2X receptors by treating strips with  $\alpha\beta$ mATP reduced relaxations to ES and  $\alpha\beta$ mATP but not those to ADP $\beta$ S. The P2X receptor blocker NF 279 inhibited relaxations to low-frequency ES. The P2X<sub>7</sub> receptor blocker Brilliant blue G had no effect. Desensitisation of P2Y receptors by treating strips with ADP $\beta$ S virtually abolished all relaxations to ES and to ADP $\beta$ S without altering those to  $\alpha\beta$ mATP. The P2Y<sub>1</sub> receptor blocker MRS 2179 potently inhibited relaxations to ES and ADP $\beta$ S. These results show that ATP or a related purine is an inhibitory neurotransmitter in the circular muscle layer of the mouse jejunum. Purinergic relaxation is mediated mainly by smooth muscle P2Y<sub>1</sub> receptors. P2X receptors that are not of the P2X<sub>7</sub> subtype, are also involved, albeit to a minor degree.

### **Role of cGMP. (Partner 1)**

There is compelling evidence that NO relaxes intestinal muscle by a cyclic guanosine monophosphate (cGMP)-dependent pathway. Several studies, however, have reported that cGMP-independent pathways are also involved. We have addressed this controversy by studying the effect of specific inhibitors of soluble guanylyl cyclase (sGC) on relaxations to endogenously released NO and exogenously added NO in the murine intestine. Jejunal circular muscle (CM) and longitudinal muscle (LM) strips were mounted in organ baths for isometric tension recording. All experiments were performed on prostaglandin F<sub>2 $\alpha$</sub> -precontracted strips and in the presence of atropine, guanethidine and suramin to block cholinergic, adrenergic and purinergic responses. In CM but not in LM strips, electrical stimulation (ES, 1-8 Hz) induced frequency-dependent relaxations that were TTX-sensitive. These relaxations were nitrenergic since they were abolished by L-nitroarginine (NOS blocker). In CM and LM strips, NO and the NO donors nitroglycerin (GTN), SIN1 and nitroprusside (SNP) induced dose-dependent relaxations which were more pronounced in CM strips than in LM strips. ODQ and NS2028 (specific blockers of sGC) abolished relaxations to ES at all frequencies and abolished relaxations to NO, GTN, SIN1 and SNP at all concentrations in CM and LM strips. ODQ or NS2028 did not affect relaxations to ATP that were obtained in the absence of suramin. Our results show that CM strips relax to NO released from nitrenergic nerves and that this relaxation is completely sGC-dependent. CM and LM strips relax to exogenously added NO and to NO donors and these relaxations are also completely sGC dependent. Our results therefore do not support the hypothesis that cGMP-independent mechanisms are involved in NO-mediated relaxations in the murine small intestine.

### **Abbreviations:**

**ATP:** adenosine triphosphate; **cGMP:** cyclic guanosine monophosphate; **CM:** circular muscle; **ES:** electrical stimulation; **GTN:** nitroglycerin; **IR:** immunoreactivity; **LM:** longitudinal muscle; **NANC:** non-adrenergic non-cholinergic; **NO:** nitric oxide; **nNOS:** neuronal NOS; **NOS:** NO synthase; **PG:** prostaglandin; **SERCA:** sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup> ATPase; **sGC:** soluble guanylyl cyclase; **SK:** small conductance Ca<sup>2+</sup>-sensitive K<sup>+</sup> channel; **SNP:** nitroprusside; **TTX:** tetrodotoxin; **VIP:** vasoactive intestinal peptide.

### **Role of nitrenergic innervation in impaired gastric accommodation. (Partner 4)**

Functional dyspepsia may involve different pathophysiological mechanisms. One of these mechanisms is impaired gastric accommodation, which involves gastric nitrenergic inhibitory nerves, but also other factors. We have made a survey of the heterogeneity of functional dyspepsia and found by cluster analysis that four factors are involved. Factor 1 is characterised by nausea, vomiting, early satiety, and weight loss and factor 2 by postprandial fullness and bloating. Both factor 1 and 2 are associated with delayed

emptying, but only factor 1 is associated with younger age, female sex, and sickness behaviour. Factor 3 is characterised by pain symptoms and associated with gastric hypersensitivity and several psychosocial dimensions including medically unexplained symptoms and health-related quality of life dimensions. Factor 4, characterised by belching, is also associated with hypersensitivity, but is unrelated to psychosocial dimensions.

In functional dyspepsia delayed gastric emptying involves an abnormal gastric distribution of the meal and is associated with postprandial fullness and vomiting. *Helicobacter pylori* infection does not play a role in the development of these symptoms but unsuppressed postprandial phasic contractility in the proximal stomach is associated with severe bloating. We also developed tests to assess symptoms related to gastroparesis and to impaired accommodation.

We have continued our study of the mechanisms underlying impaired gastric accommodation using pharmacological analysis, especially serotonergic pathways. As such, we studied the role of 5-hydroxytryptamine in the control of gastric fundus tone using the selective 5-hydroxytryptamine re-uptake inhibitor, paroxetine. Pretreatment with paroxetine did not alter the thresholds for perception and discomfort during isobaric (4.7 +/- 2.3 vs. 4.0 +/- 2.0 mmHg and 13.3 +/- 3.1 vs. 12.7 +/- 2.3 mmHg above the minimum intragastric distending pressure, N.S.) and isovolumetric (307 +/- 90 vs. 417 +/- 114 mL and 772 +/- 74 vs. 750 +/- 76 mL, N.S.) distensions. Paroxetine significantly enhanced the amplitude of the meal-induced fundus relaxation (136 +/- 51 vs. 255 +/- 43 mL,  $P < 0.05$ ). These data suggest that the release of 5-hydroxytryptamine, probably at the level of the enteric nervous system, is involved in the control of the accommodation reflex in humans, and that paroxetine may be beneficial to patients with impaired postprandial fundus relaxation. In another study we investigated the effect of Tegaserod, a 5-hydroxytryptamine-4 receptor agonist. Nineteen healthy volunteers (10 females; mean age, 23.9 years) were studied on three separate occasions after 7 days of treatment with placebo, tegaserod 2 mg b.d. or tegaserod 6 mg b.d. in a double-blind, randomised, three-way cross-over design. We found that tegaserod allows for larger intra-balloon volumes both before and after a meal. These findings warrant the investigation of the therapeutic potential of tegaserod in dyspeptic patients with impaired accommodation.

### **1.3. Pathogenesis of intestinal suffering and interaction between the enteric nervous system and the immune system in schistosomiasis, TNBS-induced ileitis, chemically induced inflammation and axotomy-induced neurochemical changes and ileus (working package: inflammation)**

**Pathogenesis of intestinal suffering and the neuro-immune interaction in schistosomiasis.** (Partner 1, partner 2 and partner 4 in collaboration with the Department of Veterinary Clinical Studies, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Roslin, UK, with the Department of General Surgery, University of Tuebingen, Germany, with the Department of Biomedical Science, University of Sheffield, UK, and with the Department of Medical Physiology and Surgery, UMC Utrecht, The Netherlands)

Morphological evidence has been provided of a neuro-immune interaction between mast cells and extrinsic primary afferent neurons in *S. mansoni*-infected mice. The increased density of CGRP-positive extrinsic afferents within the lamina propria of intestinal villi might also be reflected in a change in functional extrinsic afferent activity. Therefore, we examined extrinsic sensory afferent sensitivity in intestinal schistosomiasis using an *in vitro* mouse model, recently developed at the University of Tuebingen. Ileal segments with mesenteric

arcades of both infected and non-infected mice were used. Extracellular multiunit recordings were made from mesenteric paravascular nerve bundles. These experiments showed that the response of extrinsic afferents to chemical and mechanical stimulation does not appear to directly reflect the morphological changes found in *S. mansoni*-infected animals, although compensatory mechanisms cannot be excluded.

Communication between neurites and mast cells is a prototypic example of neuro-immune interaction. We previously reported a close apposition between mucosal mast cells (MMC) and a dense network of CGRP-ir extrinsic primary afferent nerve fibres in the lamina propria of *S. mansoni*-infected murine ileum. These CGRP-ir fibres originate from dorsal root ganglia (DRGs). In order to investigate the possibility of bidirectional cross-talk between MMCs and extrinsic primary afferent neurons, a primary culture of bone marrow-derived MMCs (technique developed at the University of Edinburgh) and a primary culture of spinal neurons derived from DRGs of adult mice, were set up. Using immunohistochemistry, both cultures were morphologically characterised. Degranulation properties of cultured MMCs in response to compound 48/80 (C48/80), a known mast cell degranulator, and the neuropeptides CGRP and SP were analysed using mMCP-I ELISA. Using optical recordings of intracellular calcium ( $[Ca^{2+}]_i$ ) and intracellular recordings, we examined the interaction between primary cultured MMCs and DRG neurons *in vitro*. Application of the mast cell degranulator C48/80 to fluo-4 loaded MMCs induced a transient rise in  $[Ca^{2+}]_i$  in approximately 56% of the MMCs studied. A smaller percentage of MMCs (15%) responded to GRP with a rise in  $[Ca^{2+}]_i$ . Compared to C48/80, the response to CGRP showed a shorter lag time and a smaller amplitude. The CGRP response was completely blocked by pertussis toxin, indicating involvement of G-proteins. Application of 'MMC juice' obtained by C48/80 degranulation, to fluo-4 loaded DRG neurons induced a rise in  $[Ca^{2+}]_i$ . We also showed that degranulation of MMCs by C48/80 in culture dishes containing DRG neurons, caused activation of DRG neurons after a much longer lag time. In conclusion, these results demonstrate a bidirectional cross-talk between cultured MMCs and DRG neurons *in vitro*, indicating a functional relevance for the close apposition of MMCs and CGRP-ir nerve fibres *in vivo*.

To examine the role of the neuropeptides CGRP and somatostatin, as well as alterations in the mast cell mediator content in the neuro-immune interaction, pharmacological and contractility experiments were performed on ileal segments of non-infected and infected wildtype and mMCP-1 knockout mice. So far, exogenously applied CGRP has not evoked any functional responses in wildtype or knockout mice.

### **Inflammation: enteric neurotransmission (Partner 1)**

Inflammation alters the contractile smooth muscle cell function which contributes to inflammation-induced changes in GI motility. However, chronic inflammation may also affect the synaptic signalling in the enteric nervous system. While it is well known that tachykinergic neurotransmission is disturbed during inflammation, the exact pathways leading to this disturbance are incompletely understood. We therefore aimed to study in detail which of the known neurokinin receptors (NK1, NK2 and NK3) are functionally active in the murine small intestine. We studied the involvement of tachykinins in the excitatory neurotransmission of the ileum. Circular ileal muscle strips were mounted in organ baths for isometric tension recording and responses were expressed as % of contraction to 50mM KCl. Substance P (SP) induced sustained, dose-dependent contractions. These were mimicked by the specific NK1 and NK2 receptor agonists septide and beta-A-NKA, respectively, while the NK3 receptor agonist senktide had no contractile effect. Contractions to SP and beta-A-NKA were not significantly affected by L-NNA (NOS blocker) or atropine, while those to septide were slightly inhibited by atropine and significantly enhanced by L-NNA. In the presence of LNNA+atropine, TTX (blocks nerve-conductance) had no further effect on the response to SP, septide or beta-A-NKA. Electrical stimulation (ES, 1-8 Hz) of

the muscle strips induced frequency-dependent contractions that were transient at 1 and 2 Hz and sustained at 4 and 8 Hz. All contractions were significantly enhanced by L-NNA. In the presence of L-NNA, atropine significantly reduced contractions to 1 and 2 Hz but had no effect on those to 4 and 8 Hz. The residual contractions to ES, obtained in the presence of LNNA+atropine, were not affected by propranolol or phentolamine but abolished by TTX. NK receptor desensitisation, which was achieved by prolonged incubation of separate muscle strips with septide, beta-A-NKA or senktide, significantly reduced contractions to ES. Strips incubated with septide still contracted to beta-A-NKA and vice versa, indicating that there was no cross-desensitisation. Our results suggest that a tachykinergic neurotransmitter is released from enteric nerves innervating the circular muscle of the ileum and that NK1, NK2 and NK3 are functionally active in this tissue. Activation of NK1, NK2 and NK3 receptors exerts a neurogenic effect involving nitrenergic, cholinergic and tachykinergic nerves. In addition, activation of NK1 and NK2 receptors also exerts a direct smooth muscle effect.

### **Expression of Connexin 43 and Connexin 40 during *Schistosoma mansoni* infection.** **(Partner 1)**

Connexin 43 and Connexin 40 are connexin proteins that form a major constituent of gap-junction proteins. Expression of these proteins was studied during acute and chronic stages of *Schistosoma mansoni* infection, with the aim to observe muscle immunoreactivity in mouse ileum during the course of disease evolution. To this end, double immunostaining for connexin 43 and c-Kit was performed using whole mounts and sections (10µm thick, fixed/unfixed) from uninfected and infected mouse ileum.

Connexin 43 only stained in the circular muscle layer but not in the longitudinal muscle layer of the ileum, which raises the question whether only the smooth muscle cells in the circular muscle layer and not those in the longitudinal layer are connected by Connexin 43. Furthermore, comparison between non-infected and infected animals showed more intense connexin 43 staining in the circular smooth muscle layer at 8 weeks of infection.

Single staining for connexin-40 on fresh, frozen ileum sections of control and infected animals revealed the presence of this protein in the endothelium of large blood vessels only. Cx40 IR did not appear not to be upregulated during inflammation.

### **Reactivity of SSTR antibodies to the *Schistosoma mansoni* egg and granuloma.** **(Partner 1)**

Somatostatin is part of an immunoregulatory circuit that is instrumental in limiting interferon-gamma production at sites of chronic inflammation. In murine schistosomiasis, parasite egg-induced granulomas express somatostatin receptor type 2 (SSTR2) on T cells. The type-2 receptor has been reported to be the exclusive somatostatin receptor present in this inflammation, and has been associated with the immunoregulatory network at play within the granulomas. The presence of the somatostatin receptor type 3 (SSTR3) has been reported on muscle cells during inflammation. Our aim was to identify immunocytochemically the presence of these somatostatin receptors (SSTR2 and SSTR3) in fresh frozen ileum sections infected with *Schistosoma mansoni*. Preliminary data revealed the presence of both receptors on the surface of the parasite eggs.

### **Study of somatostatin levels during the evolution of a schistosomiasis infection in low- and high-pathology mice.** (Partner 1 and partner 4)

The neuropeptide somatostatin exerts an antifibrotic effect on hepatic stellate cells *in vitro*, and reduces fibrosis and morbidity in *Schistosoma mansoni*-infected animals. It has been proven a highly effective drug in controlling variceal bleeding and reducing portal pressure. The aim of the present study was to investigate the role of somatostatin in the evolution of *S. mansoni*-caused disease severity in two inbred mice strains, C57BL/6J and C3H/HeN. The C3H strain

shows a more severe pathology after *S. mansoni* infection, with larger granulomas, a higher degree of hepatic fibrosis and a higher mortality rate (high-pathology strain), as compared to the BL/6 strain (low-pathology strain).

Our hypothesis is that there may be a direct correlation between the development of severe morbidity in *S. mansoni*-infected mouse strains and the inability to generate required somatostatin levels.

40 C57BL/6J and 40 C3H/HeN mice were infected with 30 cercariae and sacrificed at 8 weeks (acute stage of infection) or 16 weeks (chronic stage of infection) post-infection. Five controls were included for each group. Plasma samples were taken by cardiac puncture and somatostatin levels were determined. The degree of liver pathology was examined quantitatively by egg count, measurement of granuloma volume and determination of hepatic hydroxyproline as a value for hepatic fibrosis.

The C3H/HeN strain showed a more severe pathology with significantly larger granulomas, a higher degree of hepatic fibrosis and a higher mortality rate than the C57BL/6J strain. Within each strain the mice displayed a significantly higher degree of hepatic fibrosis in the chronic stage than in the acute stage of infection. Somatostatin levels in the plasma of the C57BL/6J strain rose significantly at 16 weeks post-infection (controls  $146.00 \pm 12.96$  ng/ml vs. infected  $275.62 \pm 23.48$  ng/ml;  $p=0.004$ ), whereas no difference was observed at 8 weeks post-infection (controls  $160.80 \pm 17.24$  ng/ml vs. infected  $191.53 \pm 12.56$  ng/ml;  $p=0.238$ ). In the C3H/HeN strain, higher somatostatin concentrations were found at 8 weeks post-infection (controls  $160.40 \pm 15.12$  ng/ml vs. infected  $204.94 \pm 7.85$  ng/ml;  $p=0.023$ ) but not at 16 weeks post-infection (controls  $191.20 \pm 15.43$  ng/ml vs  $260.30 \pm 29.49$  ng/ml;  $p=0.142$ ). The rise in somatostatin levels from 8 to 16 weeks post infection in infected animals was higher in C57BL6/J than in C3H/HeN mice ( $p=0.05$ ).

Somatostatin levels in the plasma may be one of the regulatory factors determining the progression to severe (C3H/HeN) or low (C57BL/6J) pathology during *S. mansoni* infection. During the evolution of infection somatostatin increased in the 'low-pathology' C57BL/6J mice. C3H/HeN mice only showed an increase in somatostatin in the acute stage, whereas in the chronic stage - when liver pathology worsens and mortality increases - the levels of this neuropeptide were not elevated. A possible modulatory role of host somatostatin levels in determining pathology caused by *S. mansoni* infection is suspected.

### **Inflammation: afferent nerve activation and septic ileus. (Partner 1)**

Sepsis is often associated with GI motility disturbances. We investigated the role of nicotinic synapses and extrinsic afferent neurons in septic ileus in mice. Septic ileus was induced by an i.p. injection of lipopolysaccharides (LPS). Eighteen hours later, the mice received an intragastric injection of 0.1 ml Evans blue. After 15 min, gastric emptying (GE) was measured spectrophotometrically. Small intestinal transit was measured as the migration of Evans blue. In the first series, mice received an i.p. injection of the nicotinic receptor blocker hexamethonium or saline, 1 h before Evans blue injection. In the second series, mice were pretreated, 14 days before administration of LPS or saline, with a neurotoxic dose of capsaicin or its solvent. In the third series of experiments, mice were treated with saline or the CGRP antagonist CGRP 8-37, 1 h before Evans blue injection. LPS induced a significant delay in GE and transit in saline- and solvent-treated mice. Hexamethonium accelerated GE but not transit in control mice. In LPS mice, hexamethonium reversed the delay in GE to control values and partially restored the delay in transit. Capsaicin had no effect on GE and transit in control mice but completely reversed the endotoxin-induced delay in GE and tended to improve the delay in transit. CGRP 8-37 had no effect on GE in control mice but decreased transit in control mice. In LPS mice, CGRP 8-37 reversed the delay in GE and improved the delay in transit. These results indicate a role for nicotinic synapses in the pathogenesis of septic ileus and support a role for capsaicin-sensitive afferent neurons and their neurotransmitter CGRP in gastric septic ileus.

### **Inflammation: oxidative stress and septic ileus. (Partner 1)**

Building on our previous results on the role of oxidative stress in septic ileus in mice, additional experiments were performed on the colocalisation of iNOS in residential macrophages in the external muscle layers of gastric and ileal tissues from control mice, septic mice and septic mice treated with PEG-SOD or PEG-CAT. These findings were also quantified. LPS induced a significant increase in iNOS-positive residential macrophages in the muscular layers of the stomach and ileum from 0 in controls to 8 % in the stomach of LPS mice and 7 % in the ileum of LPS mice. PEG-SOD or PEG-CAT treatment resulted in a significant decrease of these percentages to 3-4 % in both tissues. We also performed additional immunohistochemical experiments on the presence of nitrotyrosine and hydroxynonenal (HNE) as hallmarks of nitrosative and oxidative stress in the stomach and ileum of control, septic mice and septic mice treated with PEG-SOD and PEG-CAT. Immunohistochemistry showed the presence of nitrotyrosine and HNE in the gastric and ileal mucosa and submucosa of LPS-treated mice. Treatment with PEG-SOD or PEG-CAT of LPS mice diminished the presence of nitrotyrosine or HNE in both tissues.

### **Inflammation: pancreatitis and gastrointestinal motility. (Partner 1)**

Acute pancreatitis is often associated with GI motility disturbances. However, the underlying mechanisms remain largely unknown. We therefore studied the effect of acute necrotising pancreatitis on the contractility of isolated muscle strips from the gastric fundus and jejunum. Acute necrotising pancreatitis was induced by feeding young female mice a choline-deficient (CD) ethionine (0.5%) supplemented diet (CDE) for 72 hours. Enteric nerve-mediated contractions to electrical field stimulation (EFS, 0.5-8 Hz) and contractions to carbachol (Cch), substance P (SP), serotonin (5HT), prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) and KCl were studied in control, CD and CDE mice. The severity of acute pancreatitis was assessed by histological examination. Only mice with a histological severity score  $\leq 4$  after CDE were included in the CDE group. EFS, Cch, SP, 5HT,  $PGF_{2\alpha}$  and KCl induced contractions in jejunal and gastric fundus muscle strips from control, CD and CDE mice. However, all jejunal contractions were significantly decreased in CDE mice with acute pancreatitis, whereas jejunal contractility in CD mice was comparable to that in control mice. On the other hand, acute pancreatitis did not affect the contractility of gastric fundus muscle strips since contractions to EFS, Cch, SP,  $PGF_{2\alpha}$  and KCl in the gastric fundus were similar in control, CD and CDE mice. Our results show that acute necrotising pancreatitis leads to a decreased contractility of the jejunum while the contractility of the gastric fundus remains normal during pancreatitis. These results suggest that acute necrotising pancreatitis leads to a disturbed contractile activity of specific GI regions.

### **Protective influence of superoxide dismutase on nitrgic neurotransmission. (Partner 2)**

Previous experiments in our laboratory suggest that Cu/Zn superoxide dismutase (SOD) plays an essential role in the protection of the nitrgic neurotransmitter. However, following inhibition of endogenous Cu/Zn SOD with diethyldithiocarbamate, the inhibitory effect of the superoxide anion generator 6-anilino-5,8-quinolinedione (LY83583) on the nitrgic relaxant response induced by EFS of pig gastric fundus circular muscle strips was only partly restored by exogenous administration of Cu/Zn SOD. This might be related to the incapability of exogenous Cu/Zn SOD to penetrate intracellularly, while endogenous Cu/Zn SOD is active at both the intra- and extracellular level. We therefore studied the protective effect of polyethylene-glycol-superoxide dismutase (PEG-SOD), which is able to penetrate intracellularly. The protective effect of PEG-SOD versus LY83583 was not more pronounced than that of Cu/Zn SOD itself, illustrating that extracellular interaction of the endogenous nitrgic neurotransmitter with superoxide radicals is vital in determining its breakdown.

Addition of a second antioxidant together with Cu/Zn SOD (reduced glutathione, uric acid, bilirubin or catalase) did not further reverse the inhibitory effect of LY83583. This finding underlines the crucial role of endogenous Cu/Zn SOD in the protection of the nitrergic neurotransmitter.

### **Nitrergic-purinergetic interaction in rat distal colon. (Partner 2)**

The IAP project also comprises the study of the negative/positive influence of NO, produced by iNOS, in motility disturbances in inflammatory bowel diseases. The model of inflammatory bowel disease will be addition of dextrane sulphate sodium to the drinking water of rats, whereby inflammatory lesions are induced in the distal colon. The role of NO in relaxant responses of rat distal colon has been insufficiently elaborated upon in the literature, giving rise to contradictory reports; furthermore, ATP was shown to be colocalised in NANC neurons of rat colon. Prior to applying dextrane sulphate sodium, we therefore first studied the involvement of NO and ATP and their possible reciprocal interactions in inhibitory neurotransmission of normal rat distal colon. The soluble guanylate cyclase inhibitor ODQ reduced the duration and amplitude of the relaxant responses induced by exogenous NO. The blocker of small-conductance  $\text{Ca}^{2+}$ -sensitive  $\text{K}^+$  channels (SK channels), apamin, only reduced the duration of the response to NO, but when added to ODQ, increased the inhibitory effect of ODQ on both duration and amplitude. The relaxant responses to exogenous ATP were reduced by the nerve conduction blocker TTX, apamin, the NO synthesis inhibitor  $\text{N}^{\text{G}}$ -nitro-L-arginine methyl ester (L-NAME) and the P2Y purinoceptor antagonist reactive blue 2. The relaxant responses induced by electrical field stimulation, which are due to liberation of the endogenous inhibitory neurotransmitters, were abolished by tetrodotoxin but not altered by L-NAME, ODQ or reactive blue 2 alone. Apamin alone reduced the duration, while apamin plus L-NAME nearly abolished the relaxations. ODQ plus apamin, as well as reactive blue 2 plus L-NAME, reduced the duration. These observations indicate that SK channels are involved in the relaxant effect of NO, and that NO increases their sensitivity via generation of cGMP. ATP induces relaxation via P2Y receptors, which initiates a pathway leading to activation of SK channels; ATP also releases neuronal NO. Both NO and ATP are involved in inhibitory neurotransmission and both pathways must be blocked to inhibit the relaxations induced by EFS.

### **Abbreviations:**

**5HT:** serotonin; **ATP:** adenosine triphosphate; **C48/80:** compound 48/80; **[ $\text{Ca}^{2+}$ ]:** intracellular calcium; **CAT:** catalase; **Cch:** carbachol; **CD:** choline-deficient diet; **CDE:** choline-deficient ethionine supplemented diet; **cGMP:** cyclic guanosine monophosphate; **CGRP:** calcitonin gene-related peptide; **DRG:** dorsal root ganglion; **ES:** electrical stimulation; **EFS:** electrical field stimulation; **GE:** gastric emptying; **GI:** gastrointestinal; **HNE:** 4-hydroxy-2-nonenal; **iNOS:** inducible nitric oxide synthase; **ir:** immunoreactive; **L-NAME:**  $\text{N}^{\text{G}}$ -nitro-L-arginine methyl ester; **LPS:** lipopolysaccharides; **MMC:** mucosal mast cell; **mMCP-I:** mouse mast cell protease I or  $\beta$ 1-chymase; **NANC:** non-adrenergic non-cholinergic; **NK:** neurokinin; **NO:** nitric oxide; **ODQ:** soluble guanylate cyclase inhibitor; **PEG:** polyethylene-glycol; **PGF<sub>2 $\alpha$</sub> :** prostaglandin F<sub>2 $\alpha$</sub> ; **SK:** small conductance  $\text{Ca}^{2+}$ -sensitive  $\text{K}^+$ ; **SOD:** superoxide dismutase; **SP:** substance P; **TTX:** tetrodotoxin.

### **Effects of TNBS-induced colitis on non-adrenergic non-cholinergic innervation. (Partner 4)**

Electrical stimulation of colonic muscles elicits a response during the stimulation period, and a transient excitation after the stimulus. Post-stimulus or "rebound" excitation has been linked to pathways involving inhibitory neurotransmitters, prostaglandins and substance P (SP), but the mechanism is incompletely understood. Because rabbit colitis is characterised by a loss of inhibitory neurotransmission we hypothesised that it might affect the rebound

response. We therefore characterised rebound responses in non-inflamed and inflamed tissue by comparing the effect of antagonists/blockers of putative (nNO, ATP, SP, prostaglandins) and new (serotonin) neurotransmitters. NANC conditions increased rebound responses in non-inflamed strips, while this effect was reduced or abolished in inflamed strips. Rebound responses were reduced by pretreatment with the NO synthase inhibitor, L-NAME, under NANC conditions in non-inflamed strips but not affected in inflamed tissue. In contrast, the P(2) purine receptor antagonist, suramin, did not affect rebound responses in inflamed or non-inflamed strips. The effect of the cyclo-oxygenase inhibitor, indomethacin, on rebound responses was reversed from excitatory to inhibitory by inflammation. Under NANC conditions, rebound contractions were also reduced by the NK-1 antagonist, SR140333, both in normal and in inflamed strips. The most pronounced reduction in rebound responses in inflamed and non-inflamed strips under normal conditions was observed with the 5-hydroxytryptamin (1,2) antagonist, methiothepin. Our data show that rebound responses are mainly non-cholinergic and involve NO, SP, serotonin and inhibitory prostaglandins. In inflamed tissue the nitrenergic pathway is absent, excitatory prostaglandins prevail and the cholinergic and tachykinergic components are relatively more important. However, an important serotonergic contribution remains. Our data further suggest that inflammation damages different neural pathways to a different extent and is most selective for nitrenergic neuronal populations.

#### **1.4. Role of VPAC1, VPAC2, PAC1, glucagon and CGRP in the digestive tract (working package: VPAC-PAC)**

**Role of VIP and VPAC1, VPAC2 and PAC1 receptors in the chicken oviduct.** (Partner 1 and partner 3 in collaboration with the Department of Biological Structures, Functions and Technology, University of Naples Federico II, Italy)

As a side project resulting from the ongoing collaboration within the IUPA-framework, a morphological and pharmacological study was performed to elucidate the possible involvement of VIP and corresponding receptors in the mechanism of oviposition in the chicken oviduct. These data have been submitted as a original manuscript, and a revision including additional pharmacological experiments as requested by the referees has now been resubmitted.

The presence, distribution and smooth muscle motor effects of galanin and pituitary adenylate cyclase activating peptide (PACAP) were studied in the nerves of the vaginal part of the oviduct of egg-laying hens. Galanin and PACAP immunoreactivity were found in both neuronal perikarya and nerve fibres within the wall of the vaginal segment. Both populations showed a similar distribution pattern. Particularly the circular muscle and the intramural vascular net were richly innervated. A few galanin- and PACAP-IR nerve fibres extended up to the mucosal folds. Multiple labelling showed galanin to be colocalised with PACAP as well as with vasoactive intestinal polypeptide (VIP) and nitric oxide synthase (NOS) in a large, partly intrinsic neuronal subpopulation innervating the smooth muscle wall. Pharmacological *in vitro* experiments showed that isolated vaginal muscle strips had a spontaneous basal activity that was not affected by the neuronal conductance blocker tetrodotoxin (TTX). Galanin induced concentration-dependent contractions that were TTX-insensitive. PACAP, VIP, nitric oxide (NO) and the NO donor nitroglycerin caused concentration-dependent relaxations that were TTX-insensitive. Electrical field stimulation of isolated muscle strips induced frequency-dependent relaxations that were blocked by TTX and reduced by the NOS blocker L-nitroarginine. These data provide evidence that this region contains a largely intrinsic, neuronal subpopulation, capable of releasing multiple NANC motor agents for the control of local muscular activities. In addition, we provided pharmacological evidence that VIP, NO and PACAP exert an inhibitory and galanin an excitatory action on isolated muscle strips of the vaginal part of the chicken oviduct. Our results suggest that these NANC

neurotransmitters play an important role in the regulation of neuromuscular activity in this region.

### **VPAC1/VPAC2 receptor phosphorylation, sequestration and activation. (partner 3)**

Using the selective monoclonal antibody recognising the amino-terminal domain of the VPAC1 receptor, we continued the identification of the receptor residues that are phosphorylated by agonist stimulation, and studied the relationships between phosphorylation and receptor internalisation. We demonstrated that phosphorylation is not a prerequisite for agonist-dependent internalisation, and that the mechanism is clathrin-dependent and not lipid-raft-dependent. In contrast to the VPAC2 receptor, receptor recycling of the VPAC1 receptor to the membrane was not detected. Chimaeric constructs between the VPAC1 and VPAC2 receptors are currently tested to delineate the receptor domains responsible for receptor recycling. This important topic has not yet been studied in the GPCR B family of receptors.

We also continued to define the precise contribution of the third intracellular loop to G-protein coupling. We demonstrated that a basic sequence in the proximal part of the loop is indispensable (in conjunction with an acidic residue in the proximal domain of the intracellular carboxy-terminal tail) for adenylate cyclase stimulation but not for calcium stimulation, whereas another basic residue cluster located distally in IC3 is a prerequisite for calcium stimulation. We are currently also investigating the properties of a receptor mutant unable to couple to the calcium effector.

### **Agonist-induced internalisation of the human VPAC2 receptor. (partner 3)**

The agonist-induced internalisation of the human VPAC2 receptor was also studied by fluorescence-associated cell sorting using the monoclonal antibody developed in our laboratory. Internalisation was clathrin-dependent and the receptors were recycled to the membranes within 60 minutes by a mechanism completely blocked by monensin. A strict parallelism was observed between the efficacy of the agonist to stimulate adenylate cyclase activity and that to induce internalisation: N-hexanoyl VIP, considered a “super agonist”, was the most efficient analogue for internalisation. The antagonist PG 99-465 was not active. The effect of N-hexanoyl-VIP could be due to its slow dissociation rate. These results are of vital importance to drug design in that VPAC2 agonists are developed for the treatment of type 2 diabetes. Highly important in this respect is consideration of the balance between functional efficacy of the drug and possible unfavourable effects on receptor internalisation limiting its use.

### **Study of the expression of the VPAC2 receptor in gastrointestinal stromal tumours. (Partner 3 and partner 5 in collaboration with the University of Bern)**

It was recently shown by Professor Reubi (University of Bern, Switzerland) that 80% of the gastrointestinal stromal tumours (GIST) express a huge density of VPAC2 receptors. We have been contacted by Professor Reubi with the request to develop a selective tracer suitable for *in vivo* tumour identification. Apart from the strong practical and medical interest, which is directly linked to our research on new selective ligands, the scientific interest is directly related to other aspects of our programme: GIST are derived from interstitial cells of Cajal, and it will be of interest to study the control of expression of the VPAC2 receptor in these cells and in tumours. Multiple positive contacts have been made to obtain the necessary material.

## 1.5. Role of ghrelin/ghrelin receptors (in relation to motilin and motilides) in the digestive tract and (working package: ghrelin)

### Structural relationship between ghrelin and motilin. (Partner 3 and partner 4)

The structural relationship between motilin and the growth hormone secretagogue receptors (GHS-R), and between their respective ligands, motilin and ghrelin, prompted us to investigate whether ghrelin and the GHS-R agonist growth hormone-releasing peptide-6 (GHRP-6), could interact with the motilin receptor. The interaction was evaluated in the rabbit gastric antrum through binding studies of membrane preparations and contraction studies of muscle strips. We were able to show that ghrelin is unable to induce contractions via the motilin receptor. However, GHRP-6 was found to enhance neural contractile responses, partially via interaction with the motilin receptor on noncholinergic nerves with tachykinins as mediator, and partially via another receptor that may be a GHS-R subtype on cholinergic nerves that corelease tachykinins.

Within the framework of the structure-activity studies and of the study of the role of motilin in different species, we identified the motilin precursor in cats. Additionally, because the mechanism of action of motilin, in particular the role of central pathways, remains incompletely understood, we decided to study the effects of central administration of motilin in the rat. Gastric motility was monitored in conscious rats, whereas extracellular electrical activity recordings of the hippocampus were performed on anaesthetized rats to measure the influence of microinjection of motilin and CCK-8 into the hippocampus and into the cerebral ventricles. We found that hippocampal neurons are sensitive to gastric distension, and that motilin injection into the hippocampus increased the amplitude of gastric contractions. The hippocampal motilin-induced stimulation of gastric motility ( $30.6 \pm 5.5\%$ ) was completely abolished by subdiaphragmatic vagotomy ( $-2.8 \pm 4.4\%$ ) but unaffected by the intravenously applied receptor blockers atropine, phentolamine and propranolol. *In vivo* extracellular recordings of gastric distension-responsive neurons revealed that intracerebroventricular administration of motilin increased firing while CCK-8 inhibited firing. Our findings suggest that the stimulation of gastric motility by motilin administered in the hippocampus reflects the existence of a functional interaction between the hippocampus and a vago-vagus reflex running via a non-cholinergic and non-adrenergic efferent pathway.

It is known that there is a refractory period following stimulation with motilin. We have studied this phenomenon down to the molecular level, both *in vivo* and in several *in vitro* models. The *in vitro* data will be published in 2004. The *in vivo* data relate to the refractory characteristics of motilin stimulation of antral phase III, fasting gallbladder emptying and interdigestive pyloric and small intestinal motility from duodenum to ileum. Eight fasting, healthy male volunteers received, on separate subsequent days, repeated infusions of <sup>13</sup>leucine-motilin [8 pmol/(kg min) for 5 min] or saline at 30 min after phase III in the duodenum. Interdigestive motility of the antrum, pylorus, duodenum, jejunum and ileum was measured for maximum 10 h by using a 21-lumen perfused catheter. Gallbladder motility was measured by ultrasonography. Motilin infusions induced antral phase III, but only after a preceding phase III of duodenal origin. Under this condition, the time interval to phase III at the duodenal recording site was  $30 \pm 13$  (SEM) min after motilin, compared to  $79 \pm 14$  min after saline ( $P < 0.01$ ), and to  $121 \pm 13$  min for motilin infusion following an antral phase III ( $P < 0.001$ ). Motilin did not affect small intestinal motility or isolated pyloric pressure waves (IPPWs). However, the number of IPPWs was significantly affected by the origin of the preceding phase III, irrespective of whether motilin or saline was infused. Gallbladder volume decreased significantly within 10 min after each motilin infusion. This study demonstrates differential regional effects of motilin. Motilin initiates antral phase III, but stimulation is subject to a refractory period which is clearly prolonged after a preceding antral phase III. Motilin induced gallbladder emptying, however, is not subject to a refractory state. Small intestinal phase III as well as pyloric IPPWs are not affected by motilin.

### **Effect of ghrelin on septic ileus in mice. (Partner 1 and partner 4)**

In an experimental model of septic ileus in mice, we investigated whether the orexigenic peptide ghrelin and the ghrelin analogue GHRP-6 had prokinetic effects and whether they could reverse the sepsis-induced inhibition of gastric emptying and intestinal transit. Mice were injected i.p. with lipopolysaccharides (LPS) or saline (control). After 16-17 h mice were pre-treated with saline, ghrelin or GHRP-6, 1 h before intragastric administration of Evans blue. Fifteen min after Evans blue administration and after assessment of the behaviour scale, mice were killed and gastric emptying, transit and rectal temperature were measured. In control mice, ghrelin and GHRP-6 accelerated gastric emptying, but failed to increase transit significantly. Septic mice developed a delay in gastric emptying and transit, hypothermia and a deterioration of the behaviour scale. In septic mice, ghrelin expedited gastric emptying without any effect on transit, while GHRP-6 significantly accelerated gastric emptying dose-dependently but failed to increase transit significantly. Ghrelin and GHRP-6 had no effect on endotoxin-induced hypothermia or deterioration of the behaviour scale. Therefore, the beneficial effect of ghrelin and mainly of GHRP-6 offers potential therapeutic options in the treatment of septic gastric ileus.

### **Effect of ghrelin on gastrointestinal motility in rats. (Partner 1 and partner 4)**

Ghrelin and motilin form a new family of structurally related peptides. We compared the motility effects of ghrelin, the ghrelin receptor agonist, GHRP-6, and motilin in rats *in vivo* and *in vitro*. Ghrelin, GHRP-6 or motilin was injected i.p. and the effect on gastric emptying and transit was measured 20 min after intragastric application of Evans blue. In antral and fundic strips the effect of motilin, ghrelin or GHRP-6 was studied during electrical field stimulation (EFS). Ghrelin and GHRP-6 accelerated gastric emptying in rats by 2.8- and 2.6-fold, respectively, whereas motilin was ineffective. Both ghrelin and GHRP-6 but not motilin expedited transit by 1.3-fold at all doses tested. In fundic or antral strips, neural responses consisted of an on-relaxation that was reversed into a cholinergic contraction by addition of the NO-synthase blocker, L-NAME. The post-stimulus off-contraction was cholinergically mediated. In antral strips in the presence but not in the absence of L-NAME the on-responses were enhanced by ghrelin and GHRP-6 but not by motilin. The off-responses were only augmented by GHRP-6. In fundic strips GHRP-6 and ghrelin but not motilin reduced the EFS-induced on-relaxation. The effect of ghrelin was more pronounced in the presence of L-NAME. Ghrelin and GHRP-6 potentiated the off-contraction. In conclusion, ghrelin and GHRP-6 but not motilin accelerate gastric emptying and transit by activating cholinergic enteric pathways in addition to the known vagal pathways. Ghrelin may be the surrogate of motilin in rats.

#### **Abbreviations:**

**EFS:** electrical field stimulation; **L-NAME:** N<sup>G</sup>-nitro-L-arginine methyl ester; **LPS:** lipopolysaccharides; **NO:** nitric oxide.

### **Ghrelin synthesis. (Partner 3)**

The pharmacology of the ghrelin receptor was studied by synthesis of new ligands tested on wildtype and point-mutated receptors. The 3D structure of the receptor, proposed by Ingrid Langer, is based on the structure of CCK receptors. This model was evaluated taking into account our functional results obtained by the combined approach of receptor mutation and ligand modification. The following major findings (not yet published) were obtained: the orientation of the transmembrane helices allows the constitution of two hydrophobic pockets gated by a glutamic residue. The hydrophobic amino-terminal part of ghrelin consisting in octanoyl function in position 3, Phe 4, and Leu 5, occupies one of the pockets and the ligand is stabilised by ionic interaction between the acidic function of the above-mentioned glutamic acid

(E 124) and the positive charge of the amino terminus of ghrelin. A systematic mutation of the residues present in the pockets is actually in process (mutants F119A, F121A, F220A, F312A, F93A). An interesting and unexpected finding is the surprisingly high affinity of glutamine 4 ghrelin (Q4 ghrelin), which actually does not fit in with the proposed model. The possibility to replace a hydrophobic residue in that position by a polar neutral one may form the starting point of an entirely new family of ligands. Indeed, when the Phe4 residue is replaced by Cys, Ala, Tyr, D-Phe, ghrelin activity is markedly reduced; however, when replaced by His, it is only slightly reduced, but preserved when substituted with Meth, Leu, Ileu, Val, and Tryp. Gln 4 is clearly an exception. We also explored the consequences of reinforcing the amino-terminal-positive charge (Sarcosine 1 Ghreline, slightly more potent than ghrelin) or a limited delocalisation of the charge ( $\beta$ -Ala1 ghrelin was as potent and efficient as ghrelin) and also the substitutions permitted on the Ser 3 residues. Replacement of Ser by any hydrophobic residue led to inactive compounds, decanoylation and dodecanoylation of Ser 3 reduced the peptide potency when compared with the octanoylated peptide, whereas the peptide acylated with isobutyric acid conserved (reduced) activity. It appears that the presence of an ester or an amide function is required. A new ligand with an homoserine in position three esterified by heptanoic acid is actually synthesised and will be tested soon.

#### **Radioimmunoassay of ghrelin. (Partner 3 and Partner 4)**

The previous report mentioned the development of an antiserum recognising the amino-terminal part of ghrelin (including the octanoyl group), i.e., the biologically active form, as well as a second antibody recognising the carboxy terminus of ghrelin and identifying both octanoylated and deoctanoylated forms. These antibodies were used to measure plasma ghrelin (collaboration with Inge Depoortere). We also identified cell lines that normally synthesise and process the ghrelin precursor.

#### **Degradation of ghrelin. (Partner 3)**

A combination of HPLC purification, radioimmunoassays and mass spectrometry was used to analyse the metabolites of ghrelin generated in plasma and in contact with liver, kidney and gastric mucosa membrane. In contact with plasma, desoctanoylation is observed without proteolysis. Based on the effect of inhibitors, the candidate enzyme belongs to the family of the carboxyesterases, which is distinct from the cholinesterases and arylesterases. However, contact with the different tissues results in rapid proteolysis, with a preferential cleavage at the amino terminus, suggesting that the metabolites generated are inactive.

We also started - partly with a new grant from the FNRS - a research project on severe protein malnutrition in rats. One of the topics is the evolution of ghrelin levels in the stomach and blood during induction of fasting and in case of a switch to a balanced diet. This investigation line will be discussed with the other partners since other parameters in gut metabolism could be of interest.

#### **Effects of motilin/ghrelin on the visceral nociceptive respons. (Partner 4 and partner 6)**

Recent developments lead us to start the investigation of the effect of motilin and ghrelin on visceral pain induced by colorectal distension in guinea pigs. A visit of a PhD student to Toulouse had to be postponed to 2004 because of practical problems.

Experiments were started in Toulouse based on a jointly established protocol. Rats were surgically prepared for abdominal muscle electromyography and were anaesthetised by intraperitoneal administration of acepromazine (0.6 mg/kg) and ketamine (120 mg/kg). On the day of experiment, starting at least three days after surgery, animals were maintained anaesthetised with pentobarbital and the electrical activity of the abdominal muscles was recorded during rectal distension using an inflatable balloon. The balloon was inflated progressively with tepid water by steps of 0.5 ml, from 0 to 2 ml. Each step lasted 5 min. To

detect possible leakage, the volume of water introduced into the balloon was checked by complete removal at the end of the distension period. We observed that guinea pig motilin, and ghrelin, but not human motilin, at the dose of 50 mg/kg, significantly reduced the number of abdominal contractions induced by the distending volumes of 1, 1.5, and 2 ml. Lower doses (1 and 10 mg/kg) of the three peptides were ineffective. These data indicate for the first time that motilin and the motilin related peptide may decrease visceral nociceptive response to distension suggesting a new physiological role for these peptides.

## 1.6. Role of ICCs in the control of gastrointestinal motility under normal and pathological conditions (working package: ICC).

**Interstitial cells and SK3 channels.** (Partner 1 and partner 5 in collaboration with the Laboratory of Electrobiolgy, University of Antwerp, Belgium)

It is well known that ICCs act as pacemakers and conductors of slow-wave activity in the gut musculature. Apart from ICCs, the gut also contains interstitial cells that are not ICCs but that have a fibroblast-like structure. These fibroblast-like cells show IR for small-conductance calcium-activated potassium (SK3) channels. We here studied the role of SK3 channels in intestinal motility in transgenic SK3<sup>T/T</sup> mice in which SK3 channels are selectively suppressed by treatment with dietary doxycycline (DOX). Gastric emptying and intestinal transit of the dye Evans blue was studied *in vivo*. *In vitro* electrical and mechanical experiments were performed on isolated intestinal muscle strips. Western blot and SK3 IR confirmed loss of SK3 protein in the gut of DOX-treated SK3<sup>T/T</sup> (DOX-SK3<sup>T/T</sup>) mice. Suppression of SK3 channels did not significantly alter gastric emptying but significantly enhanced intestinal transit. DOX treatment of wildtype SK3<sup>+/+</sup> mice did not affect gastric emptying or intestinal transit. Loss of SK3 channels did not influence electrical slow wave frequency of small intestinal muscle cells. Mechanical contractions to carbachol and to electrical stimulation (ES) of enteric nerves (2-4 Hz) were similar in gastric fundus and jejunal muscle strips of SK3<sup>T/T</sup> and DOX-SK3<sup>T/T</sup> mice. When gastric fundus and jejunal muscle strips were precontracted with prostaglandin F2 $\alpha$ , ES induced nerve-mediated relaxations that were mimicked by exogenous NO and ATP. Relaxations to ES and NO in the gastric fundus and jejunum did not differ between SK3<sup>T/T</sup> and DOX-SK3<sup>T/T</sup> mice. However, when compared with these in SK3<sup>T/T</sup> mice, relaxations to ATP were significantly smaller in DOX-SK3<sup>T/T</sup> mice. This decreased relaxation did not result from DOX treatment itself since DOX treatment of SK3<sup>+/+</sup> mice did not alter intestinal relaxations to ATP. The amplitude and frequency of slow waves in the smooth muscle cells of the small intestine of transgenic (SK3<sup>T/T</sup>) and wildtype (SK3<sup>+/+</sup>) mice were studied with a standard microelectrode technique. When compared, resting membrane potential of the smooth muscle cells and amplitudes of the slow waves did not differ between the different groups. Only the frequency of the slow waves differed significantly between untreated SK3<sup>+/+</sup> mice and untreated SK3<sup>T/T</sup> mice. Furthermore, the effect of apamin (a blocker of SK2 and SK3 channels) was tested on the smooth muscle cells of untreated SK3<sup>+/+</sup> and DOX-SK3<sup>T/T</sup> mice. No significant differences could be observed between the different experimental conditions.

Our results show that SK3 channels play a role in murine intestinal motility. Loss of SK3 channels enhances intestinal transit *in vivo* and inhibits relaxations to ATP in gastric fundus and jejunal muscle strips *in vitro*. No evidence was found, however, that SK3 channels directly control electrical slow-wave frequency or enteric motor nerve activity in the murine intestine.

**Possible involvement of NKCC1 in the generation of spontaneous electrical slow wave activity in the murine small intestine.** (Partner 1 and partner 5 in collaboration with the Laboratory of Electrobiolgy, University of Antwerp, Belgium and Johnson & Johnson Pharmaceutical Research and Development, Beerse, Belgium)

Suppression subtractive hybridisation and RT-QPCR revealed the Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporter (NKCC1) on ICCs of the mouse small intestine. To test the involvement of NKCC1 in spontaneous slow-wave activity, we performed intracellular recordings of smooth muscle cells in the presence of bumetanide, a blocker of NKCC1. Addition of 100 μM bumetanide leads to a rapid change in the spontaneous slow-wave pattern measured with intracellular electrodes. The cells significantly depolarise within a few minutes, whereas the amplitude of the slow wave decreases. Apart from this change in amplitude, also the shape of the slow waves alters dramatically. Addition of bumetanide to the superfusion solution results in a significant decrease in both upstroke and downstroke of the slow wave, whereas the plateau phase disappears. Also the duration of the slow waves, measured as the time halfway the maximal amplitude, decreases significantly. In some cases, the slow waves disappear completely after several minutes, which is not due to a decrease in frequency, but to a decrease in the amplitude of the slow waves. Washout of bumetanide results in a slow and incomplete recovery of the slow waves. These findings indicate that NKCC1 plays a role in the spontaneous slow-wave activity in the murine small intestine.

**Slow-wave measurements in smoothelin knockout animals.** (Partner 1 and partner 5 in collaboration with the Laboratory of Electrobiolgy, University of Antwerp, Belgium and the Cardiovascular Research Institute Maastricht, The Netherlands)

Smoothelin is a cytoskeletal protein that is present in vascular as well as in visceral contractile smooth muscle cells. Smoothelin knockout animals have, apart from many other problems, problems with the GI tract, *i.e.*, they have diverticula in the small and large intestine and often a dilated duodenum. Furthermore, transit studies revealed a decreased transit of food in these animals. In an attempt to unravel the mechanism responsible for this behaviour, we performed intracellular recordings of the smooth muscle of the small intestine to record slow waves. This study revealed that the resting membrane potential of the smooth muscle cells of knockout animals is significantly depolarised compared with that in wildtype animals. Furthermore, the amplitude of the slow waves is significantly decreased in the knockout animals and the frequency of the slow waves is very irregular in these animals. Further studies are needed to unravel the mechanism underlying these processes.

**Abbreviations:**

**ATP:** adenosine triphosphate; **DOX:** doxycycline; **ES:** electrical stimulation; **ICC:** interstitial cell of Cajal; **IR:** immunoreactivity; **GI:** gastrointestinal; **NKCC1:** Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporter; **NO:** nitric oxide; **SK:** small conductance calcium activated potassium channel.

**The role of interstitial cells of Cajal in gastrointestinal motility caused by *Schistosoma mansoni* infection.** (Partner 1 and partner 5)

Interstitial cells of Cajal (ICC) act as pacemakers that generate slow waves and function as a relay between smooth muscle cells of the GI tract. In the mouse GI tract, the ICC-myenteric plexus lodged between the longitudinal and circular muscle layers, and the ICC-deep muscular plexus situated between the circular muscle layer and the submucosa, can be detected by kit immunoreactivity (IR). Faussone-Pellegrini et al. (2002) reported alterations in ICCs during and after inflammation induced by *Nippostrongylus brasiliensis* in rats (Neurogastro. Motil. 14:83-95). At day 30 post-infection when inflammation peaked, a complete loss of the ICC-deep muscular plexus in the jejunum was observed, causing alterations in motility.

The aim of our study was to determine if ICCs are similarly affected during acute and chronic *Schistosoma mansoni* infections, and thereby mediate the disturbed GI motility patterns caused by schistosomiasis.

To this end, immunohistochemistry was performed using whole mounts and sections (10µm thick, fixed/unfixed) from uninfected as well as 8- and 15-week-infected mouse ileum. Primary antibodies detected Kit IR (by binding to tyrosine kinase receptor), VACHT (vesicular acetylcholine transporter), SNAP-25 (a presynaptic marker for secretory granules), PGP-9.5 (protein gene product), SK3 (ionic channel marker for non-kit, fibroblast-like cells). The secondary antibodies used were either donkey anti-goat FITC/Texas Red or donkey anti-rabbit Texas Red. Single/Double immunofluorescence staining was done and staining observed by confocal microscopy.

Both ileum whole mounts and sections indicated that muscle thickness (Cx43 IR) and inflammatory infiltrate increased with infection. Kit-ir ICCs and SK3-ir cells were present at all normal locations, as seen in controls and during acute (after 8 weeks) and chronic (after 15 weeks) stages of infection. No disappearance of either ICC population was observed, which is in contrast to observations in the *N. brasiliensis* model of inflammation.

A preferential (although not exclusive) location of inflammatory infiltrate in contact with SK3-ir cells (non-Kit fibroblast-like cells) in the muscle layers was observed.

## 2. Structure and functioning of the network

### 2.1. Collaborative work performed

Joint research project of **partner 1** and **partner 4** with Prof. Tang Ming, Qingdao Medical College, P.R. China; project BIL 01/13 "The role of ghrelin, the recently discovered motilin-related peptide, in the regulation of gastric motility in rodents" has been continued. The theme of this project complements work package "Ghrelin". Meanwhile, this bilateral project has led to two PhD theses. The theme of this project complements work package "Ghrelin" and an extension will be applied for in 2004.

The detailed interaction between the partners (depicted in blue) has been indicated in each subtopic listed in item 1 "Description of the research activities in 2003".

The implementation of "groupware" has taken longer than expected but should be operational soon.

**All partners** presented their most recent findings during a special session of the Belgian Gastroenterology Week - Bruges, 20 February 2003 .

### 2.2. Colloquia, seminars, workshops

**Meeting of the Research Group "Gastrointestinal Regulatory Mechanisms"**  
Bruges, 20 February 2003

Programme: see addendum

### 2.3. Formal and informal contacts

Informal contacts have been organised between members of the partner teams as required by the collaborative projects.

On 17 October 2003, a formal meeting of all members in the presence of Mrs. Veronique Feys and one foreign consultant, Prof. R. DeGiorgio, (the other 2 foreign consultants, i.e. Drs. J. Rumessen and H. Allescher were excused) took place in Antwerp.

The members of the IAP meet regularly at different congresses, seminars and workshops and at public defences of several theses. The promoters meet formally during the colloquium which is organised every year in Knokke or Bruges during the Belgian week of Gastroenterology.

## 3. Publications

### 3.1. Publication list of each IAP partner (full papers only describing work performed within the framework of this IUPA)

#### Partner 1

Wu M., Van Nassauw L., Kroese A., Adriaensen D., Timmermans J.-P.: Myenteric nitroergic neurons along the rat esophagus: evidence for regional and strain differences in age-related changes. *Histochem. Cell Biol.* 119: 395-403, 2003

De Jonge F., Van Nassauw L., Adriaensen D., Van Meir F., Miller H.R.P., Van Marck E., Timmermans J.-P.: Effect of intestinal inflammation on capsaicin-sensitive afferents in the ileum of *Schistosoma mansoni* infected mice. *Histochem. Cell Biol.* 119: 477-484, 2003

Masliukov P.M., Shilkin V.V., Nozdrachev A.D., Timmermans J.-P.: Histochemical features of neurons in the cat stellate ganglion during postnatal ontogenesis. *Auton. Neurosci.* 106: 84-90, 2003

De Man J.G., Seerden T.C., De Winter B.Y., Van Marck E.A., Herman A.G., Pelckmans P.A.: Alteration of the purinergic modulation of enteric neurotransmission in the mouse ileum during chronic intestinal inflammation. *Brit. J. Pharmacol.* 139: 172-184, 2003

De Man J.G., De Winter B.Y., Seerden T.C., De Schepper H.U., Herman A.G., Pelckmans P.A.: Functional evidence that ATP or a related purine is an inhibitory NANC neurotransmitter in the mouse jejunum: study on the identity of P2X and P2Y purinoceptors involved. *Brit. J. Pharmacol.* 140:1108-1116, 2003

De Winter B.Y.: Study of the pathogenesis of paralytic ileus in animal models of experimentally induced postoperative and septic ileus. *Verh. Koninkl. Acad. Geneesk. Belg.* 65: 293-324, 2003

Chatterjee S., Mbaye A., Van Marck E.: Lower levels of the circulating neuropeptide somatostatin in *Schistosoma mansoni* infected patients may have pathological significance. *Trop. Med. Int. Health* 8: 33-36, 2003

Wu M.: Morphological study on neuronal and non-neuronal elements in relation to motility in the porcine and rat esophagus. PhD-thesis, University of Antwerp, 2003

De Jonge F., De Laet A., Van Nassauw L., Brown J.K., Miller H.R.P., van Bogaert P.-P., Timmermans J.-P., Kroese A.B.A.: In vitro activation of murine DRG-neurons by CGRP mediated mucosal mast cell degranulation. *Amer. J. Physiol. Gastrointest. Liver Physiol.* (in press)

Brown D.R., Timmermans J.-P.: Lessons from the porcine enteric nervous system. *Neurogastroenterol. Motil.* (in press)

Storr M., Sibaev A., Marsicano G., Lutz B., Schusdziarra V., Timmermans J.-P., Allescher H.D.: Cannabinoid receptor type1 modulates excitatory and inhibitory neurotransmission in mouse colon. *Amer. J. Physiol. - Gastrointest. Liver Physiol.* (in press)

De Jonge F., Van Nassauw L., Van Meir F., Miller H.R.P., Van Marck E., Timmermans J.-P.: Extrinsic sensory nerve fibres interact with mucosal mast cells in the ileal wall of *S. mansoni*-infected mice. Proc. Scientific Meeting Belgian Society for Parasitology (in press)

Chatterjee S., Mbaye A., Alfidja A.T., Weyler J., Scott J.T., Van Damme P., Van De Vijver K., Deelder A., Van Marck E.A.E.: Circulating levels of the neuropeptide hormone somatostatin may determine hepatic fibrosis in *Schistosoma mansoni* infections. Acta Tropica (in press).

## Partner 2

De Backer O., Leclere P.G., Lefebvre R.A.: Pharmacological characterization of pre- and postsynaptic prostanoid receptors in pig gastric fundus. Neuropharmacol. 45: 684-690, 2003

Janssen P., Prins N.H., Moreaux B., Meulemans A.L., Lefebvre R.A.: In vivo characterization of 5-HT<sub>1A</sub> receptor-mediated gastric relaxation in conscious dogs. Brit. J. Pharmacol. 140: 913-920, 2003

De Backer O., Colpaert E.E., Lefebvre R.A.: Influence of polyethylene-glycol-superoxide dismutase and combined depletion and repletion of antioxidants on nitrenergic relaxation in the pig gastric fundus. Eur. J. Pharmacol. (in press)

Van Crombruggen K., Lefebvre R.A.: Nitrenergic-purinergic interactions in rat distal colon. Neurogastroenterol. Motil. (in press)

Van Geldre L.A., Lefebvre R.A.: Nitrenergic relaxation in rat gastric fundus : influence of mechanism of induced tone and possible role of sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup> ATPase. Life Sci. (in press)

## Partner 3

Van Craenenbroeck M., Gregoire F., De Neef P., Robberecht P., Perret J.: Ala-scan of Ghrelin (1-14): Interaction with the recombinant human Ghrelin receptor. Peptides (in press)

Langer I., Grégoire F., Nachtergaele I., De Neef P., Vertongen P., Robberecht P.: Hexanoylation of a VPAC<sub>2</sub> receptor-preferring ligand markedly increased its selectivity and potency. Peptides (in press)

## Partner 4

Bisschops R., Vanden Berghe P., Bellon E., Janssens J., Tack J.: Electrical stimulation reveals complex neuronal input and activation patterns in single myenteric guinea pig ganglia. Amer. J. Physiol. Gastrointest. Liver Physiol. 284: G1084-1092, 2003

Depoortere I., Thijs T., Thielemans L., Peeters T.L.: Mechanisms involved in the loss of excitatory post-stimulus responses by inflammation. Naunyn Schmiedebergs Arch. Pharmacol. 367: 245-252, 2003

Fischler B., Tack J., De Gucht V., Shkedy Z.I., Persoons P., Broekaert D., Molenberghs G., Janssens J.: Heterogeneity of symptom pattern, psychosocial factors, and pathophysiological mechanisms in severe functional dyspepsia. Gastroenterology 124: 903-910, 2003

Guan Y., Tang M., Jiang Z., Peeters T.L.: Excitatory effects of motilin in the hippocampus on gastric motility in rats. *Brain Res.* 984: 33-41, 2003

Koek G.H., Sifrim D., Lerut T., Janssens J., Tack J.: Effect of the GABA(B) agonist baclofen in patients with symptoms and duodeno-gastro-oesophageal reflux refractory to proton pump inhibitors. *Gut* 52: 1397-1402, 2003

Lee K.J., Vos R., Janssens J., Tack J.: Differential effects of baclofen on lower oesophageal sphincter pressure and proximal gastric motility in humans. *Aliment. Pharmacol. Ther.* 18: 199-207, 2003

Luiking Y.C., Akkermans L.M., van der Reijden A.C., Peeters T.L., van Berge-Henegouwen G.P.: Differential effects of motilin on interdigestive motility of the human gastric antrum, pylorus, small intestine and gallbladder. *Neurogastroenterol. Motil.* 15:103-111, 2003

Piessevaux H., Tack J., Walrand S., Pauwels S., Geubel A.: Intra-gastric distribution of a standardized meal in health and functional dyspepsia: correlation with specific symptoms. *Neurogastroenterol. Motil.* 15: 447-455, 2003

Revicki D.A., Rentz A.M., Dubois D., Kahrilas P., Stanghellini V., Talley N.J., Tack J.: Development and validation of a patient-assessed gastroparesis symptom severity measure: the Gastroparesis Cardinal Symptom Index. *Aliment. Pharmacol. Ther.* 18: 141-150, 2003

Sarnelli G., Caenepeel P., Geypens B., Janssens J., Tack J.: Symptoms associated with impaired gastric emptying of solids and liquids in functional dyspepsia. *Amer. J. Gastroenterol.* 98: 783-788, 2003

Sarnelli G., Cuomo R., Janssens J., Tack J.: Symptom patterns and pathophysiological mechanisms in dyspeptic patients with and without *Helicobacter pylori*. *Dig. Dis. Sci.* 48: 2229-2236, 2003

Simren M., Silny J., Holloway R., Tack J., Janssens J., Sifrim D.: Relevance of ineffective oesophageal motility during oesophageal acid clearance. *Gut* 52: 784-790, 2003

Simren M., Tack J.: Functional dyspepsia: evaluation and treatment. *Gastroenterol. Clin. North Amer.* 32: 577-599, 2003

Simren M., Vos R., Janssens J., Tack J.: Acid infusion enhances duodenal mechanosensitivity in healthy subjects. *Amer. J. Physiol. Gastrointest. Liver Physiol.* 285: G309-G315, 2003

Simren M., Vos R., Janssens J., Tack J.: Unsuppressed postprandial phasic contractility in the proximal stomach in functional dyspepsia: relevance to symptoms. *Amer. J. Gastroenterol.* 98: 2169-2175, 2003

Tack J., Bisschops R., Koek G., Sifrim D., Lerut T., Janssens J.: Dietary restrictions during ambulatory monitoring of duodenogastroesophageal reflux. *Dig. Dis. Sci.* 48: 1213-1220, 2003

Tack J., Broekaert D., Coulie B., Fischler B., Janssens J.: Influence of the selective serotonin re-uptake inhibitor, paroxetine, on gastric sensorimotor function in humans. *Aliment. Pharmacol. Ther.* 17: 603-608, 2003

Tack J., Caenepeel P., Piessevaux H., Cuomo R., Janssens J.: Assessment of meal induced gastric accommodation by a satiety drinking test in health and in severe functional dyspepsia. *Gut* 52: 1271-1277, 2003

Tack J., Vantrappen G., Huyberechts G., Sifrim D., Janssens J., Van Overstraeten R.: Validation of a new method of measuring esophageal acid exposure: comparison with 24-hour pH monitoring. *Dig. Dis. Sci.* 48: 16-21, 2003

Tack J., Vos R., Janssens J., Salter J., Jauffret S., Vandeplassche G.: Influence of tegaserod on proximal gastric tone and on the perception of gastric distension. *Aliment. Pharmacol. Ther.* 18: 1031-1037, 2003

Xu L., Depoortere I., Thielemans L., Huang Z., Tang M., Peeters T.L.: Sequence, distribution and quantification of the motilin precursor in the cat. *Peptides* 24: 1387-1395, 2003

Xu L.: Studies on the structure and the expression of motilin and mutagenesis of the motilin receptor. PhD-thesis, University of Leuven, 2003

Lee K.J., Vos R., Janssens J., Tack J.: Influence of duodenal acidification on the sensorimotor function of the proximal stomach in humans. *Amer. J. Physiol. Gastrointest. Liver Physiol.* 286: G278-G284 (in press)

## **Partner 5**

Khelif K., De Laet M.H., Chaouachi B., Segers V., Vanderwinden J.M.: Achalasia of the Cardia in Allgrove's (triple-A) Syndrome: histopathological study of 10 cases. *Amer. J. Surg. Pathol.* 27: 667-672, 2003

Rumessen J.J., Vanderwinden J.M.: Interstitial cells in the musculature of the gastrointestinal tract: Cajal and beyond. *Int. Rev. Cytol.* 229: 115-208, 2003

Sibaev A., Franck H., Vanderwinden J.M., Allescher H.D., Storr M.: Structural differences in the enteric neural network in murine colon: impact on electrophysiology. *Amer. J. Physiol. Gastrointest. Liver Physiol.* 285: G1325-G1334, 2003

Sokolow S., Manto M., Gailly P., Molgo J., Vandebrouck C., Vanderwinden J.M., Herchuelz A., Schurmans, S.: Impaired neuromuscular transmission and skeletal muscle fiber necrosis in mice lacking Na/Ca exchanger 3. *J. Clin. Invest.* (in press)

Saur D., Vanderwinden J.M., Seidler B., Schmid R.M., De Laet M.H., Allescher H.D.: Single-nucleotide promoter polymorphism alters transcription of neuronal nitric oxide synthase exon 1c in infantile hypertrophic pyloric stenosis. *Proc. Natl. Acad. Sci. U.S.A.* (in press)

## **3.2. Joint publications of the IAP-network**

### **Partner 1, partner 2 and partner 4**

Van Geldre L.A., Fraeyman N.H., Peeters T.L., Timmermans J.-P., Lefebvre R.A.: Further characterisation of particulate neuronal nitric oxide synthase in rat small intestine. *Aut. Neurosci.* (in press)

### **Partner 1 and partner 4**

De Jonge F., Van Nassauw L., De Man J.G., De Winter B.Y., Van Meir F., Depoortere I., Peeters T.L., Pelckmans P.A., Van Marck E., Timmermans J.-P.: Effects of *Schistosoma*

mansoni infection on somatostatin and somatostatin receptor 2A expression in mouse ileum. *Neurogastroenterol. Motil.* 15: 149-159, 2003

De Winter B.Y., De Man J.G., Seerden T.C., Depoortere I., Herman A.G., Peeters T.L., Pelckmans P.A.: Effect of ghrelin and growth hormone releasing peptide-6 on septic ileus in mice. *Neurogastroenterol. Motil.* (in press)

### **Partner 1 and partner 5**

Wu M., Majewski M., Wojtkiewicz J., Vanderwinden J.-M., Adriaensen D., Timmermans J.-P.: Anatomical and neurochemical features of the extrinsic and intrinsic innervation of the striated muscle in the porcine esophagus: evidence for regional and species differences. *Cell Tissue Res.* 311: 289-297, 2003

Vanderwinden J.-M., Timmermans J.-P., Schiffmann S.N.: Glial cells, but not interstitial cells, express P2X7, an ionotropic purinergic receptor, in rat gastrointestinal musculature. *Cell Tissue Res.* 312: 149-154, 2003

### **Partner 3 and partner 4**

Depoortere I., Thijs T., Thielemans L., Robberecht P., Peeters T.L.: Interaction of the growth hormone-releasing peptides ghrelin and growth hormone-releasing peptide-6 with the motilin receptor in the rabbit gastric antrum. *J. Pharmacol. Exp. Ther.* 305: 660-667, 2003

Thielemans L., Depoortere I., Perret J., Robberecht P., Detheux M., Peeters T.L.: Desensitization of the human motilin receptor by motilides. (in press)

# ADDENDUM

THURSDAY, FEBRUARY 20<sup>th</sup> 2003

---

**RESEARCH GROUP (OG-FWO VLAANDEREN)  
"GASTROINTESTINAL REGULATORY MECHANISMS"**

## FIRST SESSION

MODERATORS: **T.L. PEETERS**

**J.-M. VAN DER WINDEN**

- 9.00 FOETAL LIVER SYNGENIC TRANSPLANTATION (FIRST REPORT).  
V. Coulic, P. Delrée, C. De Prez, S. Bakari, L. Lasser, E. Dekoster (Brussels, ULB & Loverval, GPI)
- 9.15 SPECIFIC RADIOIMMUNOASSAY FOR THE DETECTION OF THE BIOLOGICAL ACTIVE FORM OF GHRELIN.  
C. De Vriese, M.D. Martin-Martinez, F. Grégoire, P. De Neef, P. Robberecht, C. Delporte (Brussels, ULB)
- 9.30 THE GHRELIN RECEPTOR : STRUCTURE FUNCTION RELATIONSHIP OF SHORTENED PEPTIDES AND ANALOGUES.  
M. Van Craenenbroeck, F. Grégoire, P. De Neef, J. Perret, P. Robberecht (Brussels, ULB)
- 9.45 ACTIVATION OF MYENTERIC NEURONS BY GHRELIN AND GHRP-6 IN THE GUINEA-PIG SMALL INTESTINE.  
R. Bisschops, P. Vanden Berghe, I. Depoortere, T. Peeters, J. Janssens, J. Tack (Leuven, KUL)
- 10.00 EFFECT OF GHRELIN ON GASTRIC EMPTYING AND INTESTINAL TRANSIT IN CONTROL AND SEPTIC MICE.  
B.Y. De Winter, J.G. De Man, T.C. Seerden, A.G. Herman, P.A. Pelckmans (Antwerp, UA)
- 10.15 GENE EXPRESSION PROFILING OF INTERSTITIAL CELLS OF CAJAL BY SUPPRESSION SUBTRACTIVE HYBRIDISATION.  
M. Wouters, K. Smans, A. de Kerchove d'Exaerde, J.-M. Vanderwinden (Beerse, Johnson & Johnson Pharmaceutical Research and Development & Brussels, ULB)

## 10.30 COFFEE BREAK

## SECOND SESSION

MODERATORS: **J.-P. TIMMERMANS**

**P. ROBBERECHT**

- 11.00 **INVITED LECTURE : ROLE OF PAR-2 IN VISCERAL HYPERSENSITIVITY AND INFLAMMATION**  
**L. BUENO (TOULOUSE, FRANCE)**
- 11.30 DIVERGENT ROLE OF THE PERIPHERAL CRF<sub>1</sub> AND CRF<sub>2</sub> RECEPTOR IN THE MODULATION OF VISCERAL PAIN IN THE FREELY MOVING RAT.  
N. Ongenae, A. Meulemans, B. Coulie, M. Nijssen (Beerse, Johnson & Johnson Pharmaceutical Research and Development)
- 11.45 NITRERGIC-PURINERGIC INTERACTIONS IN RAT DISTAL COLON MOTILITY.  
K. Van Crombruggen, R.A. Lefebvre (Gent, RUG)
- 12.00 POSSIBLE ROLE OF SERCA IN THE NITRERGIC RELAXATION OF RAT GASTRIC FUNDUS.  
L.A. Van Geldre, R.A. Lefebvre (Gent, RUG)
- 12.15 EFFECT OF SILDENAFIL ON ESOPHAGEAL FUNCTION IN MAN.

## 12.30 LUNCH

### THIRD SESSION

MODERATORS: **R. LEFEBVRE**

**E. VAN MARCK**

- 14.00 **INVITED LECTURE: MODIFICATION OF GUINEA-PIG INTESTINAL PERISTALSIS IN VITRO BY OPIATE AND PROSTANOID RECEPTOR LIGANDS.**  
**P. HOLZER (GRAZ, AUSTRIA)**
- 14.30 CLONING AND EXPRESSION PROFILING OF CANINE NK<sub>1</sub>, NK<sub>2</sub> AND NK<sub>3</sub> RECEPTORS.  
P. Peeters, B. Moreaux, R. De Hoogt, J. Van Den Berg, P. Verhasselt, A. Meulemans, B. Coulie (Beerse, Johnson and Johnson Pharmaceutical Research and Development)
- 14.45 STUDY ON THE NON-ADRENERGIC NON-CHOLINERGIC EXCITATORY NEUROTRANSMITTER IN MOUSE ILEAL CIRCULAR MUSCLE.  
H.U. De Schepper, J.G. De Man, B.Y. De Winter, T.C. Seerden, A.G. Herman, P.A. Pelckmans (Antwerp, UA)
- 15.00 EFFICACY DISTRIBUTION OF 5-HT<sub>7</sub> RECEPTORS THROUGHOUT THE CANINE GASTRIC CORPUS.  
P. Janssen, N.H. Prins, A. Meulemans, R.A. Lefebvre (Beerse, Johnson & Johnson Pharmaceutical Research and Development & Gent, RUG)
- 15.15 GLIAL CELLS, BUT NOT INTERSTITIAL CELLS, EXPRESS P2X<sub>7</sub>, A IONOTROPIC PURINERGIC RECEPTOR, IN THE RAT GASTROINTESTINAL MUSCULATURE.  
J.-M. Vanderwinden, J.-P. Timmermans, S.N. Schiffmann (Brussels, ULB & Antwerp, UA)

## 15.30 COFFEE BREAK

### FOURTH SESSION

MODERATORS: **I. DEPOORTERE**

**P. PELCKMANS**

- 16.00 EFFECTS OF INTESTINAL SCHISTOSOMIASIS ON SOMATOSTATIN AND SOMATOSTATIN RECEPTOR 2A EXPRESSION IN MURINE ILEUM.  
F. De Jonge, L. Van Nassauw, J.G. De Man, B.Y. De Winter, F. Van Meir, I. Depoortere, T.L. Peeters, P.A. Pelckmans, E. Van Marck, J.-P. Timmermans (Antwerp, UA & Leuven, KUL)
- 16.15 THE NEUROPEPTIDE SOMATOSTATIN MAY DETERMINE FIBROSIS IN SCHISTOSOMIASIS.  
S. Chatterjee, A. Mbaye, J. Weyler, P. Van Damme, A. Deelder, E.A.E. Van Marck (Antwerp, UA; Saint-Louis, Senegal, Medical Region & Leiden, LUMC)
- 16.30 DIFFERENCES IN THE ABILITY OF MOTILIDES TO INDUCE MOTILIN RECEPTOR INTERNALIZATION UNDERLY THEIR DESENSITIZING CAPACITY.  
L. Thielemans, J. Perret, I. Depoortere, P. Robberecht, T.L. Peeters (Leuven, KUL & Brussels, ULB)
- 16.45 AcHis-Dphe2-VIP/GRF – A VPAC1 RECEPTOR ANTAGONIST – IS AN INVERSE AGONIST ON TWO CONSTITUTIVELY ACTIVE TRUNCATED VPAC1 RECEPTORS.  
P. Vertongen, C. Langlet, I. Langer, N. Gaspard, J. Cnudde, P. Robberecht (Brussels, ULB)

**17.00 END OF THE MEETING**