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5-HT Actions in the Human Submucous Plexus

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“A good set of bowels is worth more to a man than any quantity of brains”
(Josh Billings, 1818-1885)

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Abbreviations

5-BOIP	5-benzoxy-indalpine
5-CT	5-carboxamido-tryptamine
5-HT	5-hydroxytryptamine
5-HTP-DP	N-acetyl-5-hydroxytryptophyl-5-hydroxytryptophanamide
5-MeOT	5-methoxytryptamine
5-OHIP	5-hydroxy-indalpine
AC	Adenylyl cyclase
ACh	Acetylcholine
AP	Action potential
ATP	Adenosine triphosphate
CAP	Compound action potential
cAMP	Cyclic adenosine monophosphate
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
EC	Enterochromaffin
EFS	Electrical field stimulation
ENS	Enteric nervous system
f/sEPSP	Fast/slow excitatory postsynaptic potential
GERD	Gastroesophageal reflux disease
GI	Gastrointestinal
GP	Guinea Pig
GPCR	G protein coupled receptor
IBS	Irritable bowel syndrome
IPAN	Intrinsic primary afferent neuron
MP	Myenteric plexus
MSORT	Multi-site optical recording technique
NO	Nitric oxide
PAR	Protease-activated receptor
PLC	Phospholipase C
SMP	Submucosal plexus
SOM	Somatostatin
T/G/C	Tissue/Ganglia/Cells
TK	Tachykinines
VIP	Vasoactive intestinal peptide

1. INTRODUCTION

1.1. Serotonin

The term “serotonin” was first introduced in the year 1948 by Irvine Page together with the chemist Arda Green and the biochemist Maurice Rapport. This name reflects the circumstances of its discovery when these scientists isolated this substance with vasoconstrictor effects during their studies on Hypertension in bovine blood serum (“serum” - “tonus”) (Rapport et al., 1948).
Actually this substance had already been found much earlier. In 1868 Carl Ludwig stated the existence of a substance contracting blood vessels (Ludwig and Schmidt, 1868) and in the early 1930s Vittorio Erspamer isolated a substance from the mucosa of the gastrointestinal (GI) tract which had a contractor effect on the smooth muscles and called it “enteramin”. It was also Erspamer who later showed that his “enteramin” and Page’s “serotonin” are in fact the same substances (Erspamer and Asero, 1952; Erspamer, 1953). It turned out that the two substances are also identical to another substance inducing the aggregation of thrombocytes named “thrombocytin” (Rand and Reid, 1952). Meanwhile Rapport worked on the clarification of the actual chemical structure of the newfound compound and was able to show that it is a monoamin that derives from the amino acid tryptophan, decarboxylised and hydroxilised to 5-Hydroxytryptamin (5-HT) (Rapport, 1949).
It took until the mid-60s to discover the probably most prominent function of 5-HT: Its role as neurotransmitter (Dahlstrom and Fuxe, 1964).

1.1.2 Appearance and distribution

Phylogenetically 5-HT belongs to the oldest neurotransmitters of all. It probably evolved at least 700-800 million years ago in the Precambrian era (Peroutka and Howell, 1994), which is also the age when the first complex metazoans appeared on our planet. Thus it is not surprising that nowadays serotonin can be found throughout the fauna and also the flora (Kang et al., 2008). Even in higher fungi and unicellular organisms the presence of serotonin has been shown (McGowan et al., 1983; Muszyńska et al., 2009).
5-HT belongs not only phylogenetically, but also ontogenetically to the earliest neurotransmitters. It starts to show its influence already during the early stages of the embryonic development, where it regulates processes of differentiation and the neurons of the serotonergic system are among the first populations of neurons to

evolve (Kriegebaum et al., 2010). In humans serotonergic neurons first occur in the third to fourth week of embryonic development (Cordes, 2005; Kriegebaum et al., 2010). The involvement of 5-HT has been shown not only during the neurogenesis of the early brain but also in the adult neurogenesis (Lesch, 2001).

In the adult human, serotonergic neurons can be found in many places in the body. In the Central Nervous System (CNS), most of them are located in the raphe nuclei of the midbrain, from where projections into the whole brain occur. Nevertheless 5-HT is also produced in other regions of the brain such as the tegmentum of the brain stem.

The presence of 5-HT in the CNS leads to its profound influences on central regulatory functions, like the already mentioned blood pressure regulation, but also appetite, circadian rhythmicity, fear, sleep, motoric activity, memory, aggression, mood, stress regulation, pain perception, or sexual behaviour (Gray and Roth, 2007; Ayala, 2009)

Despite all these functions, the major part of the body's serotonin is found outside of the CNS (>99%), most of it being produced in the enterochromaffin (EC) cells of the gastrointestinal (GI) tract, though several neurons of the Enteric Nervous System (ENS) are known to be serotonergic, too (Gershon et al., 1965; Gershon et al., 1977; Kurian et al., 1983).

The EC cells store the 5-HT in their apical or basal secretory granula (Rubin et al., 1971). Some of it is also released into the blood where it is mainly kept within the thrombocytes and plays an important role in the case of vascular injuries by strengthening the platelet aggregation reaction (Swank et al., 1963). Further 5-HT involvement has been shown in the context of immunofunctionality, for example during the T-cell mediated immune response or in context with allergic responses (Geba et al., 1996; Fiorica-Howells et al., 2000; Wilhelm, 2005; Rudd et al., 2005; El-Nour et al., 2007). 5-HT also fulfils diverse functions in the lung, heart, kidney, pancreas, taste buds, the ciliary body of the eye, testicles and mammary glands (Kriegebaum et al., 2010).

The total amount of 5-HT in the human body has been estimated to be around 10 mg (Kim and Camilleri, 2000). Of these, 95% can be found in the intestines (Figure 1) where 5-HT is involved in the regulation of GI motility and secretion. Thus, in the next chapter one of the most important acting stages of serotonin action will be introduced: the Enteric Nervous System.

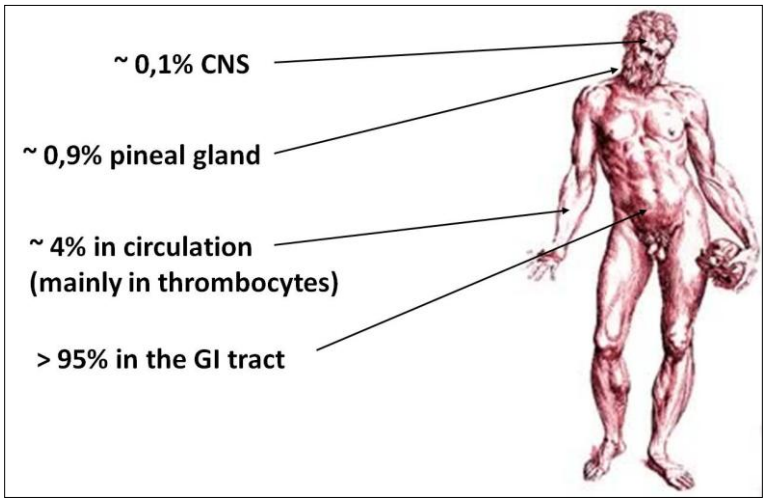

Figure 1. Distribution of serotonin in the human body.
Only around 5% of the approx. 10 mg of serotonin in the human body can be found outside the gastrointestinal tract, being located either in circulation, within the pineal gland or other parts of the Central Nervous System (mainly within the neurons of the raphe nuclei). Inside the intestines, 95% of the serotonin is stored in enterochromaffin cells, the rest acts inside neurons of the enteric nervous system. Image from “De humani corporis fabrica”, Andreas Vesalius (1514-1564).

1.2. The Enteric Nervous System

The gut is supposed to process incoming food and absorb nutrients, as well as to dispose indigestible material. Therefore it is very important for the gut to maintain coordinated motility which guarantees an optimal mixing of the food with digestive enzymes and a continuous proximal to distal transport of the luminal content. These processes are aided by the secretion of liquid into the gut lumen. Both processes, secretion and motility, are controlled by a neuronal system that, though it is connected to and also receives input from the CNS, can function completely independent from it (Trendelenburg, 1917). This is a feature that is unique for an organ within the human body.
The ENS was first named by J.N. Langley, who defined for the first time the idea that the GI tract has a nervous system of its own, calling it the “enteric nervous system” (Langley, 1922). His classification of the nervous systems into sympathetic, parasympathetic and enteric nervous system is still used. Today we know that the ENS contains more than 100 million neuronal cells. This is noticeably more than in the spinal cord and also more than all other peripheral nerve cells together (Standring, 2009), which makes the ENS the largest accumulation of neurons outside the brain. For this and for its ability to function in isolation, the ENS is often referred to as “the second brain” (Gershon, 1998).

The ENS consists of two major layers (see Figure 2): The myenteric plexus is located between the inner circular and the outer longitudinal smooth muscle layers and mainly coordinates motility along the gut wall. The second layer of neurons is called submucous plexus, because it lies directly beneath the mucosal layer, which separates the underlying gut layers from the gut lumen. Its main function is the regulation of mucosal secretion and absorption. It also innervates the lamina propria and the muscularis mucosae.

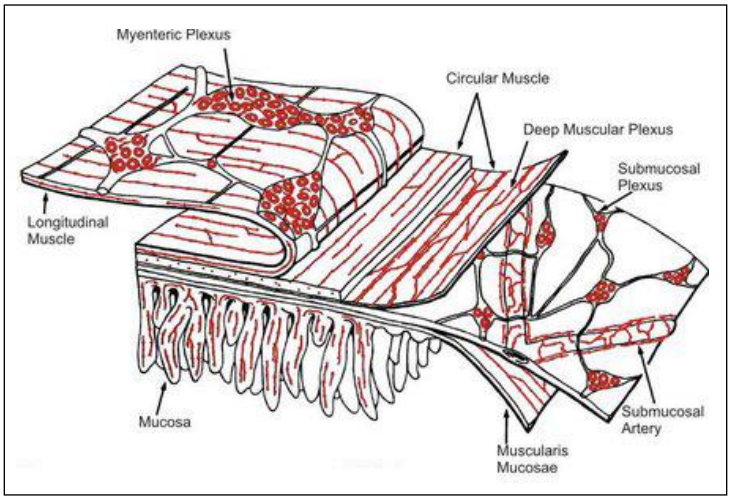

Figure 2: The myenteric and submucous plexus within the gut wall.
The myenteric plexus with its numerous ganglia and connecting fibres is located between the longitudinal and circular layers of muscle. The finer nerve bundles and ganglia of the submucous plexus lie between the circular muscular fibers and the longitudinal muscle fibers of the muscularis mucosae (Furness and Costa, 1980).

The neurons of both plexus form complex networks and fulfil different functions therein. Functionally, the sensory neurons are the first neurons in a reflex pathway which encode information about the nature and intensity of the stimulus. Interneurons connect neurons within neural pathways. Both descending and ascending interneurons have been shown (Costa et al., 1996). Muscle motor neurons are excitatory and inhibitory efferent nerve cells innervating the longitudinal, the circular smooth muscle and the muscularis mucosae throughout the digestive tract. Other motorneurons innervate blood vessles or cells of the mucosa.
One of the most important functions of the ENS is the ability to react efficiently to physiological stimuli, including movement of the villi or distortion of the mucosa, contraction of intestinal muscle and changes in the chemistry of the contents of the gut lumen via enteric reflex pathways. According to one of the most frequently mentioned models, the intrinsic afferent neurons, often also called intrinsic primary afferent neurons (IPANs) are the first neurons in intrinsic reflexes transducing changes of intraluminal conditions (Furness et al., 1998). They influence the patterns

of motility, secretion across the mucosal epithelium and local blood flow. Other studies suggest new classes of mechanosensitive interneurons, while showing that the after-hyperpolarising IPANs are not the only primary afferent neurons. In guinea pig colon for example colonic distention caused activation of tonically firing S-neurons, while there was no response from the “IPANs” (Spencer and Smith, 2004; Smith et al., 2007). One of the most recent concepts introduces mechanosensitive neurons which are multifunctional. These multifunctional enteric neurons can perform sensory as well as intregrative and motorfunctions (Mazzuoli and Schemann, 2009).

1.3. Serotonin signal transduction - The 5-HT receptors

In the mammalian nervous system 5-HT acts as a very important neurotransmitter mediating many functions in the body. This is reflected by the large family of 5-HT receptors coupled to a large number of signalling pathways. The first indications that there must be more than one receptor mediating the physiological effects of 5-HT go back to the late 50s. A classification into M and D receptors was suggested based on the antagonistic effect of morphin (M) or dibenzylin (D) respectively (Gaddum and Picarelli, 1997). This classification, however, did not last long because of the unspecific effects of the ligands. In fact, it turned out that this system did not discriminate between different receptors but between the neuronal effects of 5-HT and the transmitters direct effect on the smooth muscle (Lewis, 1960; Day and Vane, 1963).

The introduction of the radioligand binding method and improved molecularbiological techniques allowed the discovery of many 5-HT receptors during the following decades. Based on data on amino acid sequences and gene structure a new classification of the receptors was possible. The most accepted 5-HT receptor classification to date is the IUPHAR (International Union of Basic and Clinical Pharmacology) nomenclature. It divides the receptors into subtypes based on certain criteria such as ion channel coupling, amino acid sequence, intracellular effector mechanisms or affinity for 5-HT (Hoyer et al., 1994; Hoyer et al., 2002; also see Figure 3).

The multitude of 5-HT mediated effects in the GI tract and elsewhere in the human body is only possible due to the involvement of several 5-HT receptors. The 5-HT receptor family consist of at least 14 different receptor isoforms (not including receptors which have not yet been cloned, such as the 5-HT_{1P} receptor), divided into seven subfamilies from 5-HT_1 to 5-HT_7 which are listed in Table 1. With the exception

of the 5-HT_3 receptor, which is a ligand gated ion channel, all other 5-HT receptors belong to the metabotropic G protein coupled receptor (GPCR) family.

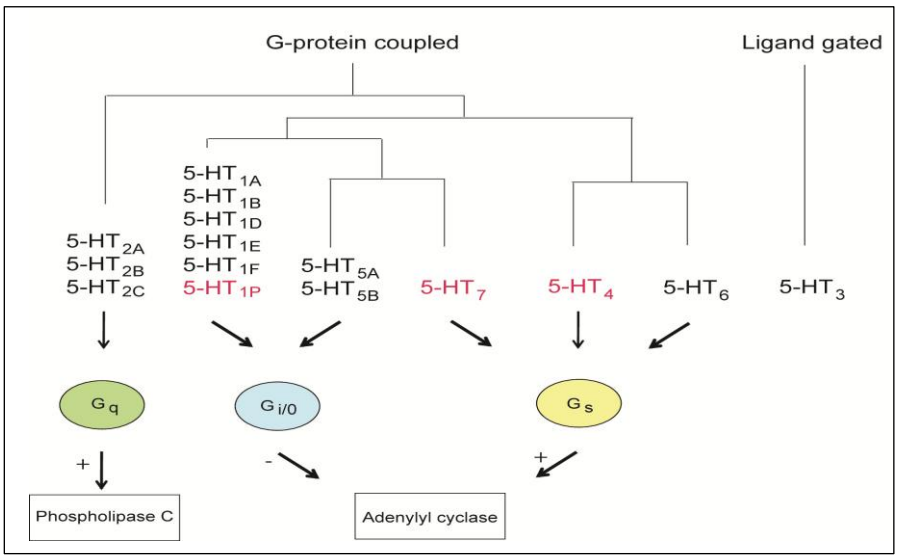

Figure 3. Phylogenetic tree of the serotonin receptor family.
The serotonin receptors can be divided into seven families. All 5-HT receptors except for the 5-HT_3 receptor, which is a ligand-gated Na+/K+-ion-channel, belong to the class of G-protein-coupled receptors. The receptors which were analysed in this thesis are marked with red. Abbreviations: G-protein: guanine nucleotide-binding protein (Gq, Gi/0, or Gs), (+): activation; (-): inhibition (modified after Manzke, 2004).

5-HT has been demonstrated to be crucial for signal transduction and processing within enteric circuits as well as in the initiation of the peristaltic reflex (Bulbring and Crema, 1959; Cooke et al., 1997; Hansen, 2003). There is evidence for the involvement of 5-HT_1, 5-HT_3, 5-HT_4, 5-HT_7 and 5-HT_{1P} receptors influencing the motor functions of the gut (Read and Gwee, 1994; Briejer et al., 1995; Galligan, 1996; Prins et al., 1999). From experiments in guinea pig it is known that especially the neural 5-HT_3, 5-HT_4 and 5-HT_{1P} receptor play a crucial role within the modulation of the peristaltic activity (Foxx-Orenstein et al., 1996; Grider and Piland, 2007). In contrast to the 5-HT_4 and 5-HT_{1P} receptor subtypes the 5-HT_3 receptor does not seem to be involved in the initiation of the peristaltic reflex in human tissue (Foxx-Orenstein et al., 1996; Björnsson et al., 1998; Grider et al., 1998; Nicholas and Spencer, 2010). The 5-HT_4 receptor is also involved in pathways inducing secretion (Kellum et al., 1994; Budhoo et al., 1996). Additionally the 5-HT_7 receptor might be especially important for the peristaltic activity, since experiments in guinea pig showed that it mediates muscle relaxation (Carter et al., 1995; Hoyer et al., 2002). Smooth muscle 5-HT_7 receptors and their relaxing influence have also been demonstrated in human (Janssen et al., 2002). Especially the submucous plexus has been suggested to play an important role in the regulation of the peristaltic reflex as

well as response patterns of lamina propria cells (Foxx-Orenstein et al., 1996; Branchek et al., 1988). Also in context with the mediation of mucosal functions the role of 5-HT in the submucous plexus has not been investigated in detail. Michel et al. (2005) were able to show that submucous neurons display a quick transient 5-HT response to 5-HT stimulation, which is mediated by the 5-HT_3 receptor. This is, up to now, the only electrophysiologically demonstrated 5-HT receptor subtype in the human submucous plexus. 5-HT stimulation, however, does also trigger a slow response, which is not mediated by the 5-HT_3 receptor (Michel et al., 2005). For a better understanding of the enteric reflex pathways it is important to know which other receptors subtypes are involved which is why the possible presence of 5-HT_4, 5-HT_7 and 5-HT_{1P} receptors on the submucous plexus in human was investigated within this project. In the following chapter these receptors will be presented in further detail.

Table 1. Serotonin receptor families and their functions.
Abbreviations: 5-HT: serotonin, IP3: inositol triphosphate, DAG: diacyclglycerol, cAMP: cyclic adenosine monophosphate. Sources: Hannon and Hoyer, 2008; Berger et al., 2009.

Family	Action	Type	Mechanism of action
5-HT_1	Inhibitory	$G_{i/0}$ protein coupled	Decreasing intracellular concentration of cAMP
5-HT_2	Excitatory	G_q protein coupled	Increasing intracellular concentration of IP3 and DAG
5-HT_3	Excitatory	Ligand gated Na^+/K^+ channel	Depolarization of cell membrane
5-HT_4	Excitatory	G_s protein coupled	Increasing intracellular concentration of cAMP
5-HT_5	Inhibitory	$G_{i/0}$ protein coupled	Decreasing intracellular concentration of cAMP
5-HT_6	Excitatory	G_s protein coupled	Increasing intracellular concentration of cAMP
5-HT_7	Excitatory	G_s protein coupled	Increasing intracellular concentration of cAMP

1.3.1. The 5-HT_4 receptor

Overview

In 1988 in neuronal tissue of mice embryos a 5-HT receptor subtype was found which was different to the other known receptors up to then (5-HT_1, 5-HT_2 and 5-HT_3) (Dumuis et al., 1988). Consequently, the newfound receptor was named 5-HT_4. This name is still the same in the current IUPHAR nomenclature. Though later several splice variants were discovered in rodents, it was not until 1997 that the 5-HT_4 receptor was eventually cloned in human (Blondel et al., 1997; Claeysen et al., 1997b; Van den Wyngaert et al., 1997).
On the cellular level the activation of the 5-HT_4 receptor inhibits neuronal potassium currents, resulting in increased neuronal excitability (Fagni et al., 1992). By this

mechanism it has been shown to modulate release of neurotransmitters such as acetylcholine, dopamine, GABA and 5-HT itself, enhancing synaptic transmission. In the CNS this may for example affect the development of memory (Ciranna, 2006). The highest level of central nervous 5-HT_4 receptors can be found in the striatum of the cerebrum, the frontal cortex and within the hippocampus (Bonaventure et al., 2000). This leads to a lot of discussion in the literature about physiological and pathophysiological properties and especially about its involvement in Alzheimer´s disease (e.g. Barnes and Sharp, 1999; Langlois and Fischmeister, 2003).
The 5-HT_4 receptor has also been shown in peripheral tissues like the bladder, heart, blood vessels, adrenal gland and in the GI tract (Hegde et al., 1995). In the latter it is located on EC cells, enterocytes, smooth muscles and presynaptically on enteric neurons (Gershon, 1999; 2005). As heteroreceptors on cholinergic neurons in the ENS (Linnik et al., 1991; Meulemans et al., 1993), it mediates an increased release of acetylcholine which in turn excites peristalsis and leads to motility enhancing effects. This finding stimulated the development of several prokinetic drugs targeting the 5-HT_4 receptor such as Metoclopramid® or very recently Resolor® (Tack et al., 2009). Further it is involved in irritable bowel syndrome (IBS), gastroparesis, dyspepsia and gastroesophageal reflux disease (GERD) (Sanger, 1996; Kahrilas et al., 2000; Quigley, 2000).

5-HT_4 receptor ligands

In general the 5-HT_4 agonists include a 4-amino-5-chloro-2-methoxybenzoic acid group as a common structural component. One of these agonists is cisapride. Despite its co-activity for 5-HT_3 receptors it was often used in animal model studies and served as a gastroprokinetic agent in clinical practice. Another partial 5-HT_4 receptor agonist, tegaserod, found its application in the treatment of symptoms of IBS and constipation (Müller-Lissner et al., 2005; Tack et al., 2005). Meanwhile, however, both substances have been withdrawn from the market due to cardiac side effects or adverse cardiovascular events.
There are several other partial 5-HT_4 agonist commonly used in both *in vivo* and *in vitro* studies such as 5-methoxytryptamine (5-MeOT). Unlike these, newer substances such as SC-53116 and even more so prucalopride are highly selective for the 5-HT_4 receptor only (Flynn et al., 1992; Briejer et al., 2001; Bureau et al., 2010). In clinical studies prucalopride (for the chemical structure see Appendix III) has been shown to significantly and consistently improve the symptoms and gut functions of patients with chronic constipation. In this context, it has recently been approved for marketing, brand name being “Resolor®” (Tack et al., 2009; Quigley et

al., 2009; Camilleri and Bharucha, 2010). A number of 5-HT_4 receptor agonists are being developed for their prokinetic action (Camilleri and Bharucha, 2010; Bowersox et al., 2011).

Relevant substances that have been used as antagonists for 5-HT_4 receptors are tropisetron, GR113808, piboserod and SB-204070. Tropisetron, however, has a higher affinity for the 5-HT_3 receptor with a pK_i of 9-10 compared to its pK_i of 6-6.5 for the 5-HT_4 receptor. GR113808 was one of the first high affine 5-HT_4 receptors (pK_i 9-9.5), though it still shows some affinity for 5-HT_3 (pK_i > 6) (Bureau et al., 2010). SB-204070 is one of the most studied 5-HT_4 antagonists. It shows low affinity for other 5-HT receptors as well as dopamine or adrenergic receptors (pK_i < 5-7) and is described as a potent and selective 5-HT_4 antagonist (Wardle et al., 1994). However, because of its ester function it is characterized by a limited interval of action as well as poor bioavailability (Bureau et al., 2010).

Piboserod (for the chemical structure see Appendix III) is one of the most active antagonists of 5-HT_4. As the lead substance in this context, it is undergoing evaluation for possible treatment of atrial fibrillation, symptomatic heart failure and IBS (e.g. De Ponti and Tonini, 2001; Kjekshus et al., 2009). It has been demonstrated to be highly selective for the 5-HT_4 receptor (Gaster et al., 1995; Bureau et al., 2010).

1.3.2. The 5-HT_7 receptor

Overview

The 5-HT_7 receptor belongs to the most recently identified 5-HT receptors. It was first cloned in 1993 from rat and later from human cDNA (Shen et al., 1993; Ruat et al., 1993; Bard et al., 1993). It stimulates adenylate cyclase activity though coupling to the Gs protein, which leads to an increase in the production of the second messenger cAMP (Bard et al., 1993; Adham et al., 1998; Graveleau et al., 2000)

There are at least four splice variants present for the 5-HT_7 receptor (5-$HT_{7(a)}$, 5-$HT_{7(b)}$, 5-$HT_{7(c)}$, 5-$HT_{7(d)}$), though the 5-$HT_{7(c)}$ receptor isoform has yet to be verified in human native tissue (Heidmann et al., 1997; Thomas and Hagan, 2004). Between the three expressed isoforms no major pharmacological differences have been identified to date.

In the CNS the 5-HT_7 receptor has been detected in many regions of the brain such as the cortex, septum, cerebellum, striatum, thalamus, hippocampus, olfactory complex, mesencephalon and in the amygdala (To et al., 1995; Bhalla et al., 2002; Thomas and Hagan, 2004). For the peripheral regions it has been described in lung, heart, blood vessels, extravascular smooth muscles, kidney, liver, spleen, pancreas,

ovaries, placenta, testis and retina (Pootanakit and Brunken, 2000; Krobert et al., 2001; Terrón and Martínez-García, 2007).
Despite this high abundance of the 5-HT_7 receptor in the mammalian body, the physiological role of the 5-HT_7 receptor is still poorly understood. Due to the abundance in certain regions of the brain, it is often associated with thermoregulation, sleep, learning and memory. It has also been suggested to be involved in the control and regulation of the circadian rhythm (Moyer and Kennaway, 1999; Ehlen et al., 2001). In the GI tract, as in the blood vessels, the 5-HT_7 receptor is involved in smooth muscle relaxation (Carter et al., 1995; Prins et al., 1999; Vanhoenacker et al., 2000).

5-HT_7 receptor ligands

Unfortunately no highly selective agonists for the 5-HT_7 receptor have been described until today. However, the receptor exhibits a high affinity for 5-carboxy-tryptamine (5-CT, pK_i 9.5, for the chemical structure see Appendix III) and for the 5-HT_4 receptor agonist 5-methoxytryptamine (5-MeOT, pK_i 8.3) as well as a considerable affinity for the 5-HT_{1A} receptor agonist 8-OH-DPAT (pK_i 7.4) (Wesolowska, 2002).
In the case of the 5-HT_7 receptor antagonists a range of clinically utilised agents exists. However, insufficient selectivity and varying behaviour as antagonists or inverse agonists on the different receptor splice variants complicate the search for a suitable tool. As an example, mesulergine and metergoline have been described to be antagonists of the 5-$HT_{7(a)}$ and 5-$HT_{7(d)}$ receptor isoforms, but these drugs display marked inverse agonist effects on the 5-$HT_{7(b)}$ splice variant (Krobert and Levy, 2002).
One of the few antagonists showing sufficient selectivity is SB-269970. It displays a 100-fold higher selectivity versus the 5-HT_7 receptor compared to all other 5-HT receptors except the 5-HT_{5A} receptor (50-fold). This is why SB-269970 is widely utilised for *in vitro* as well as *in vivo* studies (Thomas and Hagan, 2004), despite recent findings showing that it might also block the α2-adrenergic receptor (Foong and Bornstein, 2009).

1.3.3. The 5-HT_{1P} receptor

Overview

Like the other members of the 5-HT_1 receptor class, the 5-HT_{1P} receptor is Gi/0-protein linked. Its activation leads to a slow depolarisation of neurons (Mawe et al., 1986; Wang et al., 1996). Since it has still not yet been cloned, it is not listed in the IUPHAR nomenclature. So far it has been exclusively detected in the periphery (thus 1P), where it mediates peristaltic and secretory reflexes in the submucosa (Branchek et al., 1988; Gershon, 2000; Tack et al., 2007). Stimulated by 5-HT released from the EC cells 5-HT_{1P} receptors activate the intrinsic afferent neurons which in turn trigger gut contraction or relaxation (Kirchgessner et al., 1992). The 5-HT_{1P} receptor has also been found on enteric motor neurons in the myenteric plexus of guinea pigs (Tack et al., 1992; Michel et al., 1997). Further selective binding sites have been demonstrated in heart, pancreas and skin of rodents (Branchek et al., 1988; Kirchgessner et al., 1992)

Since the cDNA sequence of the 5-HT_{1P} receptor is still unknown, evidence of its existence in human must come from electrophysiological studies. In the past this has proven to be problematic because of the limited availability of intact human gut tissue containing the enteric nervous system as well as difficult accessibility of the vital neuronal plexus from the intact gut wall.

5-HT_{1P} receptor ligands

The only known selective high affinity agonist for the 5-HT_{1P} receptor is 5-hydroxy-indalpine (5-OHIP, chemical structure displayed in Appendix III) with a pK_i of 8.7, which equals the affinity of 5-HT itself. Other less potent or less selective agonists like 6-OHIP, sumatriptan, or bufotenine have been used in animal models to demonstrate 5-HT_{1P} receptor activity (Branchek et al., 1988; Hoyer et al., 1994; Tack et al., 2007)

Binding of 5-HT_{1P} agonists is displaced by a dipeptide of 5-hydroxytryptophan named N-acetyl-5-hydroxytryptophyl-5-hydroxytryptophanamide (5-HTP-DP), the commonly used antagonist for the 5-HT_{1P} receptor (Takaki et al., 1985; Mawe et al., 1986). The only other 5-HT_{1P} antagonist commonly described in literature is the prokinetic drug renzapride (Tack et al., 2007), which also has 5-HT_4 agonistic as well as 5-HT_3 antagonistic properties.

1.4. Serotonin in the gut - The functional relevance of 5-HT receptors in the ENS

The ENS is equipped with chemo- and mechanosensitive neurons, which can transfer information from sensors like the EC cells to both myenteric and submucous plexus. The local stimuli that induce the 5-HT release can, for example, be an increased intraluminal pressure which may occur during distension of the gut (Bulbring and Crema, 1959; Ferrara et al., 1987) or a pH-decrease in the lumen (Resnick and Gray, 1962; Kellum et al., 1983). 5-HT is believed to activate 5-HT_{1P} and presynaptic 5-HT_4 receptors on the sensory ending of afferent neurons. This, via inter- and motor neurons, can induce muscle contraction in ascending and muscle relaxation in descending direction, which leads to propulsion of the intraluminal content. In this context also neuronal 5-HT is involved. Additionly, paracrine 5-HT activates serotonergic receptors on secretomotor neurons, enterocytes and smooth muscle cells (Kirchgessner et al., 1992; Pan and Gershon, 2000; De Ponti, 2004, Tonini and Pace, 2006).

Besides the crucial role of the 5-HT_{1P} receptor in the regulation of the peristaltic reflex, other 5-HT receptors are involved too. 5-HT_3 and 5-HT_4 receptors for example have an excitatory effect on the involved enteric neurons that are targets of paracrine and neuronal 5-HT (De Ponti, 2004; Tonini and Pace, 2006). A recent study on tryptophan-hydroxylase-2 knock-out mice suggests that constitutive gastrointestinal motility depends rather on neuronal than on EC cell released 5-HT (Li et al., 2011), a concept that gains more and more attention lately and might lead to a change in the understanding of the peristaltic reflex in future. Studies on rodents even demonstrated, that after complete removal of the mucosa the initiation of peristalsis and the propagation of the intraluminal content was still possible (Spencer et al., 2011). This would mean that the ENS and especially the myenteric plexus possess the ability to process local stimuli independently from the input coming from the EC cells or the mucosa. There seems to exist an intrinsic neural circuitry that generates a pacemaker mechanism responsible for the cyclic colonic motor activity driving the propulsion of the intraluminal content (Keating and Spencer, 2010). The role of the paracrine 5-HT as well as the role of the submucous plexus within this concept remains an open question at the moment.

Some epithelial cells in the mucosa can also be directly activated by 5-HT, since they possess 5-HT receptors (5-HT_3 and 5-HT_4) themselves. This causes ion secretion by increasing the cAMP level inside the cells (Albuquerque et al., 1998; Ning et al., 2004). In guinea pig ileum a muscle relaxation can be triggered by 5-HT_7 receptors (Carter et al., 1995; Hoyer et al., 2002).

5-HT further stimulates the ENS indirectly as 5-HT_3 receptors are present on sensory vagal afferents involved in the modulation of the visceral sensibility. In rodents, the 5-HT_3 receptor is also involved in water and electrolyte secretion resulting in a reduction of the GI transit time (Goldberg et al., 1996; Gershon and Tack, 2007). For the human ENS, *in vitro* findings underline the key role of 5-HT_4 and 5-HT_{1P} receptors for the peristaltic reflex, whereas the 5-HT_3 receptor does not seem to be essentially involved (Foxx-Orenstein et al., 1996). This is in agreement with the data showing that distension induced peristaltic activity in healthy volunteers does not depend on 5-HT_3 pathways (Björnsson et al., 1998). All in all it seems to be evident that 5-HT plays a crucial role within the signal transduction through the mucosa as well as within signal processing within the myenteric and submucous plexus and thus in the gut's ability to react to interluminal changes. It is essentially involved in peristalsis as well as secretion.

1.5. 5-HT receptors in the human submucous plexus – Aims of the study

The existence of 5-HT_3, 5-HT_4 and to a lesser extent also 5-HT_{1P} and 5-HT_2-receptors on enteric neurons has been demonstrated (Briejer and Schuurkes, 1996; Gershon, 1999; Prins, 2001). However, our knowledge on signalling cascades and functional relevance of 5-HT receptors are mainly based on studies in enteric neurons of rodents. A few functional studies revealed that these receptors may have different roles in the human intestine. In the human submucous plexus the action of the 5-HT_3-receptor has been studied by Michel et al. (2005) using a fast imaging technique in combination with a voltage sensitive dye to monitor directly the membrane potential changes in neurons of human submucous plexus from surgical specimens of 21 patients. Local microejection of 5-HT directly onto ganglion cells resulted in a transient excitation of enteric neurons characterized by increased spike discharge, which could be mimicked by the 5-HT_3 receptor agonist 2-methyl-5-HT. Using the specific 5-HT_3 receptor antagonist tropisetron however, the response to 5-HT partly persisted in some neurons, suggesting the involvement of 5-HT_4, 5-HT_7 or 5-HT_{1P} receptors in the ENS 5-HT signalling (Michel et al., 2005).
In general however, the role of 5-HT_4, 5-HT_7 and 5-HT_{1P} receptors in the modulation of enteric neuronal activity in the human gut remains largely unknown. For our understanding of 5-HT evoked signalling in the human ENS and its pathophysiological role in functional GI disorders like IBS, it is important to fill this

knowledge-gap. Hence this study aims to investigate the presence and behaviour of the 5-HT_4, 5-HT_7 and 5-HT_{1P} receptors in the human submucous plexus.

In this respect also more information on possible differences in sensitivity for 5-HT (and other mediators) between myenteric and submucous plexus would be relevant. Based on the finding of Buhner at al. that mediators like 5-HT released from mucosal biopsy samples of IBS patients activate human enteric neurons, it can be inferred that 5-HT plays a role in this pathophysiology (Buhner et al., 2009). In context with the concept that an altered mucosa-nerve signalling may be an underlying mechanism of IBS, another study by Buhner et al. showed that the nerve sensitising effect of supernatant from mucosal biopsies of IBS patients was more prominent in the submucous than in the myenteric plexus, suggesting differential involvement of 5-HT, histamine and PAR-2 receptors (Buhner et al., 2010). Thus this thesis also aims to inverstigate into differences in sensitivity between the myenteric and the submucous plexus in guinea pig when exposed to these individual components of the IBS supernatant, which might explain these plexus related efficacies.

2. MATERIAL AND METHODS

2.1. Tissue treatment and preparation

Guinea Pig samples

For all animal experiments male “Dunkin Hartley” guinea pigs (Charles River laboratories, Kisslegg, Germany; Harlan GmbH, Borchen, Germany) were used. The experimental animals were kept under standardized conditions in species-appropriate airflow cabinets (Ehret Uniprotect, Emmendingen, Germany). The guinea pigs were fed with a standard diet (Rohfaserpellets, Altromin Spezialfutter GmbH & Co. KG, Germany) and had water available ad libitum. The animals were kept at 20-24°C and 60% humidity. The daily rhythm was set by a timer to 14 hours of light (from 7 am to 9 pm) and 10 hours of dark (SOPs 163-165 Animal Care). After spending one to two weeks in the cabinets for acclimatization, the animals were killed by cervical dislocation followed by exsanguinations (SOP162). This method was approved by the local Animal Ethical Committee and is according to the German law for animal protection and animal welfare guidelines. At slaughter the animals had an average weight of 355 g ± 6 g.

Immediately after killing, the abdomen was opened with barb forceps (FST # 11023-10, Fine Science Tools, Heidelberg, Germany) and rough scissors (FST # 14001-13, Fine Science Tools). Forceps with flat corrugated tips (FST # 11000-14, Fine Science Tools) were used to hold the abdominal wall, followed by quick removal of the ileum. For this purpose a pair of scissors with rounded tips (FST # 14010-15, Fine Science Tools) was used, in order to not damage the surrounding intestinal tissue. The ileum was then placed in a petri dish (Greiner Bio-One, Germany) and fixed to the Sylgard bottom (Sylgard® 184, Down Cornig, Wiesbaden, Germany) with fine insect pins (“Minutiennadeln” Sphinx V2A, Bioform, Nuremberg, Germany). Then the ileum was opened alongside the mesenteric border, washed several times with Carbogen aerated Krebs solution (5% CO_2 and 95% O_2; Westfahlen AG, Münster, Germany; SOP01a) and equilibrated at pH 7.4. The Krebs solution contained 117 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$ 6 H_2O, 1.2 mM NaH2PO4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$ 2 H_2O and 11 mM glucose. While being prepared the tissue was constantly perfused with a circulating pump (Ismatec ISM 827, Zurich, Switzerland) with Krebs solution. For the dissection of the tissue (SOP22), microscissors (FST # 14058-11, Fine

Science Tools) and forceps (DumostarTM 10576 and 10577, Dumont, Switzerland) were used. Myenteric plexus preparations were obtained by gently removing the mucosa, the submucosa and the circular muscle layer. For the submucous plexus preparations the mucosa was removed first. Then the preparation was turned and the submucous plexus was carefully set free from the serosal and muscle tissue layers.
Then the dissected tissue (5 x 10 mm) was pinned onto a sylgard ring (SOP35) with rectangular window (20 x 10 mm) that was placed in a self-made recording chamber with a 42-mm-diameter glass bottom (130-170 µm thickness, Sauer, Reutlingen, Germany) and continuously perfused with 37° C Krebs solution containing: 117 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl2 6 H_2O, 1.2 mM NaH2 PO4, 20 mM $NaHCO_3$, 2.5 mM $CaCl_2$ 2 H_2O and 11mM glucose (SOP01b, all chemicals purchased from Sigma-Aldrich, Schnelldorf, Germany). In some experiments with longitudinal muscle / myenterc plexus preparations, 1 µM nifedipine (SOP36) was added to reduce muscle movements. The perfusing Krebs solution was circulated at a speed of 20-25 ml/min between the reservoir (500 ml) and the recording chamber. The reservoir was kept in a water bath (WiseCircu®; Witeg, Wertheim, Germany) at constant temperature (37°C) and pH. Plastic tubes (Tygon® R3603, Ø 2.79 mm) connected the pump with the chamber.

Tissue from human patients

Human intestinal tissues samples were supplied by the Departments of Surgery at the Medical Clinic Rechts der Isar of the Technische Universität München, the Medical Clinic Großhadern of the Ludwig-Maximilians-Universität München and the Medical Clinic Freising. For this study tissue samples from 163 patients were used, 111 samples from the large and 75 samples from the small intestine. Patients receiving surgery had been previously diagnosed with carcinoma (107), diverticulitis (18), polyps (8), chronic pancreatitis (7), stoma (7), stenosis (5), fistula (3), Crohn's disease (3), perforation (2), endometriosis (2) and ileus (1).
Immediately after pathological survey, segments of macroscopically healthy gut were placed in sterile bottles containing cold oxygenated sterile Krebs solution (117 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$ 6 H2O, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$ 2 H_2O, 11 mM glucose; SOP08). During the transport to the laboratory of the Department of Human Biology the bottles were permanently kept at cold temperatures. This protocol was approved by the Ethical Committee of the Technische Universität München (# project approval 1746/07).

Immediately after arriving at the laboratory the tissue was washed three times with cold oxygenated sterile Krebs solution. After being cut along the mesenteric border the tissue was pinned flat, mucosa down, in a Sylgard lined dissection dish that was continuously perfused with ice-cold sterile Krebs solution aerated with Carbogen (SOP22). The tissue was dissected by carefully removing the mucosa and the muscle layers under a dissection microscope to obtain a preparation of the inner submucous plexus. Afterwards the preparation was cut to a size of about 20 x 10 mm and pinned onto a Sylgard ring. That ring was then placed in the above described recording chamber with continuous perfusion with 37°C Krebs solution gassed with Carbogen equilibrated at pH 7.4.

2.2. Electrophysiology

2.2.1. Optical recording with the Multi-Site Optical Recording Technique

The Multi-Site Optical Recording Technique (MSORT) is a technique that allows detecting nerve activity in the ENS with high spatial and temporal resolutions. It allows recording of actions potentials in all neurons of a given ganglion simultaneously. Details of this technique have been described previously (Neunlist et al., 1999; Schemann et al., 2002; Michel et al., 2005). An illustration of the MSORT technique is given in Figure 4. For the experiments performed in this project, the recording chamber containing the preparation (see above) was mounted onto an IX50 inverted epifluorescence microscope (Olympus, Hamburg, Germany) equipped with Hoffmann modulation optics and either a 150 W xenon arc lamp (Osram, Munich, Germany) or a green LED (PT 39 Green, Luminus Devices Inc., Billerica, USA). Controlled illumination of the preparation for the xenon lamp was achieved by a software operated shutter (Uniblitz D122, Vincent Associates, NY, USA).

The MSORT utilises the fluorescent voltage-sensitive properties of the dye 1-(3-sulfonatopropyl)-4-[ß[2-(di-n-octylamino)-6-naphthyl]vinyl]pyridinium betaine (Di-8-ANEPPS; Invitrogen, Carlsbad, CA, USA) to detect changes in membrane potential (SOP69). Di-8-ANEPPS is applied into the ganglia via local pressure application through a microejection pipette loaded with 20 µM Di-8-ANEPPS and thus incorporated into the cell membrane (SOP66). The incorporated dye will change its fluorescence linearly to the membrane potential of the cell (Fromherz and Lambacher, 1991). Di-8-ANEPPS-stained neurons were visualized with a ×20 or ×40 oil immersion objective (UAPO/340, NA=1.4, Olympus, Hamburg, Germany) using a fluorescence filter cube consisting of a 545 ± 15 nm excitation interference filter, a 565-nm dichroic mirror and a 580-nm barrier filter (AHF Analysentechnik, Tübingen, Germany).

Since any fluorescent dye recording will eventually lead to dye bleaching and phototoxicity, illumination time is a crucial factor. On one hand it has to be kept to a minimum but on the other hand it must be long enough to reveal representative responses of neurons to the applied stimuli. It turned out that recordings with durations of 1.8 s for the pharmacological experiments and 0.6 s for the electrical stimulations yielded reliable and reproducible responses. Nevertheless, in some ganglia it was possible to record for up to 5.0 s.

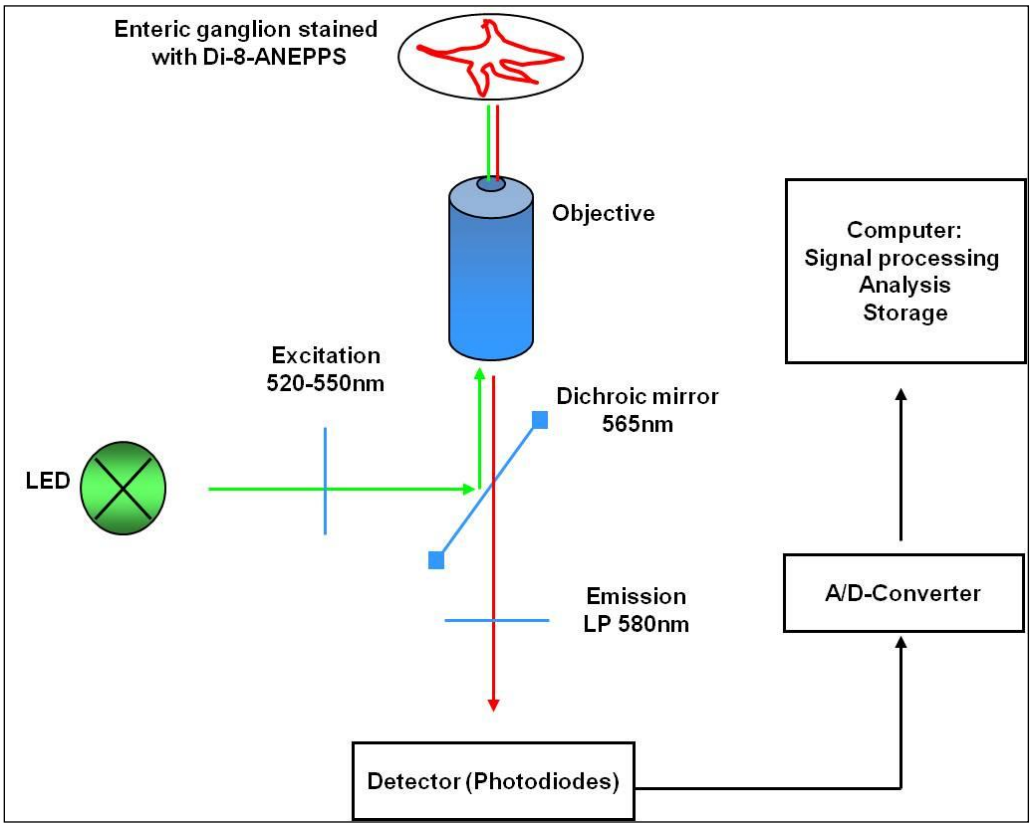

Figure 4. Illustration of the multisite optical recording technique (MSORT).
The green LED excites the Di-8-ANPEPPS incorporated in the neurons. Fluorescent changes are detected by the photodiode system. For the detection of Di-8-ANEPSS signals a modified Cy3 filter set was used. The optical signals were processed and analysed by computer. Adapted from Schemann et al., 2002.

The relative changes in fluorescence intensity (ΔF/F = change in fluorescence divided by the resting light level) are linearly related to changes in the membrane potential (Neunlist et al., 1999) and were recorded with a frequency of 1.6 kHz and processed by an array of 464 photodiodes (RedShirt Imaging, Decatur, GA, USA). The 40x objective allows a spatial resolution of 280 μm^2 per diode which ensures membrane potential recordings from all neurons in a ganglion at a single cell level. The range of the recordable fractional changes in fluorescence ranged from 0.05 % ΔF/F up to 4.0 % ΔF/F. The fluorescent images were acquired and processed by the Neuroplex 9.1.0 software (RedShirt Imaging). This technique was validated by comparison with intracellular microelectrode recordings (Neunlist et al., 1999).
The outlines of the ganglion and of the individual neurons are projected onto the image of the photodiode array allowing the identification of the origin of the optical signals. Each trace represents the signals of individual photodiodes during the recording period. The alternating current coupled photodiode system used for this

study allowed recordings of action potentials but with the compromise that slowly developing, small amplitude changes in membrane potential could not be detected. The main advantage of this technique is the possibility of recording action potentials simultaneously in a large number of neurons with high temporal and spatial resolution.

2.2.2. Staining procedure

For the staining of the individual ganglia with the fluorescent voltage-sensitive dye Di-8-ANEPPS.) 20 µM Di-8-ANEPPS dissolved in DMSO and pluronic F-127 containing Krebs solution (Stock solution: 10.3 µM Di-8-ANEPPS; 75% DMSO and 25% pluronic F-127 by weight) were injected into the ganglia by local pressure application through a microejection glass pipette. These pipettes (Science products, Hofheim, Germany), which were also used for the application of substances, were pulled with a Flaming/Brown micropipette puller (Sutter instrument Co., Novato, CA, USA; SOP66). The glass pipettes were gently positioned inside an interganglionic fibre tract and a custom made pressure ejection system was used to apply the dye. Ejection pulses lasted between 300 ms and 800 ms (ejected volume 55 ± 27 nl/s (Breunig et al., 2007)). In some cases several pulses were necessary for a proper staining. The staining was followed by a 10 to 20 min incubation time to allow the dye to incorporate into the cell membrane before starting the experiments. During the staining period it was possible to follow and to assess the progress of the labelling by briefly illuminating the ganglion. It is important to note that the dye staining does not change the electrophysiological proprieties of the nerve cells (Neunlist et al., 1999). The dye allows identification of individual cells, since it incorporates into the membrane and reveals the outline of individual cell bodies (Figure 5). The overlay of signals and ganglion image then allows the analysis of the responses from single neurons.

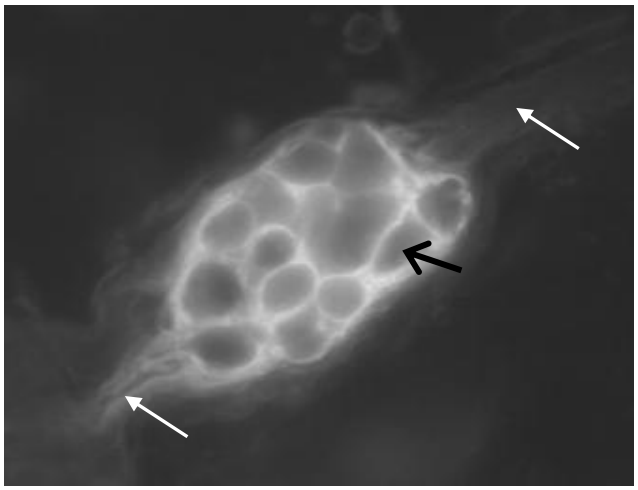

Figure 5. Human submucous ganglion stained with the voltage sensitive dye Di-8-ANEPPS.
The dye incorporates into the membrane revealing the outline of the individual cell bodies (in white). An exemplary cell body is marked with a black arrow. 15 cells are visible in this picture of a human submucous ganglion. Two interganglionic fibre tracts are marked with white arrows.

2.2.3. Application protocol

In order to apply the specific agonists onto the enteric neurons, they were first filled into a microejection glass pipette (SOP66). The filled pipette was placed into a microinjection system and positioned in close proximity (about 200 µm) to the ganglion. Openings of the glass pipette varied between 15 to 20 µm in diameter. With these parameters the applied substance is diluted by about 1:10 until it reaches the ganglion (Breunig et al., 2007).
Via local pressure ejection pulses, performed with the PicoSpritzer, the agonists were applied onto the ganglia with 0.8 to 1.0 bar and an ejection speed of 55 ± 27 nl/s.
The PAR2 agonist SLIGRL-NH2 (from D. McMaster, Peptide Synthesis Core Facility, Department of Medical Biochemistry, University of Calgary, Canada) was not applied via spritz application but by local perfusion (SOP164). Freshly oxygenated Krebs solution containing the agonist at 100 µM concentration was filled into a 1000 µl plastic syringe (Dispomed, Geinhausen, Germany), which was connected to a digital volume controlled injector (UltraMicroPump II, World Precision Instruments Inc., Sarasota, USA). A plastic tube with 1 mm in diameter (KronLab, Sinsheim, Germany) connected the syringe to a microejection glass pipette placed directly above the ganglion. The working solution was then locally perfused with a microprocessor based controller (Micro-4, World Precision Instruments Inc., Sarasota, USA) over the ganglion for 30 s at a rate of 100 nl/s followed by a recording of 1.8 s.

2.2.4. Electrical stimulation of interganglionic fibre tracts

Electrical stimulation of interganglionic fibre tracts were used to evoke fast EPSPs (fEPSPs). This was achieved with a 25 µm-diameter teflon-coated platinum electrode (Science Products, Hofheim, Germany) connected to a constant current stimulator (Stimulus Isolator A360, WPI, Berlin, Germany). Rectangular suprathreshold pulses of 600 µs duration and variable amplitude from 20 to 90 µA were used. It is important to note that the number of neurons responding does not neccessarily equal the number of vital neurons. The values for neurons responding to electrical stimulation are usually an underestimation for two reasons. Firstly, not all axons projecting to the ganglion are stimulated with this method as a particular neuron might receive input from an axon projecting to the ganglion via a different fiber tract (Neunlist et al., 1999). Secondly, most but not all enteric neurons in the human submucous plexus receive fEPSPs (70.8 ± 24.5 %, Klaus Michel, personal communication).

Nevertheless, electrical stimulation is a quick and efficient way to verify the viability of the neuronal network and crucial to investigate drug actions on synaptic transmission.

2.2.5. Analysis of presynaptic activity

Electrical stimulations were used to study presynaptic actions of 5-HT_4 agonists. The strength of individual pulses was carefully chosen between 20 and 90 µA in order to evoke subthreshold fEPSPs. Then three stimulations were applied. The first served as a control, the second electrical stimulation was given after perfusing 5-MeOT (10 µM; SOP85) for 20 min and the third after 45 min of wash-out of 5-MeOT. In the experiments performed with prucalopride (SOP109) an additional control stimulation was applied. These two control stimulations were conducted with a time difference of 5 min and allowed to assess the normal variations in fEPSP amplitudes. The next stimulation was applied after a 20 min perfusion of prucalopride (10 µM). Amplitudes and durations of fEPSPs were analysed. For the fEPSP amplitude a fEPSP to baseline ratio was calculated. This ratio measures the change light intensity (ΔF/F[%]) representing the change in membrane potential. For the prucalopride experiments also the area under the curve of the fEPSPs after electrical stimulation was measured. A representative trace is presented in the results section. For each individual neuron, only changes in amplitude, duration or area exceedeing twice the variance between the two control stimulations were considered relevant.

2.2.6. Multi-Captioning of signals to detect late onset responses

All non-5-HT_3 mediated responses are long lasting and have a late onset. Thus, one recording period of 1.8 sec may not be sufficient to reveal a GPCR mediated 5-HT response. Therefore, a different recording protocol using “Multi-Captioning” was used (SOP175). It consisted of five acquisitions, each lasting for 1.8 s; the first one started shortly before the 5-HT stimulation and the other four occurred thereafter with 1.2 s period of no recordings in between (see
Figure **7**, “Multi-Captioning after 5-HT-application”, 3.1.2). This acquisition stated a reasonable compromise between the necessity to record late onset responses and the risk of dye bleaching or phototoxicity.

2.2.7. Actions of 5-HT, histamine and PAR-2 activating peptide in the myenteric and submucous plexus

In these experiments the sensitivity of guinea-pig myenteric and submucous neurons to 5-HT (SOP46), histamine (SOP57) and the PAR-2 agonist SLIGRL-NH2 (SOP164) was compared. For this purpose, from the colon of each animal both myenteric and submucous plexus were prepared and the above mentioned substances were spritzed onto the ganglia of both plexus in random order. Only experiments where the ganglia of both plexus showed a response to electrical stimulation or nicotine (SOP90) were considered for evaluation.

2.2.8. Organ bath experiments

After killing the guinea pigs, the entire stomach was removed and immediately placed in ice-cold oxygenated Krebs solution (SOP102). Subsequently, the stomach was opened along the greater curvature, thoroughly washed and pinned mucosal side up in Sylgard-coated Petri dishes. The mucosa was then carefully removed. Then muscle strips (1.5 cm^2) were cut parallel to the circular or longitudinal muscle axis and mounted in 25 ml organ baths where they were maintained in oxygenated Krebs solution at 37°C. One edge of each muscle strip was attached to an isometric tension

transducer connected to a Quad Bridge and a MacLab/4S analog/digital converter (MacLab, AD Instruments, Spechbach, Germany).
After mounting in the organ baths (SOP101), tissues were equilibrated with a preload set at 15 mN for 45 min. To ascertain tissue viability, electrical field stimulations (EFS) were performed using a Grass SD9 stimulator set (Quincy Mass, USA) at a constant supramaximal voltage of 100 V, with a pulse frequency of 10 Hz and a pulse width of 0.6 ms for 10 s. All tissues used in the experiments were vital and responded to EFS with an initial contraction followed by an inhibition of muscle contractility. After each EFS response, tissues were thoroughly rinsed and allowed to equilibrate for approximately 25 min. 5-BOIP was added to the organ baths at a final concentration of 10 µM. 20 min after each application the tissues were rinsed three times with fresh Krebs buffer and allowed to recover for approximately 30 min until the tissues had reached previous tone. All responses were recorded and analysed employing LabChart 6 software (AD Instruments, Spechbach, Germany) on a Windows XP-based computer. In the guinea pig corpus the response to EFS can be divided in several components. The initial response to the EFS is the contractile on-response which commences together with the EFS. Once the stimulus stops, many preparations show an off-response which may consist of relaxatory or contractile response. The duration of the off-response can exceed that of the on-response. Its end is defined by the time the muscle tone returns to baseline level.

2.3. Reproducibility tests

For spritz application two stimulations with identical protocol were applied 15 min apart from each other. For 5-HT and 5-BOIP the differences between the two stimulations were not significant for both cell count and AP frequency. Prucalopride, 5-MeOT and 5-CT did not evoke APs after spritz application (for further details see Chapter 3 - Results and Appendix IV).

2.4. Pharmacology

2.4.1. Agonists

An overview on all serotonergic agonists and antagonists used in this study can be found in Table 2 at the end of this chapter. All mentioned concentrations of substances for microejection in this thesis relate to the concentration at which they are filled into the glass pipette. As mentioned above (Chapter 2.2.3), after ejection the applied substance is diluted by about 1:10 until it reaches the ganglion.

Serotonin (Serotonin creatinine sulphate monohydrate (5-HT), Sigma H-7752, Sigma-Aldrich, Schnelldorf, Germany; SOP46) served as an agonist for all serotonergic receptors. Serotonin was dissolved in Krebs and stored at a 10 mM stock solution at 4°C for maximally 1 week. It was microejected at a concentration of 1 mM for 400 ms.

Two 5-HT_4 receptor agonists were used: 5-methoxytryptamine (5-MeOT, Ref. Number: M-6628, Sigma-Aldrich, Schnelldorf, Germany; SOP85) and prucalopride (Ref. Number: GW686036A, GlaxoSmithKline, Harlow, UK; SOP109). 100 mM stock solutions were stored at 4°C for one to two weeks. Working concentrations were 100 µM for 5-MeOT and 10 µM for prucalopride. Stock and working solutions for both substances were diluted in Krebs solution.

The stock solution of the 5-HT_7 receptor agonist 5-carboxytryptamin (5-CT, Ref. Number: GA/64436 Tocris (BioTrend), Cologne, Germany; SOP91) was prepared in distilled water at a concentration of 100 mM. The working concentration was mostly 50 µM in Krebs solution, although concentrations of 1 µM, 5 µM, 10 µM, 100 µM, 500 µM and 1 mM were also tested.

For the 5-HT_{1P} receptor the only reliable agonist known is 5-hydroxyindalpine (5-OHIP), which is not commercially available. Some older samples from GlaxoSmithKline, Harlow, UK, Solvay Pharma, Hannover, Germany and Kali Chemie Pharma, Hannover, Germany; SOP93) seemed to have lost their efficacy as spritz application on human submucous or guinea pig myenteric neurons did not evoke any responses. Likewise, these samples had no effect on muscle activity. Therefore, new 5-OHIP was synthesized (M.Müller, Department of Biological Chemistry of the Techische Universität München, Germany, see Appendix II). At the very end of this study, however, it turned out that the assembled substance was not 5-hydroxy-indalpine, but a very similar molecule: 5-benzoxyindalpine (5-BOIP, for chemical structure see Appendices II and III)). Thus the discovery of 5-BOIP as a possible novel 5-HT_{1P} agonist was by accident. The details on the synthesis of 5-BOIP can be found in Appendix II. For the experiments, 5-BOIP was always freshly prepared

(SOP93) and dissolved at a stock solution of 100 mM in Krebs solution and stored at 4°C for 24 hours. The working solution was primarily 50 µM, but also 5 µM and 100 µM concentrations were tested.
In several experiments nicotine, the agonist for nicotinergic acetylcholine receptors, served as a positive control for the viability of the neuronal network. The nicotine (N-5260, Sigma-Aldrich, Schnelldorf, Germany) stock solution (10 mM in Krebs solution) was stored at -20°C until being used for application at a concentration of 100 µM for 200 ms (SOP90).
The histamine stock solution (1 mM) was stored in aq. bidest at -20°C. The working solution was 100 µM in Krebs solution (SOP57).
The PAR2 agonist SLIGRL-NH2 (from D.McMaster, Peptide Synthesis Core Facility, Department of Medical Biochemistry, University of Calgary, Canada) was prepared at a 10 mM stock solution in distilled water and stored at -20°C. For the working solution a 100 µM concentration was used (SOP164).

2.4.2. Antagonists

Antagonists were added to the Krebs solution perfusing the tissue in order to test whether they reduced responses to agonists that were delivered via spritz application. Thus the antagonists were always perfused after a recording of the neurons response to the agonist. This was compared to agonist actions in the presence of the antagonist and the recovery after wash out of the antagonist.
The 5-HT_3 receptor antagonist cilansetron (Ref. Number: SCF000183, Solvay, Hannover, Germany) was perfused for 20 min at a concentration of 0.1 µM (SOP163). The standard wash-out time for cilansetron was 40 min. However, in some experiments extenden wash out periods of up to 2 hrs had to be used. The stock solution (100 µM) was kept frozen in aliquots at -20°C.
The stock solution for the 5-HT_4 receptor antagonist piboserod (Ref. Number: GW686036A, GlaxoSmithKline, Harlow, UK) was prepared at 100 µM in Krebs and stored in aliquots at 4°C for a maximum of one week (SOP110). The working solution was perfused 1 µM for 20-30 min and later washed-out for 40-60 min.
The 5-HT_{1P} receptor antagonist n-acetyl-5-hydroxytryptophyl-5-hydroxy-tryptophanamide (5-HTP-DP, from M.Müller, Department of Biological Chemistry of the Techische Universität München, Germany, see Appendix I) was dissolved at 10 mM in Krebs solution with 3-5 % DMSO to increase solubility and stored in aliquots at 4°C for at maximum one week (SOP92). For the experiments 10 µM of 5-HTP-DP were perfused for 20-30 min. The wash-out time of 5-HTP-DP was 60 min.

In the experiments with guinea pig myenteric plexus the L-type Ca^{2+} channel blocker nifedipine (Ref. Number N-7634 Sigma-Aldrich, Schnelldorf, Germany) was added to the perfusion system at a concentration of 1 µM to prevent muscle movements (SOP36).

Table 2. 5-HT receptor agonists and antagonists used in the experiments with the respective concentrations and time of application.

Abbreviations: 5-MeOT: 5-Methoxytryptamine; 5-CT: 5-Carboxytryptamine; 5-BOIP: 5-Benzoxytryptamine; 5-HTP-DP: N-acetyl-5-hydroxytryptophyl-5-hydroxytryptophanamide.

* provided by the Department of Biological Chemistry of the Techische Universität München, Germany.

Drug	Function	Company	Reference number	Application method	Concen-tration	Application Time
5-HT	5-HT receptor agonist	Sigma-Aldrich	H-7752	spritz application	1 mM	400 ms
5-MeOT	5-HT_4 receptor agonist	Sigma-Aldrich	M-6628	spritz application	1-100 µM	400 ms/ 20 min
Prucalopride	5-HT_4 receptor agonist	Glaxo-SmithKline	GW686036A	spritz application/ perfusion	1-100 µM/ 10 µM	400 ms/ 20 min
5-CT	5-HT_7 receptor agonist	Tocris (BioTrend)	GA/64436	spritz application	50 µM	400 ms
5-BOIP	Putative 5-HT_{1P} receptor agonist	*	*	spritz application	50 µM	400 ms
Cilansetron	5-HT_3 receptor antagonist	Solvay	SCF000183	perfusion	0.1 µM	20 min
Piboserod	5-HT_4 receptor antagonist	Glaxo-SmithKline	SB207266A	perfusion	1 µM	20-30 min
5-HTP-DP	5-HT_{1P} receptor antagonist	*	*	perfusion	10 µM	20-30 min

2.5. Immunohistochemistry

5-HT receptor antibody staining in the human ENS has not been very successful in the past. The 5-HT_3 receptor for example is probably the most studied receptor subtype in the ENS, but despite many efforts to the knowledge of the author a reliable commercially available antibody against the enteric 5-HT_3 receptor has not yet been found. However new antibodies against 5-HT_4 and 5-HT_7 have been designed recently by Santa Cruz. An immunohistochemical approach aimed to examine the usefulness of those antibodies in guinea pig and human tissue because these two receptors are within the main focus of this thesis. Since an antibody against the 5-HT_{1P} receptor does not exist, only the antibodies against 5-HT_4 and 5-HT_7 receptors were tested.

Human tissue specimens were fixed overnight at room temperature in a solution containing 4% paraformaldehyde and 0.2% picric acid in 0.1 mol/L phosphate buffer and then washed three times for 10 min in phosphate buffer (SOP14). Guinea pig tissues were fixed either for 4 h at room temperature or overnight at 4°C (SOP14). Until further processing the samples were stored at 4º C in phosphate-buffered saline (PBS) containing 0.1% NaN_3. For the following steps the samples were kept on continuous agitation (Agitatior, Heidolph, Schwabach, Germany) permeabilized in Triton X-100 (TX, 0.5%)/PBS/NaN_3 (0.1%)/Horse serum (HS, 4%) for 1 h at room temperature. Then the specimens were washed again for three times 10 min in phosphate buffer. For the 5-HT receptor stainings the tissues were incubated in TX(0.5%)/PBS/NaN_3/HS (4%) containing the primary antibodies goat polyclonal SR-4 (C-18) (Reference number sc-32566, Santa Cruz Biotechnology, Heidelberg, Germany) for the 5-HT_4 receptor and goat polyclonal SR-7 (S-20) (Ref. Number sc-19158, Santa Cruz Biotechnology, Heidelberg, Germany) for the 5-HT_7 receptor at dilutions from 1:200, 1:500, 1:1000, 1:5000. The incubation time was 12-16 h for guinea pig tissue and 40 to 48 h for human tissue at room temperature. Afterwards, the tissues were rinsed three times for 10 min in PBS. Then the tissues were incubated in buffered solution containing the secondary antisera donkey anti goat (Cy3) (Ref number 705165147, Dianova, Hamburg, Germany) for 1.5-2 h for the guinea pig preparations and 3.5 h for the human preparations. The final dilution of secondary antibody was 1:500 (SOP73).

Antibodies against calbindin were also used because they have been described as reliable markers for AH/Dogiel type II neurons in the guinea pig ileum, neurons that are supposed to act as sensory neurons in the ENS (Furness et al., 1988). Rabbit anti-calbindin (Ref. number AB 1778, Chemicon, Hofheim, Deutschland) was used for these experiments (SOP15). The tissues were incubated in TX (0.5%)/PBS/NaN_3/HS (4%) containing the primary antibodies at a dilution of 1:1000

for anti-calbindin for 48 h at room temperature. After washing in PBS, the tissues were incubated for 24 h in buffered solution containing the secondary antisera donkey anti rabbit conjugated to carbocyanin (Cy2; Ref. number 711225152, Dianova, Hamburg, Germany) at a dilution of 1: 200.

Finally, all specimens were again washed in PBS three times for 10 minutes, mounted on poly-l-lysine-coated slides and cover slipped with a solution of PBS (pH 7.0) /NaN_3 (0.1) containing 65% glycerol. The preparations were examined with an epifluorescence microscope (BX61WI, Olympus, Japan). Appropriate filters were used to visualize the Di-8-ANEPPS and the fluorophores separately. Pictures were acquired with a monochrome video camera (Fluo View II, Olympus) connected to computer and controlled by analySIS 3.1 (Olympus-SIS, Münster, Germany) image software. Frame integration and contrast enhancement were employed for image processing.

Table 3. Primary antibodies used in the experiments.

Antibody	**Antigen**	**Company**	**Reference number**	**Dilutions:**	
				Human	**Guinea Pig**
Goat anti-SR4 (C-18)	Cytoplasmic domain of human 5-HT_4 receptor	Santa Cruz (Heidelberg, Germany)	sc-32566	1:1,000 1:5,000	1:200 1:500 1:1,000 1:2,000 1:5,000
Goat anti-SR7 (S-20)	Peptide near N-terminus of human 5-HT_7 receptor	Santa Cruz (Heidelberg, Germany)	sc-19158	1:500 1:1,000 1:5,000	1:200 1:500 1:1,000
Rabbit anti-Calbindin	Calbindin molecule	Chemicon (Hofheim, Deutschland)	AB 1778		1:1000

2.6. Data analysis and statistics

The identification of individual neurons in the ganglia was possible from the moment that the dye incorporates into the membrane revealing the outline of individual cell bodies. Overlaying ganglion picture with the signals responses from single neurons could be analysed. Thereby the number of ganglion cells in the field of view, the number of responding cells per ganglion and the number and frequency of action potentials per cell were identified and analysed. The number of tissues (T), ganglia (G) and cells (C) are indicated as T/G/C. For the cell count the illustration of percentages always relates to the number of cells counted within the monitored ganglion, if not mentioned otherwise. For signal analysis we used Neuroplex 9.1.0 (RedShirt Imaging), Igor Pro 6.04 (Wavemetrics Inc, Lake Oswego, OR, USA), Microsoft Office Excel 2003 (Microsoft Corp., Redmont, WA, USA) and Image J 1.32j software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011).

All the statistic analyses were performed with the software Sigmastat 3.1 (Systat Software Inc, Erkrath, Germany) and Sigmaplot 9.0 (Systat Software Inc) software. All data are presented as mean ± standard deviation or median and 25/75 pecentile values when they are not normally distributed.

To detect differences in the frequency of action potential discharge or cell count between two control groups of acquisitions depending on study design and distribution of data a students' t-test, paired t-test, or a Mann-Whitney rank sum test (if data was not normally distributed) was performed. For comparing more than two groups, one way analysis of variance, or a Kruskal-Wallis repeated measures analysis of variance on ranks if the data was not normally distributed, was used. For multiple comparisons also Dunn's Method and Tukey tests were applied. For all analysis the difference between data groups was defined significant when the P-value was ≤ 0.05.

3. RESULTS

3.1. Electrophysiology of 5-HT_{1P}, 5-HT_4 and 5-HT_7 receptors

3.1.1. Preliminary experiments in the myenteric and submucous plexus

Small intestinal tissue from guinea pig was used for the preliminary experiments since it is the most commonly used animal model in this field. These experiments in both myenteric and submucous plexus consisted of direct spritz applications of nicotine (100 µM) and 5-HT (1 mM) onto the Di-8-ANEPPS stained ganglion. An example of a Di-8-ANEPPS stained ganglion is displayed in the introduction (Figure 5).

The neurons of the myenteric plexus showed an average action potential (AP) frequency of 6.6 ± 4.1 Hz in 34 out of 78 cells (44.9 %) in response to 5-HT. This was measured in tissue samples of 4 different animals where a total of 6 ganglia were tested (Tissues: 4 / Ganglia: 6 / Cells: 78). 69.7 % of the neurons (59 out of 78) responded to nicotine with an AP frequency of 9.5 ± 3.1 Hz.

In the submucous plexus of the guinea pig 54.6 % of the neurons (40 out of 73) responded to 5-HT (4/7/73). With 7.9 ± 3.0 Hz the AP frequency was significantly higher (8.4 %) than in the myenteric plexus. For the response to nicotine on the other hand, it was 8.5 ± 3.5 Hz and thus 8.9 % lower compared to the myenteric plexus with 51 out of 73 neurons responding, which means 69.9 %.

The response of the submucous neurons to the 5-HT application was also tested in human tissue (6/12/116). Here 44.7 % (52 out of 116) of the neurons responded to the 5-HT spritz application with an AP frequency of 3.9 ± 2.1 Hz. In three tissues (3/7/39) two 5-HT stimulations (1 mM, 400 ms) were applied 15 min apart from each other to check for reproducebility. The difference between the two stimulations was not significant for both cell count and AP frequency: To the first application 46.1 % of the neurons (18 out of 39) responded with 3.7 ± 1.4 Hz. To the second application 43.6 % of the neurons (17 out of 39) showed APs with a frequency of 3.5 ± 1.7 Hz.

Influence of the 5-HT_3 antagonist cilansetron

Cilansetron is a selective and high affinity 5-HT_3 receptor blocker (see also Introduction - Table 2). Indeed in 80.5 % of the human neurons the responses to 5-HT could be blocked by cilansetron. In 19.5 % of the neurons however there were still some APs.

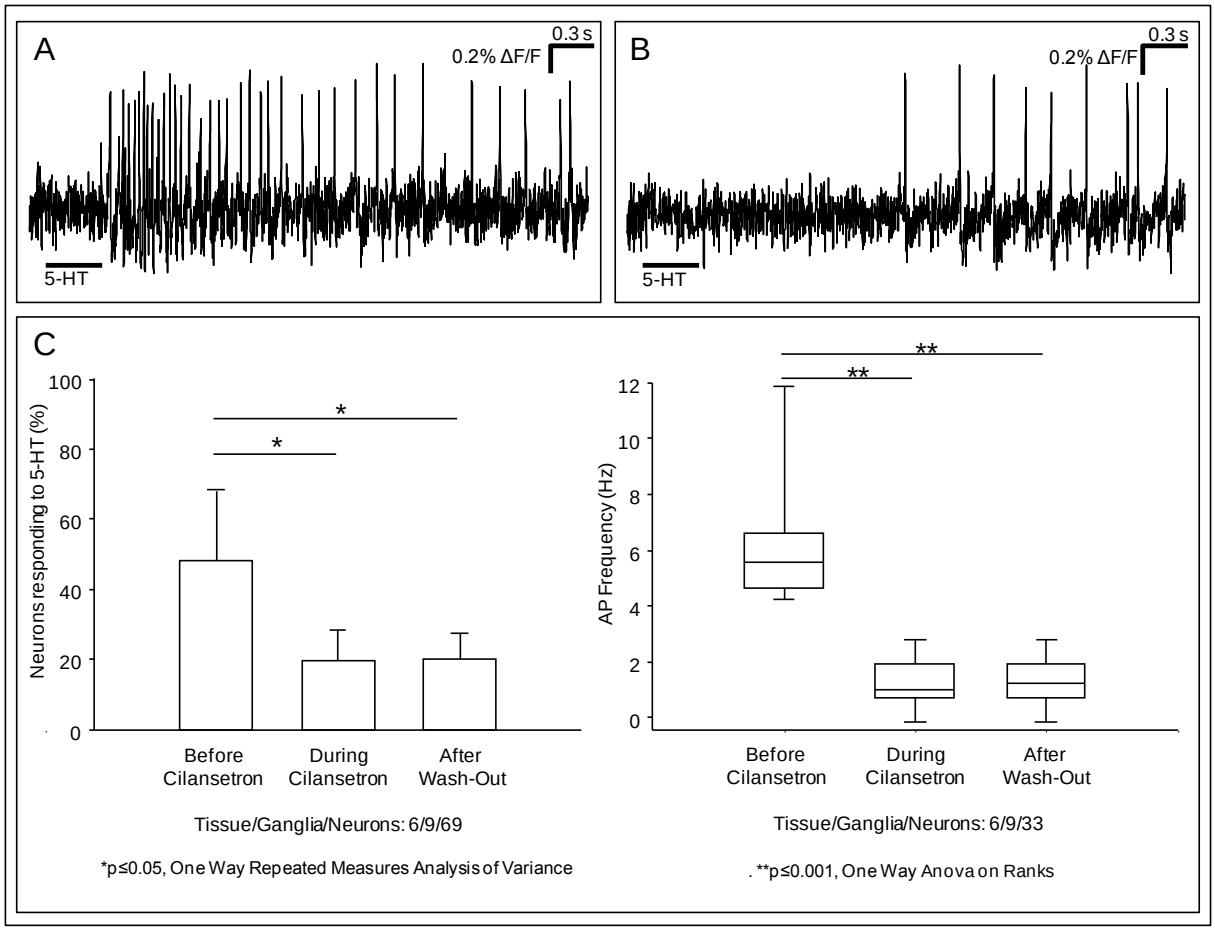

Figure 6. Effects of 5-HT spritz application before and after cilansetron perfusion.
A: Trace representing the neuronal response of a submucous neuron to the spritz application of 5-HT (1 mM) onto the ganglion. The time and duration of the spritz application (400 ms) is marked by a black bar directly below the trace. Shortly after the application of 5-HT the neuron starts to fire APs at a high rate as can be seen by the many spikes. B: The neuronal response to 5-HT is strongly reduced by the 5-HT_3 antagonist cilansetron (0.1 µM, but a late onset response still persists. C: Cilansetron reduces the AP frequency (right) as well as the number of neurons responding to 5-HT (left) significantly, but the response does not recover after wash-out. There are several neurons in which the 5-HT response is not completely blocked by the 5-HT_3 receptor antagonist.

The duration of the recording in the displayed traces was 5.021 s. Though some of the later APs do indeed persist after the perfusion of cilansetron, it is obvious that the major part of the response to 5-HT is blocked by cilansetron. However, there is still a clear late neuronal response under the antagonist's influence. It was not possible to demonstrate recovery of the response with washout periods up to 2 hours (Figure 6). Herefore 69 cells of 10 ganglia from 6 tissues were analysed. The AP frequency of the cells that responded to 5-HT application was 5.6 [4.8/6.8] Hz before and 1.0 [0.7/1.9] Hz after perfusion of the antagonist. The later was not significantly different from the 1.2 [0.7/1.9] Hz after wash-out. This was also true for the percentage of cells responding: 19.5 ± 8.4 % of the neurons responded to 5-HT in cilansetron and 19.7 ± 7.2 % after wash-out. Before perfusion of the antagonist 47.2 ± 19.6 % of the neurons show APs in response to the 5-HT application.

3.1.2. Late onset 5-HT response

The investigation into the late onset response of submucous neurons to 5-HT application require long recording durations, which may lead to bleaching effects within the neurons. Also possible effects of phototoxicity can not be completly excluded. Together with the fact that a proper wash-out of cilansetron was not possible, this necessitated another way of isolating the non-5-HT_3 driven response to the 5-HT application. Thus a “multi-captioning” approach was designed to gain a better understanding of the late onset 5-HT-response, wherein the reliable recording of the late onset responses of neurons to 5-HT application turned out to be feasible. Therefor, five signal acquisitions were applied, each one for 1.8 s, starting shortly before the 5-HT application with a pause of 1.2 s in between (
Figure **7**).

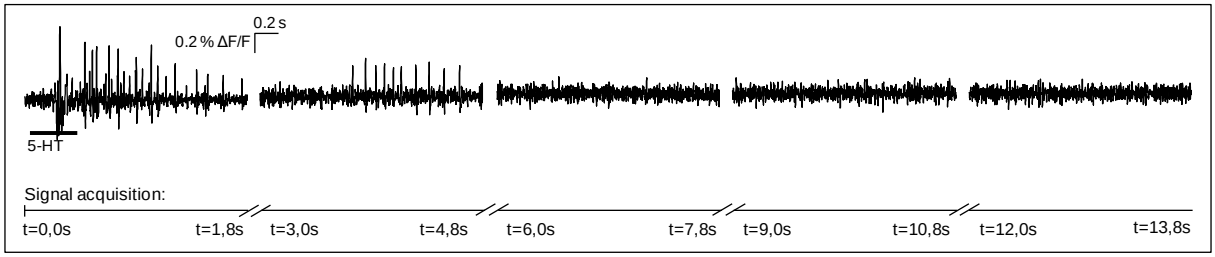

Figure 7. Multi-Captioning after 5-HT-application.
Exemplary traces of five successive acquisitions taken from one submucous neuron. The first acquisition starts at t = 0s, followed by application of 5-HT (1 mM). Recording time is 1.8 s for each acquisition, with 1.2 s pause in between respectively as indicated by the timeline at the bottom. Action potentials in response to the 5-HT application can be seen in the first (t = 0 s -1.8 s) and in the second (t = 3.0 s - 4.8 s) time frame. The spike discharge in the first timeframe represents the neurons immediate response to the 5-HT application. A later response as can be seen the second trace can be considered as late onset response. The black bar directly below the first trace marks the duration of the spritz application (400 ms).

The results from 77 neurons (5 patients, 8 ganglia) show that 42.5 % responded to 5-HT within the first 1.7 s after the start of the 5-HT application, with an AP frequency of 4.6 ± 2.1 Hz (
Figure **8**). 5-HT was applied 0.1 s after the beginning of the acquisition. In the second time frame (3.0 - 4.8 s) 21.5 % of the neurons still showed APs, the average frequency being 1.4 ± 0.4 Hz. The third to fifth frames did not have many neurons responding, with 4.2 %, 4.6 % and 0.8 % respectively. Also their AP frequency was rather low (0.2 ± 0.4 Hz; 0.3 ± 0.4 Hz and 0.1 ± 0.2 Hz, respectively). The results of

these experiments illustrate the strong response to 5-HT immediately after its spritz application, but also the late onset APs described in the chapter above. Knowing from the cilansetron experiments that the immediate 5-HT response is almost completely 5-HT_3 driven, it was concluded that the focus needed to be on the acquisition periods beyond the first time frame.

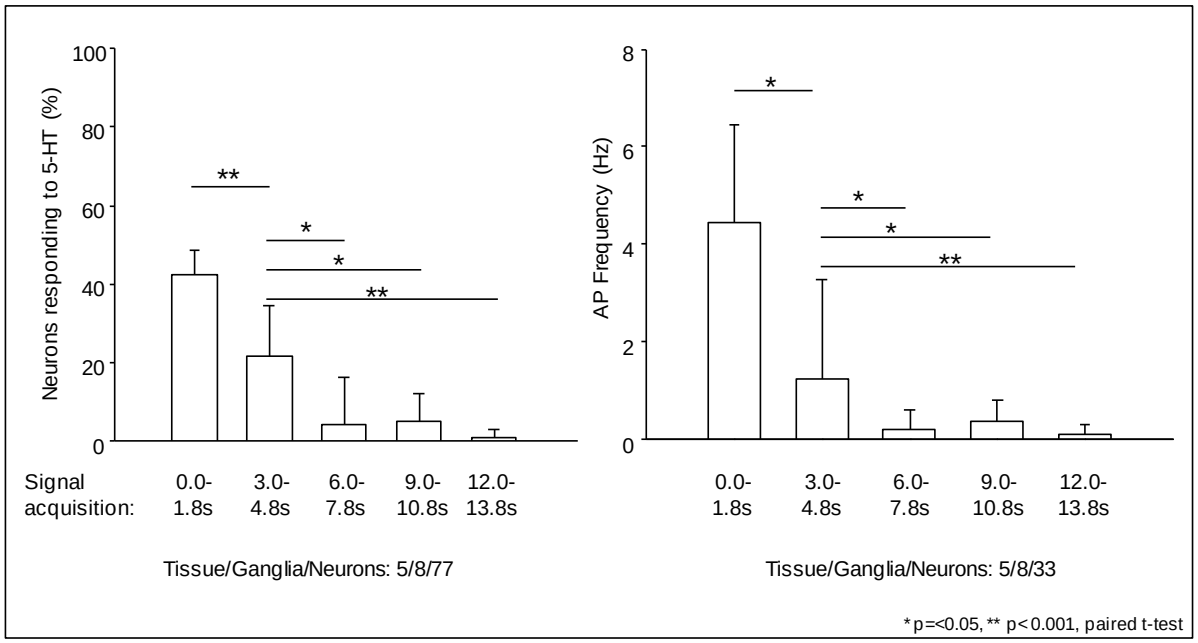

Figure 8. Multi-Captioning results.

Cell count (left) and frequency (right) of the different multi-captioning time frames for the response to 5-HT (1mM), with an electrical stimulation as control. The percentage of cells responding as well as the AP frequency are the highest immeadeatly after the 5-HT application, which is represented within the first timeframe (0.0 - 1.8 s). The response to 5-HT is still strong during the second timeframe (3.0 - 4.8 s), whereas there are one very few neurons responding to 5-HT later than 6 s after the beginning of the first acquisition. There are also no significant differences between the third, fourth and fifth period of recording for both cell count and AP frequency.

3.1.3. The 5-HT_{1P} Receptor

Effects of the 5-HT_{1P} antagonist 5-HTP-DP

The previous experiments showed that the response to 5-HT has an early and a late component. While the early component is mainly mediated by the 5-HT_3 receptor, the late response must be mediated mainly by one or maybe more different receptors. To characterise the nature of this receptor more precisely the effect of the 5-HT_{1P} antagonist 5-HTP-DP on the late response was tested.

5-HTP-DP was first tested in the guinea pig. Three times a 5-HT spritz (1 mM, 400 ms) was applied onto the Di-8-ANEPPSs stained ganglion. The experiments in cilansetron and from the “multi-captioning” suggested that the late onset response in response to the application of 5-HT occurs later than the 5-HT_3 mediated immediate response. Thus, the beginning of the recording was set to 2 s after 5-HT application and the duration of the acquisition to 3.1 s. This represents a signal acquisition timeframe of 2.1 - 5.2 s from the multi-captioning experiments, effectivly an extended version of the second timeframe in which the late onset response was most prominent. The first application took place before, the second during perfusion (20 min) of the 5-HT_{1P} antagonist (10 µM) and the third after 60 min wash-out. The percentage of neurons responding as well as the AP frequency evoked by 5-HT is significantly reduced by 5-HTP-DP (

Figure **9**). Out of 146 neurons from 6 ganglia and 4 animals, 33.1 ± 19.3 % responded with APs after the first application, 13.9 ± 13.3 % after the second and 17.6 ± 14.4 % after the third. In other words, 5-HTD-DP reduced the number of neurons responding by 60.4 %. In 43 out of the 48 neurons (90.0 %) that showed a late onset response to the 5-HT, 5-HTD-DP either fully blocked or reduced the 5-HT response. In 4 neurons the response was not change at all, for one neuron the response was increased during the perfusion of the antagonist.

It should be noted that a neuron was always marked as “responding” if it showed APs to 5-HT application. In 48 neurons 5-HT evoked an AP frequency of 3.4 [1.6/6.6] Hz. The spike frequency was significantly reduced to 0.8 [0.0/2.1] Hz by 5-HTP-DP, which corresponded to an average reduction in APs of 65.6 %. Among the 48 responsive neurons, 5-HTP-DP completely blocked the late onset response to 5-HT in 14 neurons and reduced the AP frequency in further 29 neurons. In five neurons the AP Frequency stayed the same and in one neuron the AP frequency was increased. In total 5-HTP-DP reduced or blocked the late onset response in 43 out of 48 neurons (89.6 %). The effect of 5-HTP-DP was reversible as the AP frequency

recovered to values of 3.1 [1.1/4.8] Hz after wash out. However, a substantial number of neurons were still unresponsive which explains that the number of 5-HT responsive neurons did not fully recover to control values.

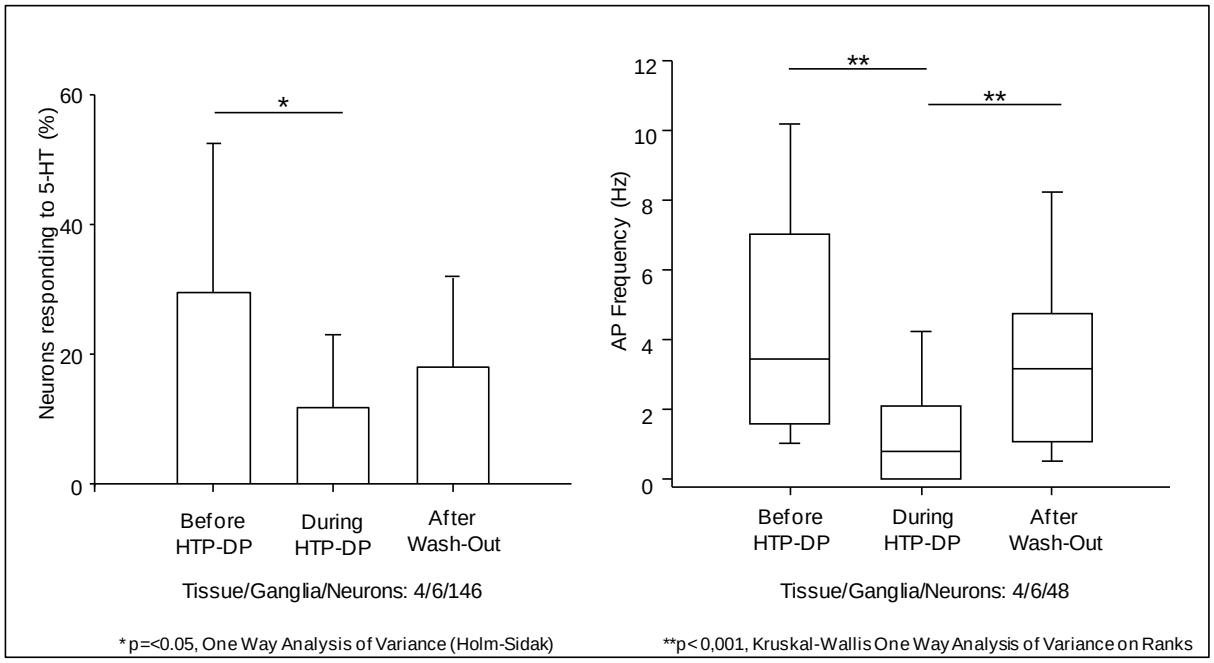

Figure 9. Influence of 5-HTP-DP on the 5-HT response in guinea pig.
Left: Percentage of neurons responding to 5-HT (1 mM). Right: AP frequency evoked by 5-HT. 5-HTP-DP (10 µM) reduces the AP-frequency as well as the number of cells responding to 5-HT. The reduction tends to disappear after wash-out. This is only significant for the frequency though.

The same approach as in the guinea pig was also used in human tissue (Figure 10). In total 76 neurons from 3 patients (6 ganglia) were tested. The number of neurons showing a late onset responde to 5-HT application here was higher than the results from the cilansetron experiments would suggest: 44.2 ± 6.7 % of the neurons (34 out of 76) showed a late onset response to 5-HT before 5-HTP-DP. During perfusion of the antagonist it was reduced to 31.5 ± 13.7 %. This reduction is lower than in guinea pig, but still significant. After wash-out, 38.5 ± 11.5 % of the neurons showed APs to the 5-HT stimulation.
For the AP frequency the recovery after wash-out is significant, from 1.1 [0.6/4.2] Hz to 3.2 Hz ± 2.0 Hz. The same is true for the reduction by 5-HTP-DP, from 4.0 [2.6/5.8] Hz to the already mentioned 1.1 [0.6/4.2] Hz, which means a decrease in the spike frequency of 72.5 %. Out of the 34 neurons with a late onset response all but 1 neuron showed a reduction in AP frequency during 5-HTP-DP, allthough it was blocked completely in only 9 neurons (26.5 %).

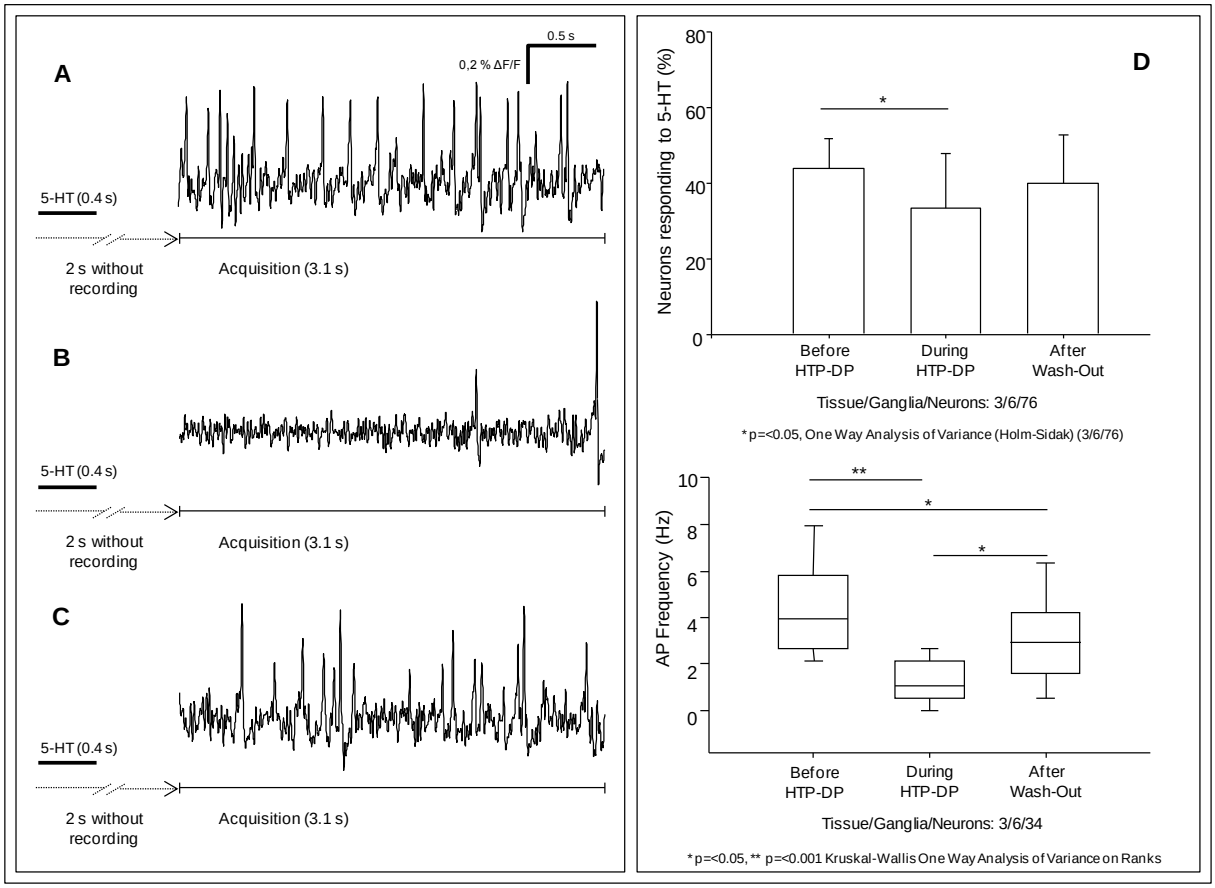

Figure 10. Influence of 5-HTP-DP on the late onset 5-HT-response in human.

A: Response to the standard 5-HT (1 mM, 400 ms) spritz application. 5-HT was applied 2s before the start of the acquisition, which recorded the neuron's late onset response to 5-HT. Here the neuron responded by firing APs. B: Response to the 5-HT application after perfusion of 5-HTP-DP (20 min, 10 µM). The number of APs evoked by 5-HT is highly reduced by the 5-HT_{1P} antagonist, but the neuron still fires two APs. C: Recovery of the 5-HT response after 60 min wash-out. D: Effects of 5-HTP-DP on the percentage of neurons with a late onset 5-HT-response and on the AP frequency evoked by 5-HT. 5-HTP-DP significantly reduces the AP-frequency as well as the number of cells responding to 5-HT. The reduction tends to disappear after wash-out, which is only significant for the frequency though.

Effects of the putative 5-HT_{1P} agonist 5-benzoxy-indalpine (5-BOIP)

5-BOIP was tested at three different concentrations (5 µM, 50 µM, 100 µM) in preliminary experiments in guinea pig (3/3/29) via direct spritz application onto myenteric neurons. The 5 µM concentration yielded very few responses: 5.6 ± 9.6 % of the neurons responded with an AP frequency of 0.53 ± 0.53 Hz. The response to 100 µM of BOIP was stronger, with 13.7 ± 3.8 % responding neurons firing at a frequency of 1.3 ± 1.0 Hz. The strongest response was observed with 50 µM 5-BOIP: 28.4 ± 11.7 % of the neurons responded to BOIP and the AP frequency was 1.7 Hz ± 1.0 Hz. The effects of 5-BOIP proved to be reproducible. Two 5-BOIP stimulations (100 µM, 400ms) were applied 15 min apart from each other. The difference between the two stimulations was not significant for both cell count and AP frequency: To the first application 8 neurons out of 29 (27.6 %) responded with 1.7 ± 0.9 Hz. To the second application 7 out of 29 neurons (24.1 %) showed APs with a frequency of 1.5 ± 1.0 Hz. The effect of the substance was also tested in organ bath experiments with guinea pig gastric muscle strips in analogy to the experiments with 5-OHIP performed by Michel et al. in 1997.

Electrical field stimulations were applied on mucosa-free circular muscle strips before and after adding 5-BOIP (5 µM) to the perfusion system to check for a 5-HT_{1P} induced reduction of the muscular on-response as described by Michel et al. (1997) and other possible effect of 5-BOIP on the muscle tone. 9 tissues from 6 animals were measured and analysed (

Figure **11**).

For each tissue two electrical field stimulations were applied under control condition and two further stimulations after bath application of 5-BOIP. The results for the control stimulations showed no significant difference and thus the data were pooled. The same was also true for the two stimulations after perfusion. Since 5-BOIP caused a significant increase in basal muscle tone from 39.7 ± 9.5 mN to 43.5 ± 12.2 mN (+ 9.6 %), all values for the analysis of the response to the EFS were calculated relative to the baseline. The results show that 5-BOIP indeed reduced the on-response, from +8.9 ± 4.1 mN to +4.6 ± 2.3 mN, which corresponded to a reduction of 48.3 %.

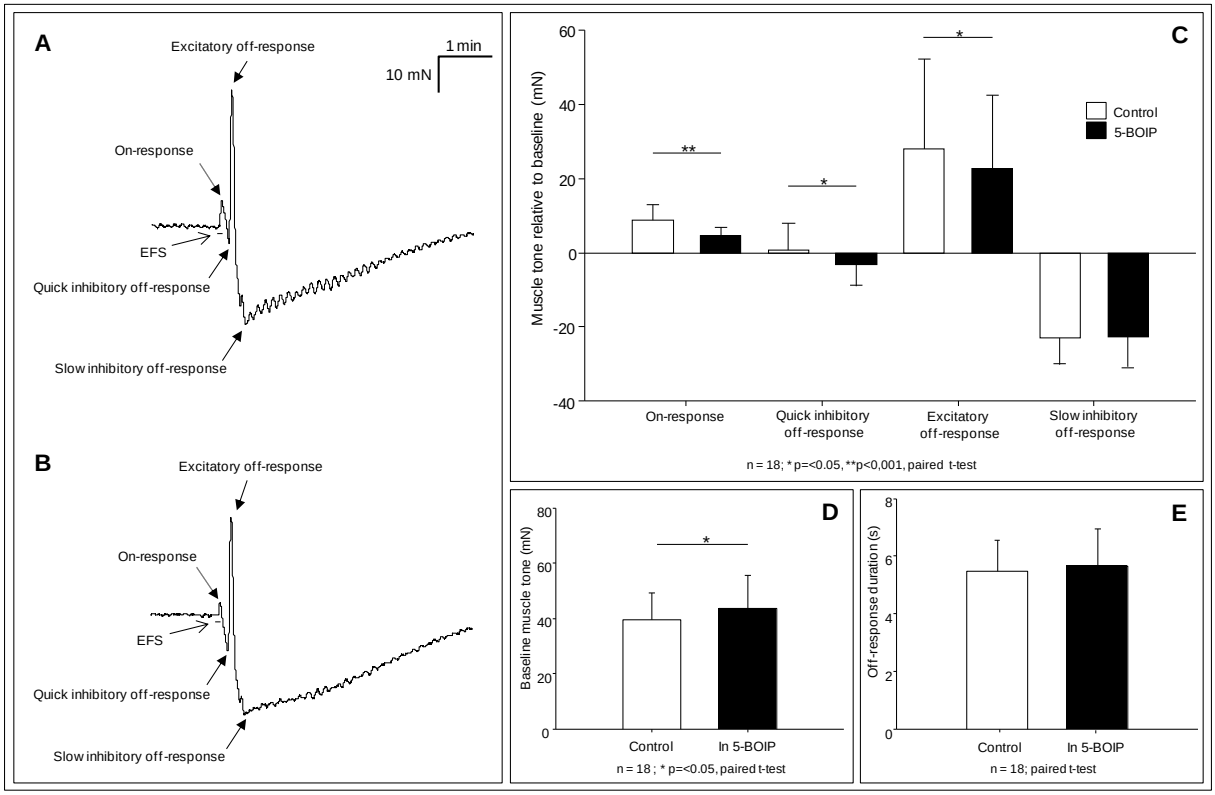

Figure 11. 5-BOIP reduced on-response as well as fast inhibitory and excitatory off-response of guinea pig gastric circular muscle tissueto electrical field stimulation in the organ bath.
A/B: Effect of the electrical field stimulation (EFS, 100 V, 10 Hz, 10 s, marked by a small bar below the trace) on the muscle tone under control conditions (A) and during 5-BOIP (B). The different parts of the response are marked with arrows. The on-response as well as the excitatory off-response are higher before the perfusion of the 5-HT_{1P} agonist than after. Also the inhibitory off-response is strenghtend by 5-BOIP. C: Results for the analysis of on- and off-response to the EFS. All values presented in this figure are relative to the baseline. 5-BOIP reduced the excitatory responses, while strengthening the quick inhibitory off-response. The quick inhibitory off-response was characterised by a sharp decrease from the peak of the on-response. However, this decrease not always went below the baseline, which is why the mean before 5-BOIP shows a positive value. The slow inhibitory off-response did not change significantly. D: Effect of 5-BOIP on baseline tone. Intrestingly there was a significant increase in the muscle tone baseline. E: The agonist did not affect the off-response's duration.

But not only the on-response was significantly altered by the 5-HT_{1P} agonist; after perfusion the inhibitory off-response was increased from +0.7 ± 7.2 mN to -3.2 ± 5.7 mN while the excitatory off-response decreased from +28.1 ± 24.0 mN to +22.8 ± 19.7 mN. The slow inhibitory off-response, however, did not change significantly (Control: -22.9 ± 7.2 mN / in 5-BOIP: -22.6 ± 8.1 mN), neither did the duration of the off-response, which was 5.5 ± 1.1 s in the control and 5.6 ± 1.3 s during 5-BOIP. Initially, the effects of 5-BOIP were tested in the guinea pig myenteric plexus. 5-BOIP (50 µM) was applied on the ganglia via spritz application. It triggered APs in 23.8 ±

6.2 % of the 192 neurons from 6 animals, with an average AP frequency of 1.6 [1.0/3.1] Hz (Figure 12). These effects were decreased to 5.4 ± 6.9 % of the neurons responding with 0.0 [0.0/0.0] Hz by the 5-HT_{1P} receptor antagonist 5-HTP-DP, which was perfused for 20 min immediately after the first application of the agonist. It has to be noted, that despite the median and the 25/75 percentile for the AP frequency during 5-HTP-DP being 0.0, nine out of 48 neurons were still responding during 5-HTP-DP at a spike frequency of 1.4 Hz [1.1/1.6]. In 5 of these neurons the AP frequency was reduced though, while two neurons showed an increase. In total this means that the AP frequency of the late onset response to 5-BOIP is reduced by the 5-HT_{1P} antagonist in in 44 out of 48 neurons (91.7 %). The number of neurons responding to 5-BOIP is reduced by 77.3 % during 5-HTP-DP. After 60 minutes of washing out the antagonist the response to 5-BOIP recovered: 19.6 ± 4.6 % of the neurons responded with an average of 1.1 [0.5/2.1] Hz.

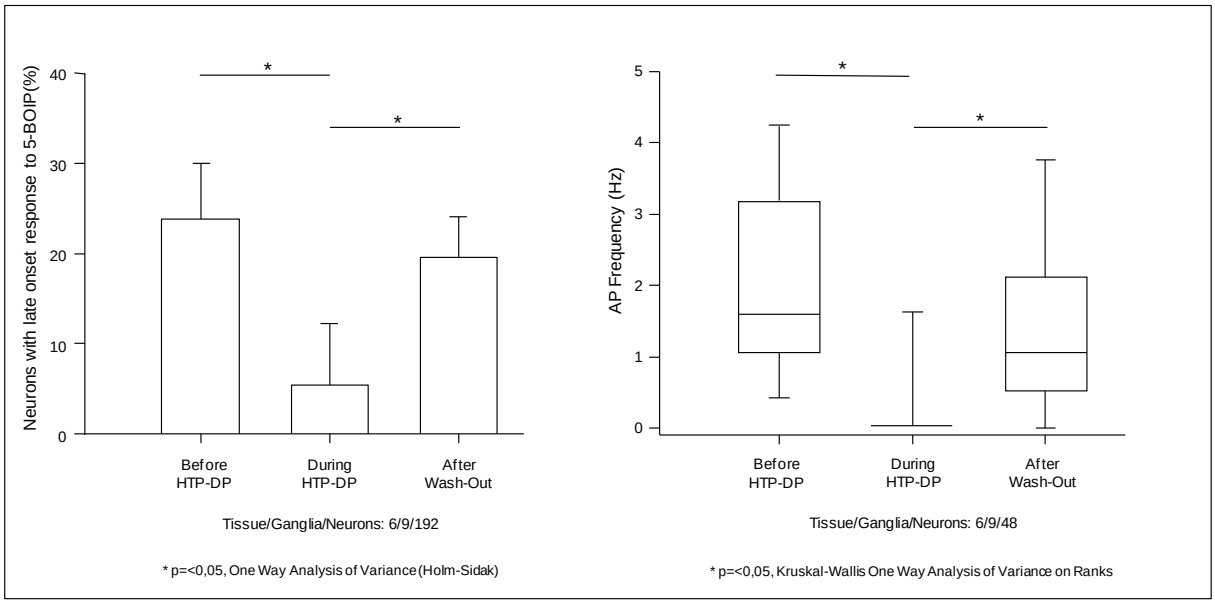

Figure 12. Effect of 5-HTP-DP on the late onset response caused by the 5-HT_{1P} agonist 5-Benzoxyindalpine (5-BOIP) in guinea pig myenteric neurons.
Number of cells responding (left) and AP frequency (right). 5-BOIP (50 µM, 400 ms) evoked a response in 23.8 % of the neurons. This response is significantly reduced after 5-HTP-DP perfusion (20 min, 10 µM) in almost all neurons, though some of them still fire few APs. But most of the AP evoked by 5-BOIP are completely blocked by 5-HTP-DP. After wash-out the response to 5-BOIP recovers significantly.

The 5-BOIP (50 µM) was then also applied on human submucous ganglia. In five neurons from five ganglia in three different tissues 5-BOIP triggered no immediate response (0-1.7 s after application). In total 61 neurons in eight ganglia of tissue from five patients were analysed (Figure 13).

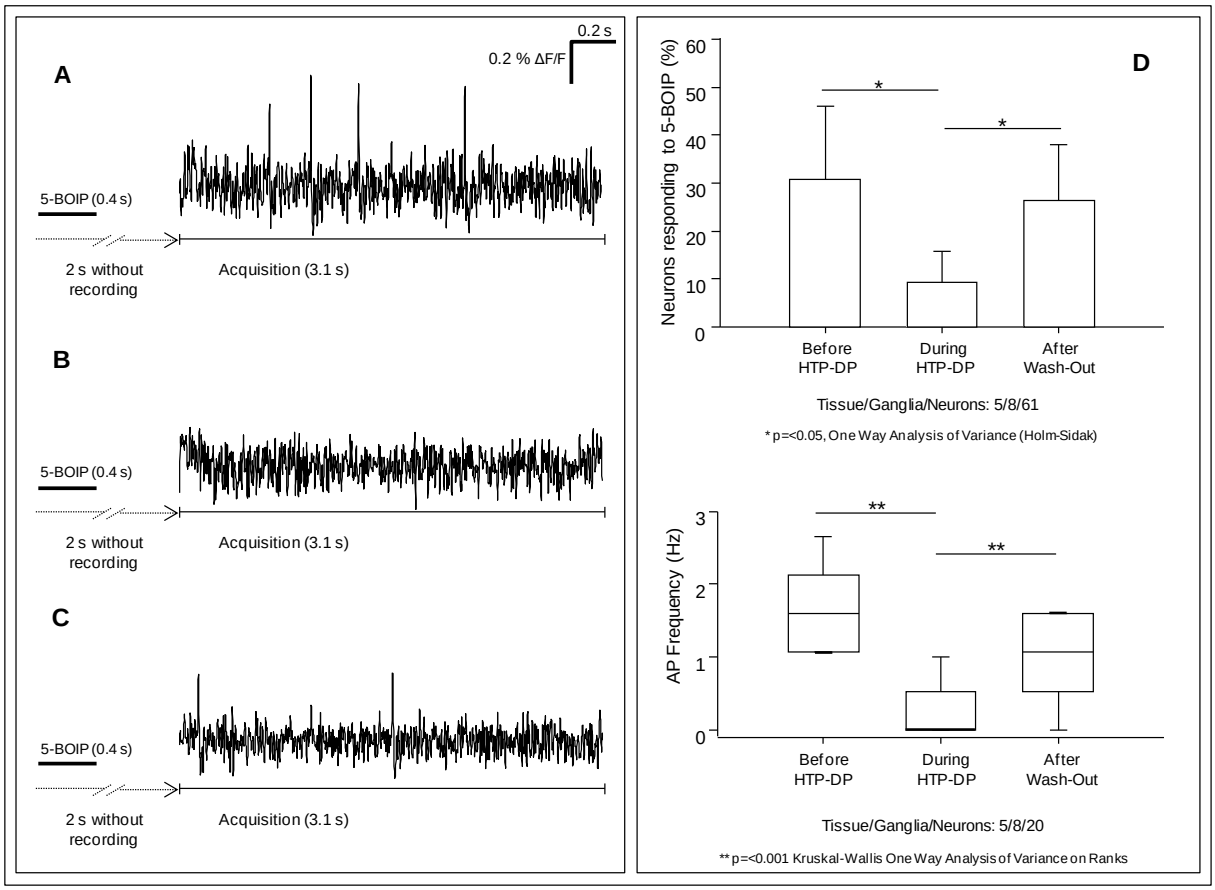

Figure 13. Effect of 5-HTP-DP on the late onset response to 5-BOIP in the human submucous plexus.

A: Late onset response to 5-BOIP (50 µM, 400ms) spritz application. Like 5-HT before, 5-BOIP was applied 2s before the start of the acquisition. The recorded traces thus display the neuron's late onset response to the agonist, which in this case consists of 4 APs. B: Response to the 5-BOIP application after perfusion of 5-HTP-DP (10 µM, 20 min). The neuronal response to the agonist is blocked by the 5-HTP-DP. C: After wash-out of the 5-HT_{1P} antagonist, the neuronal response to 5-BOIP consists of two AP. D: Effects of 5-HTP-DP on the percentage of neurons with a late onset response to 5-BOIP and on the AP frequency evoked by the agonist. 5-HTP-DP significantly reduces the AP-frequency as well as the number of cells responding to 5-BOIP. This reduction recovers significantly after the wash-out of the antagonist (60 min).

In 30.9 ± 15.2 % of the neurons the application of the agonist evoked a late onset response. The average AP frequency was 1.6 [1.0/2.1] Hz. After the perfusion of 5-HTP-DP (20 min, 10 µM) APs remained in 9.4 ± 6.4 % of the neurons, with an average frequency of 0.0 [0.0/0.5] Hz. Out of the 20 neurons with a late onset response to 5-BOIP, 6 neurons still fired APs (0.5 [0.5/0.9] Hz). But in all of them the values are lower in 5-HTP-DP than before the perfusion. So 5-HTP-DP reduces the AP frequency in all of the neurons that showed a late onset response to 5-BOIP. The percentage of neurons responding is reduced by 69.6 % (from 30.9 % to 9.4 %). With the antagonist being washed out, the response to 5-BOIP recovered to values of 26.3 ± 11.7 % and 1.0 [0.5/1.6] Hz.

3.1.4. The 5-HT$_4$ receptor

Effects of the 5-HT$_4$ receptor agonist 5-methoxy-tryptamine (5-MeOT)

Like for the 5-HT$_{1P}$ receptor, first the postsynaptic effect of the 5-HT$_4$ receptor agonist 5-methoxytryptamine (5-MeOT) was tested. After a control electrical stimulation with 20-70 µA, 5-MeOT (100 µM) was applied via spritz application on human submucous neurons (60/9/5). Though 87.8 % of the neurons showed fEPSPs after electrical stimulation, not a single one responded to the 5-HT$_4$ agonist with APs. 5-MeOT spritz application evoked no late onset response in the human submucous plexus (3/5/36). As mentioned in the introduction the 5-HT$_4$ receptor is presumed to have an influence on the presynaptic activity of enteric neurons.

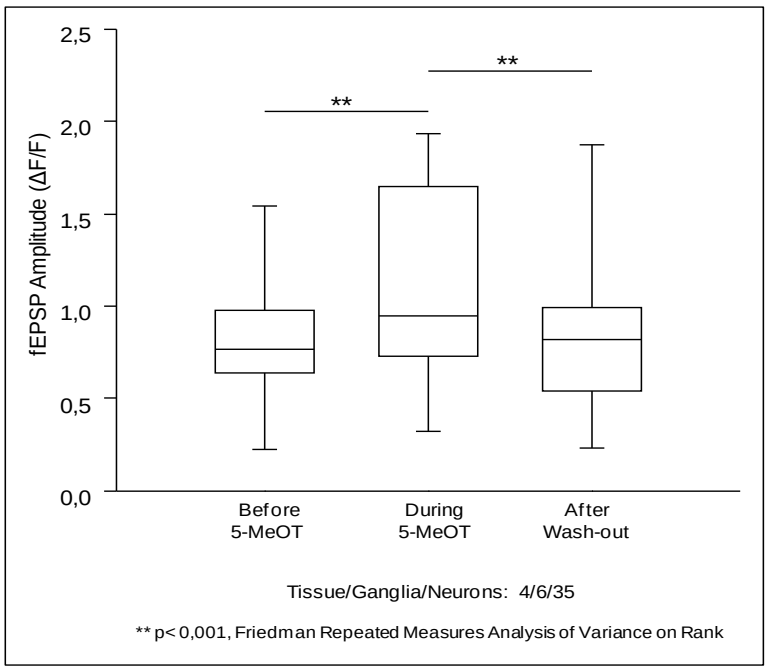

Figure 14. Effect of 5-MeOT on the fEPSP.
fEPSP amplitiudes evoked by electrical fiber tract stimulations before, in perfusion of and after wash-out of 5-MeOT (10 µM, perfused for for 20 min). There is a significant and reversible increase in fEPSP amplitude in the presence of the 5-HT$_4$ agonist 5-MeOT.

Thus, after 5-MeOT showed no effect on postsynaptic excitability of the human submucous plexus, its influence on the fEPSPs was investigated. fEPSPs were evoked by electrical stimulation of interganglionic fibre tracts as described in Materials and Methods (see chapter 2.2.4 and 2.2.5). Three stimulations were applied. The first served as a control, the second electrical stimulation was given after perfusing 5-MeOT (10 µM) for 20 minutes and a third one was applied after further 45 min of wash-out (Figure 14). 5-MeOT significantly increased fEPSP amplitude by 20.3 % in comparison to control stimulations in 35 neurons from four patients).

Effects of the 5-HT_4 receptor agonist prucalopride

The novel 5-HT_4 agonist prucalopride shows a higher specificity and affinity for the 5-HT_4 receptor than 5-MeOT. The spritz application on the ganglion was used to check for any effects on the basal activity. A representative trace for the application of prucalopride and for an electrical stimulation, which served as a control for the integrity of the neuronal network, from one neuron is shown in
Figure **15**. The electrical stimulus triggers a compound action potential (CAP), which represents the non-synaptic axonal signal, and a synaptically mediated fEPSP from the cell body (as can be seen in
Figure **15**B). But like 5-MeOT, prucalopride did not evoke any action potential after spritz application, although the fEPSPs to electrical stimulation in 70.8 ± 27.7 % showed viability of the neurons. Four ganglia with 24 neurons from three patients were analysed and concentrations from 1 µM to 100 µM were tested. Tissue from the same three patients also served for testing the late onset response to prucalopride (3/3/20). Like before the agonist did not evoke any AP, although 63.5 ± 27.6 % of the neurons showed fEPSPs to electrical stimulation.
To investigate actions of prucalopride on fEPSPs, interganglionic fibre tracts were electrically stimulated. Three stimulations were applied. The first and second stimulations were applied before the perfusion of prucalopride. The second control stimulation allowed checking for natural variability of the responses. The third electrical stimulation was applied after perfusing prucalopride (10 µM) for 20 minutes. For 35 neurons from five ganglia in four patients the duration of the fEPSPs was measured. 28.6 % (10 out of 35) of the neurons responded with increased fEPSP duration in prucalopride compared to the natural variance. For the definition of an increase in fEPSP duration and length compared to the natural variance see chapter 2.2.5 in the methods section.

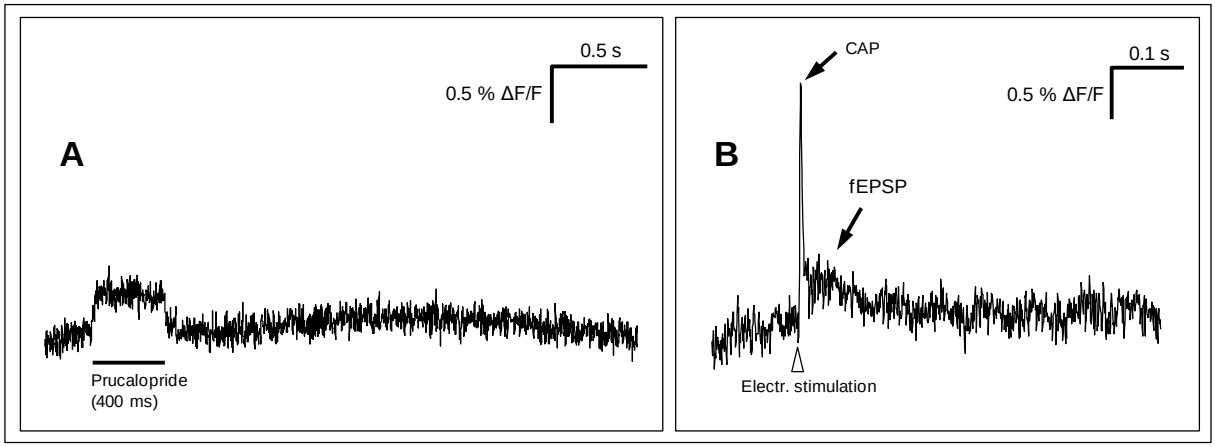

Figure 15. The 5-HT$_4$ agonist prucalopride evokes no postsynaptic effect in a human submucous neuron.

A: Spritz application of prucalopride (100 µM, 400 ms) has no effect on this neuron. The black bar below the trace displays the beginning and duration of the application. The increase in the baseline during this application is an artefact caused by tissue movement during the pressue pulse ejection. B: Electrical stimulation as control for the integrity of the neuronal network. The displayed neuron shows a compound action potential (CAP) and fEPSP in response to the electrical stimulation. The triangle marks the moment of the electrical stimulus (50 µA, 0.6 ms). The evoked CAP and fEPSP are marked with arrows.

In the presence of prucalopride the fEPSP duration increased on average by 31.9 %, from 96.8 ± 36.2 ms to 142.1 ± 60.7 ms. The variability between the two control stimulations was 14.1 ± 9.9 ms (14.6 %). The calculation of the fEPSP amplitude showed that prucalopride evoked an increase in 14 out of 35 neurons (34.0 %, 3/4/35). For these neurons the average fEPSP amplitude was 0.38 ± 0.24 % ΔF/F for the first stimulation and 0.37 ± 0.24 % ΔF/F for the second, which resulted in a variability of 0.05 ± 0.03 ΔF/F (13.1 %). The perfusion of the 5-HT$_4$ agonist significantly increased the fEPSP amplitude to 0.57 ± 0.28 % ΔF/F which corresponded to an increase in the fEPSP amplitude of 34.2 %.

The area value (Δ%s) was calculated via an area-under-curve analysis in Igor Pro 6.04 (4/5/50). Results show that prucalopride increased the area value in 34 % of the neurons (17 out of 50, Figure 16). For these neurons, the area values for the control stimulations before prucalopride amounted to 0.021 [0.013/0.028] Δ%s and 0.027 [0.012/0.034] Δ%s, resulting in a natural variance of 0.004 [0.002/0.007] Δ%s or 16.7 %. In prucalopride the area value increased to 0.059 [0.024/0.074 Δ%s]; an average increase of 46.9 %.

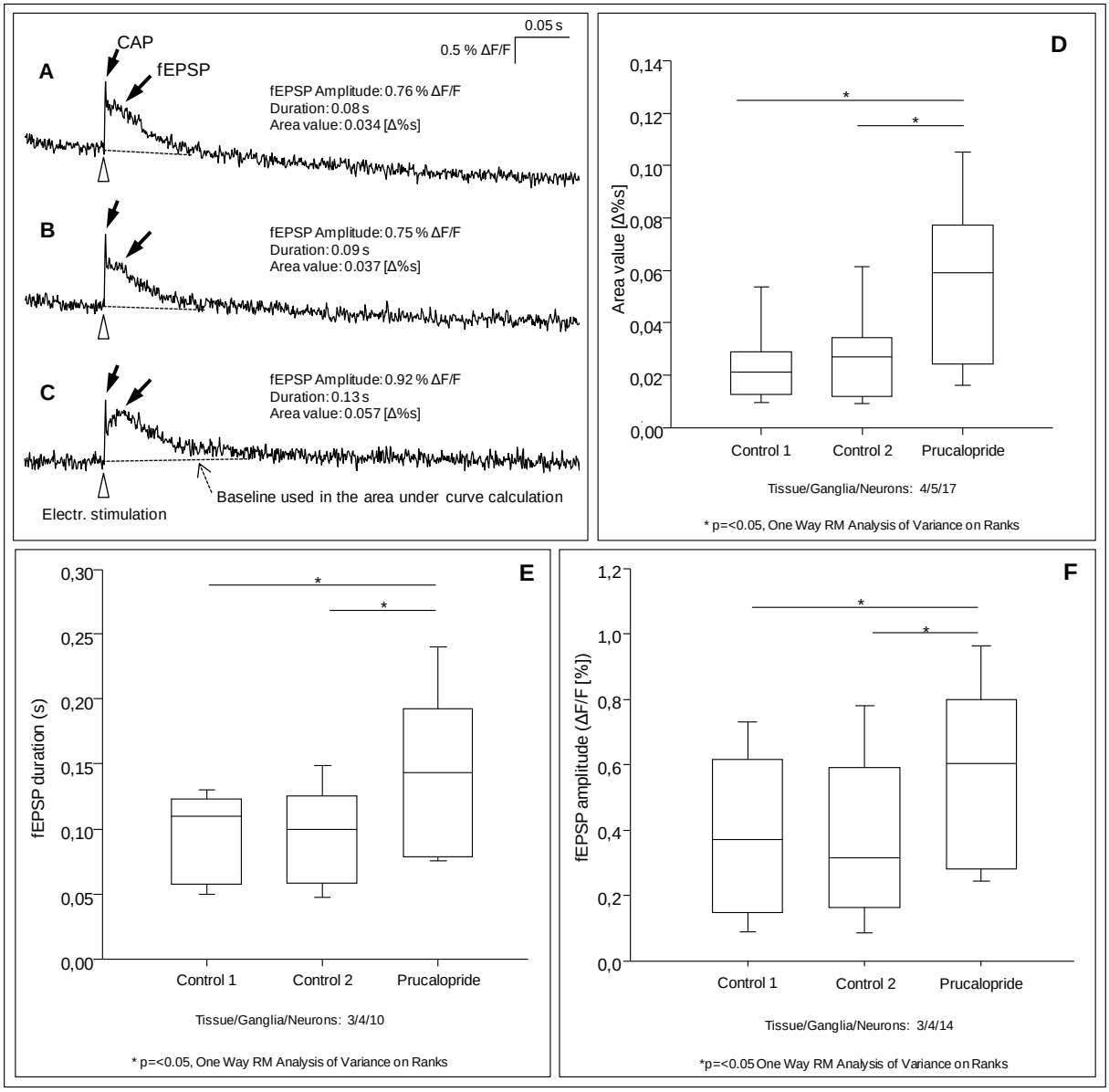

Figure 16. Area under curve calculation for the effect of prucalopride on fEPSPs in response to electrical stimulation in human submucous neurons.

A/B: Traces of the two control stimulations, which evoked CAPs and fEPSPs. Values for fEPSP amplitude, duration and area value are presented. The natural variance between the two identical control stimulations in this neuron is low, 0.01 % ΔF/F for the fEPSP's amplitude, 0.01 s for the duration and 0.003 [%Δs] for the area value. C: Electrical stimulation after perfusion of prucalopride (10 µM, 20 min). The trace shows a clear increase in fEPSP amplitude, duration and area value. For all traces the electrical stimulation (30 µA) is marked by a triangle below the trace and the arrow marks CAP and fEPSP. A dotted line marks the baseline used for the area under curve calculation in IgorPro 6.04©. D: Results of the calculation of the area value for the responsive neurons. The 5-HT$_4$ agonist prucalopride evokes an area value significantly increased to both of the controls, which show no significant difference between each other. E/F: Results for the calculation of fEPSP duration and amplitude. Prucalupride also facilitates the both duration and amplitude of the fEPSP. Again the natural variances represent no significant difference.

Effect of the 5-HT$_4$ antagonist piboserod on the response to prucalopride

The next step was to see whether the fascilitating effect of prucalopride could be blocked with the specific 5-HT$_4$ antagonist piboserod. Thus, after the two control fEPSPs followed by the perfusion of prucalopride, piboserod was perfused (1 µM, 20 min) and another electrical fibre tract stimulation was conducted (4/5/38, Figure 17). Prucalopride increased the fEPSPs in 15 neurons (39.5 %). The fEPSP amplitude for these responsive neurons rose to 0.57 ± 0.28 ΔF/F[%], an increase of 34.7 % compared to the control. The control stimulations themselves (0.39 ± 0.24 ΔF/F[%] and 0.37 ± 0.25 ΔF/F[%]) showed no significant difference. The calculated natural variability amounted to 0.05 ± 0.03 ΔF/F[%]. Piboserod reversed the effect of prucalopride on the fEPSP amplitude to a level not significantly different from the control stimulations (0.44 ± 2.7 ΔF/F[%]). After the wash-out of piboserod a significant recovery of the prucalopride induced increase in the fEPSP amplitude to 0.55 ± 0.27 ΔF/F[%]) was detectable, 33.2 % higher than the control. Lastly, the agonist was also washed out which lead again to a normalisation of the fEPSP ratio to 0.42 ± 0.22 ΔF/F[%]).

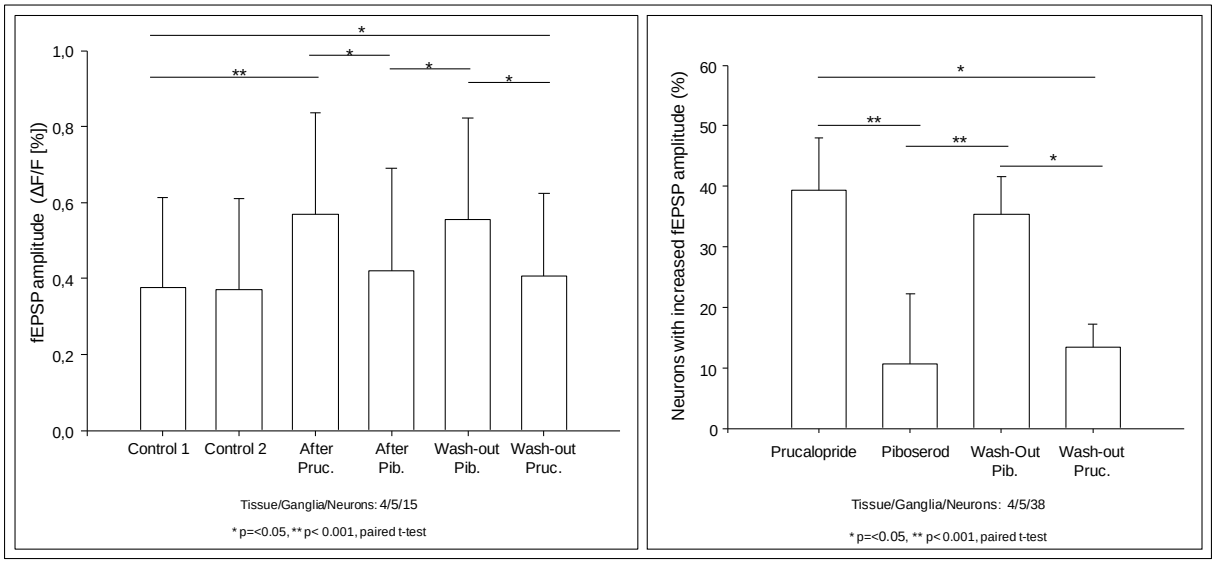

Figure 17. Piboserod reversibly blocks the agonistic effects of prucalopride in human submucous neurons.

The two control stimulations show no significant variance for the fEPSP amplitude (left). The perfusion of the 5-HT$_4$ agonist prucalopride (Pruc, 10 µM, 20 min) however caused a significant increase in the EPSP/baseline ratio that was blocked by the corresponding antagonist piboserod (Pib, 1 µM, 20 min). This blocking effect disappeared after piboserod wash-out (30 min). Also after the agonists' wash-out the ratio returned to the level of the control stimulation. The analysis of the percentage of responsive neurons per ganglion (right) shows that the increasing effect of prucalopride on the EPSP was clearly reduced by piboserod and reappeared after the antagonists' wash-out. After 30 min wash-out of prucalopride the increasing effect decreased significantly.

Piboserod blocked the effect of prucalopride in 11 out of 15 neurons (73.3 %). In the remaining four neurons, the fEPSP amplitude was reduced, too, but still higher than two times the natural variance. For the responsive neurons also the amplitude of the compound action potential was evaluated in order to check for possible effects of prucalopride on fast sodium channels. It turned out that prucalopride had no significant effects on the compound action potential (
Figure **18**): The CAP amplitude was for 1.01 ± 0.56 ΔF/F[%] for the first control stimulation, 0.93 ± 0.50 ΔF/F[%] for the second and 0.94 ± 0.57 ΔF/F[%] during prucalopride.

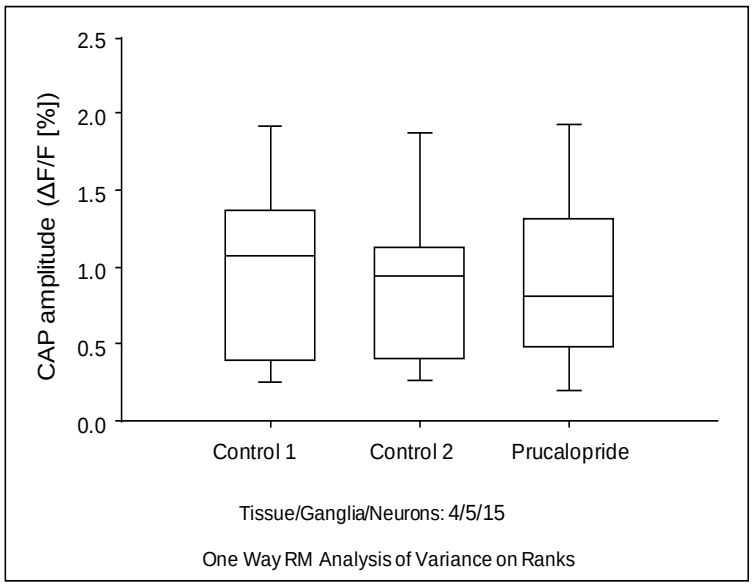

Figure 18. Influence of prucalopride on the compound action potential.
The compound action potentials (CAP) show no significant difference between the two control stimulations and the response after prucalopride perfusion (10 µM, 20 min).

The antagonist piboserod was also tested for any possible involvement in the late onset response to 5-HT. For this purpose 5-HT (1 mM) was applied via spritz application directly onto the ganglion. This evoked late onset APs in 38.2 ± 13.6 % of the submucous neurons. Then piboserod (1 µM, 20 min) was perfused with the Krebs solution. As displayed in
Figure **19** the late response to 5-HT did not change significantly neither after the perfusion of piboserod (37.5 ± 8.4 %) nor after 60 min of wash-out (36.7 ± 13.26 %).

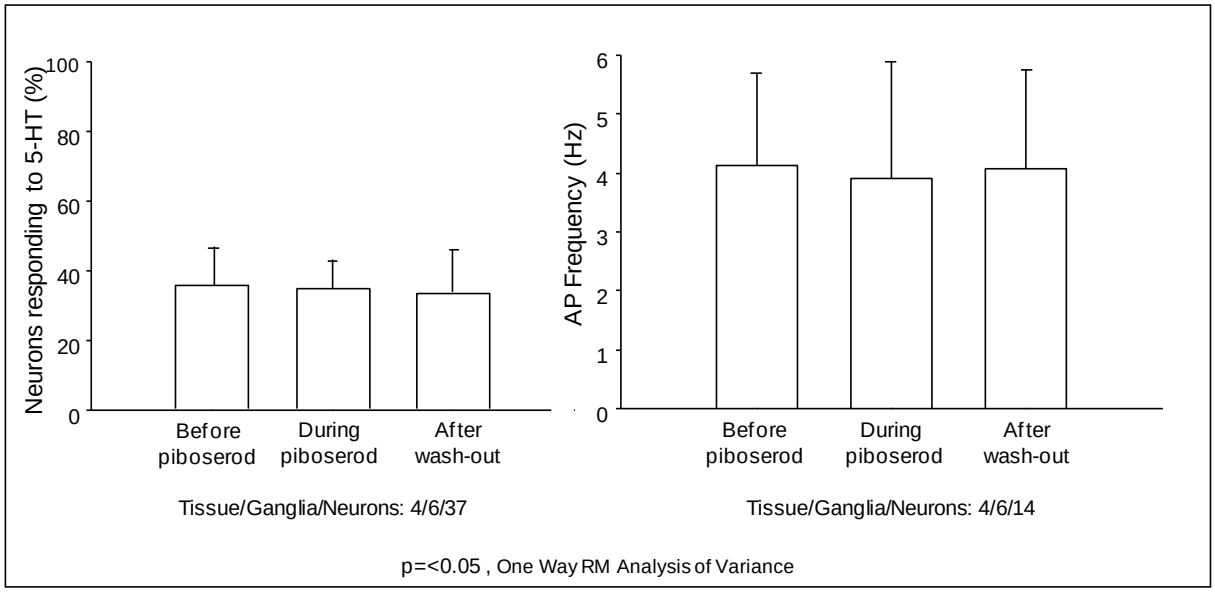

Figure 19. Effect of piboserod on the late onset 5-HT response.

The late onset response to 5-HT (1 mM, 400 ms) for both percentage of cells responding (left) and AP frequency of the responsive cells (right) shows no significant changes to perfusion of the 5-HT_4 antagonist piboserod (1 µM, 20 min).

3.1.5. The 5-HT_7 receptor

Actions of the 5-HT_7 receptor were supposed to be revealed by the 5-HT_7 receptor agonist 5-Carboxytryptamin (5-CT). It was applied via spritz application in both guinea pig myenteric (14/21/479) and human submucous neurons (6/9/71). A concentration of 50 µM was used for the working solution, later also other concentrations ranging from 1 µM up to 1 mM were tested (in human: 3/4/21; in guinea pig: 2/3/66).

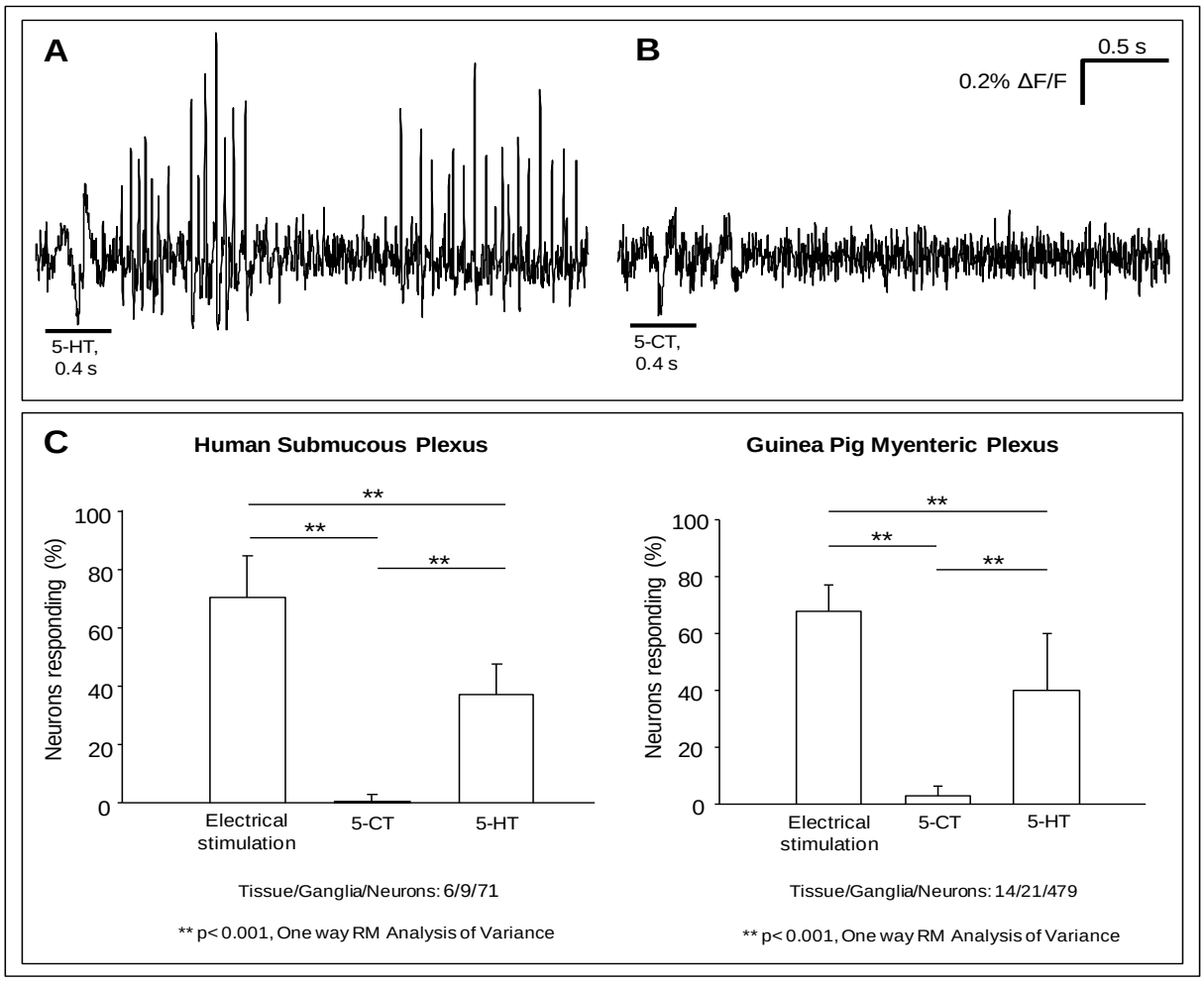

Figure 20. Effect of 5-CT on human submucous and guinea pig myenteric neurons.
A/B: Response of the same human submucous neuron to 5-HT (A, 1 mM, 400 ms) and 5-CT (B, 50µM, 400 ms). 5-HT evokes a clear response in this neuron, whereas the 5-CT application triggers no APs at all. Blackbars below the traces mark the duration of the application. C: In both human submucous plexus (left) and in guinea pig myenteric plexus (right) almost no effect of the 5-HT_7 receptor agonist was traceable, although 5-HT and electrical stimulation evoke clear responses as would have been expected.

An electrical stimulation of a neuronal fibre tract was applied to serve as a positive control for the integrity of the neuronal network and additionly 5-HT was spritzed onto the ganglia 10 to 20 min after application of 5-CT. In the guinea pig 5-CT evoked APs in 2.6 ± 8.8 % of the neurons, whereas 40.1 ± 10.1 % of the same neurons responded to 5-HT (Figure 20).

The AP frequency of the 5-CT responsive neurons was 0.8 ± 0.4 Hz. 67.7 % of the neurons showed fEPSPs in response to electrical fiber tract stimulation. In human submucous plexus only 0.7 ± 2.2 % of the neurons responded to the spritz application of 5-CT with an AP frequency of 0.5 ± 0.5 Hz. Statistically the percentage of neurons responding to 5-CT in human was not significantly different from 0 ($p=0.34$, one sample t-test, performed with Graph Pad© Online Calculator). However, 37.2 ± 10.5 % of the analysed neurons were responsive to 5-HT and 70.2 ± 14.4 % to electrical stimulation.

3.2. Immunohistochemistry

The following chapter describes the results of the antibody staining with the new Santa Cruz antibodies against 5-HT_4 and 5-HT_7 receptors in both guinea pig and human tissue. For every tissue the “neuron specific enolase antibody” (NSE) served as a pan-neuronal marker. Every antibody was tested in guinea pig and human tissue preparations. For both guinea pig and human tissue, the antibodies were used in myenteric as well as in the submucous plexus preparations. For human tissue additionally, cross section preparations were stained.

3.2.1. 5-HT_7 receptor stainings

In the myenteric plexus of guinea pig ileum, the SR-7 antibody revealed a staining at dilutions of 1:200, 1:500 and 1:1000. The analysis of 108 ganglia in four tissues revealed 776 out of 3054 cells that were stained by SR-7 (25.4 %). In the submucous plexus of the guinea pig ileum the 5-HT_7 receptor also seems to be present (Figure 21). Here 91 out of 798 cells from 76 ganglia in 3 tissues (11.4 %) showed a staining by the SR-7 antibody. The antibody seemed to deliver the best results at a dilution of 1:500 or 1:1000. For the human submucous and myenteric plexus, no reliable SR-7 staining could be obtained. In total five tissues of human colon and one sample of human ileum were stained. In cross section (3 colon, 3 ileum) some staining was observed in epithelial cells (Figure 21 C2/3), however, their identity was not determined.

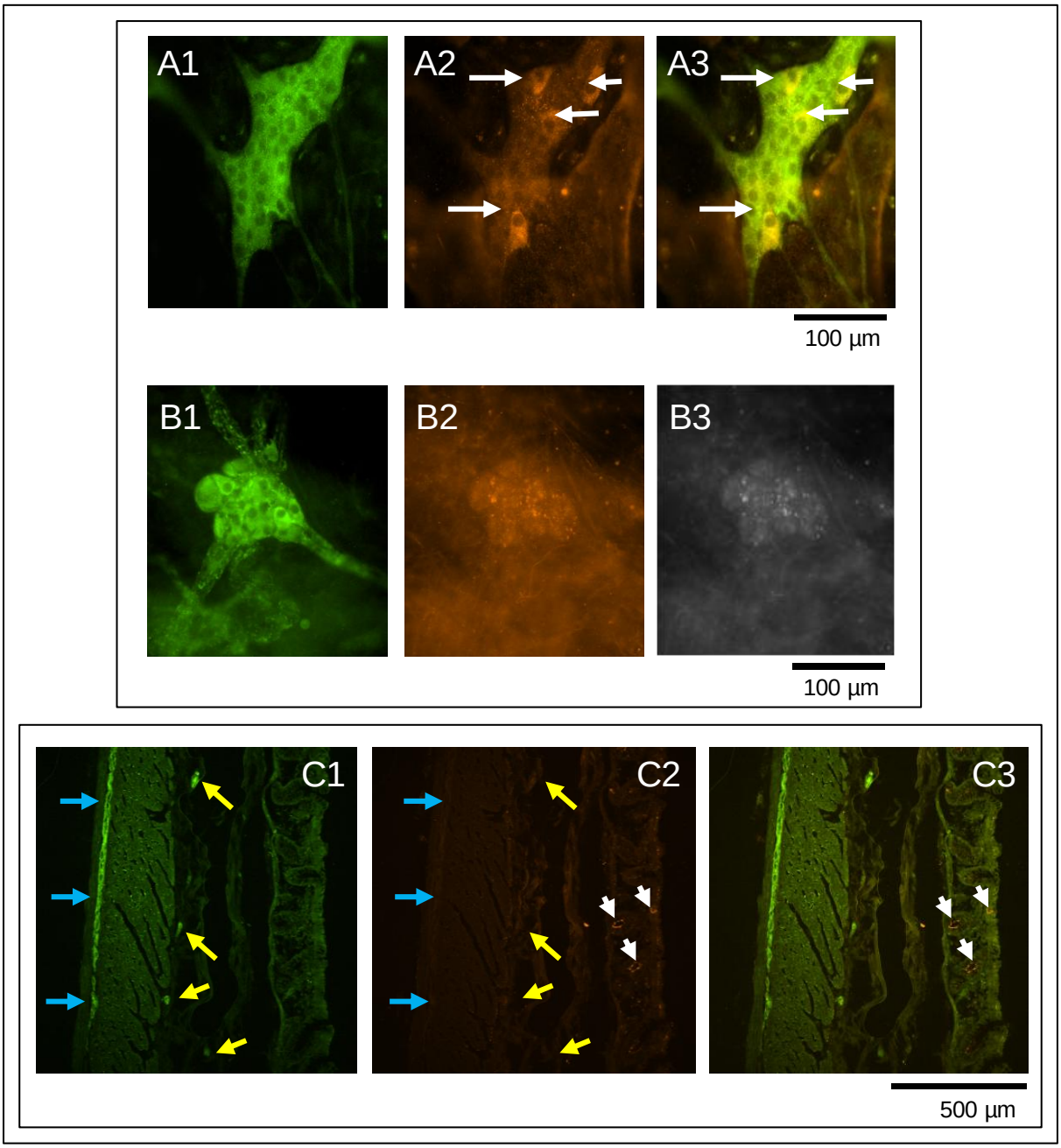

Figure 21. 5-HT$_7$ Receptor staining in guinea pig and human tissue.
A: 5-HT$_7$ receptor antibodies stained several cells in the submucous plexus of guinea pig ileum. From the displayed ganglion's 32 NSE positive cells (A1, rabbit anti-NSE, 1:2000) there are four cells that show a staining with SR-7 (A2, goat anti-SR-7, 1:500). This can also be seen in the overlay (A3, merged picture+Cy3). B: The SR-7 antibody reveals no specifically stained cells in the inner submucous plexus of human sigmoid colon (B1: rabbit anti-NSE, 1:2000; B2: goat anti-SR-7, 1:1000; B3 "autoflourescence"). C: 5-HT$_7$ receptor antibodies in a wholemount section of human colon staining no cells in both myenteric and submucous plexus. Blue arrows mark the position of the layer with NSE stained Myenteric cells, yellow arrows the position of NSE stained submucous cells. The NSE stained neurons (C1, rabbit anti-NSE, 1:2000) are not stained by the 5-HT$_7$ antibody (C2, goat anti-SR-7, 1:1000). There were however some SR-7 stainings in the mucosal layer, illustrated by white arrows. These results can also be seen in the overlay picture (C3).

3.2.2. 5-HT$_4$ receptor stainings

In the myenteric and submucous plexus of the guinea pig the 5-HT$_4$ receptor antibody (SR-4) revealed unspecific staining, even at dilutions of 1:2000 or 1:5000 (Figure 22). Likewise, the SR-4 antibody appeared to yield unspecific staining in human submucous and myenteric plexus (tissue from six patients) even at 1:5000 dilution, the NSE positive cells still show only unspecific staining by the SR-4 antibody.

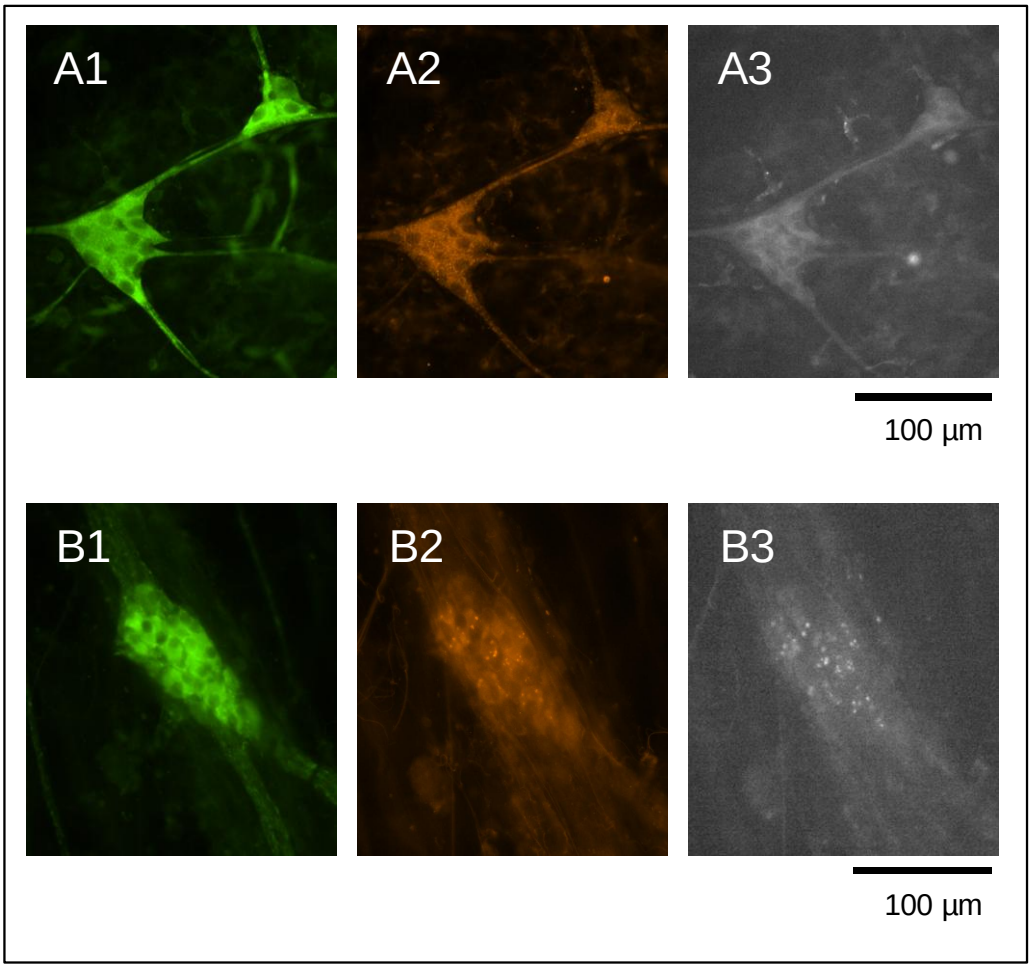

Figure 22. Unspecific staining of neurons and background by the SR-4 antibody in the submucous plexus of guinea pig and human.
A: Submucous ganglion in guinea pig ileum. NSE staining (A1, rabbit anti-NSE, 1:2000), SR-4 staining (A2, goat anti-SR-7) and the autofluorescence control (A3). The SR-4 antibody causes autofluorescence in the neurons, but there are no specific SR-4 stainings visible. B: NSE staining (B1, rabbit anti-NSE, 1:2000), SR-4 staining (B2, goat anti-SR-4, 1:5000) and autoflourescence control for a human submucous ganglion. Like before in the guinea pig the NSE positive cells show unspecific staining by the SR-4 antibody, which stained a lot of connective tissue too.

No SR4-staining was detected cross-sections of the human intestine (four tissues). Like for the 5-HT_7 receptor antibody, unidentified SR-4 positive cells were present in the epithelium (Figure 23). A co-staining with 5-HT antibodies was performed. It turned out that the SR-4 positive areas do not overlap with the 5-HT stained one.

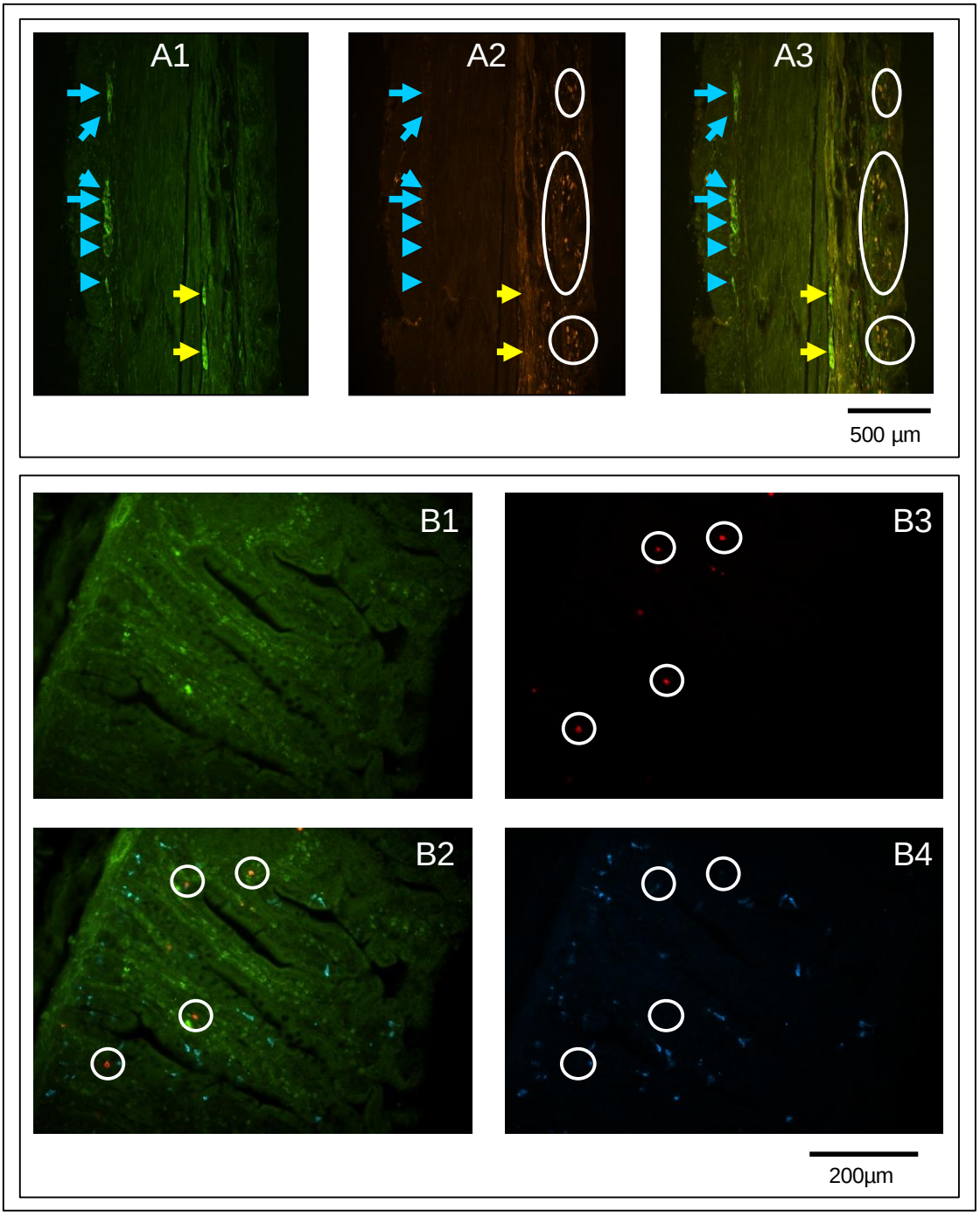

Figure 23. Effects of 5-HT_4 receptor antibodies in cross section preparations of human.
A: NSE staining (A1, rabbit anti-NSE, 1:2000), SR-4 staining (A2, goat anti-SR-7, 1:5000) and overlay picture (A3) of a cross section preparation through a human gut wall. Blue arrows mark the layer with NSE stained myenteric cells, yellow arrows NSE positive submucous cells. In the SR-4 staining

unspecific effects on connective tissue appear in the submucous layer, but none of the marked neurons of both plexus show a positive reaction for SR-4. However, many SR4-stained structures in the mucosa are clearly visible, highlighted by white circles in picture A2 and A3. B: High resolution pictures of the mucosal region of a cross section preparation with co-localisation of SR-4 and 5-HT antibody stainings (human ileum, B1 rabbit anti-NSE, 1:2000; B2: goat anti-SR-4, 1:5000; B3: rat anti-5-HT 1:100; D: overlay picture). Several SR-4 positive structures were found in the mucosa (highlighted with white circles). They are not co-localised with the 5-HT staining as can be seen in the overlay picture (C4).

3.3. Comparing serotonin, histamine and PAR-2 actions between the myenteric and the submucous plexus

An altered mucosa-nerve signalling involving serotonin, histamine and tryptase may be an underlying mechanism of IBS (Buhner et al., 2009; Buhner and Schemann, 2012). Therefore, sensitivity of myenteric and submucous neurons of guinea-pig colon to the individual mediators was investigated. For all experiment both plexus were taken from the same animal. 5-HT was applied to 14 ganglia of 7 guinea pigs. With a median of 55.2 [53.8/56.3] % of the neurons responding, the submucous plexus was significantly more sensitive to the 5-HT spritz application than the myenteric plexus. Here 5-HT evoked APs in 38.6 [36.4/44.0] % of the neurons. The application of histamine and of the PAR-2 agonist SLIGRL on the other hand yielded no significant differences. In the myenteric plexus 21.3 [15.5/37.3] % of the neurons responded to histamine (8/12/279) and 38.2 [33.3/44.2] % to SLIGRL (7/12/230).

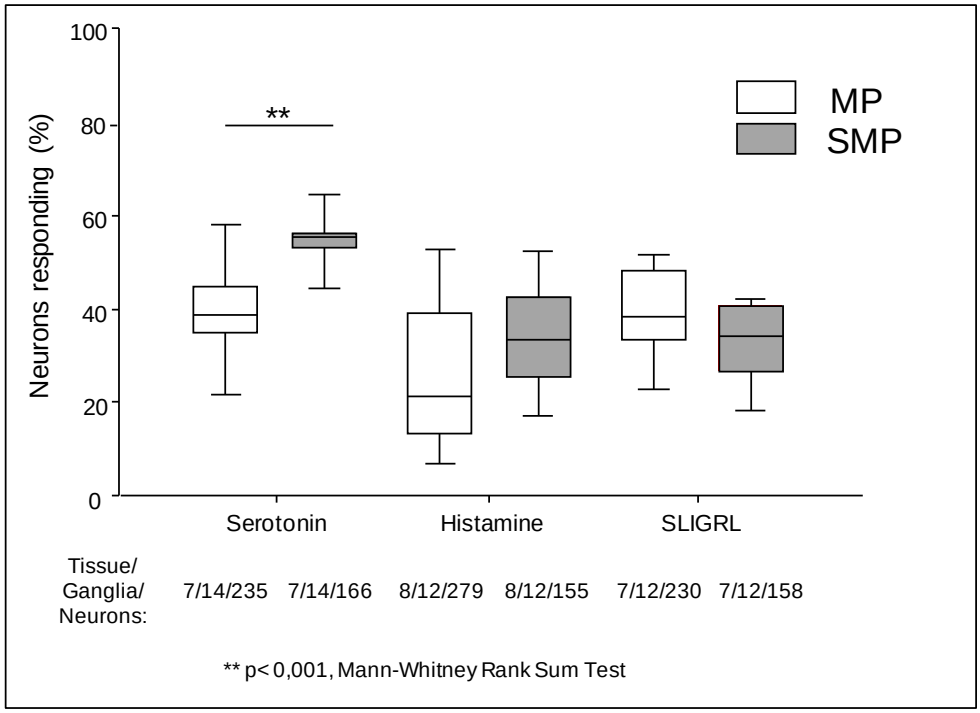

Figure 24. Comparison between myenteric and submucous Plexus: Percentage of neurons responding to 5-HT, histamine and SLGRL application.

The percentage of neurons responding to 5-HT is significantly higher in the SMP. For histamine and SLIGRL the difference between the two plexus is not significant.

The application of histamine and of the PAR-2 agonist SLIGRL on the other hand yielded no significant differences. In the myenteric plexus 21.3 [15.5/37.3] % of the neurons responded to histamine (8/12/279) and 38.2 [33.3/44.2] % to SLIGRL (7/12/230). In the submucous plexus histamine activated 33.3 [15.5/42.0] % of the neurons (8/12/155) on average while SLIGRL evoked APs in 34.2 [29.3/39.1] % (7/12/158) (Figure 24). Not only that the submucous plexus contained more 5-HT responsive neurons than the myenteric plexus, but those submucous neurons also fired more action potentials in response to 5-HT. 91 out of 166 submucous neurons fired in response to a 5-HT spritz at a frequency of 8.5 [6.4/11.1] Hz, whereas 92 out of 235 myenteric neurons responded with a lower spike frequency of 6.4 [4.2/8.0] Hz. The AP frequency in response to histamine was comparable between the two plexus: the firing rate was 3.7 [2.6/5.3] Hz in 52 out of 155 neurons for the submucous plexus and 3.2 [/2.1/5.1] Hz in the myenteric plexus (67 out of 279 neurons). Likewise, SLIGRL evoked a similar response in that 54 out of 158 submucous neurons discharged APs at 2.7 [2.1/4.9] Hz and 87 out of 230 myentric neurons at 2.6 [1.6/5.4] Hz (Figure 25).

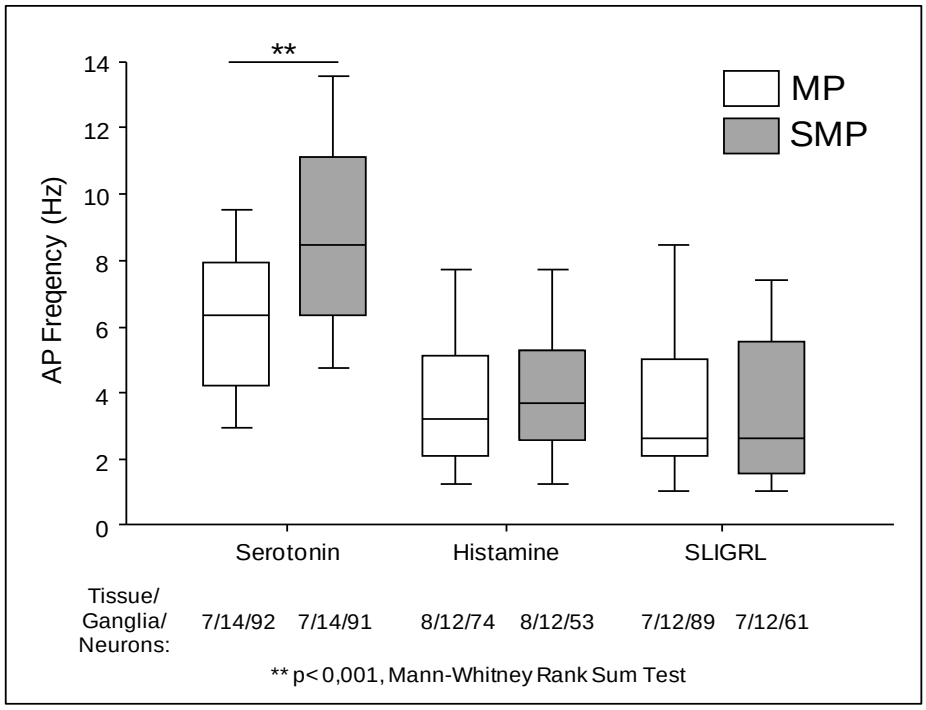

Figure 25. Comparison between submucous and myenteric plexus: AP frequency.
The AP frequency for 5-HT is significantly higher in the SMP than in the MP. For histamine and SLIGRL there were no significant differences.

Another interesting result was the different response to nicotine. The agonist for nicotinergic acetylcholine receptors served as a control for the viability of the neuronal network as well as to rule out that activation of ionotropic receptors would per se exhibit a stronger activation of submucous neurons. It turned out that nicotine activated 79.8 [77.3/86.7] % of myenteric neurons (483 out of 604) but with 71.8 [67.5/77.5] % (303 out of 421) significantly less submucous neurons. The nicotine evoked firing rate was also significantly higher in myenteric neurons compared to submucous neurons 9.3 [7.4/11.7] % (14/31/483) versus 7.9 [5.6/10.7] % (14/31/303).

4. DISCUSSION

4.1. 5-HT$_4$ and 5-HT$_7$ receptor activity

4.1.1. 5-HT$_4$ receptor electrophysiology

The electrophysiological data of this study demonstrates that the two 5-HT$_4$ receptor agonists 5-MeOT and prucalopride evoke no action potentials when applied directly onto human submucous ganglia. This agrees with previous studies in animal models, where only presynaptic activity of 5-HT$_4$ receptors could be detected, suggesting that they increase the strength of neurotransmission in prokinetic pathways of acetylcholine and CGRP (Craig and Clarke, 1990; Hoyer et al., 1994; Briejer and Schuurkes, 1996; LePard et al., 2004; Liu et al., 2005; Gershon and Liu, 2007). Presynaptic localisation of 5-HT$_4$ receptors was further demonstrated by electron microscopic immunocytochemistry using a 5-HT$_4$ receptor antibody in mouse tissue (Gershon and Liu, 2007). Presynaptic modulation of cholinergic neurotransmission has also been shown in humans, in the fundus and the oral third of the corpus (Leclere and Lefebvre, 2002).

Accordingly, in the present study 5-MeOT and prucalopride both presynaptically facilitated fEPSPs in the human submucous plexus. Activating the 5-HT$_4$ receptor increased both amplitude and duration of the fEPSPs in response to electrical stimulation of submucous neurons. Unspecific effects of prucalopride on sodium channels can be excluded since the compound action potential remained unchanged. Since prucalopride proved to be the more efficient agonist, it was also used for the experiments with the 5-HT$_4$ receptor antagonist piboserod. It was shown that the antagonist blocked the effects of prucalopride in the human submucous plexus. This antagonising action was reversible after wash-out, though very few cells still showed a slightly increased EPSP/baseline ratio.

The results of the present study reveal that 5-HT$_4$ receptors are involved in the presynaptic modulation of neurotransmission in the human submucous plexus. Functional studies on ionsecretion (Ussing chamber, isolated human tissue) already suggested that release of mucosal 5-HT induced by mucosal stroking activates 5-HT$_4$ receptors on enteric sensory neurons, although so far not they have been able to demonstrate a functional role of neuronal 5-HT$_4$ receptors in the human ENS (Borman and Burleigh, 1996)(Kellum et al., 1999).

Possible other functions of 5-HT$_4$ receptors could be sensory, e.g. on CGRP neurons (Foxx-Orenstein et al., 1996) or indirectly in the regulation of blood flow. It has also

been demonstrated that the 5-HT_4 receptor is involved in chloride secretion in the human jejunal mucosa via a nonneural pathway (Kellum et al., 1994; Budhoo et al., 1996).

In general, presynaptic receptors can be of several types, including members of the ionotropic and metabotropic ion channels (Miller, 1990; McGehee and Role, 1996). Their activation can regulate transmitter release. The presynaptic receptors can work in several ways. Calcium influx, for example, triggers the final release of synaptic vesicles, which is why changes in calcium influx due to regulation of these channels can influence transmitter release (Miller, 1990; Wu and Saggau, 1997; Miller, 1998).

Another mechanism would be the activation of presynaptic ion channels (Miller, 1998). Stimulation of a presynaptic receptor could modulate the action potential in this region of the neuron or change its activation threshold, e.g. via activation of potassium or chloride channels. For the 5-HT_4 receptor, it has already been suggested that they exert their prokinetic action by increasing the strength of neurotransmission at cholinergic synapses (Liu et al., 2005). Still, further studies will be needed to identify the pathways downstream of the receptor.

The observed changes in duration and amplitude of the EPSPs due to the 5-HT_4 receptor agonists suggest that the 5-HT_4 receptor might, like in the animal model, facilitate the initiation of the peristaltic reflex as mentioned above. Such effects have also been demonstrated by mucosal application of 5-HT_4 receptor agonists like prucalopride and tegaserod in human jejunum (Grider et al., 1998).

These results are especially interesting in context with functional gastrointestinal disorders, such as IBS, which are often associated with altered enteric serotonergic signalling. 5-HT_3 and especially 5-HT_4 receptors seem to be efficient targets in the treatment of symptoms of IBS. 5-HT_3 receptor antagonists like cilansetron for example seem to modulate visceral sensitivity and slow intestinal transit and seem to be effective in diarrhea predominant IBS (Brown et al., 1993; Scarpignato and Pelosini, 1999; Camilleri, 2001; Ford et al., 2009). 5-HT_4 receptor agonists such as tegaserode, prucalopride or mosapride can relieve abdominal pain and improve intestinal transit in constipation predominant IBS (Camilleri, 2001; De Ponti and Tonini, 2001; Kale-Pradhan and Wilhelm, 2007; Ford et al., 2009; Kanazawa et al., 2011) and the 5-HT_4 antagonist piboserode is also being investigated for a diarrhea predominant IBS (De Ponti and Tonini, 2001).

The importance of the 5-HT_4 receptor in this context is underlined by the finding that 5-HT_3 receptor antagonists inhibit CGRP release as well as ascending contraction and descending relaxation of circular muscle induced by mucosal stimulation in guinea pig colon but not in human, whereas the 5-HT_4 receptor antagonists can fulfil this function in both tissues (Foxx-Orenstein et al., 1996).

It has further been shown that 5-HT_4 agonists have a prokinetic action and can relieve symptoms like constipation and intestinal discomfort, not only in case of IBS but also chronic constipation (Prather et al., 2000; De Ponti and Tonini, 2001; Müller-Lissner et al., 2005; Lacy et al., 2009). The increase in excitability by 5-HT_4 agonists shown in this study would imply a fascilitation of excitatory motor pathways leading to this procinetic effects.

The results of the present study provide experimental evidence for these mechanisms. They are also the first functional demonstration of 5-HT_4 receptors in the human submucous plexus.

4.1.2. 5-HT_7 receptor electrophysiology

Previous studies in rodents revealed that 5-HT_7 receptors mediate intestinal smooth muscle relaxation and accommodation (Tonini et al., 2005). 5-HT is involved in smooth muscle accommodation in the preparatory phase of peristalsis by direct activation of 5-HT_7 receptors. It has been suggested that abnormal stimulation of the 5-HT_7 receptor may contribute to certain clinical syndromes such as IBS, and that the 5-HT_7 receptor may be a reasonable candidate for therapeutic intervention in some patients (Vanhoenacker et al., 2000, Tonini et al., 2005; Zou et al., 2007).
However, as the results of this study showed, there seem to be no 5-HT_7 receptors in the human submucous plexus. The 5-HT_7 agonist 5-CT evoked no responses in the submucous ganglia. This could mean that 5-CT is not potent enough to activate the 5-HT_7 receptors in the human submucous plexus. This argument is supported by the fact that 5-CT only evoked a very weak response in the guinea pig myenteric plexus, although here the receptor has already been demonstrated (Monro et al., 2005). On the other hand, with a pK_i value of 9.5 the 5-HT_7 receptor exhibits a high affinity for 5-CT. Another possible reason would be receptor internalisation due to tissue handling, though this may be ruled out because then the other 5-HT receptors would also be affected. 5-HT_7 receptors also could only have sub-threshold effects in which case further studies would be needed.
The most likely explanation for the lack of signals after 5-CT application however, would be that the 5-HT_7 receptor simply is not expressed, or if present, does not play a major role in the human submucous plexus. An immunohistochemical study could shed light onto these questions; unfortunately so far no 5-HT_7 receptor staining has been achieved in the human ENS, which also includes the efforts within this thesis. Possibly 5-HT_7 receptors simply are not expressed in the human ENS, or at least not in the submucous plexus. In the case of mRNA in human enteric tissue, reports of 5-HT_7 receptor expression are limited to the circular muscle of the human colon (Irving et al., 2007).
Evidence of a functional role in human alimentary tract tissue has been obtained, because the 5-HT_7 receptor was shown to mediate relaxation of human colonic circular smooth muscle (Prins et al., 1999). Although 5-HT_4 receptors are known to mediate the 5-HT induced relaxation of human colon circular muscle (Tam et al., 1995; McLean et al., 1995), Prins and collegues showed that after 5-HT_4 receptor blockade 5-HT still induced a relaxation in segments of colon obtained from human patients. This relaxation was blocked by mesulergine, a 5-HT_7 receptor antagonist, leading to the conclusion that the relaxation of human colon by 5-HT is also mediated

by smooth muscle 5-HT_7 receptors. The inability of tetrodoxin to affect this relaxation suggests that the receptor is located directly on the smooth muscle, which fits together with the mRNA expression data by Irving et al. mentioned above (Prins et al., 1999). A similar effect on enteric neurons has never been demonstrated.

From the electrophysiological point of view a possible next step for clarifying the role of the 5-HT_7 receptor in human could be an investigation into the effects of 5-CT human myenteric tissue. Also, the 5-HT_7 receptor antagonist SB-269970 could be an interesting tool to test in both plexus, but first the usefulness of 5-CT as 5-HT_7 receptor agonist in the human ENS has to be verified, unless a new selective 5-HT_7 receptor agonist is found.

4.1.3. 5-HT_4 and 5-HT_7 receptor immunohistochemistry

For both the 5-HT_4 receptor and the 5-HT_7 receptor, electrophysiological and immunohistochemical experiments were performed. The Santa Cruz antibodies SR-4 and SR-7 were applied in guinea pig and human tissue. Successful use of the antibodies has been demonstrated in human tissue (Ruat et al., 1993; Gelernter et al., 1995; Eglen et al., 1995; Claeysen et al., 1997a). The experiments in the guinea pig myenteric plexus showed that the SR-7 antibody does not stain the 5-HT_7 receptor in the corpus region of the stomach. However this receptor could be shown in the ileum. In the submucous plexus of the ileum, although there was unspecific staining, SR-7 antibody marked some neurons. Interestingly, other experiments with calbindin co-staining in both plexus indicate that the 5-HT_7 receptors in the guinea pig ileum could be co-localised with this marker for putative intrinsic afferent neurons as previously described by Monro et al (Monro et al 2005). This claim is based on the finding that calbindin is a chemical marker for intrinsic afferent neurons in the guinea pig ileum (Furness et al., 1990). Nevertheless, these experiments can only be considered as preliminary indicators until a much larger number of experiments are performed, including a specific blocking peptide to absolutely rule out the possibility of false-positive results due to non-specific binding of the antibody.

In human tissue the SR-7 antibody did not show any 5-HT receptor staining within the enteric neurons. The SR-4 receptor antibody caused unspecific staining in both plexus of the guinea pig. In the human intestine, similarly to the SR-7 results, the antibody was not able to stain the 5-HT_4 receptors.

As Wouters et al. observed, immunoreactivity for the 5-HT_4 receptor had never been demonstrated although this would be expected by functional data in the human ENS (Wouters et al., 2007). Regarding animal models, some authors mention specificity problems with 5-HT receptor antibodies in the ENS (Poole et al., 2006). One of the rare examples for 5-HT_4 receptor staining in non-human enteric neuronal tissue is the co-localisation of 5-HT_4 receptors with calbindin in myenteric ganglia of the guinea pig ileum (Poole et al., 2006), where the 5-HT_4 receptor staining is visible inside the neurons. However, non-specific staining inside and also outside of the ganglion seemingly could not be avoided.

There are no immunohistochemistry results published on 5-HT_4 or 5-HT_7 receptors in the human ENS despite several antibodies being available which have been successfully used in the human CNS (e.g. (Wai et al., 2011; Eglen et al., 1995; Teitler and Herrick-Davis, 1994). Interestingly, most of the studies published on 5-HT_4 or 5-HT_7 receptor antibodies are blotting or ELISA experiments rather than

immunoflourescence. It seems that reliable immunofluorescence staining of 5-HT_4 and 5-HT_7 receptors remains a challenge.

In order to examine the behaviour of the two antibodies on the different gut layers, wholemount preparations of the human gut wall were also stained. Again, neither the myenteric nor the submucous plexus displayed any 5-HT receptor staining. There where however for SR-7 and even more for SR-4 stainings in the mucosal layer which is not surprising since projections of submucous neurons are described to reach the mucosa (Song et al., 1992). However, the specificity problem remains.

It remains unknown whether the difficulties to achieve staining is related to different expression of the receptor splice variants in the ENS compared to the CNS. The 5-$HT_{4(d)}$ variant for example does not exist in the CNS but seems to be limited to human small intestine and colon (Blondel et al., 1998). Also, little is known about the quantitative differences in expression of 5-HT_4 and 5-HT_7 receptor splice variants, probably because pharmacology and function of especially the human 5-HT_7 splice variants are very difficult to discriminate (Krobert et al., 2001). On the other hand it seems unlikely that different splice variants are an important factor, since out of the eleven known 5-HT_4 receptor splicing products, seven variants (a,b,c,d,e,f,g and i), as well as all three human 5-HT_7 variants (a,b,d) are expressed in the human intestine (Coupar et al., 2007). Another reason would be environmental factors hindering the antibodies from reacting with the target. Poor primary antibody potency or target accessibility for enteric 5-HT receptors could play a role. The strong background staining observed might be due to endogenous enzymes or antibody cross-reactivity. Possibly designing 5-HT_4 and 5-HT_7 antibodies specifically with enteric instead of central 5-$HT_{4/7}$ receptors might improve the chances. However, the next logical step would probably be to investigate further into methods of improving accessibility of the receptors.

4.2. 5-BOIP and 5-HT$_{1P}$ receptor activity

The results of this study show that neurons of human submucous plexus still responded to the application of 5-HT after perfusion of the 5-HT$_3$ antagonist cilansetron. The blocking effect of cilansetron was significant only for the early response. This is in accordance with the results of an earlier study (Michel et al., 2005) and implies that the “late response” of submucous neurons to 5-HT application must be driven by receptors other than 5-HT$_3$. For all of these experiments, a proper wash-out of cilansetron was not possible, which suggests a very high affinity of the submucous 5-HT$_3$ receptors for cilansetron rendering the wash-out durations used insufficient. Cilansetron might even act as an irreversible antagonist in the human submucous plexus.

The results from the Multi-Captioning showed that it was possible to record the late response to 5-HT in the presence of cilansetron 2 to 5 seconds after the 5-HT application. Besides identifying the timing of the late response, the use of the Multi-Captioning also had another advantage: recording for a shorter period limits the possible phototoxic effect of the fluorescent dye. In summary, these experiments suggested the involvement of other receptors such as the 5-HT$_{1P}$ receptor. Results from animal model studies suggested that the 5-HT-induced slowly developing, long lasting activation in enteric neurons was mediated by the 5-HT$_{1P}$ receptor and involved in the initiation of the peristaltic reflex (Takaki et al., 1985; Mawe and Gershon, 1993). The only pharmacological demonstration of the putative 5-HT$_{1P}$ receptor in human so far was a study by Tack et al. (2000), who used the anti-migraine drug sumatriptan to cause a relaxation of the proximal stomach. Sumatriptan acts as an agonist at 5-HT$_{1P}$ receptors on guinea-pig gastric myenteric neurons and was able to restore impaired accommodation and to improve early satiety in patients with functional dyspepsia (Fiorica-Howells et al., 1993; Tack et al., 1998).

The results of the experiments with the 5-HT$_4$ and 5-HT$_7$ receptors show no direct involvement of these receptors in the late response, but as observed in the cilansetron experiments, the 5-HT$_3$ receptor sometimes might still trigger few action potentials within the timeframe of the late response, although the largest part of the late response is clearly not 5-HT$_3$ mediated.

In this study, the 5-HT$_{1P}$ antagonist 5-HTP-DP successfully reduced the late response of human submucous neurons as well as guinea pig myenteric neurons to 5-HT application. This indicates that the late response might indeed be driven by the

5-HT_{1P} receptor. The antagonist, however, did not block all of the late APs. Since 5-HTP-DP is known to be a specific and efficient 5-HT_{1P} receptor antagonist, this could mean that although the 5-HT_{1P} receptor may be mainly responsible for the late response, other receptors could still be involved. Alternatively, the affinity of the human 5-HT_{1P} receptor for 5-HTP-DP might simply not be strong enough for a complete block. This would indicate that the structure of the human 5-HT_{1P} receptor could differ slightly from the guinea pig one.

The accidentally discovered 5-BOIP, a substance closely related to the established 5-HT_{1P} agonist 5-OHIP, caused late onset postsynaptic activation of neurons in both guinea pig myenteric and human submucous plexus. The close resemblance is also true for the organ bath experiments. 5-OHIP has been described to dose dependently evoke a relaxation of the gastric smooth muscle (Michel et al., 1997). 5-BOIP also caused a relaxation, which was larger than the one described for 5-OHIP (50 % versus 38 %). Furthermore, 5-BOIP is more stable than 5-OHIP, since 5-BOIP lacks acidic proton at position 5 and therefore formation of oxidized quinine imine species is significantly less likely (personal communication by Dr. Müller, Technische Universität Munich, Institute of Biological Chemistry). The chemical structures of both 5-OHIP and 5-BOIP are displayed in Appendix III. Since working under oxygen-free conditions is not possible while performing experiments in living tissue, the usage of 5-BOIP instead of 5-OHIP would solve the problem that 5-OHIP is quite susceptible to oxidation (see Appendix II) and further allow longer and safer storage. However, in order to thoroughly compare the two substances, dose response experiments with both substances on tissue from the same animals should be performed.

As explained in the introduction, the effects of 5-OHIP can be antagonised by 5-HTP-DP. The reduction of 77.3 % for the number of responding neurons and of 86.8 % in AP frequency in guinea pig myenteric plexus in response to 5-BOIP after perfusion of the 5-HT_{1P} antagonist shows that the same can be said for 5-BOIP. In human tissue, the reduction was not as strong as in guinea pig, but with 69.6% for the cell count and 68.8% for the frequency significant. This evidence of 5-BOIP being a specific 5-HT_{1P} agonist is supported by the fact that it did not evoke an early response after spritz application.

Thus 5-BOIP might have the potential to become an effective therapeutic tool. The 5-HT_{1P} receptor is, as mentioned before, involved in both contractile and relaxing actions of the peristaltic reflex and in the stomach this receptor activates nitrergic neurons that are responsible for relaxation (Foxx-Orenstein et al., 1996; Takaki et al., 1985). So specific activation of the 5-HT_{1P} by a new, agonist - which is more stable and less complex to synthesise than its precursor - could improve peristalsis and transit in the gut and modulate gastric accommodation. This would be relevant in diseases such as chronic obstipation or functional dyspepsia.

In contrast to the significant effects of 5-HTP-DP and 5-BOIP, neither the 5-HT_7 receptor agonist 5-CT nor the 5-HT_4 receptor agonist prucalopride or the 5-HT_4 receptor antagonist piboserod showed any influence on the late response. The observation that 5-HT_7 receptors do not seem to be involved in the late response in human submucous tissue together with the results for 5-HTP-DP and 5-BOIP contradict the suggestion that the “orphan” 5-HT_{1P} receptor may actually be the 5-HT_7 receptor (Monro et al., 2005). Like in most of the animal models the mediation of slow excitatory response in human seems to be associated with the 5-HT_{1P} receptor only (Mawe et al., 1986; Mawe and Gershon, 1993; Wade et al., 1996). It has to be considered though, that in one study slow EPSPs in myenteric AH neurons of the guinea pig ileum were reduced by the 5-HT_7 receptor antagonist SB 269970 (Monro et al., 2005).

In summary, the results from both organ bath and electrophysiology strongly pointed to 5-BOIP being a potent 5-HT_{1P} agonist. It caused postsynaptic late onset activation of neurons. Further, 5-HTP-DP significantly reduced the late response of human submucous neurons to 5-HT application. Together, these results strongly suggest the involvement of the 5-HT_{1P} receptor not only in guinea pig myenteric but also in the human submucous plexus.

Many questions about the 5-HT receptors still remain unanswered until today, mainly because of the sheer number of serotonin receptors together with the unsufficient specificity of many agonists and antagonist. The present study thus confirms the physiological role of 5-HT_4 and 5-HT_{1P} receptors in the submucous plexus of the ENS for human. The 5-HT_7 receptor, however, seems to play no significant role in this context

4.3. Comparing serotonin, histamine and PAR-2 actions between the myenteric and the submucous plexus

The experiments that compared the 5-HT, histamine and PAR-2 sensitivity between the myenteric and the submucous plexus in guinea pig showed that the submucous plexus is more sensitive to 5-HT than the myenteric plexus while the sensitivity to histamine and the PAR-2 agonist SLIGRL was not significantly different. Previously, it was shown that biopsy supernatants from IBS patients activate human submucous neurons (Buhner et al., 2009). Receptors for 5-HT – as well as for histamine and proteases - are functionally expressed in both submucous and myenteric plexus (Gao et al., 2002; Reed et al., 2003; Michel et al., 2005; Wood, 2006; Wouters et al., 2007; Breunig et al., 2007). Nevertheless, this excitation seems to be primarily a feature of submucous neurons, which may be related to differential expression profile of receptors activated by soluble mediators in the supernatants (Buhner et al., 2010). This predominant action in submucous plexus suggests an altered mucosa-nerve signalling in IBS. The number of neurons responding to 5-HT was significantly higher in the submucous than in the myenteric plexus. In addition, 5-HT evoked a stronger spike discharge in the submucous plexus. These results are clearly no experimental artefacts (e.g. easier accessibility of substances to the submucous plexus in the used setup) as control experiments with nicotin showed a slightly but significantly higher sensibility in the myenteric plexus. This also renders the possibility of ionotropic receptors in general causing a stronger activation of submucous neurons unlikely.
Further, for histamine and the PAR2 activating peptide SLIGRL no significant difference between the two plexus was detectable, they both displayed similar actions in myenteric and submucous neurons.
As already mentioned above, a previous study by Buhner et al. (2009) showed that mediators released from colonic mucosal biopsy samples of patients with IBS excite neurons of the human submucosal plexus. It was further demonstrated that IBS supernatant-evoked excitation is mediated by proteases, histamine, and 5-HT. First, 5-HT, histamine and tryptase levels in supernatants correlated with the spike discharge induced by the supernatants. Second, mast cells density as well as histamine and tryptase levels in supernatants were higher in IBS than in controls. And third, the activation induced by IBS supernatants could be suppressed by a 5-HT_3 receptor antagonist as well as by histamine receptor (H1-H3) antagonists and protease inhibition. It also turned out that the nerve sensitising effects of IBS supernatants are more prominent in the submucous than in the myenteric plexus,

suggesting differential involvement of serotonin, histamine and PAR-2 receptors (Buhner et al., 2009). Neuronal activating effects have also been shown for IBS supernatants on visceral afferents in rats and mice leading to visceral hypersensitivity in mice (Barbara et al., 2007; Cenac et al., 2007).

The finding that in the present study the response to 5-HT is higher in the submucous plexus than in the myenteric plexus could be explained by channel properties of myenteric $5HT_3$ receptor differing from those expressed on submucous neurons (Derkach et al., 1989)(Zhou and Galligan, 1999). Differences in $5\text{-}HT_3$ receptor subunits or particular pharmacological properties of myenteric $5\text{-}HT_3$ receptors might play a role (Zhou and Galligan, 1999).

Taken together, this may suggest that 5-HT is prominently involved in the nerve sensitising effects in the submucous plexus caused by the IBS supernatants. It has to be considered though that the molecular basis for the the plexus related efficacies still remains a matter of discussion and that other mechanisms may also contribute to this sensitising effect. The already mentioned differences in the study of Buhner et al (2009) seem to be larger than can be explained by the differences in 5-HT in the present study.

Thus - although all 5-HT, histamine and PAR-2 have been proposed to play a major role in IBS pathophysiology (Vergnolle, 2005; Azpiroz et al., 2007; Kraneveld et al., 2008) - there are quite likely still other components of the IBS supernatant that might be involved in the nerve sensitizing action, such as interleukins, TNF-alpha or other protease actions (Liebregts et al., 2007; Karanjia et al., 2009; Zhao et al., 2011). This corresponds with the result of Buhner et al., where proteases appeared to play a dominant role (Buhner et al., 2009). Also synergistic actions between 5-HT, histamine and proteases caused by particular receptor clustering in the submucous plexus would be a possible explanation for the prominent activation of IBS supernatants in submucous rather than myenteric neurons (Buhner et al., 2009). Again this could also involve other mediators which are increased in the intestinal mucosa of IBS patients as metioned above.

Recent results from Buhner et al. (Buhner et al., 2012, revision submitted) suggest that the plexus related activation profile is a functional correlate of the intimate cross-talk between immune or epithelial cells and submucous neurons. Thus the interactions between 5-HT, among other mucosal mediators, and submucous nerves might represent an attractive therapeutic target in the treatment of functional bowel disorders like IBS.

5. SUMMARY

English summary:

The enteric nervous system (ENS) controls the peristaltic reflex, modulates motility as well as secretion in the gut and has the ability to react efficiently to physiological stimuli, including movement of the villi or distortion of the mucosa, contraction of intestinal muscle and changes in the chemistry of the contents of the gut. Within the ENS, serotonin (5-HT) acts as an important neurotransmitter. The multitude of 5-HT mediated effects in the gastrointestinal (GI) tract and elsewhere in the human body is only possible due to the involvement of several 5-HT receptors. From experiments in rodents it is known that neural 5-HT_4, 5-HT_{1P} and 5-HT_7 receptors are crucial for the modulation of neuronal activity in the ENS. Especially the submucous plexus plays an important role in the regulation of mucosal functions as well as response patterns of lamina propria cells. The role of 5-HT_4, 5-HT_7 and 5-HT_{1P} receptors in the modulation of enteric neuronal activity in the human gut, however, remains largely unknown. For our understanding of 5-HT evoked signalling in the human ENS and its pathophysiological properties in functional GI disorders like Irritable Bowel Syndrome (IBS), it is important to fill this gap of knowledge. Thus this study aimed to investigate the presence and functions of 5-HT_4, 5-HT_7 and 5-HT_{1P} receptors in the human submucous plexus as well as differences in sensitivity between the myenteric and the submucous plexus in guinea pig when exposed to components of supernatant from mucosal biopsies of IBS patients (5-HT, histamine, protease-activated receptor 2 (PAR2)-activating peptide).

Fast neuroimaging (MSORT) with the voltage sensitive dye Di-8-ANEPPS was used to record activity of enteric neurons of both human and guinea pig tissue when exposed to 5-HT_4, 5-HT_7 and 5-HT_{1P} agonists and antagonists. Also organ bath experiments and Immunohistochemistry were applied to provide insights in to receptor expression and function.

The results show that the two 5-HT_4 receptor agonists 5-methoxytryptamine and prucalopride evoke no action potentials when applied directly onto human submucous ganglia. However, the activation of the 5-HT_4 receptor increased both amplitude and duration of fast excitatory postsynaptic potentials (fEPSPs) in response to electrical stimulation of interganglionic fiber tracts. These effects could be reversibly blocked by the 5-HT_4 receptor antagonist piboserod. The results are the first functional demonstration of 5-HT_4 receptors in the human submucous plexus.

The fascilitation of fEPSPs may represent the neurophysiological correlate of the prokinetic actions of 5-HT_4 agonists in clinical studies.

The direct response to 5-HT in human submucous neurons has a bimodal time course. The fast component is 5-HT_3 mediated while the receptor mediating the late response was unknown so far. This late response was blocked by the 5-HT_{1P} antagonist 5-HTP-DP in human submucous neurons as well as in guinea pig myenteric neurons. Furthermore, the in this study discovered substance 5-benzoxy-indalpine (5-BOIP), a substance closely related to the established 5-HT_{1P} agonist 5-hydroxy-indalpine (5-OHIP), was identified as a potential 5-HT_{1P} agonist. Like 5-OHIP it caused late onset postsynaptic activation of neurons in both guinea pig myenteric and human submucous plexus. These effects could be reversibly blocked by 5-HTP-DP. In organ bath experiments 5-BOIP caused a relaxation which was even stronger than the one described for 5-OHIP.

The experiments with the 5-HT_7 agonist 5-carboxytryptamine suggest that this receptor does not play a major role in the human submucous plexus. Preliminary immunohistochemical experiments indicate that antibody staining for 5-HT_7 receptors and potentially also of 5-HT_4 receptors might be worth further investigation, in human tissue however reliable 5-HT receptor staining remains a big challenge.

Comparing the 5-HT, histamine and PAR-2 sensitivity between the myenteric and the submucous plexus in guinea pig showed that the submucous plexus is more sensitive to 5-HT than the myenteric plexus while the sensitivity to histamine and the PAR-2 agonist SLIGRL were not significantly different. Interactions between 5-HT, among other mucosal mediators, and submucous nerves might represent an attractive therapeutic target in the treatment of functional bowel disorders like IBS.

Zusammenfassung:

Das Enterische Nervensystem (ENS) kontrolliert den peristaltischen Reflex, moduliert Motilität ebenso wie Sekretion im Darm und hat die Fähigkeit effizient auf physiologische Stimuli zu reagieren, einschließlich Bewegung der Villi oder mukosaler Distorsion, intestinaler Muskelkontraktion und Veränderungen in der Chemie des Darminhalts. Innerhalb des ENS agiert Serotonin (5-HT) als wichtiger Neurotransmitter. Die Vielzahl an 5-HT vermittelten Effekten im gastrointestinalen (GI) Trakt und anderswo im menschlichen Körper in nur möglich aufgrund der Beteiligung etlicher 5-HT-Rezeptoren. Aus Versuchen in Nagern ist bekannt, dass neurale 5-HT_4-, 5-HT_{1P}- und 5-HT_7-Rezeptoren entscheidend an der Modulation von neuraler Aktivität im ENS beteiligt sind. In der Regulation mukosaler Funktionen sowie in den Antwortmustern von Zellen der Lamina propria spielt der submuköse Plexus (SMP) eine wichtige Rolle. Die Rolle der 5-HT_4-, 5-HT_{1P}- und 5-HT_7-Rezeptoren innerhalb der Modulation der enterischen neuralen Aktivität ist allerdings weitestgehend unbekannt. Für unser Verständnis von 5-HT-vermittelter Signalübertragung im humanen ENS und dessen pathophysiologische Eigenschaften in funktionalen Störungen des GI-Trakts wie beispielsweise Reizdarmsyndrom (Irritable Bowel Syndrome - IBS) ist es wichtig, diese Wissenslücke zu schließen. Daher war die Analyse von Präsenz und Funktionen von 5-HT_4-, 5-HT_{1P}- und 5-HT_7-Rezeptoren im humanen SMP Ziel dieser Studie. Ebenso wurden Unterschiede in Sensitivität zwischen dem myenterischen und dem submukösen Plexus im Meerschweinen in Reaktion auf Komponenten von Überständen (5-HT, Histamin, protease-activated receptor 2 (PAR2)- aktivierendes Peptid) mukosaler Biopsien von IBS-Patienten untersucht.

„Fast Neuroimaging“ (MSORT) mit dem spannungssensitiven Farbstoff Di-8-ANEPPS diente der Aufnahme der Aktivität enterischer Neurone von menschlichem und Meerschweingewebe, welches 5-HT_4, 5-HT_7 und 5-HT_{1P} Agonisten und Antagonisten ausgesetzt wurde. Des Weiteren wurden Organbad- und Immunhistochemie Experimente durchgeführt, um weitere Einblicke in Rezeptorexpression und -funktion zu erhalten.

Die Ergebnisse zeigen dass die beiden 5-HT_4-Rezeptoragonisten 5-Methoxytryptamin und Prucalopride keine Aktionspotentiale auslösen, wenn sie direkt auf humane submuköse Ganglien appliziert werden. Allerdings erhöht die Aktivierung des 5-HT_4-Rezeptors im humanen submukösen Plexus sowohl die Amplitude als auch die Dauer schneller exzitatorische postsynaptischer Potentiale (fast excitatory postsynaptic potentials -fEPSPs) in Antwort auf elektrische Stimulation interganglionärerer Faserstränge. Diese Effekte konnten mit dem 5-HT_4-Rezeptorantagonist Piboserod reversibel geblockt werden. Es handelt sich bei

diesen Ergebnissen um die erste funktionale Demonstration von 5-HT_4-Rezeptoren im humanen submukösen Plexus. Die Faszilitation von fEPSPs könnte die neurophysiologische Entsprechung der prokinetischen Aktivitäten von 5-HT_4 Agonisten darstellen.

Die direkte Antwort auf 5-HT zeigt einen bimodalen Zeitablauf: Die schnelle Komponente ist 5-HT_3 vermittelt, während der Rezeptor, welcher die späte Antwort vermittelt bisher unbekannt war. Diese späte Antwort wurde durch den 5-HT_{1P}-Antagonist 5-HTP-DP sowohl im humanen SMP als auch im myenterischen Plexus des Meerschweins geblockt. Darüber hinaus wurde die während dieser Studie entdeckte Substanz 5-Benzoxy-indalpin (5-BOIP) - eine Substanz nah verwandt mit dem etablierten 5-HT_{1P}-Agonist 5-hydroxy-indalpin (5-OHIP) - als potenzieller 5-HT_{1P}-Agonist identifiziert. Wie 5-OHIP löste sie eine spät einsetzende postsynaptische Aktivierung von Neuronen im myenterischen Plexus des Meerschweins wie auch im humanen SMP aus. Diese Effekte konnten durch 5-HTP-DP reversibel blockiert werden. In Organbadexperimenten löste 5-OHIP eine Relaxation aus, die sogar stärker war als sie für 5-OHIP beschrieben ist.

Die Experimente mit dem 5-HT_7-Agonist 5- Carboxytryptamin legen nahe, dass dieser Rezeptor keine entscheidende Rolle im humanen SMP darstellt. Vorläufige immunhistochemische Experimente weisen darauf hin, dass Antikörperfärbungen für 5-HT_7-Rezeptoren und möglicherweise auch für 5-HT_4-Rezeptoren weitere Forschungsanstrengungen wert sein könnten. Im humanen Gewebe jedoch bleiben verlässliche 5-HT-Rezeptorfärbungen eine große Herausforderung.

Beim Vergleich von Sensitivität für 5-HT, Histamin und PAR2 zwischen myenterischem und submukösem Plexus im Meerschwein zeigte sich, dass letzterer empfindlicher für 5-HT ist, während sich für Histamin und für den PAR2-Agonisten SLIGRL keine signifikante Unterschiede ergaben. Interaktionen zwischen 5-HT, unter anderen mucosalen Mediatoren, und submukösen Nerven könnten ein attraktives therapeutisches Target bei der Behandlung funktionaler Störungen des Darms wie IBS darstellen.

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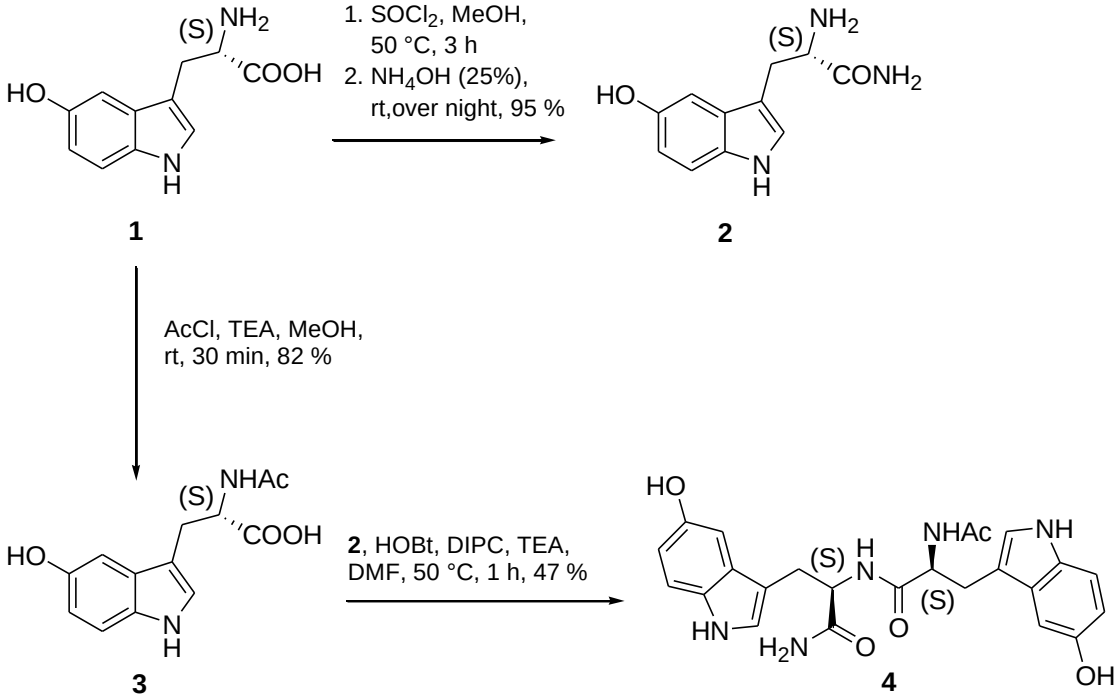
7. APPENDICES

Appendix I - Synthesis of N-acetyl-5-hydroxytryptophyl-5-hydroxytryptophan amide (5-HTP-DP)

Experiments designed and performed and by M.Müller, Department of Biological Chemistry of the Techische Universität München, Germany



Experimental Part:

Synthesis of (*2S*)-Amino-(5-hydroxyindolyl)-propanamide

2.0 g (9.1 mmol) (S)-5-hydroxytryptophan (**1**) were dissolved in 100 ml dry MeOH and cooled to 0 °C. 6.6 ml (91 mmol) $SOCl_2$ were added dropwise over 5 min. The mixture was then stirred for 3 h at 50 °C. The crude solid was evaporated in vacuo and mixed with 20 ml NH_4OH (25 %) at room temperature and stirred over night. The mixture was evaporated and purified by flash chromatography (SiO_2, CH_2Cl_2-MeOH 2:1 + 1 % TEA) and yielded 2.2 g (8.4 mmol, 95 %) yellow foam.

^{1}H-NMR (500 MHz, d6-DMSO, □/ppm):

10.85 (s, 1 H), 8.60 (br, 1 H), 7.53 (s, 1 H), 7.13 (d, J=8.5 Hz, 2 H), 7.07 (d, J=2.1 Hz, 1 H), 6.92 (d, J=2.1 Hz, 1 H), 6.01 (dd, J=8.5 Hz, J=2.1 Hz, 1 H), 3.62-3.50 (m, 2 H), 3.03 (dd, J=14.3 Hz, J=5.0 Hz, 1 H), 2.75 (dd, J=14.4 Hz, J= 5.0 Hz, 1 H)

ESI-MS (0.1 % HCOOH): m/z = 218.10 $[M-H]^-$, 264.20 $[M-H+HCOOH]^-$, 437.30 $[2xM-H]^-$

Synthesis of N-acetyl-(*2S*)-amino(5-hydroxyindolyl)-propionic acid

2.0 g (9.1 mmol) (S)-5-hydroxytryptophan (**1**) and 6.3 ml (45.4 mmol) TEA were dissolved in 200 ml dry Methanol. 1.6 ml (22.7 mmol) AcCl were added dropwise at room temperature and vigorously stirred for 30 min at room temperature. The reaction was mixed with portions of water and extracted three times with ethyl acetate. The combined aequos layers were frozen and dried in vacuo. Flash chromatography (SiO_2, CH_2Cl_2-MeOH 2:1 + 1 % TEA) yielded 1.9 g (7.4 mmol, 82 %) pale brown solid.

^{1}H-NMR (500 MHz, d6-DMSO, □/ppm):

10.97 (s, 1 H), 8.29 (d, J=8.0 Hz, 1 H), 7.96 (s, 1 H), 7.33 (d, J=8.5 Hz, 1 H), 7.21 (d, J=2.2 Hz, 1 H), 7.18 (d, J=2.0 Hz, 1 H), 6.81 (dd, J=8.4 Hz, J=1.9 Hz, 1 H), 4.45 (q, J=6.0 Hz, 1 H), 3.30-3.15 (m, 1 H), 3.14-3.06 (m, 1 H), 1.80 (s, 3 H)

ESI-MS (0.1 % HCOOH):

m/z = 261.30 $[M-H]^-$, 523.40 $[2xM-H]^-$, 545.30 $[2xM-H+Na]^-$

1.9 g (7.4 mmol) of **3** were dissolved in 50 ml dry DMF. 100 mg (740 □mol) of $HOBt \cdot H_2O$, 1.3 ml (8.1 mmol) DIPC and 5.2 ml (37.2 mmol) NEt_3 were added at ambient temperature and the mixture stirred for 0.5 h. 1.6 g (7.4 mmol) of **2** were added to the mixture and stirred over night. After adding additional 100 □L DIPC the mixture was stirred for 1 h at 50 °C and cooled to room temperature. Small portions of water were added and the resulting white precipitate was filtered off. The aequos layer was directly frozen and lyophilized in vacuo. Flash chromatography (SiO_2, CH_2Cl_2-MeOH 4:1) yielded 1.51 g (3.3 mmol, 47 %)of a white solid.

^{1}H-NMR (500 MHz, d4-MeOH, □/ppm):

7.18 (d, J=8.7 Hz, 1H), 7.15 (d, J=8.7 Hz, 1 H), 7.03 (s, 1 H), 6.94 (d, J=2.3 Hz, 1 H), 6.92 (s, 1 H), 6.83 (d, J=2.3 Hz, 1 H), 6.71-6.66 (m, 2 H), 4.52 (q, J=6.6 Hz, 2 H), 3.15-2.95 (m, 4 H), 1.78 (s, 3 H)

ESI-MS (0.1 % HCOOH):

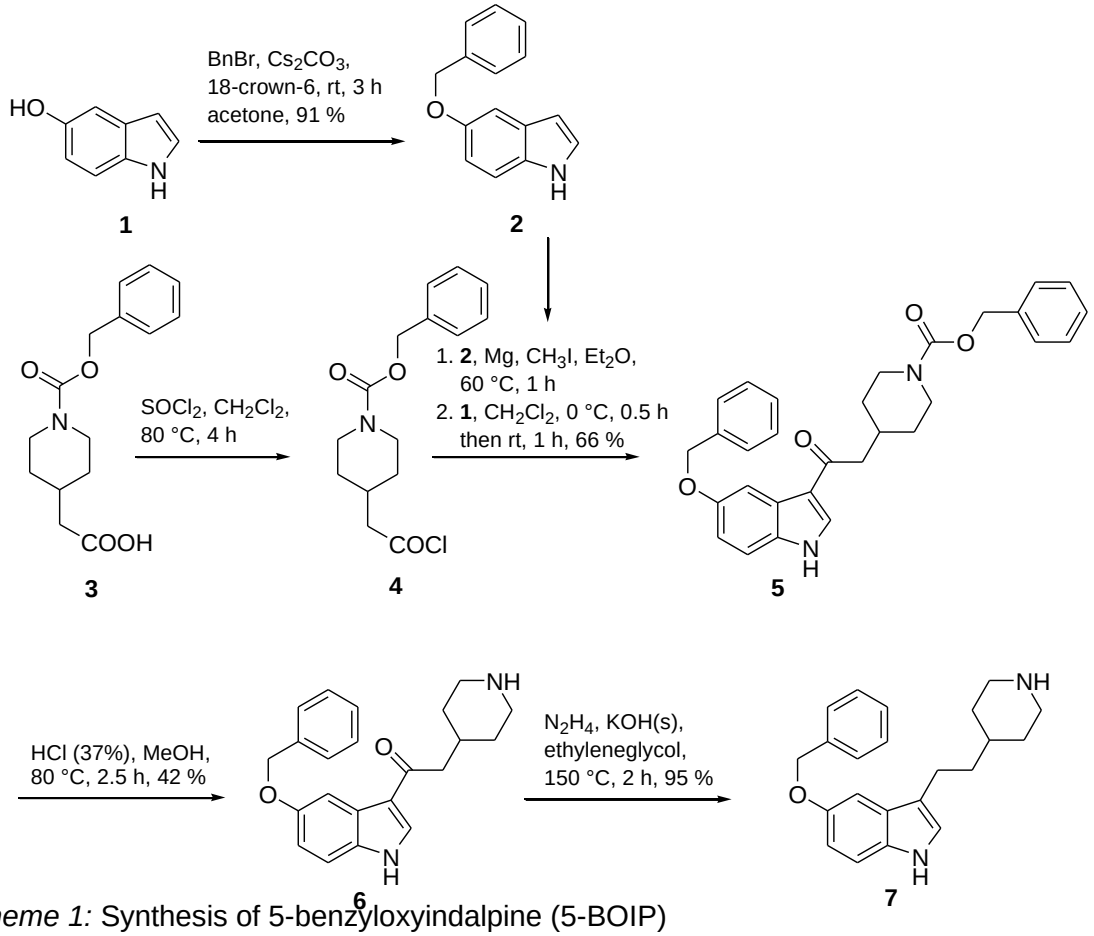
m/z = 464.40 $[M+H]^+$, 486,40 $[M+Na]^+$

Appendix II - Synthesis of 5-benzyloxyindalpine (5-BOIP)

Experiments designed and performed and by M.Müller, Department of Biological Chemistry of the Techische Universität München, Germany

Introduction:

5-Benzyloxyindalpine (**7**) was synthesized in 5 steps starting from commercially available 5-hydroxy-indole which was first alkylated with benzylbromide using cesium carbonate and crown ether. The protected indole (**2**) was coupled in a *Grignard* reaction with acid chloride of commercially available piperidinyl acetic acid (**4**). Removal of carbamate protecting group in **5** was performed with concentrated HCl to provide **6**. In a last step, the carbonyl group was transformed to methylene group in a *Wolff-Kishner* reduction to yield 5-benzyloxyindalpine **7** (*scheme 1*).

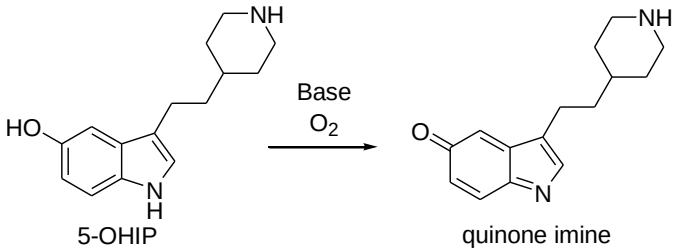


Scheme 1: Synthesis of 5-benzyloxyindalpine (5-BOIP)

Stability of 5-OHIP and 5-BOIP:

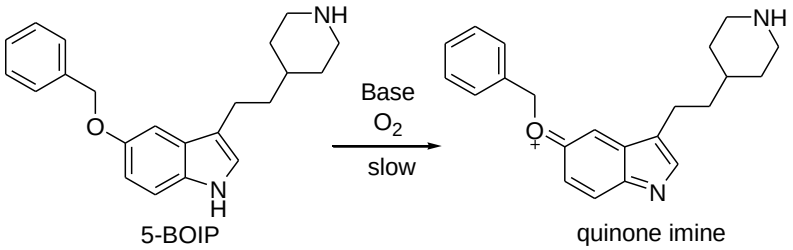
5-OHIP is prone to oxidation to the corresponding quinone imine species resulting from oxidation by oxygen in the air (*scheme 2*). Hence, working under oxygen-free conditions prolongs lifetime of 5-OHIP. *Wolf-Kishner* reduction requires harsh conditions comprising elevated temperature and strong basic medium. Therefore

degassing the solutions prior to use reduces amount of oxidized 5-OHIP. To further increase the yield of 5-OHIP an alternate milder reduction protocol of the carbonyl in **5** is highly recommended.



Scheme 2: Oxidation of 5-OHIP

5-BOIP is more stable since 5-BOIP lacks acidic proton at position 5 and therefore formation of oxidized quinine imine species is significantly hampered (*scheme 3*).



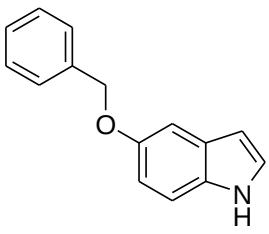
Scheme 3: Oxidation of 5-BOIP

General recommendations for working with 5-OHIP/5-BOIP:

- Working under oxygen-free/inert atmosphere (N_2, argon)
- Storage as solid since oxidation is enhanced in aequos and basic medium

Experimental Part:

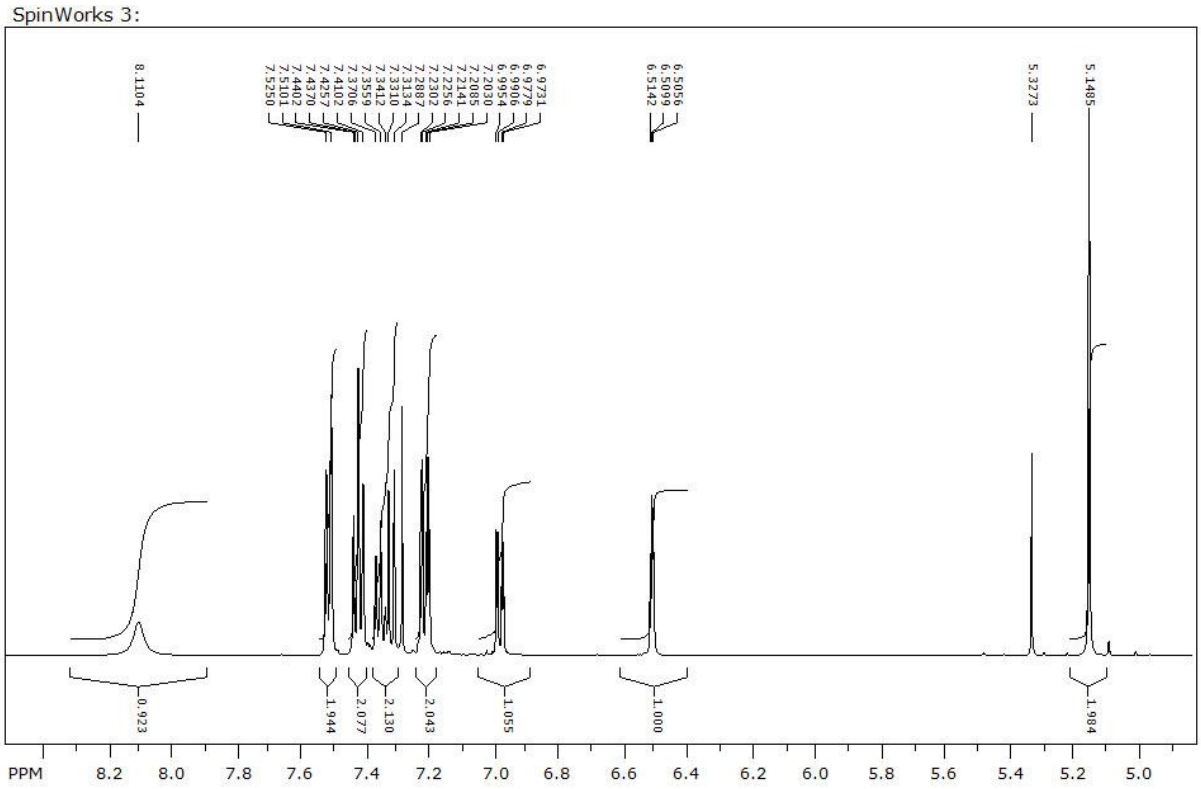
Synthesis of 5-Benzyloxyindole (**2**): (MM372)



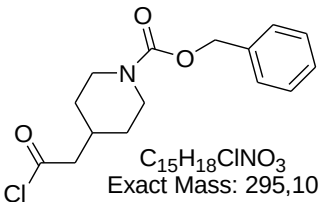
$C_{15}H_{13}NO$
Exact Mass: 223,10

0.5 g (3.8 mmol) 5-Hydroxyindole (**1**) were dissolved in 5 ml acetone and mixed with 535 □L (4.5 mmol) benzylbromide, 2.45 g (7.5 mmol) Cs_2CO_3 and 1.2 g (4.5 mmol) 18-crown-6 at room temperature. After stirring for 2 h, the mixture was diluted with water, smoothly acidified with 1 M HCl and extracted three times with CH_2Cl_2. The combined organic layers were dried with Na_2SO_4, filtered and evaporated. The crude product was purified by flash chromatography (SiO_2, cyclohexane-ethyl acetate 4:1) and yielded 762 mg (3.4 mmol, 91 %) of pale yellow solid.

^{1}H-NMR (500 MHz, $CDCl_3$): 8.11 (br, 1 H), 7.52 (d, J=7.5 Hz, 2 H), 7.41 (t, J=7.8 Hz, 2 H), 7.37-7.30 (m, 2 H), 7.22 (d, J=2.3 Hz, 1 H), 7.21 (t, J=2.8 Hz, 1 H), 6.98 (dd, J=8.8 Hz, J=2.4 Hz, 1 H), 6.51 (br, 1 H), 5.15 (s, 2 H).



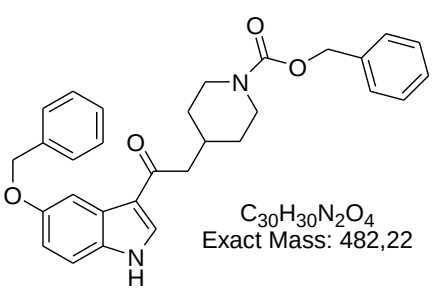
Synthesis of 2-(4-Benzyloxycarbonyl)-piperidinylacetic acid chloride (**4**): (MM371)



$C_{15}H_{18}ClNO_3$
Exact Mass: 295,10

502 mg (1.8 mmol) of 2-(4-Benzyloxycarbonyl)-piperidinylacetic acid (3) were dissolved in anhydrous CH_2Cl_2 under argon and mixed with 10 ml $SOCl_2$. After stirring for 4 h at 80 °C, the solution was directly evaporated. The residue was mixed three times with anhydrous CH_2Cl_2 and evaporated. The residue was further mixed three times with anhydrous toluene and evaporated in vacuo to yield 535 mg (1.8 mmol, 99 %) of white solid. The product was used without further purification.

Synthesis of 5-Benzyloxy-3-[2-(4-(benzyloxycarbonyl)piperidinyl)ethyl]indole (**5**): (MM373)

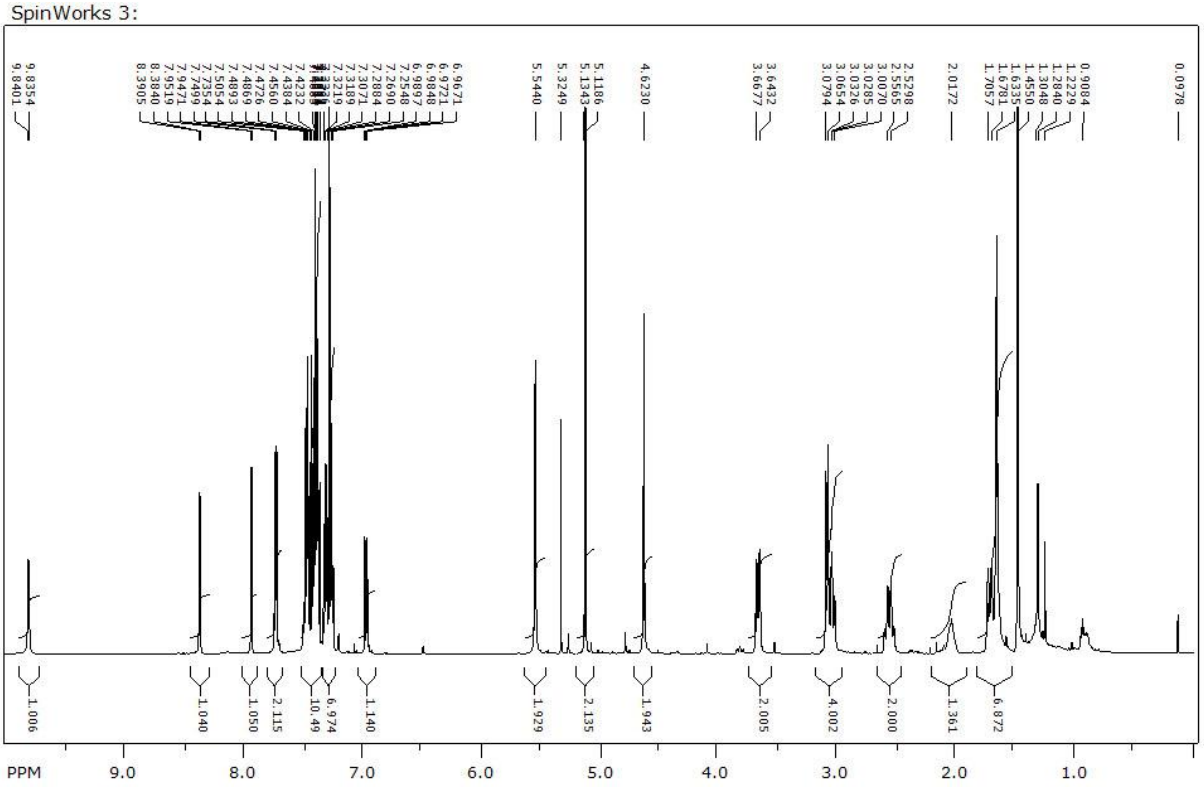


$C_{30}H_{30}N_2O_4$
Exact Mass: 482,22

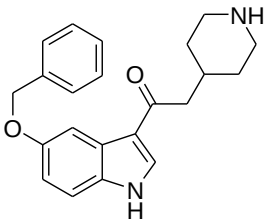
110 mg (4.5 mmol) Mg and few crystals I_2 were mixed with 10 ml dry Et_2O and stirred for 10 min at 60 °C under argon atmosphere. 283 L (4.5 mmol) of

CH_3I in 10 ml dry Et_2O were added dropwise at 60 °C and the mixture stirred further 2.5 h at 60°C. The mixture was cooled to room temperature and 485 mg (2.2 mmol) of 2 in 10 ml dry Et_2O were added dropwise. After subsequent stirring for 1 h at 60°C, the solution was cooled to 0 °C and 535 mg (1.8 mmol) of 4 in 10 ml anhydrous CH_2Cl_2 were added dropwise (yellow suspension). After stirring 0.5 at 0 °C and 1 h at room temperature, the solution was diluted with water, acidified with 1 M HCl (yellow) and extracted three times with CH_2Cl_2. The combined organic layers were dried with Na_2SO_4, filtered and evaporated in vacuo. The crude yellow product was purified by flash chromatography (SiO_2, CH_2Cl_2-MeOH 9:1) to yield 574 mg (1.2 mmol, 66 %) of white solid.

^{1}H-NMR (500 MHz, $CDCl_3$): 9.84 (br, 1 H), 8.39 (d, J=3.3 Hz, 1 H), 7.95 (d, J=2.4 Hz, 1 H), 7.75 (d, J=7.3 Hz, 2 H), 7.50-7.36 (m, 10 H), 7.35-7.25 (m, 3 H), 6.98 (dd, J=8.8 Hz, J=2.5 Hz, 1 H), 5.54 (s, 2 H), 5.12 (s, 2 H), 4.62 (s, 2 H), 3.65 (d, J=12.3 Hz, 2 H), 3.10-3.00 (m, 4 H), 2.54 (q, J=13.4 Hz, 2 H), 2.02 (br, 1 H), 1.68 (d, J=13.8 Hz, 2 H) → 4 excess protons!



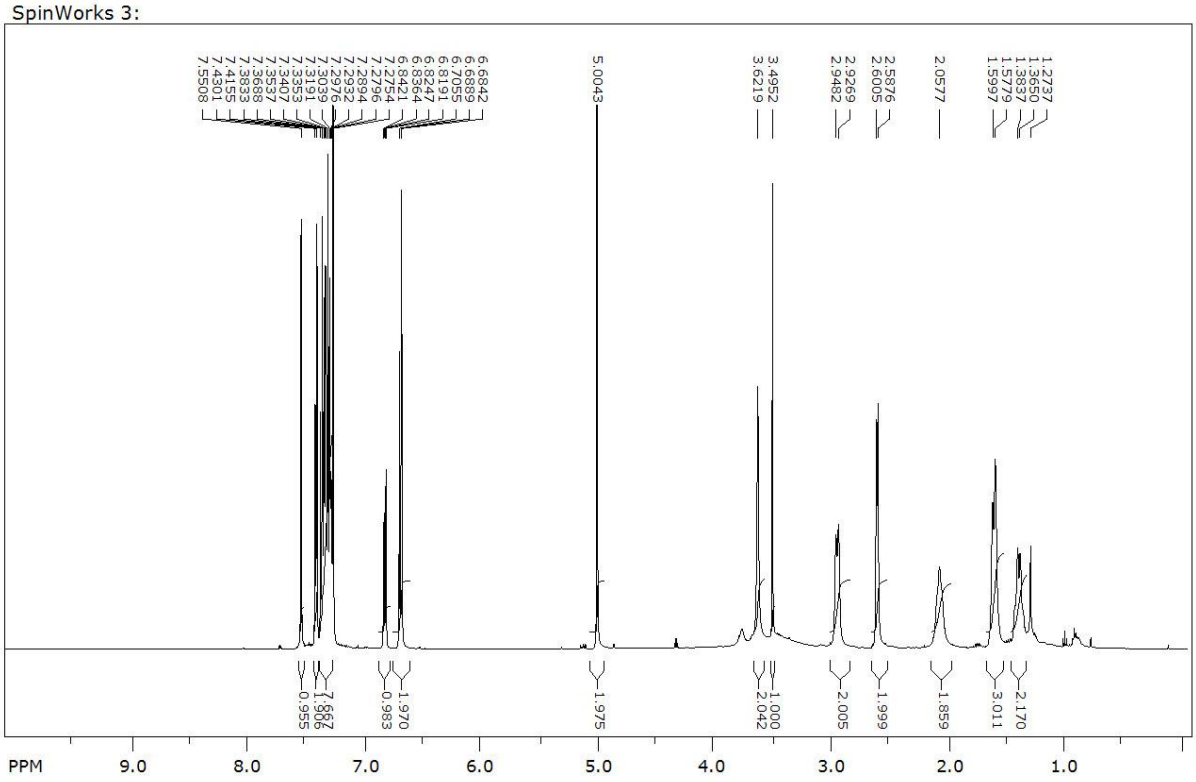
Synthesis of 5-Benzyloxy-3-[2-(4-piperidinyl)ethyl]indole (**6**): (MM374)



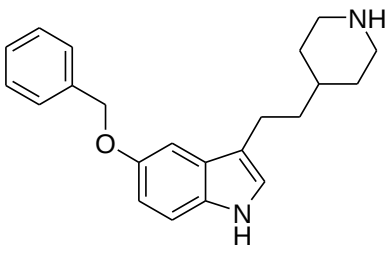
$C_{22}H_{24}N_2O_2$
Exact Mass: 348,18

574 mg (1.2 mmol) of **5** were dissolved in 5 ml MeOH and 5 ml HCl (37 %) and stirred for 2.5 h at 80 °C. The mixture was cooled to room temperature and directly evaporated in vacuo to yield 184 mg (0.5 mmol, 42 %) of slight pink solid.

^{1}H-NMR (500 MHz, $CDCl_3$): 7.55 (s, 1 H), 7.42 (d, J=7.3 Hz, 2 H), 7.40-7.25 (m, 5 H), 6.83 (dd, J=8.7 Hz, J=2.9 Hz, 1 H), 6.71-6.68 (m, 2 H), 5.00 (s, 2 H), 3.62 (s, 2 H), 3.50 (s, 1 H), 2.93 (d, J=10.7 Hz, 2 H), 2.59 (d, J=6.5 Hz, 2 H), 2.06 (br, 2 H), 1.64-1.52 (m, 2 H), 1.37 (q, J=9.4 Hz, 2 H) → 2 excess protons!



Synthesis of 5-Benzyloxyindalpine (**7**): (MM375)

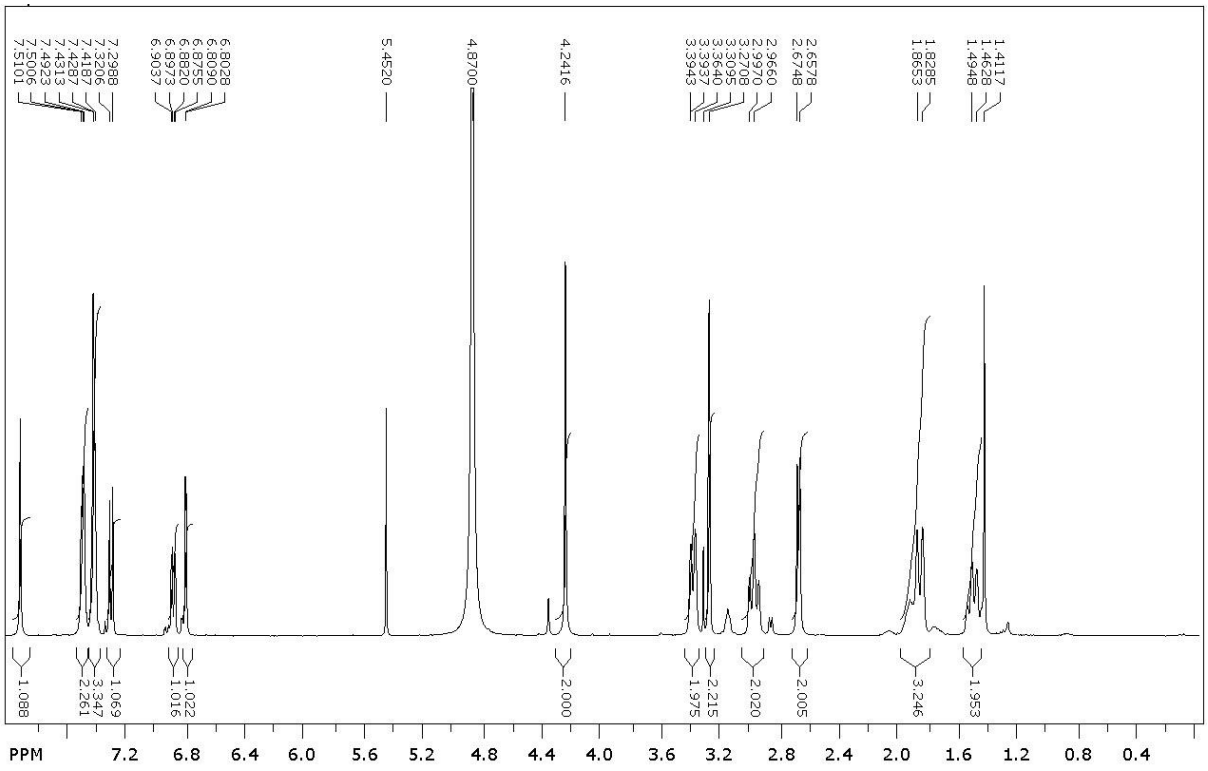


$C_{22}H_{26}N_2O$
Exact Mass: 334,2045

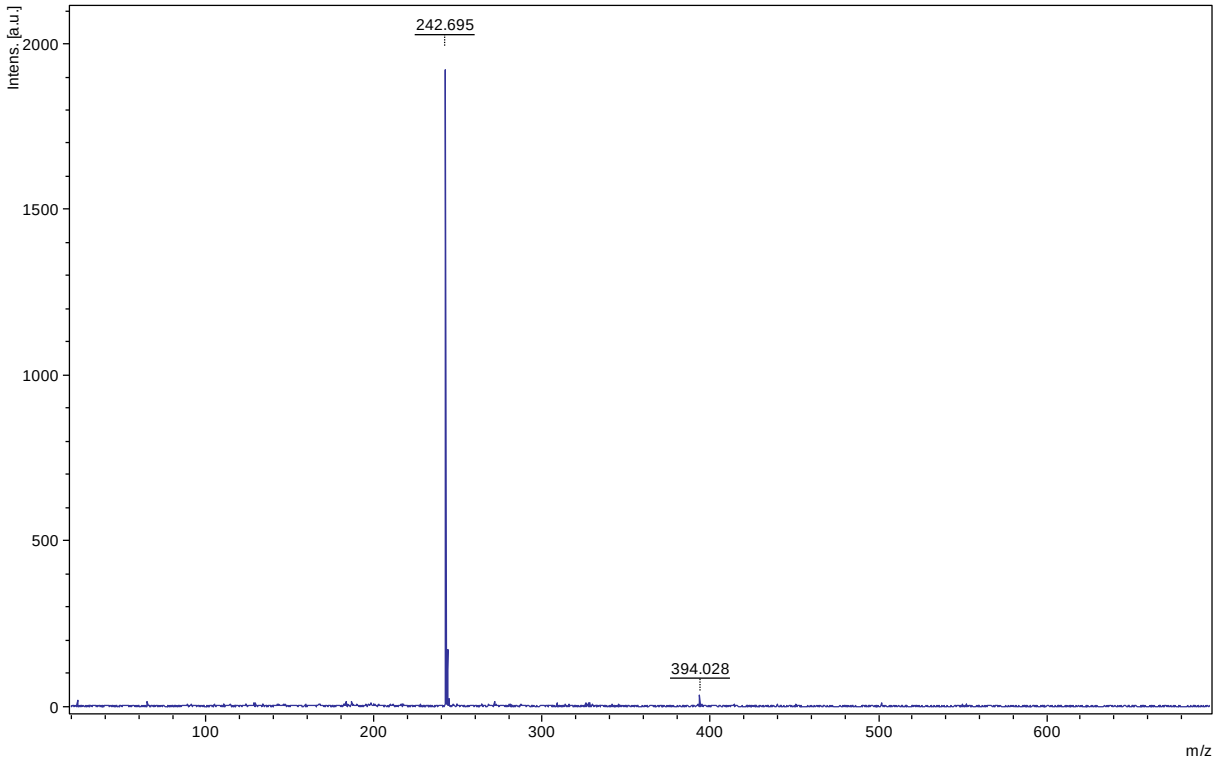
184 mg (0.5 mmol) of **6** were dissolved in 10 ml ethylene glycol, mixed with 2 ml $N_2H_4 \cdot H_2O$ and 1 g (17.8 mmol) solid KOH and stirred for 2 h at 150 °C. The solution was cooled to room temperature, diluted with water and extracted three times with ethyl acetate. The combined organic layers were dried with Na_2SO_4, filtered and

evaporated in vacuo. The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2-MeOH 7:3) to yield 130 mg (0.5 mmol, 95 %) of brown solid. Portion of the crude brown solid was further purified by semi-preparative HPLC (RP-8 select-B) to yield 20 mg of pale brown solid.

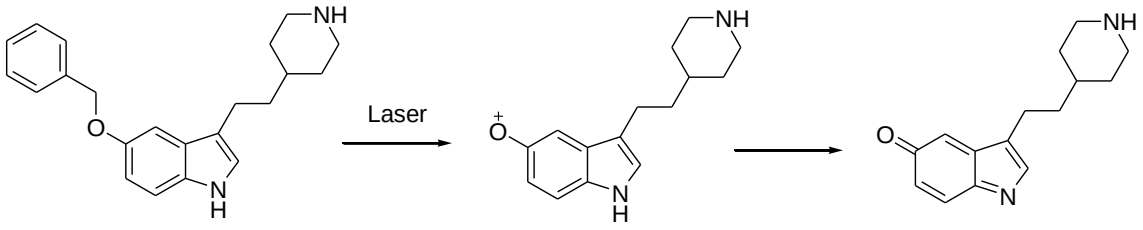
^{1}H-NMR (400 MHz, d4-MeOD): 7.99 (s, 1 H), 7.51-7.7.41 (m, 5 H), 7.31 (d, J=7.4 Hz, 1 H), 6.95 (dd, J=8.7 Hz, J=2.5 Hz, 1 H), 6.86 (d, J=2.5 Hz, 1 H), 4.24 (s, 2 H), 3.38 (d, J=11.9 Hz, 2 H), 3.27 (br, 2 H), 3.00 (t, J=12.2 Hz, 2 H), 2.66 (d, J=6.8 Hz, 2 H), 2.00-1.90 (br, 1H), 1.85 (d, J=15 Hz, 2 H), 1.48 (q, J=12.6 Hz, 2 H). Sum of protons is 24, NH exchanged in d4-MeOD (1H-NMR silent).



MS (MALDI-TOF, matrix: 2,3,4-Trihydroxyacetophenon): m/z = 242.695



Remarks: MALDI-TOF displays mass peak at m/z = 242.7 for 5-BOIP and not at m/z = 334 as expected. Irradiation (laser beam) of 5-BOIP leads to cleavage of the benzyl group and subsequent oxidation to the corresponding quinone imine of 5-OHIP (photooxidation).



$C_{22}H_{26}N_2O$
Exact Mass: 334,2045

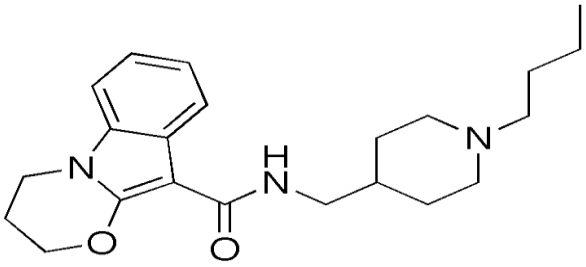
$C_{15}H_{19}N_2O^+$
Exact Mass: 243,15

$C_{15}H_{18}N_2O$
Exact Mass: 242,14

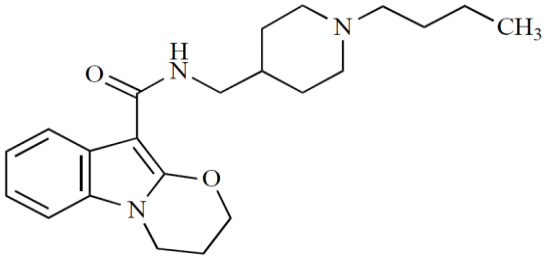
Appendix III - Chemical Structures

1. Chemical structures of the 5-HT_4 receptor antagonist piboserod, the 5-HT_4 receptor agonists 5-methoxytryptamine and prucalopride, and the 5-HT_7 receptor agonist 5-carboxytryptamine:

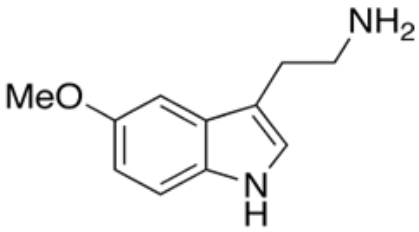
Piboserod



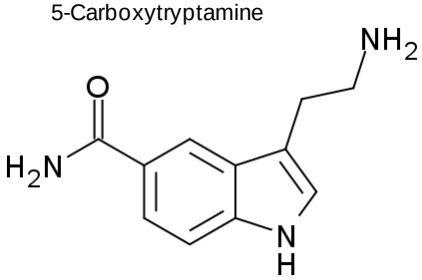
Prucalopride



5-Methoxytryptamine

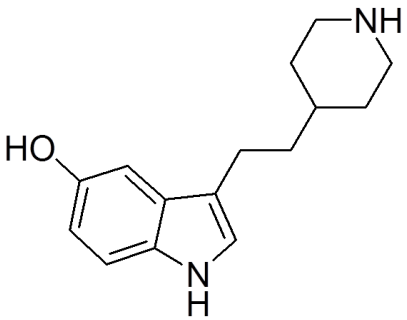


5-Carboxytryptamine

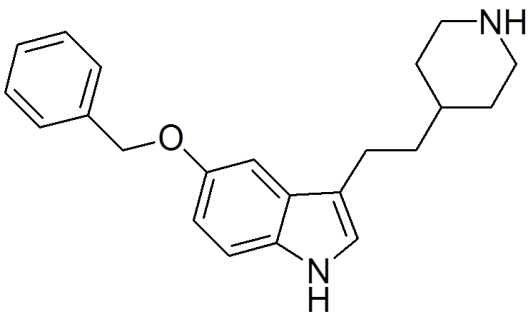


2. Comparison of the established 5-HT_{1P} receptor antagonist 5-hydroxytryptamine and the new potential 5-HT_{1P} agonist 5-benzoxytryptamine (for further information also see Appendix II):

5-Hydroxytryptamine (5-OHIP)



5-Benzoxytryptamine (5-BOIP)



Appendix IV - Collection of Data and Values

Average AP Frequency to application of 5-HT (Hz) (Guinea Pig)			
Myenteric Plexus		**Submucous Plexus**	
Cell No.	**Frequency (Hz)**	**Cell No.**	**Frequency (Hz)**
1	2,12	1	11,67
2	5,84	2	6,37
3	2,65	3	10,08
4	2,12	4	2,65
5	3,18	5	3,18
6	6,37	6	3,34
7	3,71	7	3,81
8	2,12	8	5,72
9	3,18	9	6,19
10	4,24	10	6,67
11	6,37	11	7,15
12	6,90	12	7,62
13	12,20	13	8,12
14	9,02	14	12,73
15	15,38	15	14,32
16	9,55	16	13,26
17	11,14	17	7,96
18	6,37	18	6,90
19	2,12	19	8,49
20	13,79	20	6,37
21	10,61	21	5,73
22	14,85	22	6,21
23	4,77	23	7,16
24	5,84	24	11,67
25	10,61	25	13,85
26	13,79	26	8,12
27	7,43	27	6,21
28	5,84	28	10,98
29	4,24	29	7,16
30	2,12	30	6,68
31	3,71	31	10,50
32	6,37	32	4,77
33	3,71	33	5,84
34	2,12	34	7,64
		35	8,49
Mean	**6,60**	36	3,18
T/G/C	**4/6/78**	37	8,49
		38	9,02
		39	12,73
		40	8,12
		Mean	**7,88**
		T/G/C	**4/7/73**

Average AP Frequency to application of nicotine (Hz) (Guinea Pig)			
Myenteric Plexus		**Submucous Plexus**	
Cell No.	**Frequency (Hz)**	**Cell No.**	**Frequency (Hz)**
1	7,00	1	3,71
2	10,50	2	5,31
3	8,75	3	4,24
4	11,09	4	5,31
5	4,67	5	4,77
6	9,34	6	2,65
7	8,75	7	6,90
8	12,25	8	12,73
9	11,09	9	5,31
10	5,25	10	15,92
11	8,75	11	9,55
12	7,00	12	15,92
13	18,09	13	10,61
14	7,00	14	11,67
15	7,00	15	13,26
16	10,50	16	10,61
17	8,75	17	10,08
18	7,00	18	4,24
19	3,50	19	10,08
20	12,84	20	4,24
21	5,25	21	14,85
22	10,50	22	4,77
23	5,84	23	9,55
24	6,42	24	11,14
25	7,00	25	11,14
26	16,34	26	7,96
27	9,92	27	9,02
28	10,95	28	13,79
29	6,87	29	3,71
30	12,12	30	9,55
31	11,54	31	11,14
32	7,45	32	12,20
33	12,70	33	7,96
34	8,04	34	9,02
35	8,62	35	12,20
36	8,04	36	7,96
37	8,62	37	8,49
38	5,70	38	12,73
39	11,54	39	5,31
40	7,45	40	6,37
41	12,70	41	12,20
42	9,20	42	7,96
43	6,28	43	8,49
44	8,04	44	8,49
45	8,62	45	5,84
46	9,20	46	6,37
47	5,70	47	9,55
48	9,20	48	6,90
49	14,45	49	4,77

50	15,04	50	3,71
51	12,12	51	3,18
52	8,62		
53	6,87	**Mean**	**8,50**
54	15,04	**T/G/C**	**4/7/73**
55	10,37		
56	6,28		
57	16,21		
58	9,20		
59	14,45		
Mean	**9,45**		
T/G/C	**4/6/78**		

Cells responding to 5-HT in GPMP (%)		**Cells responding to nicotine in GPMP (%)**	
Ganglion number	**%**	**Ganglion number**	**%**
1	46,87	1	
2	50,00	2	80,00
3	37,50	3	73,330
4	32,140	4	86,36
5	45,830	5	65,21
6	57,140	6	
Mean	**44,52**	**Mean**	**76,23**
T/G/C	**4/6/78**	**T/G/C**	**4/6/78**

Cells with 5-HT response (%) in Human SMP		**Average AP Frequency to 5-HT (Hz) in Human SMP**	
Ganglion	**%**	**Ganglion**	**%**
1	40,00	1	3,71
2	37,50	2	2,65
3	43,33	3	3,71
4	58,33	4	8,49
5	47,50	5	4,24
6	38,66	6	6,90
7	25,00	7	3,71
8	55,00	8	3,18
9	50,00	9	2,12
10	42,33	10	1,59
11	55,56	11	2,12
12	42,86	12	4,24
Mean	**44,67**	**Mean**	**3,89**
T/G/C	**6/12/116**	**T/G/C**	**6/12/52**

Effects of cilansetron on the AP frequency to 5-HT stimulation in the Human SMP					
Before cilansetron		**In cilansetron**		**After wash-out**	
AP Frequency (Hz)	**Ganglion**	**AP Frequency (Hz)**	**Ganglion**	**AP Frequency (Hz)**	**Ganglion**
5,84	1	1,59	1	1,59	1
4,24	2	0,00	2	0,00	2
4,77	3	0,53	3	0,00	3
5,84	4	0,53	4	1,59	4
5,31	5	2,12	5	1,59	5
5,84	6	1,59	6	1,06	6
9,02	7	3,18	7	1,06	7
12,20	8	0,00	8	0,00	8
5,31	9	1,06	9	1,06	9
4,24	10	1,06	10	2,12	10
6,26	**Mean**	**1,17**	**Mean**	**1,01**	**Mean**
5,57	**Median**	**1,06**	**Median**	**1,22**	**Median**
T/G/C 6/9/33					

Effects of cilansetron on the percentage of neurons to 5-HT stimulation responding in the Human SMP					
Before cilansetron		**In cilansetron**		**After wash-out**	
Neurons resonding to 5-HT (%)	**Ganglion**	**Neurons resonding to 5-HT (%)**	**Ganglion**	**Neurons resonding to 5-HT (%)**	**Ganglion**
50,00	1	25,00	1	20,00	1
34,00	2	14,29	2	22,58	2
45,45	3	16,13	3	25,00	3
85,71	4	12,50	4	3,85	4
43,75	5	14,29	5	24,00	5
71,43	6	24,00	6	28,57	6
40,00	7	36,84	7	20,00	7
54,55	8	19,05	8	13,64	8
25,00	9	25,00	9	25,00	9
22,60	10	7,69	10	14,29	10
47,25	**Mean**	**19,48**	**Mean**	**19,69**	**Mean**
T/G/C 6/9/69					

Multi-Captioning of the neuronal response to 5-HT in the Human SMP							
Ganglion	**No of cells (total)**	**Cells with EPSP**	**% Cells with EPSP**	**Cells with 5-HT response in take 1**	**% Cells with 5-HT response take 1**	**Cells with 5-HT response in take 2**	**% Cells with 5-HT response take 2**
1	16	14	87,50	7	43,75	3	18,75
2	9	7	77,78	5	55,56	2	22,22
3	8	7	87,50	3	37,50	1	12,50
4	7	7	100,00	3	42,86	0	0,00
5	10	8	80,00	4	40,00	2	20,00

6	14	12	85,71	6	42,86	3	21,43
7	8	7	87,50	3	37,50	3	37,50
8	5	4	80,00	2	40,00	2	40,00
Total	**77**	**66**	**85,75**	**33**	**42,50**	**16**	**21,55**

Ganglion	No of cells (total)	Cells with 5-HT response in take 3	% Cells with 5-HT response take 3	Cells with 5-HT response in take 4	% Cells with 5-HT response take 4	Cells with 5-HT response in take 5	% Cells with 5-HT response take 5
1	16	0	0,00	1	6,25	1	6,25
2	9	3	33,33	1	11,11	0	0,00
3	8	0	0,00	0	0,00	0	0,00
4	7	0	0,00	0	0,00	0	0,00
5	10	0	0,00	0	0,00	0	0,00
6	14	0	0,00	0	0,00	0	0,00
7	8	0	0,00	0	0,00	0	0,00
8	5	0	0,00	1	20,00	0	0,00
Total	**77**	**3**	**4,17**	**3**	**4,67**	**1**	**0,78**

Ganglion	Average 5-HT frequency in take 1 (Hz)	Average 5-HT frequency in take 2 (Hz)	Average 5-HT frequency in take 3 (Hz)	Average 5-HT frequency in take 4 (Hz)	Average 5-HT frequency in take 5 (Hz)	
1	3,71	1,06	0,00	1,06	0,53	
2	2,65	1,06	1,06	0,53	0,00	
3	3,71	0,53	0,00	0,00	0,00	
4	8,49	0,00	0,00	0,00	0,00	
5						
6	6,90	4,24	0,00	0,00	0,00	
7	3,71	1,06	0,00	0,00	0,00	
8	3,18	1,59	0,00	0,53	0,00	
						T/G/C:
Total	**4,62**	**1,36**	**0,15**	**0,30**	**0,08**	**5/8/77**

Effect of 5-HTP-DP on the late onset response to 5-HT in GPMP - AP Frequency (Hz)			
Cell Number	**Before 5-HTP-DP**	**During 5-HTP-DP**	**After Wash-Out**
1	11,12	4,24	10,59
2	12,18	10,06	12,71
3	9,00	3,71	7,94
4	10,06	0,53	1,06
5	11,65	1,06	3,71
6	11,12	7,94	13,23
7	0,00	0,00	2,12
8	9,53	5,82	6,88
9	5,29	3,71	4,76
10	4,24	0,00	2,12
11	4,76	2,65	4,24

12	4,24	0,53	3,18
13	2,65	0,00	1,59
14	1,59	1,59	1,06
15	1,59	0,53	2,12
16	3,18	1,06	4,24
17	3,71	1,06	3,71
18	4,24	1,59	3,71
19	2,65	0,00	7,94
20	7,41	3,18	4,76
21	9,00	2,12	4,76
22	7,41	4,24	5,29
23	5,29	1,59	3,18
24	7,94	4,24	4,24
25	0,53	0,53	0,53
26	1,06	0,53	1,06
27	2,12	1,06	1,59
28	1,06	0,00	0,53
29	1,59	1,06	1,59
30	1,06	0,00	0,53
31	0,53	0,53	1,06
32	1,06	0,00	0,53
33	3,71	0,00	7,41
34	2,12	0,00	1,59
35	1,06	0,00	1,06
36	1,06	0,00	0,53
37	2,65	0,00	2,12
38	2,12	0,00	1,06
39	1,59	0,00	1,06
40	0,53	0,00	2,65
41	2,12	0,00	1,06
42	4,76	0,00	4,76
43	4,24	1,06	4,76
44	0,00	2,12	6,88
45	7,94	1,59	11,65
46	4,24	1,59	3,71
47	2,65	2,12	5,82
48	5,82	1,06	1,59
Median	**3,44**	**1,06**	**3,18**
Mean	**4,26**	**1,54**	**3,82**
T/G/C	**4/6/48**		

Effect of 5-HTP-DP on the late onset response to 5-HT in GPMP - Cells count							
Ganglion Number	**No. of Cells**	**Cells responding before 5-HTP-DP**	**Cells responding before 5-HTP-DP (%)**	**Cells responding during 5-HTP-DP**	**Cells responding during 5-HTP-DP (%)**	**Cells responding after wash-out**	**Cells responding after wash-out (%)**
1	19	6	31,58	7	36,84	7	36,84
2	26	3	11,54	2	7,69	1	3,85
3	21	7	33,33	4	19,05	6	28,57
4	32	8	25,81	5	16,13	7	22,58
5	26	18	69,23	1	3,85	0	0,00
6	22	6	27,27	0	0,00	3	13,64
Total	**146**	**48**	**33,13**	**3,17**	**13,93**	**4**	**17,58**
T/G/C:	**4/6/146**						

Effect of 5-HTP-DP on the late onset response to 5-HT in Human SMP - AP Frequency (Hz)			
Cell Number	**Before 5-HTP-DP**	**During 5-HTP-DP**	**After Wash-Out**
1	6,35	1,06	7,41
2	2,12	2,12	1,59
3	9,00	4,24	8,47
4	7,94	1,59	7,94
5	6,88	1,06	4,24
6	7,94	2,65	3,18
7	4,24	0,00	3,71
8	3,18	1,06	1,59
9	3,71	1,59	1,59
10	3,71	2,12	3,18
11	2,65	0,53	2,12
12	4,24	1,06	3,71
13	7,94	2,12	5,29
14	2,12	0,00	0,53
15	2,65	0,00	2,12
16	3,71	1,59	3,71
17	2,65	0,00	1,06
18	2,12	1,06	1,59
19	4,24	2,12	2,65
20	2,65	1,59	0,53
21	4,24	1,06	2,12
22	3,71	0,00	4,24
23	3,71	1,59	2,12
24	5,82	0,53	4,24
25	2,12	0,00	0,00
26	1,59	0,00	2,65
27	4,76	1,06	2,65
28	3,18	2,12	3,18

29	5,82	1,06	5,29
30	6,88	2,65	4,76
31	2,12	0,00	0,00
32	5,29	2,65	3,71
33	4,76	1,06	3,71
34	4,24	2,12	2,65
Median	**3,97**	**1,06**	**2,91**
Mean	**4,36**	**1,28**	**3,16**
T/G/C:	**4/6/34**		

Effect of 5-HTP-DP on the late onset response to 5-HT in Human SMP – Cell count

Ganglion Number	No. of Cells	Cells responding before 5-HTP-DP	Cells responding before 5-HTP-DP (%)	Cells responding during 5-HTP-DP	Cells responding during 5-HTP-DP (%)	Cells responding after wash-out	Cells responding after wash-out (%)
1	12	5	41,67	4	33,33	3	25,00
2	11	5	45,45	3	27,27	6	54,55
3	13	6	46,15	4	30,77	5	38,46
4	9	3	33,33	3	33,33	3	33,33
5	11	6	54,55	5	45,45	6	54,55
6	16	7	43,75	3	18,75	4	25,00
TOTAL	**72**	**32**	**44,15**	**3,67**	**31,49**	**4,50**	**38,48**
T/G/C	**3/6/76**						

Effect of 5-HTP-DP on the late onset response to 5-BOIP in GPMP

Cell Number	Frequency before HTP-DP	Frequency during HTP-DP	Frequency after wash-out 60 min
1	4,24	0,00	0,00
2	2,12	0,00	1,59
3	4,77	1,06	2,65
4	1,59	0,00	2,12
5	2,65	1,06	2,65
6	0,53	0,00	0,00
7	0,53	0,00	0,00
8	3,18	0,00	3,71
9	1,59	0,00	1,06
10	3,71	0,00	1,59
11	2,12	0,00	2,12
12	1,06	0,00	1,06
13	1,06	0,00	1,06
14	4,77	0,00	5,84
15	4,24	0,00	3,71

16	2,65	0,00	2,65
17	1,06	1,59	
18	3,18	0,00	4,24
19	2,12	0,53	3,18
20	1,06	0,00	2,12
21	1,59	0,00	1,06
22	6,37	0,00	4,24
23	3,18	0,00	1,06
24	2,12	0,00	1,59
25	4,24	0,00	0,00
26	2,12	0,00	0,00
27	0,53	0,00	1,59
28	1,06	1,59	2,12
29	1,06	0,00	0,00
30	3,71	1,59	1,06
31	3,18	1,59	0,00
32	0,53	0,00	0,00
33	2,65	3,18	2,65
34	0,00	0,00	1,59
35	1,59	0,00	1,59
36	0,53	0,00	1,06
37	1,06	0,00	0,53
38	1,59	0,00	0,53
39	1,59	0,00	0,53
40	1,06	1,06	1,06
41	2,12	0,00	2,12
42	1,59	0,00	0,53
43	1,06	0,00	0,00
44	1,59	0,00	0,00
45	0,53	0,00	0,00
46	1,06	0,00	1,06
47	1,06	0,00	4,24
48	2,65	0,00	1,06
Mean	**2,08**	**0,28**	**1,55**
Median	**1,59**	**0,00**	**1,06**
		T/G/C:	**6/9/48**

AP Frequency for Neurons responding to BOIP in 5-HTP-DP in GPMP	
Cell Number	**Frequency (Hz)**
1	1,06
2	1,06
3	1,59
4	0,53
5	1,59
6	1,59
7	1,59
8	3,18
9	1,06
Mean	**1,29**
Median	**1,59**
T/G/C:	**6/9/9**

Effect of 5-HTP-DP on the late onset response to 5-BOIP in GPMP- Cell count							
Ganglion Number	**No. of cells (total)**	**Cells responding before 5-HTP-DP**	**Cells responding before 5-HTP-DP (%)**	**Cells responding during 5-HTP-DP**	**Cells responding during 5-HTP-DP (%)**	**Cells responding after wash-out**	**Cells responding after wash-out (%)**
1	23	7	30,43	2	8,70	4	17,39
2	26	4	15,38	0	0,00	6	23,08
3	24	4	16,67	1	4,17	3	12,50
4	18	4	22,22	2	11,11	4	22,22
5	23	5	21,74	0	0,00	3	13,04
6	20	7	35,00	4	20,00	5	25,00
7	17	4	23,53	0	0,00	4	23,53
8	22	5	22,73	1	4,55	4	18,18
9	19	5	26,32		0,00	4	21,05
Total:	**192**	**45**	**23,78**	**10**	**5,39**	**37**	**19,56**
						T/G/C:	**6/9/192**

AP Frequency for Neurons responding to BOIP in 5-HTP-DP in human	
Cell	**Frequency (Hz)**
1	0,53
2	1,06
3	0,53
4	0,53
5	0,53
6	1,06
Mean	**0,70**
Median	**0,53**
T/G/C:	**3/5/6**

Effect of 5-HTP-DP on the late onset response to 5-BOIP in human - Frequency (Hz)			
Cell Number	**Frequency before HTP-DP**	**Frequency during HTP-DP**	**Frequency after wash-out 60 min**
1	2,12	0,00	1,06
2	1,06	0,00	0,53
3	1,59	0,00	1,06
4	1,06	0,53	1,59
5	2,12	0,00	1,06
6	1,59	0,00	0,00
7	1,06	0,00	1,59
8	1,06	0,00	1,06
9	1,06	0,00	0,00
10	2,12	0,00	2,12
11	2,65	1,06	1,06
12	2,12	0,00	0,53
13	1,06	0,00	0,53
14	2,12	0,53	1,59
15	0,00	0,00	0,53
16	2,65	0,00	1,59
17	1,59	0,53	1,06
18	1,06	0,53	1,06
19	1,06	0,00	0,00
20	3,71	1,06	1,59
Mean	**1,62**	**0,22**	**0,98**
Median	**1,59**	**0,00**	**1,06**
		T/G/C:	**5/8/20**

Effect of 5-HTP-DP on the late onset response to 5-BOIP in human - Cell count							
Ganglion Number	**No. of cells (total)**	**Cells responding before 5-HTP-DP**	**Cells responding before 5-HTP-DP (%)**	**Cells responding during 5-HTP-DP**	**Cells responding during 5-HTP-DP (%)**	**Cells responding after wash-out**	**Cells responding after wash-out (%)**
1	9	3	42,86	1	14,29	2	28,57
2	7	3	33,33	1	11,11	2	22,22
3	7	1	14,29	0	0,00	1	14,29
4	9	5	55,56	1	11,11	4	44,44
5	4	1	25,00	0	0,00	1	25,00
6	6	1	16,67	1	16,67	1	16,67
7	7	3	42,86	1	14,29	3	42,86
8	12	2	16,67	1	8,33	2	16,67
Total	**61**	**19**	**30,90**	**6**	**9,47**	**16**	**26,34**
						T/G/C:	**5/8/61**

Cells with response to 5-MeOT in the human SMP		
Immediate Response:		
Ganglion Number	**Cells responding to electrical stimulation (%)**	**Cells responding to MeOT (%)**
1	100,00	0,00
2	88,89	0,00
3	100,00	0,00
4	77,78	0,00
Mean	**91,67**	**0,00**
Late onset response:		
Ganglion Number	**Cells responding to electrical stimulation (%)**	**Cells responding to MeOT (%)**
5	75,00	0,00
6	100,00	0,00
7	60,00	0,00
8	88,89	0,00
9	100,00	0,00
Mean	**82,96**	**0,00**
	T/G/C:	**6/9/60**

Cells with response to prucalopride spritz application in human SMP				
Ganglion Number	**Cells with response to el. Stimulation (%)**	**Cells with response to prucalopride (1 µM) (%)**	**Cells with response to prucalopride (50 µM) (%)**	**Cells with response to prucalopride (100 µM) (%)**
1	50,00	0,00	0,00	0,00
2	100,00	0,00	0,00	0,00
3	44,44	0,00	0,00	0,00
4	88,89	0,00	0,00	0,00
Mean	**70,83**	**0,00**	**0,00**	**0,00**
5	69,57	0,00	0,00	0,00
6	87,50	0,00	0,00	0,00
7	33,33	0,00	0,00	0,00
Mean	**63,47**	**0,00**	**0,00**	**0,00**
			T/G/C:	**3/7/44**

Effect of prucalopride in the human SMP - fEPSP length all cells			
Cell Number	**fEPSP length Control 1 (s)**	**fEPSP length Control 2 (s)**	**fEPSP length during prucalopride (s)**
1	0,108	0,096	0,137
2	0,143	0,124	0,207
3	0,121	0,096	0,213
4	0,117	0,082	0,185
5	0,209	0,063	0,249
6	0,112	0,104	0,079
7	0,079	0,091	0,101
8	0,106	0,112	0,139
9	0,226	0,106	0,422
10	0,041	0,034	0,036
11	0,066	0,104	0,087
12	0,129	0,149	0,170
13	0,079	0,090	0,128
14	0,132	0,088	0,141
15	0,086	0,118	0,113
16	0,105	0,147	0,121
17	0,079	0,119	0,106
18	0,046	0,039	0,045
19	0,042	0,040	0,043
20	0,049	0,059	0,091
21	0,050	0,050	0,081
22	0,072	0,089	0,060
23	0,013	0,017	0,017
24	0,067	0,078	0,079

25	0,058	0,084	0,071
26	0,059	0,056	0,075
27	0,061	0,083	0,059
28	0,040	0,051	0,050
29	0,130	0,146	0,243
30	0,101	0,142	0,142
31	0,053	0,046	0,077
32	0,043	0,075	0,059
33	0,092	0,084	0,094
34	0,127	0,103	0,114
35	0,084	0,033	0,103
Mean	**0,089**	**0,085**	**0,118**
Median	**0,079**	**0,089**	**0,096**
Increase:	**24,56 %**		
		T/G/C:	**3/4/35**

Effect of prucalopride in the human SMP- fEPSP length in responsive neurons (increase > 2x natural variance)				
Cell Number	**fEPSP length Control 1 (s)**	**fEPSP length Control 2 (s)**	**Natural variance [s]**	**fEPSP length during prucalopride (s)**
1	0,121	0,096	0,025	0,213
2	0,117	0,082	0,035	0,185
3	0,112	0,104	0,009	0,079
4	0,106	0,112	0,006	0,139
5	0,129	0,149	0,020	0,170
6	0,108	0,119	0,011	0,148
7	0,049	0,059	0,010	0,091
8	0,059	0,056	0,004	0,075
9	0,130	0,146	0,016	0,243
10	0,053	0,046	0,007	0,077
Mean	**0,099**	**0,097**	**0,014**	**0,142**
Median	**0,110**	**0,100**	**0,010**	**0,143**
Increase in lenght (%):	**31,93**			
Cells with increase in length:10/35=28,6%				
			T/G/C:	**3/4/35**

Effect of prucalopride in the human SMP - fEPSP area all cells			
Cell Number	**fEPSP area Control 1 (%s)**	**fEPSP area Control 2 (%s)**	**fEPSP length during prucalopride (%s)**
1	0,064	0,019	0,035
2	0,039	0,075	0,095
3	0,013	0,011	0,010
4	0,032	0,034	0,059
5	0,028	0,026	0,016
6	0,017	0,021	0,023
7	0,011	0,015	0,007
8	0,031	0,027	0,063
9	0,014	0,012	0,016
10	0,034	0,038	0,057
11	0,132	0,134	0,146
12	0,144	0,118	0,150
13	0,004	0,010	0,022
14	0,023	0,034	0,060
15	0,043	0,060	0,059
16	0,017	0,043	0,085
17	0,013	0,015	0,024
18	0,022	0,025	0,014
19	0,011	0,013	0,014
20	0,008	0,009	0,017
21	0,019	0,022	0,023
22	0,012	0,012	0,011
23	0,021	0,024	0,094
24	0,025	0,035	0,067
25	0,014	0,018	0,028
26	0,015	0,011	0,024
27	0,023	0,018	0,019
28	0,014	0,012	0,020
29	0,085	0,091	0,061
30	0,022	0,032	0,095
31	0,043	0,036	0,077
32	0,016	0,018	0,012
33	0,039	0,038	0,041
34	0,046	0,046	0,034
35	0,012	0,009	0,029
36	0,016	0,025	0,020
37	0,018	0,020	0,027
38	0,021	0,026	0,022
39	0,041	0,040	0,034
40	0,010	0,012	0,070
41	0,053	0,075	0,042
42	0,042	0,030	0,029
43	0,031	0,025	0,041
44	0,024	0,029	0,033
45	0,016	0,022	0,024
46	0,012	0,028	0,021
47	0,027	0,030	0,041

48	0,013	0,021	0,009
49	0,036	0,045	0,041
50	0,013	0,022	0,027
Mean	**0,030**	**0,032**	**0,042**
Median	**0,021**	**0,025**	**0,029**
Increase:	**22,81 %**		
		T/G/C:	**4/5/50**

Effect of prucalopride in the human SMP - fEPSP area in responsive neurons (increase > 2x natural variance)				
Cell	**fEPSP area Control 1 (%s)**	**fEPSP area Control 2 (%s)**	**Natural variance [%s]**	**fEPSP length during prucalopride (%s)**
1	0,032	0,034	0,002	0,059
2	0,031	0,027	0,004	0,063
3	0,034	0,038	0,005	0,057
4	0,132	0,134	0,002	0,146
5	0,023	0,034	0,011	0,060
6	0,017	0,043	0,027	0,085
7	0,013	0,015	0,002	0,024
8	0,011	0,013	0,001	0,014
9	0,008	0,009	0,001	0,017
10	0,021	0,024	0,003	0,094
11	0,025	0,035	0,010	0,067
12	0,015	0,011	0,004	0,024
13	0,014	0,012	0,002	0,020
14	0,022	0,032	0,010	0,095
15	0,012	0,009	0,003	0,029
16	0,010	0,012	0,002	0,070
17	0,027	0,030	0,003	0,041
Mean	**0,026**	**0,030**	**0,005**	**0,057**
Median	**0,021**	**0,027**	**0,003**	**0,059**
Increase in area (%):	**46,92**			
Cells with increase in area:17/50=34,00%				
			T/G/C:	**4/5/50**

Effect of prucalopride in human SMP - fEPSP amplitude in neurons (all cells)			
Cell Number	**Baseline/ EPSP ratio Control 1 (dF/F[%])**	**Baseline/ EPSP ratio Control 2 (dF/F[%])**	**Baseline/ EPSP ratio during prucalopride (dF/F[%])**
1	0,26	0,34	0,59
2	0,29	0,21	0,22
3	0,23	0,11	0,08
4	0,61	0,66	0,72
5	0,57	0,49	0,70
6	0,65	0,32	0,60
7	0,63	0,57	0,89
8	0,32	0,41	0,27
9	0,25	0,26	0,28
10	0,46	0,71	0,73
11	0,26	0,19	0,26
12	0,16	0,19	0,40
13	0,09	0,09	0,08
14	0,40	0,20	0,06
15	0,06	0,08	0,25
16	0,74	0,42	0,28
17	0,44	0,39	0,33
18	0,19	0,22	0,23
19	0,12	0,12	0,11
20	0,14	0,18	0,28
21	0,15	0,11	0,24
22	0,23	0,18	0,19
23	0,43	0,36	0,77
24	0,16	0,18	0,12
25	0,39	0,38	0,41
26	0,12	0,09	0,29
27	0,13	0,11	0,10
28	0,04	0,10	0,22
29	0,17	0,21	0,23
30	0,11	0,15	0,07
31	0,31	0,27	0,63
32	0,14	0,12	0,16
33	0,57	0,72	0,68
34	0,60	0,56	0,49
35	0,44	0,39	0,46
Mean	**0,31**	**0,29**	**0,35**
Median	**0,26**	**0,21**	**0,28**
Increase in amplitude (%):		**12,56**	
		T/G/C	**3/4/35**

Effect of prucalopride in human SMP - fEPSP amplitude in responsive neurons (increase > 2x natural variance)				
Cell Number	**Baseline/ EPSP ratio Control 1 (dF/F[%])**	**Baseline/ EPSP ratio Control 2 (dF/F[%])**	**Natural variance (dF/F[%])**	**Baseline/ EPSP ratio during prucalopride (dF/F[%])**
1	0,14	0,18	0,04	0,28
2	0,15	0,11	0,04	0,24
3	0,43	0,36	0,07	0,77
4	0,12	0,09	0,03	0,29
5	0,31	0,27	0,04	0,63
6	0,16	0,19	0,03	0,40
7	0,06	0,08	0,02	0,25
8	0,61	0,66	0,05	0,72
9	0,57	0,49	0,08	0,70
10	0,63	0,57	0,06	0,89
11	0,25	0,26	0,01	0,28
12	0,76	0,75	0,01	0,92
13	0,70	0,81	0,11	1,01
14	0,51	0,42	0,09	0,58
Mean	**0,39**	**0,37**	**0,05**	**0,57**
Median	**0,37**	**0,32**	**0,04**	**0,61**
Increase in amplitude:	**34,17%**			
Cells with increase in amplitude:14/35=40,00%				
			T/G/C:	**3/4/35**

Effects of prucalopride and piboserod - fEPSP amlitude in human SMP						
Cell Number	**fEPSP amlitude Control 1 (dF/F[%])**	**fEPSP amlitude Control 2 (dF/F[%])**	**fEPSP amlitude during prucalopride (dF/F[%])**	**fEPSP amlitude during piboserod (dF/F[%])**	**fEPSP amlitude after wash-out of piboserod (dF/F[%])**	**fEPSP amlitude after wash-out of prucalopride (dF/F[%])**
1	0,26	0,34	0,59	0,22	0,69	0,30
2	0,29	0,21	0,22	0,09	0,21	0,17
3	0,23	0,11	0,08	0,04	-0,02	-0,17
4	0,61	0,66	0,72	0,64	0,83	0,59
5	0,57	0,49	0,70	0,61	0,63	0,51
6	0,65	0,32	0,60	0,18	0,51	0,46
7	0,63	0,57	0,89	0,71	0,87	0,44
8	0,32	0,41	0,27	0,25	0,25	0,25
9	0,25	0,26	0,28	0,06	0,18	0,09
10	0,46	0,71	0,73	0,65	0,80	0,66
11	0,26	0,19	0,26	0,38	0,28	0,46
12	0,16	0,19	0,40	0,14	0,36	0,22
13	0,09	0,09	0,08	0,02	-0,04	-0,02
14	0,40	0,20	0,06	-0,02	0,03	0,00
15	0,06	0,08	0,25	0,10	0,27	0,13
16	0,74	0,42	0,28	0,65	0,46	0,33
17	0,44	0,39	0,33	0,30	0,24	0,29
18	0,19	0,22	0,23	0,16	0,25	0,20
19	0,12	0,12	0,11	0,18	0,20	0,27
20	0,14	0,18	0,28	0,21	0,26	0,22
21	0,15	0,11	0,24	0,41	0,40	0,34
22	0,23	0,18	0,19	0,10	0,12	0,70
23	0,43	0,36	0,77	0,53	0,75	0,42
24	0,16	0,18	0,12	0,42	0,30	0,29
25	0,39	0,38	0,41	0,31	0,25	0,41
26	0,12	0,09	0,29	0,24	0,29	0,33
27	0,13	0,11	0,10	0,16	0,22	0,24
28	0,04	0,10	0,22	0,12	0,28	0,21
29	0,17	0,21	0,23	0,27	0,30	0,41
30	0,11	0,15	0,07	0,13	0,21	0,09
31	0,31	0,27	0,63	0,36	0,45	0,41
32	0,14	0,12	0,16	0,13	0,22	0,27
33	0,51	0,42	0,58	0,35	0,49	0,48
34	0,60	0,56	0,49	0,48	0,32	0,11
35	0,44	0,39	0,46	0,35	0,41	0,39
36	0,76	0,75	0,92	0,84	0,90	0,80
37	0,70	0,81	1,01	0,89	0,98	0,84
38	0,57	0,72	0,68	0,59	0,00	0,00
Mean	**0,34**	**0,32**	**0,39**	**0,32**	**0,37**	**0,32**
Median	**0,28**	**0,24**	**0,28**	**0,26**	**0,29**	**0,30**
					T/G/C	**4/5/38**

Effects of prucalopride and piboserod – fEPSP amlitude in responsive neurons of human SMP						
Cell Number	**fEPSP amlitude Control 1 (dF/F[%])**	**fEPSP amlitude Control 2 (dF/F[%])**	**fEPSP amlitude during prucalopride (dF/F[%])**	**fEPSP amlitude during piboserod (dF/F[%])**	**fEPSP amlitude after wash-out of piboserod (dF/F[%])**	**fEPSP amlitude after wash-out of prucalopride (dF/F[%])**
1	0,14	0,18	0,28	0,21	0,26	0,22
2	0,15	0,11	0,24	0,41	0,4	0,34
3	0,43	0,36	0,77	0,53	0,75	0,42
4	0,12	0,09	0,29	0,24	0,29	0,33
5	0,31	0,27	0,63	0,36	0,45	0,41
6	0,16	0,19	0,4	0,14	0,36	0,22
7	0,06	0,08	0,25	0,1	0,27	0,13
8	0,61	0,66	0,72	0,64	0,83	0,59
9	0,57	0,49	0,7	0,61	0,63	0,51
10	0,63	0,57	0,89	0,71	0,87	0,44
11	0,25	0,26	0,28	0,06	0,18	0,09
12	0,76	0,75	0,92	0,84	0,9	0,8
13	0,7	0,81	1,01	0,89	0,98	0,84
14	0,51	0,42	0,58	0,35	0,49	0,48
15	0,26	0,34	0,59	0,22	0,69	0,3
Mean	**0,37**	**0,37**	**0,57**	**0,42**	**0,55**	**0,40**
	Responsive cells: 15/38 = 39.5 %					
	% increase Pruc:		34,7368421			
	% increase Pib:		11,5689382			
	% increase w/o Pib:		33,1736527			
	% increase w/o Pruc:		8,82352941			
	Nat.Variance 0.05				**T/G/C**	**4/5/38**

Effects of prucalopride and piboserod – percentage of responsive neurons per ganglion					
Ganglion	**Cells responding to electrical stimulation**	**% Cells with increased EPSP after Pruc**	**% Cells with increased EPSP after Pib**	**% Cells with increased EPSP after w/o Pib**	**% Cells with increased EPSP after w/o Pruc**
1	100,00	35,71	7,14	28,57	14,29
2	83,33	33,33	0,00	33,33	0,00
3	100,00	50,00	25,00	37,50	12,50
4	85,70	42,86	14,29	42,86	14,29
5	75,00	25,00	0,00	25,00	0,00
Mean	**88,81**	**37,38**	**9,29**	**33,45**	**8,21**
				T/G/C	**4/5/38**

Effects of prucalopride on the CAP amplitude of responsive neurons in human SMP (dF/F[%])			
Cell Number	**fEPSP amlitude Control 1 (dF/F[%])**	**fEPSP amlitude Control 2 (dF/F[%])**	**fEPSP amlitude during prucalopride (dF/F[%])**
1	0,91	0,82	0,74
2	1,08	1,01	0,81
3	1,14	1,11	1,05
4	0,24	0,26	0,17
5	0,39	0,3	0,32
6	1,98	2,03	1,99
7	0,76	0,94	0,55
8	1,68	1,25	1,69
9	0,29	0,41	0,48
10	1,37	0,91	1,17
11	1,26	1,13	1,32
12	1,14	1,35	1,42
13	1,68	1,25	1,69
14	1,15	1,45	1,63
15	0,29	0,41	0,48
Mean	**1,02**	**0,973**	**1,03**
Median	**1,14**	**1,01**	**1,05**
		T/G/C	**4/5/15**

Effect of piboserod on late onset response to 5-HT in human SMP - AP frequency of responsive cells			
Cell Number	**AP frequency to 5-HT (Hz)**	**AP frequency to 5-HT during piboserod (Hz)**	**5-HT frequency after wash-out (Hz)**
1	4,68	3,16	6,27
2	7,33	2,63	3,10
3	4,68	5,28	3,10
4	2,57	4,22	6,80
5	3,10	9,51	4,68
6	6,27	6,33	5,21
7	2,57	3,69	2,57
8	5,74	3,16	3,10
9	3,10	3,69	6,27
10	5,21	3,16	3,62
11	3,10	2,10	3,62
12	4,15	2,63	5,21
13	2,04	3,16	2,04
14	3,10	2,10	1,51
Mean	**4,12**	**3,91**	**4,08**

		T/G/C	**4/6/14**

Effect of piboserod on late onset response to 5-HT in human SMP- percentage of cells responding			
Ganglion Number	**Cells responding to 5-HT**	**Cells responding to 5-HT during piboserod (%)**	**Cells responding to 5-HT after wash-out (%)**
1	39,75	39,75	33,50
2	39,33	34,66	34,66
3	43,75	36,00	54,00
4	50,00	50,00	43,50
5	18,18	27,27	18,18
Mean	**38,20**	**37,54**	**36,77**
		T/G/C	**4/6/37**

Effect of 5-CT in the human SMP – percentage of cells responding			
Ganglion Number	**Cells responding to electric stimulation (%)**	**Cells responding to 5-CT (%)**	**Cells responding to 5-HT (%)**
1	60,00	0,00	
2	63,64	0,00	27,27
3	77,78	0,00	
4	83,33	0,00	33,33
5	66,67	0,00	
6	40,00	0,00	
7	75,00	0,00	50,00
8	85,71	0,00	28,57
9	80,00	6,67	46,67
Mean	**70,24**	**0,74**	**37,17**
		T/G/C	**6/9/71**

Effect of 5-CT in GP MP - % cells responding			
Ganglion Number	**Cells responding to electric stimulation (%)**	**Cells responding to 5-CT (%)**	**Cells responding to 5-HT (%)**
1	54,17	0,00	25,00
2	78,57	3,57	28,57
3	78,26	0,00	21,74

4	72,22	0,00	50,00
5	71,43	0,00	38,10
6	73,68	0,00	57,89
7	70,83	0,00	62,50
8	62,50	8,33	0,00
9	71,43	0,00	41,67
10	75,00	10,00	
11	72,00	4,00	
12	52,94	0,00	20,00
13	62,50	12,50	47,06
14	65,22	4,35	
15	43,48	0,00	52,17
16	78,13	3,13	43,48
17	64,71	0,00	28,13
18	75,00	6,25	64,71
19	63,16	0,00	25,00
20	72,41	3,45	84,21
21	64,71	0,00	31,03
Mean	**67,73**	**2,65**	**40,07**
		T/G/C	**14/21/479**

Effect of 5-CT - AP Frequency (Hz) of responsive neurons		
Cell Number	**Human SMP**	**GP MP**
1		0,66
2		1,59
3		0,80
4		1,06
5		0,53
6		0,53
7		0,53
8		0,80
9	0,50	1,06
Mean	**0,50**	**0,84**
T/G/C	**6/9/1**	**14/21/9**

Immunohistochemistry: Anti-SR7 staining GP MP Ileum 1:200		
Ganglion	**Cells Total**	**Cells with SR-7 staining**
1	35	13
2	22	5
3	43	14

4	27	5
5	58	14
6	40	11
7	25	6
8	27	7
9	28	7
10	67	17
11	18	3
12	22	4
13	39	13
14	15	3
15	13	3
16	29	6
17	9	1
18	13	2
19	19	3
20	15	3
21	22	2
22	15	14
23	29	4
24	17	6
25	39	7
26	26	13
27	21	3
28	37	11
29	32	11
30	27	7
31	9	0
32	36	9
Mean	**874**	**227**
	%	**25,97**

Immunohistochemistry: Anti-SR7 staining GP MP Ileum 1:1000		
Ganglion	**Cells Total**	**Cells with SR-7 staining**
1	23	7
2	25	7
3	21	1
4	31	6
5	28	2
6	12	3
7	21	5
8	47	15
9	30	6
10	44	9
11	35	14
12	28	4

13	32	6
Mean	**377**	**85**
	%	**22,55**

Immunohistochemistry: Anti-SR7 Staining GP MP Ileum 1:500 (1)		
Ganglion	**Cells Total**	**Cells with SR-7 staining**
1	35	6
2	9	2
3	28	9
4	22	4
5	8	3
6	48	9
7	21	6
8	22	9
9	31	6
10	58	20
11	11	0
12	21	3
13	20	6
14	37	19
15	31	8
16	17	3
17	35	12
18	7	0
19	18	6
20	43	11
21	39	7
22	34	4
23	37	10
24	35	8
25	47	13
26	14	3
27	6	1
Total	**734**	**188**
	%	**25,61**

Immunohistochemistry: Anti-SR7 Staining GP MP Ileum 1:500 (2)		
Ganglion	**Cells Total**	**Cells with SR-7 staining**
1	23	9
2	49	11
3	22	5

4	24	4
5	63	17
6	16	4
7	19	3
8	33	8
9	16	0
10	24	13
11	27	6
12	39	7
13	40	11
14	22	7
15	38	9
16	29	8
17	41	14
18	26	5
19	36	6
20	45	9
21	39	10
22	31	8
23	26	9
24	10	2
25	9	2
26	27	7
27	30	10
28	21	6
29	33	8
30	48	13
31	34	12
32	11	3
33	33	13
34	9	3
35	35	5
36	41	9
Total	**1069**	**276**
	%	**25,82**

Immunohistochemistry: Anti-SR7 Staining GP SMP Ileum 1:500 (1)		
Ganglion	**Cells Total**	**Cells with SR-7 staining**
1	12	2
2	11	2
3	6	0
4	14	3
5	10	2
6	7	3
7	4	0
8	16	0

9	10	0
10	6	2
11	14	3
12	4	1
13	14	4
14	3	1
15	6	0
16	7	2
17	18	1
18	13	2
19	13	0
20	4	1
21	6	0
22	14	1
23	9	2
24	11	2
25	11	1
26	13	3
Total	**256**	**38**
	%	**14,84**

Immunohistochemistry: Anti-SR7 Staining GP SMP Ileum 1:500 (2)		
Ganglion	**Cells Total**	**Cells with SR-7 staining**
1	13	4
2	15	2
3	23	3
4	7	0
5	12	1
6	8	0
7	9	1
8	12	1
9	6	0
10	16	2
11	12	2
12	10	0
13	11	3
14	8	0
15	16	0
16	10	2
17	4	1
18	15	0
19	18	0
20	14	1
21	4	0
22	8	1
23	11	1

24	15	0
25	16	3
Total	**293**	**28**
	%	**9,56**

Immunohistochemistry: Anti-SR7 Staining GP SMP Ileum 1:1000		
Ganglion	**Cells Total**	**Cells with SR-7 staining**
1	12	1
2	7	0
3	6	0
4	8	1
5	15	1
6	7	0
7	8	2
8	16	2
9	10	3
10	12	0
11	14	1
12	12	1
13	6	2
14	6	1
15	13	2
16	8	0
17	13	1
18	5	0
19	3	1
20	19	0
21	14	0
22	7	2
23	9	1
24	6	2
25	13	1
Total	**249**	**25**
	%	**10,04**

Comparing 5-HT actions between MP and SMP of GP – Percentage of cells with 5-HT response			
Ganglion	**Cells responding in MP (%)**	**Cells responding in SMP (%)**	**Ganglion**
1	40,85	55,56	**1**
2	30,22	50,00	**2**
3	47,29	58,82	**3**

4	36,36	56,25	**4**
5	39,13	54,50	**5**
6	17,24	56,25	**6**
7	26,09	55,56	**7**
8	44,00	47,06	**8**
9	39,00	70,00	**9**
10	46,67	54,83	**10**
11	36,36	53,85	**11**
12	38,14	55,56	**12**
13	36,67	54,83	**13**
14	68,75	41,67	**14**
Mean	39,05	54,62	
Median	**38,57**	**55,19**	
T/G/C	**7/14/235**	**7/14/166**	

Comparing 5-HT Actions between MP and SMP of GP - Average frequency of APs to 5-HT-Stimulation			
Cell	**AP frequency in the MP (Hz)**	**AP frequency in the SMP (Hz)**	**Cell**
1	13,79	15,38	**1**
2	3,18	5,31	**2**
3	3,71	3,18	**3**
4	8,49	11,67	**4**
5	2,12	4,77	**5**
6	5,84	9,55	**6**
7	2,65	5,31	**7**
8	2,12	5,84	**8**
9	3,18	6,37	**9**
10	6,37	14,85	**10**
11	3,71	9,02	**11**
12	2,12	4,24	**12**
13	3,18	4,24	**13**
14	4,24	3,18	**14**
15	6,37	8,49	**15**
16	6,90	7,96	**16**
17	12,20	11,14	**17**
18	11,67	11,67	**18**
19	7,96	9,02	**19**
20	7,43	8,49	**20**
21	5,31	10,08	**21**
22	11,14	15,92	**22**
23	6,37	12,20	**23**
24	7,96	4,24	**24**
25	7,43	8,49	**25**
26	4,24	7,96	**26**

27	6,37	11,14	**27**
28	7,96	10,08	**28**
29	6,37	4,77	**29**
30	5,84	7,96	**30**
31	12,73	6,37	**31**
32	9,02	16,45	**32**
33	7,96	6,37	**33**
34	6,37	6,37	**34**
35	3,71	9,55	**35**
36	6,37	7,96	**36**
37	4,77	6,37	**37**
38	6,37	3,18	**38**
39	5,84	11,67	**39**
40	7,96	4,77	**40**
41	6,37	9,55	**41**
42	4,24	5,31	**42**
43	9,55	5,84	**43**
44	9,02	6,37	**44**
45	6,37	14,85	**45**
46	5,84	9,02	**46**
47	6,37	7,96	**47**
48	6,90	4,24	**48**
49	6,37	9,02	**49**
50	4,24	5,84	**50**
51	5,84	6,37	**51**
52	4,24	4,77	**52**
53	10,08	9,55	**53**
54	6,37	15,38	**54**
55	8,49	5,31	**55**
56	11,14	6,37	**56**
57	7,43	4,77	**57**
58	5,84	9,02	**58**
59	4,24	12,73	**59**
60	2,12	14,32	**60**
61	3,71	13,26	**61**
62	6,37	16,45	**62**
63	3,71	11,14	**63**
64	2,12	8,49	**64**
65	7,43	11,67	**65**
66	6,37	13,26	**66**
67	11,14	12,20	**67**
68	6,37	10,08	**68**
69	7,96	9,02	**69**
70	11,67	5,84	**70**
71	4,24	4,24	**71**
72	5,84	11,67	**72**
73	2,12	7,43	**73**
74	3,71	3,71	**74**
75	7,96	12,73	**75**
76	6,37	6,37	**76**
77	7,43	11,14	**77**

78	4,24	12,73	**78**
79	3,71	6,90	**79**
80	7,96	4,77	**80**
81	6,37	9,55	**81**
82	2,12	6,37	**82**
83	5,84	14,32	**83**
84	2,65	5,31	**84**
85	4,24	5,84	**85**
86	2,65	6,37	**86**
87	4,77	10,08	**87**
88	6,37	12,20	**88**
89	5,84	9,55	**89**
90	3,18	5,31	**90**
91	3,71	5,84	**91**
92	12,20		
Mean	**6,18**	**8,59**	
Median	**6,37**	**8,49**	
T/G/C	**7/14/92**	**7/14/91**	

Comparing histamine actions between MP and SMP of GP - Cells with response to histamine			
Ganglion	**Cells responding to histamine in the MP (%)**	**Cells responding to histamine in the SMP (%)**	**Ganglion**
1	6,06	31,25	**1**
2	8,70	16,67	**2**
3	12,00	17,65	**3**
4	21,00	50,00	**4**
5	52,38	26,25	**5**
6	36,36	42,86	**6**
7	18,75	33,33	**7**
8	16,67	37,50	**8**
9	52,94	41,67	**9**
10	40,00	53,33	**10**
11	26,25	33,33	**11**
12	21,50	25,00	**12**
Mean	26,05	34,07	
Median	**21,25**	**33,33**	
T/G/C	**8/12/279**	**8/12/155**	

Comparing histamine actions between MP and SMP of GP - Average frequency of APs to histamine stimulation			
Cell	**AP frequency in the MP (Hz)**	**AP frequency in the SMP (Hz)**	**Cell**
1	1,93	1,28	**1**
2	1,28	1,28	**2**
3	1,93	1,93	**3**
4	3,21	4,49	**4**
5	4,49	3,85	**5**
6	7,70	8,34	**6**
7	1,93	2,57	**7**
8	3,21	3,21	**8**
9	1,93	4,49	**9**
10	3,21	2,57	**10**
11	7,70	14,12	**11**
12	2,57	1,93	**12**
13	1,93	2,57	**13**
14	3,21	2,57	**14**
15	3,21	3,85	**15**
16	4,49	7,70	**16**
17	13,48	4,49	**17**
18	3,21	5,78	**18**
19	7,70	8,34	**19**
20	5,78	3,21	**20**
21	5,13	4,49	**21**
22	3,85	3,21	**22**
23	7,70	4,49	**23**
24	7,06	5,13	**24**
25	5,78	1,28	**25**
26	3,21	3,85	**26**
27	5,13	3,21	**27**
28	1,28	3,85	**28**
29	8,99	0,64	**29**
30	3,85	3,21	**30**
31	1,28	1,93	**31**
32	1,93	7,06	**32**
33	5,13	3,21	**33**
34	2,57	1,28	**34**
35	5,13	1,93	**35**
36	1,28	7,70	**36**
37	4,49	3,21	**37**
38	0,64	6,90	**38**
39	2,57	3,18	**39**
40	1,93	1,06	**40**
41	3,21	3,18	**41**
42	1,28	6,90	**42**
43	1,93	5,31	**43**
44	7,06	9,02	**44**
45	1,93	4,24	**45**

46	4,49	3,71	**46**
47	2,57	7,43	**47**
48	1,28	5,84	**48**
49	2,65	4,24	**49**
50	2,12	1,06	**50**
51	4,77	3,71	**51**
52	6,90	1,06	**52**
53	2,65	5,31	**53**
54	4,24		
55	3,18		
56	2,12		
57	2,65		
58	4,24		
59	2,65		
60	2,12		
61	3,18		
62	3,85		
63	2,12		
64	1,06		
65	2,65		
66	4,49		
67	7,06		
68	8,99		
69	3,85		
70	5,13		
71	7,70		
72	5,13		
73	7,70		
74	4,49		
Mean	3,98	4,16	
Median	**3,21**	**3,71**	
T/G/C	**8/12/279**	**8/12/155**	

Comparing nicotine actions between MP and SMP of GP - Cells with response to nicotine (%)			
Ganglion	**MP**	**SMP**	**Ganglion**
1	86,36	38,46	**1**
2	65,22	77,27	**2**
3	78,00	69,33	**3**
4	90,16	80,00	**4**
5	76,19	73,00	**5**
6	77,57	64,00	**6**
7	100,00	75,00	**7**
8	80,95	71,63	**8**
9	81,81	68,59	**9**
10	84,85	77,78	**10**
11	86,96	77,27	**11**

12	79,31	76,47	**12**
13	65,22	100,00	**13**
14	80,00	87,50	**14**
15	73,33	44,44	**15**
16	86,36	80,00	**16**
17	65,22	66,67	**17**
18	77,27	38,46	**18**
19	73,33	64,29	**19**
20	87,08	75,00	**20**
21	86,31	70,63	**21**
22	79,59	87,50	**22**
23	80,95	68,23	**23**
24	87,38	70,63	**24**
25	76,50	87,50	**25**
26	65,65	69,00	**26**
27	88,89	75,33	**27**
28	80,00	45,56	**28**
29	94,44	79,33	**29**
30	61,54	70,00	**30**
31	92,00	80,95	**31**
32	70,00	45,71	**32**
33	95,45	84,21	**33**
Mean	80,42	70,90	
Median	**79,80**	**71,80**	
T/G/C	**14/31/483**	**14/31/303**	

Average Frequency of APs to Nicotine - stimulation (Hz)			
Cell	**MP**	**SMP**	**Cell**
1	4,77	10,08	**1**
2	20,69	14,32	**2**
3	10,08	15,38	**3**
4	10,61	18,04	**4**
5	5,84	8,49	**5**
6	16,45	9,02	**6**
7	5,84	2,12	**7**
8	1,06	7,96	**8**
9	0,53	11,14	**9**
10	5,84	1,06	**10**
11	4,24	13,79	**11**
12	3,18	12,73	**12**
13	2,65	11,14	**13**
14	4,24	11,67	**14**
15	12,73	14,32	**15**
16	4,24	7,96	**16**
17	7,43	5,84	**17**
18	8,49	2,12	**18**

19	7,96	9,02	**19**
20	13,79	3,18	**20**
21	13,79	4,77	**21**
22	11,14	3,71	**22**
23	14,85	4,77	**23**
24	14,32	4,24	**24**
25	10,61	2,12	**25**
26	11,14	5,84	**26**
27	14,85	11,14	**27**
28	14,32	4,77	**28**
29	12,32	13,79	**29**
30	7,84	8,49	**30**
31	10,64	13,79	**31**
32	9,52	9,02	**32**
33	11,20	10,08	**33**
34	9,52	11,14	**34**
35	10,08	9,55	**35**
36	12,32	9,02	**36**
37	7,84	3,71	**37**
38	10,64	9,02	**38**
39	9,52	3,71	**39**
40	11,20	12,89	**40**
41	9,52	3,92	**41**
42	10,08	8,40	**42**
43	8,40	9,52	**43**
44	8,96	9,52	**44**
45	7,84	6,72	**45**
46	11,76	7,84	**46**
47	10,64	12,32	**47**
48	7,28	3,36	**48**
49	8,40	8,40	**49**
50	8,96	9,52	**50**
51	7,84	10,64	**51**
52	11,76	6,72	**52**
53	11,20	7,84	**53**
54	7,28	10,64	**54**
55	9,41	6,72	**55**
56	9,02	7,28	**56**
57	7,43	11,20	**57**
58	11,67	4,48	**58**
59	14,85	5,60	**59**
60	6,37	10,64	**60**
61	9,55	6,72	**61**
62	7,96	7,28	**62**
63	10,08	7,28	**63**
64	4,24	5,04	**64**
65	8,49	5,60	**65**
66	7,96	8,28	**66**
67	11,14	5,84	**67**
68	10,08	4,24	**68**
69	4,77	3,18	**69**

70	7,96	2,65	**70**
71	6,37	2,12	**71**
72	16,45	3,18	**72**
73	6,37	4,77	**73**
74	6,37	5,84	**74**
75	9,55	4,24	**75**
76	7,96	2,12	**76**
77	6,37	3,71	**77**
78	3,18	6,37	**78**
79	11,67	3,71	**79**
80	4,77	2,12	**80**
81	9,55	7,43	**81**
82	5,31	6,37	**82**
83	5,84	11,14	**83**
84	6,37	6,37	**84**
85	14,85	7,96	**85**
86	9,02	11,67	**86**
87	9,96	4,24	**87**
88	6,24	5,84	**88**
89	11,02	2,12	**89**
90	10,49	3,71	**90**
91	6,77	7,96	**91**
92	11,55	6,37	**92**
93	7,31	7,43	**93**
94	7,84	4,24	**94**
95	7,31	3,71	**95**
96	7,84	7,96	**96**
97	5,18	6,37	**97**
98	10,49	6,12	**98**
99	6,77	9,84	**99**
100	11,55	6,65	**100**
101	8,37	8,24	**101**
102	5,71	6,65	**102**
103	7,31	8,77	**103**
104	7,84	10,37	**104**
105	8,37	9,84	**105**
106	5,18	7,18	**106**
107	8,37	7,71	**107**
108	13,14	7,18	**108**
109	13,67	10,37	**109**
110	11,02	6,65	**110**
111	7,84	11,96	**111**
112	6,24	9,31	**112**
113	13,67	7,18	**113**
114	9,43	7,18	**114**
115	5,71	7,18	**115**
116	14,73	7,18	**116**
117	8,37	6,65	**117**
118	13,14	7,71	**118**
119	14,73	13,02	**119**
120	8,90	12,49	**120**

121	16,32	11,43	**121**
122	15,26	8,24	**122**
123	13,67	6,12	**123**
124	9,96	11,96	**124**
125	17,38	8,77	**125**
126	7,31	10,37	**126**
127	7,84	13,02	**127**
128	13,67	8,24	**128**
129	6,77	11,96	**129**
130	11,55	12,49	**130**
131	7,31	8,77	**131**
132	7,84	14,61	**132**
133	8,37	13,02	**133**
134	6,77	11,96	**134**
135	9,55	8,77	**135**
136	15,38	15,14	**136**
137	5,31	5,59	**137**
138	6,37	7,18	**138**
139	14,85	11,96	**139**
140	9,02	8,77	**140**
141	12,73	11,43	**141**
142	14,32	6,65	**142**
143	13,26	9,84	**143**
144	7,96	11,96	**144**
145	6,90	9,84	**145**
146	16,45	7,43	**146**
147	6,37	9,55	**147**
148	9,02	2,65	**148**
149	12,20	4,24	**149**
150	10,08	9,55	**150**
151	10,08	6,37	**151**
152	12,20	6,37	**152**
153	6,37	9,02	**153**
154	7,96	7,96	**154**
155	6,90	5,84	**155**
156	16,45	4,24	**156**
157	11,14	9,02	**157**
158	8,49	4,24	**158**
159	11,67	6,90	**159**
160	15,38	9,02	**160**
161	12,20	7,96	**161**
162	9,55	7,43	**162**
163	7,43	9,55	**163**
164	9,02	6,37	**164**
165	11,14	3,71	**165**
166	7,96	3,71	**166**
167	7,43	10,08	**167**
168	6,90	7,96	**168**
169	9,02	6,90	**169**
170	11,67	7,96	**170**
171	10,08	9,02	**171**

172	7,96	8,49	**172**
173	9,02	6,90	**173**
174	7,43	4,77	**174**
175	9,55	5,84	**175**
176	6,90	7,96	**176**
177	8,49	5,31	**177**
178	4,77	4,77	**178**
179	9,55	3,71	**179**
180	9,02	5,84	**180**
181	12,61	7,96	**181**
182	8,90	5,84	**182**
183	8,37	3,85	**183**
184	14,73	0,64	**184**
185	13,14	2,57	**185**
186	12,08	1,28	**186**
187	5,18	8,99	**187**
188	6,77	3,85	**188**
189	6,24	4,24	**189**
190	7,84	2,12	**190**
191	5,18	3,71	**191**
192	9,43	6,37	**192**
193	13,67	9,52	**193**
194	19,51	2,65	**194**
195	8,37	2,12	**195**
196	8,90	3,18	**196**
197	11,02	4,77	**197**
198	9,96	5,84	**198**
199	11,55	9,52	**199**
200	13,82	9,52	**200**
201	8,52	6,72	**201**
202	6,39	7,84	**202**
203	16,78	12,32	**203**
204	15,66	3,36	**204**
205	13,97	8,40	**205**
206	14,53	5,21	**206**
207	17,35	6,49	**207**
208	10,59	7,13	**208**
209	8,34	3,28	**209**
210	4,40	5,85	**210**
211	11,72	5,21	**211**
212	5,53	3,28	**212**
213	7,22	6,49	**213**
214	6,09	6,50	**214**
215	7,22	5,38	**215**
216	6,65	4,81	**216**
217	4,40	6,50	**217**
218	8,34	15,51	**218**
219	13,97	6,50	**219**
220	7,22	9,88	**220**
221	16,78	11,01	**221**
222	11,16	10,44	**222**

223	16,78	16,63	**223**
224	11,72	16,63	**224**
225	12,84	13,82	**225**
226	13,97	17,76	**226**
227	12,28	17,20	**227**
228	11,72	13,26	**228**
229	6,09	13,82	**229**
230	11,72	17,76	**230**
231	6,09	17,20	**231**
232	7,22	15,08	**232**
233	6,31	10,32	**233**
234	11,07	13,29	**234**
235	12,25	12,10	**235**
236	12,25	13,89	**236**
237	13,18	12,10	**237**
238	10,53	12,70	**238**
239	10,00	15,08	**239**
240	11,59	10,32	**240**
241	17,42	13,29	**241**
242	11,71	12,10	**242**
243	9,59	13,89	**243**
244	6,94	12,10	**244**
245	8,00	12,70	**245**
246	7,47	10,92	**246**
247	7,34	11,51	**247**
248	13,71	13,03	**248**
249	7,87	9,32	**249**
250	14,36	10,79	**250**
251	10,00	5,84	**251**
252	8,40	11,14	**252**
253	11,06	4,77	**253**
254	5,34	13,79	**254**
255	9,59	8,49	**255**
256	6,94	13,79	**256**
257	9,06	3,18	**257**
258	7,34	4,77	**258**
259	10,53	3,71	**259**
260	10,53	4,77	**260**
261	4,69	4,24	**261**
262	7,34	2,12	**262**
263	7,87	6,50	**263**
264	16,36	12,10	**264**
265	7,87	13,89	**265**
266	6,81	12,10	**266**
267	7,87	12,70	**267**
268	11,29	10,92	**268**
269	7,46	11,51	**269**
270	7,99	3,85	**270**
271	13,82	0,64	**271**
272	6,92	5,85	**272**
273	11,70	5,21	**273**

274	9,17	3,28	**274**
275	12,35	6,49	**275**
276	7,99	3,71	**276**
277	11,91	5,84	**277**
278	10,79	7,96	**278**
279	7,43	5,84	**279**
280	9,70	7,96	**280**
281	8,11	5,84	**281**
282	6,52	12,49	**282**
283	3,33	2,65	**283**
284	9,05	4,24	**284**
285	8,52	9,55	**285**
286	14,88	6,37	**286**
287	4,40	6,37	**287**
288	11,72	9,02	**288**
289	5,53	8,77	**289**
290	13,94	10,37	**290**
291	14,87	13,02	**291**
292	16,99	8,24	**292**
293	6,91	11,96	**293**
294	6,91	6,12	**294**
295	10,63	9,84	**295**
296	12,75	6,65	**296**
297	6,91	8,24	**297**
298	12,10	6,65	**298**
299	5,73	8,77	**299**
300	11,04	11,96	**300**
301	7,32	7,96	**301**
302	8,51	6,37	**302**
303	9,99		
304	11,16		
305	15,40		
306	6,26		
307	16,22		
308	7,44		
309	10,10		
310	9,57		
311	13,16		
312	8,95		
313	9,51		
314	6,49		
315	12,11		
316	7,74		
317	10,63		
318	8,39		
319	7,86		
320	14,34		
321	11,69		
322	15,40		
323	14,87		
324	7,61		

325	12,87		
326	8,39		
327	11,19		
328	8,95		
329	9,51		
330	8,51		
331	6,91		
332	8,91		
333	7,85		
334	10,51		
335	17,93		
336	8,38		
337	3,93		
338	10,59		
339	10,19		
340	7,53		
341	7,53		
342	11,39		
343	7,94		
344	10,30		
345	8,71		
346	14,42		
347	5,94		
348	10,19		
349	12,85		
350	9,65		
351	12,55		
352	8,19		
353	12,11		
354	10,73		
355	6,72		
356	7,12		
357	7,66		
358	8,20		
359	12,45		
360	7,63		
361	10,73		
362	10,20		
363	8,07		
364	14,56		
365	15,08		
366	8,07		
367	11,49		
368	7,54		
369	8,19		
370	9,25		
371	8,72		
372	13,38		
373	4,60		
374	11,92		
375	13,91		

376	10,73		
377	7,01		
378	4,89		
379	7,67		
380	8,07		
381	9,37		
382	8,60		
383	8,07		
384	11,26		
385	11,91		
386	11,90		
387	7,54		
388	16,56		
389	11,79		
390	17,62		
391	8,31		
392	8,75		
393	10,99		
394	9,31		
395	12,11		
396	4,59		
397	8,31		
398	3,53		
399	6,72		
400	8,19		
401	10,96		
402	6,72		
403	10,43		
404	9,90		
405	5,66		
406	7,12		
407	6,72		
408	10,31		
409	6,59		
410	7,66		
411	5,53		
412	1,41		
413	6,19		
414	6,19		
415	4,59		
416	9,90		
417	11,90		
418	7,66		
419	5,12		
420	8,31		
421	8,19		
422	8,31		
423	9,90		
424	3,53		
425	9,37		
426	11,37		

427	7,78		
428	5,12		
429	11,49		
430	3,00		
431	10,84		
432	7,12		
433	15,20		
434	9,37		
435	16,80		
436	6,72		
437	8,19		
438	10,84		
439	8,84		
440	8,31		
441	11,49		
442	0,88		
443	13,08		
444	11,55		
445	9,87		
446	10,43		
447	4,59		
448	12,02		
449	6,72		
450	14,14		
451	10,43		
452	8,19		
453	11,55		
454	6,72		
455	12,11		
456	8,84		
457	8,75		
458	15,20		
459	7,63		
460	7,78		
461	11,49		
462	9,87		
463	14,67		
464	8,19		
465	10,99		
466	9,87		
467	15,20		
468	14,67		
469	12,67		
470	8,19		
471	9,87		
472	14,14		
473	12,67		
474	10,99		
475	10,43		
476	11,55		
477	7,63		

478	9,31		
479	15,20		
480	10,79		
481	15,00		
Mean	9,65	8,06	
Median	**9,31**	**7,90**	
T/G/C	**14/31/483**	**14/31/303**	

Comparing PAR2 actions between MP and SMP of GP - Cells with response to SLIGRL (%)			
Ganglion	**MP**	**SMP**	**Ganglion**
1	41,18	32,00	**1**
2	33,33	21,70	**2**
3	25,00	33,33	**3**
4	50,00	42,15	**4**
5	37,50	35,00	**5**
6	21,74	16,67	**6**
7	33,33	38,46	**7**
8	52,17	42,11	**8**
9	37,93	30,77	**9**
10	42,31	41,18	**10**
11	38,50	25,00	**11**
12	50,00	36,84	**12**
Mean	38,58	32,93	
Median	**38,22**	**34,17**	
T/G/C	**7/12/230**	**7/12/158**	

Average Frequency of APs to SLIGRL - stimulation (Hz)			
Cell	**MP**	**SMP**	**Cell**
1	3,71	1,06	**1**
2	2,12	2,65	**2**
3	2,65	2,12	**3**
4	2,12	6,37	**4**
5	2,65	1,59	**5**
6	1,59	2,65	**6**
7	4,77	2,65	**7**
8	0,53	3,18	**8**
9	4,24	2,65	**9**
10	0,53	2,65	**10**
11	5,31	1,59	**11**
12	2,65	1,06	**12**

13	3,71	1,59	**13**
14	1,06	4,24	**14**
15	6,37	6,37	**15**
16	2,65	2,12	**16**
17	5,31	3,18	**17**
18	2,12	2,12	**18**
19	1,59	7,43	**19**
20	8,49	5,84	**20**
21	9,02	1,06	**21**
22	1,06	1,59	**22**
23	6,90	11,67	**23**
24	3,71	8,49	**24**
25	2,12	2,65	**25**
26	2,65	2,12	**26**
27	2,12	6,37	**27**
28	2,65	1,59	**28**
29	1,59	2,65	**29**
30	4,77	1,06	**30**
31	5,84	3,18	**31**
32	4,24	2,65	**32**
33	0,53	1,59	**33**
34	5,31	6,37	**34**
35	2,65	1,59	**35**
36	3,71	1,06	**36**
37	1,06	2,12	**37**
38	6,37	0,53	**38**
39	2,65	2,12	**39**
40	5,31	2,65	**40**
41	2,12	4,24	**41**
42	1,59	7,43	**42**
43	8,49	3,71	**43**
44	9,02	1,59	**44**
45	1,06	5,84	**45**
46	1,59	3,71	**46**
47	9,55	4,24	**47**
48	13,26	5,31	**48**
49	3,71	4,77	**49**
50	3,18	7,96	**50**
51	5,84	10,61	**51**
52	5,84	6,90	**52**
53	9,55		
54	2,65		
55	2,65		
56	2,12		
57	1,06		
58	2,12		
59	2,65		
60	4,77		
61	3,71		
62	2,65		
63	2,12		

64	1,06		
65	7,43		
66	11,14		
67	4,24		
68	3,18		
69	4,77		
70	4,77		
71	2,65		
72	2,12		
73	4,77		
74	3,71		
75	1,59		
76	2,12		
77	3,71		
78	2,65		
79	6,90		
80	6,90		
81	2,65		
82	1,59		
83	2,65		
84	9,02		
85	2,12		
86	4,77		
87	2,65		
88	2,12		
89	3,71		
Mean	3,87	3,70	
Median	**2,74**	**2,64**	
T/G/C	**7/12/89**	**7/12/52**	

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