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Mechanosensitivity in the myenteric plexus of the guinea pig ileum

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Abbreviations

5-HT	5-hydroxytryptamine
AHP	After-hyperpolarization
AMCA	Aminomethylcoumarin-acetat
ATP	Adenosine triphosphate
Calb	Calbindin
CGRP	Calcitonin gene-related peptide
ChAT	Choline acetyltransferase
CNS	Central nervous system
DRG	Dorsal root ganglion
EC	Enterochromaffin
ENK	Enkephalin
ENS	Enteric nervous system
EPSP	Excitatory postsynaptic potential
GI	Gastrointestinal
HT	High-threshold
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
ICC	Interstitial cell of Cajal
IGLE	Intraganglionic laminar ending
IPAN	Intrinsic primary afferent neuron
LT	Low-threshold
LTSC	Low threshold small conductance
MEC/DEG	Mechanosensory abnormal/degenerin
MP	Myenteric plexus
MSC	Mechanosensitive channels
MSORT	Multi-Site Optical Recording Technique
NeuN	Neuronal nuclei antibody
NFP	Neurofilament protein
NO	Nitric oxide
NOS	Nitric oxide syntethase
NSC	Non-selective cation
SMP	Submucosal plexus
SOM	Somatostatin
SP	Substance P
TRP	Transient receptor potential
TTX	Tetrodotoxin
VIP	Vasoactive intestinal peptide

Mechanosensitivity in the myenteric plexus of the guinea pig ileum

Introduction

Jackie Wood, professor of physiology, cell biology and internal medicine at Ohio State University said: “What Mother Nature had done, rather than packing all of those neurons in the big brain in the skull and sending long lines to the gut, is distribute the microcomputer, the little brain, right along the systems that require control”. The idea that the gut can function by its own dates back to 1899 when Bayliss and Starling made their observations in dogs and rabbits on local reflexes in the isolated or extrinsically denervated intestine (Bayliss and Starling 1899; Bayliss and Starling 1901). During the First World War the German scientist Trendelenburg showed that a self-contained, self-regulating nervous system that is embedded within the wall of the gut could function on its own without input from the brain or the spinal cord (Trendelenburg 1917). In 1921 Langley published the great book “The Autonomic Nervous System” setting the basis for the first classification of the autonomic nervous system in the parasympathetic and sympathetic divisions. He also included a third division coning the term “Enteric Nervous System”, considering the fact that this system differs from the parasympathetic and sympathetic in its anatomical and functional independence from the brain and spinal cord. For reasons that still mystify researchers today, all the data collected until this point went into hibernation for nearly half a century but are still the basis for today research. Around 1965 some neurobiologists began to realise the clinical relevance of the autonomy of the gut and since the 1980s MD Gershon coined the term “second

(or little) brain in the gut". Nowadays it is well known that the enteric nervous system (ENS) regulates all the reflex pathways that control blood flow, motility, water and electrolyte transport and acid secretion, autonomously from the central nervous system (CNS). Despite that the CNS can influence and modulate the gut activity the ability of the gut to function in isolation is one of the most intriguing phenomenons in neurogastroenterology. This requires coding of sensory stimuli by specialized cells in the gut wall, which function as sensory neurons. These neurons are capable to detect chemical and/or mechanical stimulations and to orchestrate the appropriate responses via the neuronal network constituted by the two ganglionated plexus embedded in the gut wall. A lot of studies were carried out in the last years regarding the pathways used by enteric neurons to detect chemical environmental changes and to respond to them. On the other hand, despite all the studies on mechanosensitivity carried on non-neuronal mechanism (e.g. smooth muscle, interstitial cell of Cajal) in the gut, there is a lack of knowledge regarding mechanosensory neuronal pathways. Enteric neurons seemed to be prominent candidates to relay mechanosensitivity. Surprisingly, the identity of mechanosensitive neurons in the ENS as well as the appropriate stimulus modality is unknown.

Enteric nervous system

The ENS is composed by over 100 millions nerve cells embedded in the gut wall.

The nerve cell bodies are grouped in small aggregates, the enteric ganglia, which are connected by bundles of nerve cell processes to form two major ganglionated plexuses in the tubular digestive tract: the myenteric plexus (MP), also called the *Auerbach plexus*, that mainly regulates motility, and the submucosal plexus (SMP), which is often referred to as the *Meissner plexus*, that mainly controls secretion. The MP lies between the outer longitudinal and the inner circular muscle layers of the intestine and forms a continuous network around the circumference and along the gastrointestinal (GI) tract, from the upper oesophagus to the internal anal sphincter. The myenteric ganglia vary in size, shape and orientation between animal species and from one part of the intestine to another (Furness 2006). In the ileum of the guinea pig, ganglia range in size from 5 to over 200 nerve cell bodies (Furness 2006). The SMP is significant only in the small and large intestines. In general, the interconnecting strands of the SMP are finer and the ganglia are smaller than those of the MP (Furness 2006).

Ultrastructure analysis show that enteric ganglia are remarkably compact, consisting of the cell bodies of neurons, enteric glia, and nerve cell processes (Furness 2006). Particularly an electron microscope study described the ganglia of the MP of the guinea pig ileum like very compact structures, completely surrounded by a basal lamina and isolated from the connective tissue and blood vessels (Gabella 1972). All the spaces are occupied by nervous and glial

elements, constituting a dense neuropil, with an intervening gap of 20 nm between the adjacent membranes (Gabella 1972).

Enteric neurons can be divided into different functional classes according to morphology of their cell body, projections to target, neurochemistry and pharmacological properties.

Morphological classification of the enteric neurons

The first and most enduring classification of enteric neurons by their shape was made by Dogiel more than 100 years ago: he provided a comprehensive description of neuron morphologies in the MP and SMP of the intestine from human, guinea pig, rabbit, rat, dog and cat (Dogiel 1895; Dogiel 1896; Dogiel 1899). He described three types of neurons now generally referred to as Dogiel types I, II and III.

The type I are flattened cells, slightly elongated, with stellate or angular outlines, they have 4 to 20 lamellar dendrites and long process which is likely the axon (Dogiel 1899). These neurons do not appear to belong to a single functional class: some are inhibitory motor neurons to the muscle, some are excitatory motor neurons and some are interneurons.

The Dogiel type II neurons have large oval or round cell bodies. Dogiel described them as having 3-10 dendrites and one axon (Dogiel 1899), however is now recognized that the principal processes are all axons. Between 80 and 90% of Dogiel type II neurons in guinea pig MP are immunoreactive for the calcium binding protein Calbindin (Calb) (Furness et al. 1988; Iyer et al. 1988; Song et al. 1991; Costa et

al. 1996) and nearly all are immunoreactive for choline acetyltransferase (ChAT) (Steele et al. 1991).

Type III cells were described as having 2 to 10 dendrites that became thinner and branched distant from the cell body; these dendrites appear to be relatively short, smooth but sometimes they have varicosities. The axon of these cells begins from a protrusion of the cell body or from a dendrite (Dogiel 1899). These cells are today called neurons with filamentous dendrites. Other morphological types of cells were also identified by Dogiel (1899) and in later studies (e.g. Stach 1989), but there is less of a consensus about the general use of this extended classification. Dogiel also suggested a correlation between the morphology and the functions of type I and type II neurons studying the dendrites and the axonal projection, he hypothesized that type I neurons fulfil motor whereas type II neurons have sensory functions (Dogiel 1895; Dogiel 1896; Dogiel 1899).

Electrophysiological classification of the enteric neurons

The first intracellular recordings from enteric neurons that provided the basis for classification were reported in 1970s (Nishi and North 1973; Hirst et al. 1974; Wood and Mayer 1978).

The two major electrophysiological classes of myenteric neurons are:

- AH neurons characterized by phasic spike discharge, spike component partially carried by calcium influx, slow afterspike hyperpolarization (AHP) following a single action potential discharge. They receive little fast synaptic input but can generate slow excitatory postsynaptic potentials (slow EPSPs)

in other AH neurons and S neurons. These cells have a distinctive Dogiel II morphology with large smooth cell bodies and multipolar processes. However, a few myenteric AH neurons are filamentous, uniaxonal neurons and shows fast excitatory postsynaptic potential (fast EPSPs). Many of them are Calb positive (Hirst et al. 1974).

- S neurons which exhibit tonic spike discharge. These neurons lack slow AHP, show sodium-driven action potentials and receive abundant fast EPSPs. These neurons are slowly adapting neurons considered to function as excitatory and inhibitory motoneurons or interneurons. They show a typical Dogiel type I morphology and are usually uniaxonal (Hirst et al. 1974).

While S neurons have abundant synaptic input only relatively few AH neurons generate fast EPSPs, and these are of small amplitude compared with those in S neurons (Kunze and Furness 1999; Blackshaw et al. 2007).

Functional and neurochemical classification of the enteric neuron

Since more than hundred years it has been known that the ENS contains motor neurons, interneurons, and sensory neurons organised into functional circuits (Bayliss and Starling 1899; Langley and Magnus 1905; Trendelenburg 1917).

- Motor neurons: there are excitatory and inhibitory motor neurons innervating the longitudinal, the circular smooth muscle and the muscularis mucosae throughout the digestive tract. These neurons are uniaxonal. The primary transmitters of the excitatory motor neurons are acetylcholine and tachykinins. The inhibitory neurons have multiple transmitters, including nitric oxide (NO), vasoactive intestinal peptide (VIP) and adenosine triphosphate (ATP) (Furness 2006).

  The majority of neurons innervating the circular muscle have their cell bodies in the MP. In the guinea pig they are all in myenteric ganglia (Wilson et al. 1987), in other species, including rat (Ekblad et al. 1987; Ekblad et al. 1988), dog (Sanders and Smith 1986, Furness et al. 1990a), pig (Timmermans et al. 2001), and probably human, a component of circular muscle innervation comes from submucosal ganglia. A consistent finding of retrograde labelling studies has shown that the cell bodies of inhibitory motor neurons, supplying the circular muscle layer, are located oral to the circular muscle that they innervate (Brookes et al. 1991; Brookes et al. 1996;

Brookes et al. 1997; Pfannkuche et al. 1998; Yuan and Brookes 1999) and project aborally for 0.5-25 mm in the myenteric plexus before entering the muscle. In contrast, the cell bodies of excitatory motor neurons, also supplying the circular muscle layer, mostly lie aboral to the circular muscle that they innervate, at distances up to about 8 mm (Brookes et al. 1991). However, a few cholinergic motor neurons project for short distances (up to 1 mm) aborally to the circular muscle (Brookes et al. 1991).

The longitudinal muscle motor neurons are about 25% of nerve cells in the myenteric ganglia of the guinea pig small intestine and their axons do not project more than 3 mm from their nerve cell bodies (Brookes et al. 1992). The cell bodies of the neurons supplying the longitudinal muscle layer are in the MP of small animals. In the pig the majority of them are in the MP, but some longitudinal muscle motor neurons have cell bodies in the outer SMP (Timmermans et al. 2001).

- Interneurons: these neurons have been identified in all gut regions, and vary between regions more than the other types of neurons. In the guinea pig small intestine there classes of descending interneurons and one class of ascending interneurons have been identified (Costa et al. 1996; Furness 2006).

  The ascending interneurons are all located in the myenteric plexus and have medium/large-sized cells bodies with Dogiel type I morphology with lamellar dendrites and a single orally

projecting axon. They project up to 14 mm orally from their nerve cell body. They have a unique chemical coding containing immunoreactivity for ChAT, Calretinin, Substance P (SP), Neurofilament protein (NFP), and Enkephalin (ENK) (Brookes et al. 1997). Their synaptic transmission is predominantly cholinergic, through nicotinic receptors (Furness 2006). Ascending interneurons account for 5% of all myenteric neurons (Costa et al. 1996). In the guinea pig colon there are three classes of ascending interneurons all ChAT immunoreactive, which is consistent with ascending reflexes in the colon utilizing cholinergic neuroneuronal transmission (Lomax and Furness 2000).

The descending interneurons in the guinea pig small intestine include those immunoreactive for ChAT plus Somatostatin (SOM), ChAT plus Nitric oxide synthase (NOS), VIP and others substances and those immunoreactive for ChAT plus 5-hydroxytryptamine (5-HT) (Furness 2006). Their synaptic transmission is also cholinergic, as indicated by their immunoreactivity for ChAT, but transmission in local reflexes is not purely cholinergic (Furness 2006). The ChAT /5-HT neurons do not make connections with the inhibitory muscle motor neurons; they are involved in descending excitatory reflexes (Young and Furness 1995).

The ChAT /SOM neurons are present in the myenteric plexus; they have a characteristic soma-dendritic morphology, with smooth, medium-sized cell bodies with numerous filamentous

dendrites and a single axon (Portbury et al. 1995; Song et al. 1997).

The ChAT /NOS interneurons are involved in descending inhibitory reflexes (Yuan et al. 1995).

- Intestinofugal neurons: these neurons have the cell bodies embedded in the gut wall and send their processes to the prevertebral ganglia, where they form synapses with post-ganglionic sympathetic neurons (Kuntz 1938; Szurszewski and Miller 1994). In the small intestine of the guinea pig, the intestinofugal neurons are small Dogiel type I cells with short lamellar dendrites or sometimes short filamentous dendrites, but all have a single axon (Tassicker et al. 1999). They are immunoreactive for VIP/ChAT and calcitonin gene-related peptide (CGRP) but not for NOS, unlike those in the large intestine (Anderson et al. 1995; Mann et al. 1995). The sympathetic neurons that are innervated by intestinofugal neurons inhibit motility as well as secretion (Furness 2006).

- Sensory neurons: these cells are the first neurons in a reflex pathway which encode information about the nature and intensity of the stimulus. Using the immediate early gene marker Fos, neurons in the SMP of the guinea pig small intestine that produced Fos in response to either cholera toxin or to mechanical stimulation by puffs of nitrogen onto the mucosal villi were identified (Kirchgessner et al. 1992). When the

nicotinic blocker hexamethonium was present (to reduce fast synaptic activation of neurons) one particular class of cells was labelled. These cells were immunoreactive for Calb and/or SP (or a related tachykinin) (Kirchgessner et al. 1992). Sensory neurons make up approximately 13% of all neurons in the SMP of the guinea pig small intestine (Song et al. 1992) and have been dye filled during intracellular recordings (Bornstein et al. 1989). The SMP neurons with Calb and/or tachykinin immunoreactivity have AH-like properties with multipolar morphology (Bornstein et al. 1989; Evans et al. 1994). These cells have extensive projections within the submucous ganglia, often appearing to contact other nerve cell bodies (Bornstein et al. 1989; Evans et al. 1994). They can be retrogradely labelled by tracers applied to both the mucosa (Kirchgessner et al. 1992; Song et al. 1992) and to the MP (Kirchgessner et al. 1992; Song et al. 1998) and thus project to both targets. Sensory neurons are immunoreactive for ChAT (Furness et al. 1984) and are likely to make cholinergic and non-cholinergic synapses onto other classes of SMP and MP neurons. These sensory neurons do not appear to receive fast nicotinic synaptic inputs (Bornstein et al. 1984; Evans et al. 1994). The SMP neurons with immunoreactivity for Calb and/or tachykinins have Dogiel type II morphology.

The identification of myenteric Dogiel type II neurons as sensory neurons was achieved using electrophysiological recordings. A large proportion of myenteric Dogiel type II

neurons in the guinea pig small intestine respond to mucosally applied chemicals, such as solutions with low or high pH or short-chain fatty acids (Kunze et al. 1995; Bertrand et al. 1997). It is not currently clear whether Dogiel type II cells are directly activated by mucosal chemicals or whether they are activated indirectly, perhaps via enteroendocrine cells as demonstrated for SMP primary afferent neurons. However, the observation that responses to mucosal acid were not blocked by low [Ca^{2+}], high [Mg^{2+}] Krebs solution, which blocked synaptically evoked responses, suggests that the axons of these neurons may be capable of directly transducing some chemical stimuli (Kunze et al. 1995). It is not known whether or not the SMP sensory neurons also respond to chemical stimulation.

The IPANs theory

The term intrinsic primary afferent neurons (IPANs) has been proposed and used for enteric neurons that encode for sensory stimuli and represent the first neurons in the intrinsic reflex circuits that influence motility, secretion and blood flow (Furness et al. 1998; Pan and Gershon 2000; Clerc et al. 2002). The morphological, electrophysiological and neurochemical characteristics of IPANs have been most thoroughly studied in the guinea pig ileum (Brookes and Costa 2002*;* Furness et al. 2004). In this region all IPANs identified so far appear to have Dogiel type II morphology, e.g. ovoid cell soma with a pseudo-uniaxonal or multiaxonal appearance with terminals in the myenteric and submucosal ganglia, as well as projections to the mucosa. Electrophysiologically, they are AH/type 2 nerve cells, displaying a long lasting after spike hyperpolarisation (Iyer et al. 1988; Hendriks et al. 1990; Kunze and Furness 1999). In the guinea pig small intestine, IPANs respond to electrical stimulation of interganglionic fiber tract with slow EPSPs. Fast EPSPs to these neurons are rarely recorded and when present they are of low amplitude (Kunze and Furness 1999). These features are distinct from the other major class of enteric neurons, which have unipolar morphology (Dogiel type I), receive fast EPSPs, and are considered to function as interneurons and motor neurons. Electropyhsiologically, they belong to S/type 1 neurons.

Mechanosensitivity of IPANs

In few studies it has been shown that in the guinea pig small intestine, myenteric AH neurons, unlike S neurons, respond to chemical stimulants applied to the mucosa (Smith 1994; Smith 1996; Kunze et al. 1995; Bertrand et al. 1997; Bertrand and Bornstein 2002). In addition, AH neurons in the guinea pig small intestine respond to both, stretch and contraction, with an ongoing action potential discharge (Kunze et al. 1998; Kunze and Furness 1999). This response depends on an increase in smooth muscle tension (tone) and is dependent on the opening of gadolinium-sensitive mechanosensitive channels. Action potentials were also abolished by drugs (e.g. isoprenaline or nicardipine) that abolish muscle tension despite maintained stretch (Kunze et al. 1998; Kunze and Furness 1999). Muscle tone contributes to the mechanosensitivity of enteric neurons in the guinea pig ileum. In this tissue the neural response to sustained stretch depends on opening of stretch activated channels in the muscle followed by muscle contraction and mechanical communication from the contracting muscle mostly to myenteric AH neurons and very rarely to myenteric S neurons (Kunze et al. 1999; Kunze et al. 2000).

The action potentials were generated in the processes of Dogiel type II neurons, not in the nerve cell bodies. Thus in the guinea pig ileum these AH neurons are suggested to function as intrinsic mechanosensory and/or chemosensory neurons that may be responsible for initiating ascending excitatory and descending inhibitory peristaltic reflexes (Kunze et al. 1998, Kunze et al. 1999; Kunze and Furness 1999). Other myenteric AH neurons are also

activated during the stretch by synaptic transmission from neurons that respond directly to distortion (Kunze et al. 2000). Nevertheless, some of the S neurons also seem to react directly to the stretch, thus some uniaxonal neurons could also function as intrinsic sensory neurons (Kunze et al. 1998; Kunze and Furness 1999). Moreover, enteric neurons seem to show different responses depending on which mechanosensitive region of the neuron is activated. Thus, it seems that when the deformation is on the interganglionic nerve processes that occurs when the intestine is distended or the muscle contracts, this leads to action potential discharge; while deformation of the neuron soma inhibits spike discharge in myenteric AH neurons in the guinea pig ileum; this compression of the soma by pressure causes increased opening of potassium channels and thus has an inhibitory effect which may be protective (Kunze et al. 2000). However, in other mammalian sensory neurons, mechanical distortion increases excitability of both the neurites and the cell bodies (Cunningham et al. 1995; Cunningham et al. 1997; Kraske et al. 1998; Raybould et al. 1999).

Immunoreactivity of IPANs

IPANs are cholinergic (Furness et al. 1984; Steele et al. 1991), containing the peripheral form of choline acetyltransferase (Chiocchetti et al. 2003), and express tachykinins (Kirchgessner et al. 1992; Song et al. 1991), neuromedin U (Furness et al. 1989), the neurokinin receptor 3 (Johnson et al. 1998; Jenkinson et al. 1999; Furness 2000) and the $P2X_2$ receptor (Castelucci et al. 2002).

A large proportion of IPANs is immunoreactive for the calcium-binding protein Calb (Furness et al. 1988; Iyer et al. 1988; Quinson et al. 2001). Thus, Calb is a marker for multipolar sensory neurons in the guinea pig small intestine (Furness et al. 1990b). Another study also supports the evidence that Calb immunoreactive neurons appear to be sensory neurons (Costa et al. 1996). Later, it has been shown that NeuN, a neuronal nuclei antibody, seems to be a selective marker for enteric IPANs in the guinea pig small intestine (Costa et al. 2001; Costa et al. 2002; Brody et al. 2002; Poole et al. 2002). In the MP of the guinea pig ileum quantitative data indicate that NeuN immunoreactive neurons are about 38% of all myenteric neurons (Costa et al. 2001, Costa et al. 2002). Furthermore all Calb immunoreactive neurons express NeuN (Costa et al. 2001, Costa et al. 2002). In the guinea pig ileum, NeuN labels the nuclei of almost all the enteric neurons, but the cytoplasmic expression of NeuN appears to be restricted to Dogiel type II neurons (Chiocchetti et al. 2003; Van Nassauw et al. 2005). While all Calb immunoreactive neurons express NeuN, not all NeuN immunoreactive neurons are positive for Calb. There are 2 NeuN subpopulation, one which is also immunoreactive for Calb ($NeuN^+/Calb^+$) (67±2%) and one which is not ($NeuN^+/Calb^-$).

From the IPANs theory to the multifunctional enteric neurons theory

The use of the term IPANs and in particular the concept behind it was recently challenged (Spencer and Smith 2004; Wood 2004; Smith et al. 2007; Blackshaw et al. 2007; Wood 2008). The functional distinction of AH cells as the only enteric nerve population responding to mechanical stimuli was challenged for the first time by a study from Spencer and Smith (2004). In this study, performed in the guinea pig distal colon, they show that in circumferentially stretched preparations AH neurons appear to be electrically silent, while S interneurons respond with an ongoing action potential discharge. It is also noteworthy that action potential discharge in mechanosensory S neurons does not cease when the muscle is paralyzed. Further studies performed by this group allow them to conclude that in the guinea pig AH neurons appear to be necessary for initialing peristaltic waves, where muscle tone is required, while S neurons generate a more rapidly occurring motor pattern (called “ongoing peristaltic reflex activity”), where muscle tone is not required (Smith et al. 2007). Thus, they propose the existence of two different intrinsic sensory neurons populations and suggest that unipolar S neurons may be mechanosensory and at the same time function as interneurons (Smith et al. 2007).

These findings show that mechanosensitivity in the ENS is not a unique property of a single neuron type and that mechanosensory neurons may behave differently in the various gut regions and may be

stretch, tension, stress, and/or strain sensitive. In other words they may be multifunctional and, as such, they would have specialized regions for coding sensory information whereas other regions are responsible for synaptic transmission within the ENS (Grundy and Schemann 2005). Moreover, different populations of mechanosensitive neurons may be reflection of region-specific control of muscle activity. Thus phasic muscle activity is predominant in the ileum whereas tonic muscle activity prevails in the distal colon (Blackshaw et al. 2007). The rapid accommodating AH neuron is well suited to respond to phasic activity as spike discharge is phasic and self-limited by the slow AHP. The slowly accommodating S neuron, however, is able to respond to sustained changes in muscle tone as it is capable to generate tonic spike discharge. AH neurons are present in the guinea pig distal colon but their mechanosensitivity may have been missed as Spencer and Smith recorded neuronal activity during sustained stretch rather than the immediate responses to stretching the tissue (Blackshaw et al. 2007). Mucosally projecting AH neurons appear to respond to tension (tone) of smooth muscle, whereas S interneurons respond to changes in smooth muscle length or gut diameter (Smith et al. 2005). Functionally, these sensory modalities appear analogous to those in skeletal muscle where Golgi tendon organs and muscle spindles within the same muscle bundle give complementary information about changes in muscle force and length, respectively (Smith et al. 2005). A region with predominant tonic muscle activity is the gastric fundus/corpus region; no AH neurons are present in this region (Schemann and Wood 1989). However, distension evoked,

neurally mediated muscle responses can be evoked in flat sheet gastric corpus preparation suggesting mechanosensitive S neurons may also be present in the gastric MP (Schemann et al. 2008). AH neurons have been identified in the gastric antrum, a region that primarily exhibits phasic activity (Blackshaw et al. 2007). Moreover, if we consider different regions of the gut in species other than guinea pig it is difficult to ascribe function based solely upon the morphology and electrophysiological behavior of neurons. For example in the MP of the human colon there is a relative paucity of AH neurons although distension evoked peristaltic reflex activity can be readily evoked (Brookes et al. 1987; Bjornsson et al. 1998). Another example is the MP of the pig ileum, where only 17% of Dogiel type II neurons exhibit slow AHP and the majority of myenteric plexus neurons with multiple long processes receive fast EPSPs (Cornelissen et al. 2001).

Thus, species and region-specific differences in morphology, electrophysiology, neurochemistry and neuropharmacology mean that is not possible to ascribe all sensory function to a single type of neuron (Schemann and Neunlist 2004; Grundy and Schemann 2005; Blackshaw et al. 2007).

The studies that will be presented in this thesis are based on the concept that mechanosensitive neurons in the ENS do not belong to a particular class with unique properties. We propose the existence of multifunctional mechanosensitive enteric neurons. It is important to note that this concept has ancient roots. The first idea of spontaneous pacemaker units in the gut that are multifunctional and provide simultaneous tonic input to both neurons and muscle fibers is dated

1970 (Wood 1970). After 30 years another study pointed out the idea that IPANs are multifunctional, serving both a primary afferent and an interneuronal function secretomotor purpose (Pan and Gershon 2000).
In order to validate the concept of multifunctional neurons, we aimed to identify and characterize mechanosensory neurons in the MP of the guinea pig ileum. We studied the response to these neurons to the application of physiological mechanical stimuli that mimick contractile activity rather than using sustained stretch which hardly occurs in the ileum. In the review by Smith et al. (2007), it is noted that as enteric neurons are embedded between the two muscle layers, it is extremely difficult record from intrinsic neurons while dynamically stretching the tissue. This is the reason that such experiments are carried out using sustained stretch. We think that, especially in the ileum, where phasic muscle activity is predominant, a brief mechanical stimulation seems a more physiological stimulus. Our hypothesis is that stress and strain are important stimulus modalities, therefore, mechanosensory neurons respond to deformation. It is also likely that mechanosensory neurons receive fast synaptic input suggesting that their activity can be highly modulated by other neurons and hence that there is a low stimulus fidelity which allows adjusting for rapid gain changes in sensory networks. This should be an important feature, as mechanosensitive neurons must rapidly adapt to changes in contractile activity. Local adjustment of the excitability level in a sensory network which constantly modulates the responsiveness to a particular stimulus is thus a “smart strategy” to integrate many sensory inputs for a sensible adjustment of spatially

and temporarily coordinated gut behaviour. Therefore, the gain in the sensory networks may be important in determining gut behaviour. The gain is adjusted by excitatory or inhibitory synaptic inputs from other enteric neurones, input from the parasympathetic and sympathetic innervation, crosstalk with extrinsic afferents, release of mediators from enteroendocrine cells, immune cells and is probably also influenced by glia, interstitial cells of Cajal (ICC) and muscle cells. Integration of such multiplexed information is a remarkable capability of the ENS (Blackshaw et al. 2007). The network concept is supported by the findings that the ENS contains many multitasking neurones which implies that many enteric neurones are multitargeted and multifunctional. For example, the mechanosensory myenteric S neurone in the guinea pig distal colon functions at the same time as an interneuron receiving synaptic inputs from other enteric neurones (Spencer and Smith 2004). The same is true for mechanosensitive myenteric AH neurones that make interneuronal connections and are important to gate excitability spread in sensory and interneuronal networks. In addition, in the myenteric plexus of the stomach, numerous multitargeted neurones with projections to the muscle layers, mucosa and other myenteric ganglia have been identified (Schemann et al. 1995).

Extrinsic afferent neurons

A relatively rich afferent innervation conveys sensory information from the GI tract to the CNS where gut reflex function is coordinated and integrated with behavioural responses. Afferent innervation also mediates sensations from the gut. Three anatomical divisions of extrinsic sensory neurons can be distinguished. Vagal afferents that have their cell bodies in the nodose ganglia and project centrally to the nucleus tractus solitarius, innervate the oesophagus, the stomach and, with decreasing density, the intestine. Thoraco-lumbar spinal afferents with cell bodies in the dorsal root ganglia (DRG) innervate the entire length of the GI tract. The third division consist of spinal afferents with cell bodies in the sacral (or in some species, lumbar-sacral) DRG, which innervate the distal bowel via the pelvic nerves. Central projections of these afferent neurons enter the brain stem and spinal cord and make synaptic connections with second order neurons that distribute visceral information throughout the central nervous system. There are both anatomical and functional differences between different populations of sensory afferents supplying the GI tract. Functionally, three distinct and characteristic patterns of terminal distribution can be identified within the gut wall. One population of afferent fibres has responsive endings in the serosal layer and in the mesenteric connections often in association with mesenteric blood vessels. Another population has been traced into the muscularis externa and forms endings either in the muscle layers (Berthoud and Powley 1992; Fox et al. 2000; Wang and Powley 2000) or in the MP (Berthoud et al. 1997). The third population makes endings in the

mucosal lamina propria, where they are positioned to detect material absorbed across the mucosal epithelium or released from epithelial and sub epithelial cells including enterochromaffine and immunocompetent cells (Berthoud et al. 1995; Berthoud and Patterson 1996; Williams et al. 1997; Ward et al. 2003).

The three different populations of afferent endings have different sensory modalities responding to both mechanical and chemical stimulation generated within and outside the bowel wall (Lynn and Blackshaw 1999; Grundy 2002; Blackshaw and Gebhart 2002). Nerve terminals in the serosa and mesentery are activated by distortion of the mesenteric attachments; this means that they do not signal distension or contraction of the bowel wall unless it is strong enough to evoke mesenteric or serosal distortion. Afferents within the muscle layers of the gut wall also respond to distension and contraction, but have lower thresholds for activation and reach maximal responses within physiological levels of distension. Vagal and pelvic afferents show maintained responses to distension whereas splanchnic afferents seems to be more rapidly adapting (Berthound et al. 2004). Mucosal afferents in all three pathways do not respond to distension or contraction but are exquisitely sensitive to mechanical deformation of the mucosa, such as might occur with particulate material within the lumen (Berthound et al. 2004).

Terminals in the longitudinal and circular muscle layers have been described as intramuscular arrays, consisting of several long (up to a few mm) and straight axons running parallel to the respective layer and connected by oblique or right-angled short connecting branches

(Berthound et al. 2004). These intramuscular arrays have been suggested to be in-series tension receptor endings, possibly responding to both passive stretch and active contraction of the muscle (Powley and Phillips 2002), although direct evidence for this is currently lacking. Vagal afferent terminals in the MP throughout the GI tract have been described as intraganglionic laminar endings (IGLEs) (Rodrigo et al. 1975; Neuhuber 1987). These endings are in intimate contact with the connective tissue capsule and enteric glial cells in the myenteric ganglia and have long been hypothesized to detect mechanical shear forces between the orthogonal muscle layers (Neuhuber 1987). Evidence for such a mechanosensory function of IGLEs has been elaborated by mapping the receptive field of vagal afferent endings in the oesophagus and stomach and showing morphologically that individual “hot spots” of mechanosensitivity correspond to single IGLEs with the von Frey hair technique (Zagorodnyuk and Brookes 2000; Zagorodnyuk et al. 2001). Recording from nerve bundles running between the pelvic ganglia and the colon and rectum has revealed low-threshold slowly adapting mechanoreceptors, similar to those in the oesophagus and stomach, which could be activated both by gut distension and by focal mechanical probing. Analysis of dye-filling revealed specialized endings in guinea pig myenteric ganglia which correspond to the transduction sites of rectal mechanoreceptors. These endings have been called rectal IGLEs (Zagorodnyuk and Brookes 2000). This type of low-threshold slowly adapting mechanosensitivity was not encountered for splanchnic afferents to the colon, suggesting that

IGLEs may be the transduction site specifically for the low threshold mechanoreceptors in both vagal and pelvic nerve fibres. The different stimulus response profiles of vagal, splanchnic and pelvic mechanoreceptors are compatible with the concept of vagal afferents being involved in physiological regulation, pelvic afferents being involved in both physiological regulation and pain, and splanchnic afferents mediating mainly pain (Berthound et al. 2004).

IGLEs may also respond to chemical stimuli such as acetylcholine and ATP raising the possibility that these endings also play a key in the sensory role in detecting release of mediators from within the synaptic neuropil of the myenteric ganglia or surrounding tissues (Kirkup et al. 1998). However, evidence that such chemosensory mechanisms contribute to mechano-transduction is lacking.

Mucosal terminals are most abundant in the proximal duodenum, becoming relatively sparse in the distal small intestine. Vagal, splanchnic and pelvic mucosal mechanoreceptors supply all regions of the GI tract. They are characterized by low thresholds to mechanical stimuli, such as stroking with a fine brush, relatively rapid adaptation to continuous stimulation and in most cases sensitivity to a variety of chemical stimuli (polymodal receptors) (Berthound et al. 2004). In addition to evoking direct responses, a wide range of chemical mediators may influence mechanosensitivity, particularly that of spinal afferents. These mediators can be released under inflammatory conditions, injury or ischemia from a variety of cell types, for example: platelets, leucocytes, lymphocytes, macrophages, mast cells, glia, fibroblasts, blood vessels, muscles and neurons. Each of these specific

cells (e.g. mast cells) may release several of these modulating agents, some of which may act directly on the sensory nerve terminal while others may act indirectly, causing release of other agents from other cells in a series of cascades (Berthound et al. 2004). Moreover is important to consider that both spinal and vagal afferents have collateral branches that supply blood vessels and innervate the enteric ganglia. These varicose nerve fibers contain transmitters including CGRP and SP. The activation of afferent fibers causes action potentials that are propagated centrally but those also invade axon collaterals and stimulate the release of neurotransmitters in a local axon reflex which serves to modulate blood flow and enteric reflex pathways. It is important to note that axon reflexes do not require connection to the cell body, so they are present in isolated gut segments.

Extrinsic sensory neurons theory

It is relevant to illustrate the theory of J.D. Wood and his group about sensory transmission in the gut: in his last paper (Wood 2008) he claims that there is no evidence that any neurons inside the gut, other than DRG neurons, genetically express the molecular and cellular mechanism necessary for stimulus detection, accurate coding and transmission of the sensory information as scientifically understood in sensory neurophysiology. Thus, he disputes the necessity of IPANs in the ENS. He affirms that the extrinsic innervation of the gut is extensive and that there is excessively large number of AH-type and S-type neurons present. This redundancy of sensory neurons seems to him odd because of the fact that Darwinian natural selection is generally parsimonious. So, from his point of view, the evolutionary advantage to have multiple populations of sensory neurons in the ENS alongside with highly evolved specialization for sensory detection expressed by extrinsic afferent neurons is unclear (Wood 2008). Accordingly, he thinks that the concept of mechanosensitive AH neurons is equivocal. His alternative explanation is that DRG mechanoreceptors in the myenteric ganglia were stimulated to release SP or CGRP, which in turn evoked slow EPSPs in the AH neurons (Wood 2008). Moreover, he challenge the use of the term sensory signaling for the signal from enterochromaffin (EC) cells to AH cells, considering this kind of signal as a paracrine signaling (Wood 2008).

Mechanosensitivity during evolution

There are many examples of mechanical force affecting the physiology of living cells. Because mechanical stimuli are everywhere, mechanosensation could represent one of the oldest sensory transduction processes that evolved in living organisms. The idea of mechanically gated (mechanosensitive) ion channels arose originally from studies of specialized mechanosensory neurons (Martinac 2004). As mechano-electrical molecular switches, these convert mechanical force exerted on the cell membrane into electrical or biochemical signals in physiological processes such as cellular turgor control in bacteria and touch and hearing in mammals (Martinac 2004). Since their discovery in embryonic chick skeletal muscle (Guharay and Sachs 1984) and frog muscle (Brehm et al. 1984), mechanosensitive channels (MSC) have been found in many cell types (Sachs 1988; Morris 1990; Martinac 1993). More importantly cells that transduce mechanical stimuli into electrical signals are the most common sensory receptors in vertebrates (Martinac 2004). MSC are present in the membranes of organisms from the three domains of life: bacteria, archaea, and eukarya (Pivetti et al. 2003).

Mechanical stress starts electrophysiological and biochemical responses in cells. Mechanical stress can influence physiological processes at the molecular, cellular, and systemic level. The primary target for mechanical stimulation is the plasma membrane of the cell, which can respond to variable physical stress with changes of the open probability of MSC. MSC respond to mechanical forces along the plane of the cell membrane (membrane tension), but not to hydrostatic

pressure perpendicular to it (Gustin et al. 1988; Sokabe and Sachs 1990; Sokabe et al. 1991). What makes these channels respond to membrane tension is less clear. Moreover, various membrane deformations can include different MSC types and this result in different electrophysiological cell responses (Isenberg et al. 2003).

There are three broad classes of mechanisms that may impart stretch sensitivity on a membrane ion channel:

1. Bilayer: mechanical forces are conveyed to the channel purely via the bilayer. Tension sensitivity occurs because of a difference protein area (or hydrophobic thickness and/or lateral shape) between the open and closed channel conformations (Hamill 2006).
2. Extrinsic tethered: tensions are exerted directly on the channel protein via extracellular or cytoskeletal elastic elements/gating springs. When tension is exerted on the gating spring, the open state is energetically more favourable (Hamill 2006).
3. Intrinsic tether (hybrid): in this model, the gating spring is one of the cytoplasmic domains that binds to the phospholipids and, in this way, becomes sensitive to membrane stretch (Hamill 2006; Sharif-Naeini et al. 2008).

The models do not need to be mutually exclusive, and a single channel may derive its mechanosensitivity from all three mechanisms.

Criteria to establish direct mechanical activation of ion channels have recently been reviewed (Christensen and Corey 2007). Several tests

are described to distinguish between mechanically gated channels that act as force sensors themselves and mechanically sensitive channels that are activated by second messengers downstream of the true force sensors. First, the latency of the current elicited by the stimulus should be faster than known second-messenger systems, typically less than 5 milliseconds. Currently, many of the stimuli used to demonstrate mechanosensitivity, particularly osmotic stimuli, lack the rapid rise time needed to determine a latency that is this fast. Moreover, the kinetics of channel activation should depend on the amplitude of the stimulus a larger mechanical force should result in faster channel opening. This is a simple consequence of a larger force lowering the energy barrier to channel opening (Christensen and Corey 2007).

Genetic and molecular data obtained from the studies of model organisms such as the bacterium *Escherichia coli*, the nematode worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and the mouse help to distinguish between classes of mechanically gated ion channels and interacting molecules, which are likely parts of the mechano-transducing apparatus. Among prokaryotic MSC studied to date, the best characterized are the MSC of the bacterium *Escherichia coli* (Martinac and Kloda 2003). Three types of MSC were identified in *E. coli*, which based on their conductance were named as MSCm (m for mini), MSCs (s for small) and MSCl (l for large) (Berrier et al. 1996).

MSC are found in membranes of a variety of eukaryotic cells; they have been reported in a number of other tissues including invertebrate

stretch receptor neurons, bacteria, yeast and plant cells, and for the vertebrates: liver cells, kidney cells, chick cardiac myocytes, ventricles, smooth muscles, endothelial cells, and aortic baroreceptors **(**Takahashi and Gotoh 2000). Despite much electrophysiological information about them, molecular characterization and elucidation of their roles in mechanosensory transduction in eukaryotes have been slow in comparison with the progress in our understanding of prokaryotic MSC. Nonetheless, recent work has identified and electrophysiologically characterized two members of a new family of two-pore-domain, weakly inward-rectifying K^+ channels that are mechanosensitive: TREK and TRAAK. Furthermore, mutagenesis studies in *Caenorhabditis elegans*, zebrafish and *Drosophila* have revealed that some of the ion channels belonging to the mechanosensory abnormal/degenerins (MEC/DEG) and transient receptor potential (TRP) superfamilies might also be mechanosensitive. Indeed, genetic work in worms, flies and zebrafish indicates several members of the TRP channel superfamily might play a role in the physiology of mechano- and osmosensation in these organisms (Martinac 2004).

TREK channels are polymodal K^+ channels (gated by a variety of chemical and physical stimuli) expressed in a variety of tissues, but are particularly abundant in the brain and in the heart (Patel et al. 1999). TRAAK is widely expressed in the brain, spinal cord, and retina, which indicates that it has a function wider than mechano-transduction in neuronal excitability (Patel et al. 1999).

The MEC/DEG subfamily of degenerins is responsible for swelling-induced neuronal degeneration in nematodes. The subfamily includes the MEC-4, MEC-6 and MEC-10 proteins, which are thought to function as subunits of an MSC that might have a role in touch sensitivity (Martinac 2004).

The TRP-family proteins comprise six subfamilies of cation-selective channels: the canonical TRPC (seven members), the melastatin TRPM (eight members), the vanilloid TRPV (six members), and the more distantly related mucolipin TRPML (three members), polycystin TRPP (three members), and ankyrin TRPA (one member) (Sharif-Naeini et al. 2008). The TRP channels are expressed in many tissues in numerous organisms, mediating responses to a variety of physical stimuli (light, osmolarity, temperature and pH) and chemical stimuli (odours, pheromones and nerve growth factor) (Minke and Cook 2002).

MSC have been classified as stretch-activated or stretch-inactivated. They also have been classified, on the basis of their selectivity, as cation selective, K^+ selective, or anion selective.

While considerable progress has been made in the elucidation of cellular mechanisms for transduction of light, sound, odour, and taste stimuli, understanding of mechanical transduction in the somatosensory system has lagged. Because the small size and inaccessibility of sensory nerve endings prevents direct recording of generator currents in vivo, advances in understanding somatosensory transduction mechanisms have depended on the development of in vitro models of transduction. It is generally assumed that deformation

of the membrane of peripheral nerve endings triggers the opening of mechanosensitive ion channels that generate a depolarizing current (McCarter et al. 1999). Recently, whole-cell currents activated by stretch or pressure in DRG neurons from adult rats have been identified. It was a non-selective cation current block by gadolinium and benzamil that might be mediated by a member of the degenerin/ epithelial sodium channel family (McCarter et al. 1999; Takahashi and Gotoh 2000; Drew et al. 2002).

Mechanosensitive K^+ channels where identify and characterize in rat colon sensory neurons (Su et al. 2000). The two most frequent MSC in DRG neurons were identified (Cho et al. 2002). These are two distinct mechanosensory cationic channels activated by pressure applied to patch membranes of DRG neurons (Cho et al. 2002). The two MSC exhibited different thresholds, thus named as low-threshold (LT) and high-threshold (HT) MSC, and sensitivity to pressure (Cho et al. 2002). Judging from the diverse responses of mechanoreceptors to mechanical stimuli, a variety of MSC other than LT and HT channels would be present in sensory neurons. Recently a new type of MSC in the DRG was described: a low threshold small conductance (LTSC) channel (Cho et al. 2006). LTSC channels were found mostly in small cultured sensory neurons and might play a role in mediating somatosensations, including pain (Cho et al. 2006).

Mechanosensitivity in the gut: ion channels

Mechanosensitivity underlies several of the fundamental processes that are required for effective GI function. The GI tract is required to "sense" force and translate it to a chemical or electrical signal. Mechanosensation is a property of several cell types within the GI tract. EC cells release serotonin and other messengers in response to physical stimuli (Kirchgessner et al. 1992). Intrinsic enteric nerves (Clerc and Furness 2004) and extrinsic nerves (Zagorodnyuk et al. 2001) that project to the ENS respond to mechanical stimuli and convert mechanical stimuli to an electrical signal. Also smooth muscle cells and ICCs are also able to directly sense and respond to mechanical stimuli (Kraichely and Farrugia 2007). Smooth muscle cells are the final common pathway for contraction, while ICC have several diverse functions, which include generating a pacing signal (Barajas-Lopez et al. 1989), mediating neuronal input to smooth muscle (Ward et al. 2000), setting the smooth muscle membrane potential gradient (Farrugia et al. 2003) and mechanotransduction (Kraichely and Farrugia 2007).

In GI tract several families of ion channels can be mechanosensitive. These include:

- Potassium channels: mechanosensitivity is a property of several potassium channel families. In the inward-rectifying family, the Shaker-IR channel demonstrates mechanosensitivity, with force applied to the channel, resulting in both stretch activation and

stretch inactivation (Gu et al. 2001). Mechanosensitivity is also a property of specific K channels in the two-pore domain K channel family. This family includes at least 15 members, of which TREK-1 (KCNK2), TREK-2 (KCNK10) and TRAAK (KCNK4) are currently known to be mechanosensitive (Kraichely and Farrugia 2007). TREK-1 and TREK-2 have been reported in GI smooth muscle (Kraichely and Farrugia 2007). Recently it has been shown that another type of potassium channels, the two-pore domain K^+ channel TASK1 contribute to the resting outward current in AH/type 2 neurons (Matsuyama et al. 2008).

Ca^{2+}-activated K^+ channels play an important role in the control of neuronal excitability via the generation of the afterspike hyperpolarization (Davies et al. 2006). The large-conductance Ca^{2+}-activated potassium channel is a voltage-/Ca^{2+}-gated channel found in GI smooth muscle and ICC and is also reported to be mechanosensitive (Kraichely and Farrugia 2007).

- Calcium channels: when enteric neurons fire action potentials, voltage-operated calcium channels allow extracellular Ca^{2+} to enter the cytoplasm down an electrochemical Ca^{2+} gradient, producing transient intracellular Ca^{2+} signals. These Ca^{2+} signals act as intracellular second messengers that are capable of mediating a range of cytoplasmic responses, including release of neurotransmitter, the opening of calcium-dependent ion channels to regulate neuronal excitability, and Ca^{2+}-dependent gene transcription (Smith et al 2003). In myenteric

neurons, a subset of Ca^{2+} currents during action potential firing has a characteristic role in neuronal excitability and synaptic transmission. Ca^{2+} release from intracellular stores and Ca^{2+} uptake into sequestrated compartments are also important in control of myenteric neuronal excitability and synaptic transmission. Intracellular Ca^{2+} also serves to regulate a variety of ion channels in the plasma membrane by several mechanisms, including by direct activation and inactivation of ion channels and by facilitation of a particular open state. Changes in intracellular Ca^{2+} therefore modulate the activity of many K^+, non-selective cation, Cl^- and Ca^{2+} channels themselves, placing Ca^{2+} channels in a central role in regulating GI smooth muscle motor function. Ca^{2+} channels have been classified by electrophysiological (on the basis of their conductance, activation and inactivation potentials) and pharmacological (using a variety of toxins and non-conducted ions) means into L-, N-, P-, Q-, R- and T-type channels. Within the enteric nervous system, L-, N-, P- and Q-type Ca^{2+} channels have been identified using immunohistochemical, electrophysiological and pharmacological tools (Smith et al. 2003). N-type Ca^{2+} channels have a crucial role in signal transmission of depolarization through neurotransmitter release. The membrane depolarization caused by inhibition of Na^+/K^+-ATPase releases acetylcholine from the guinea pig myenteric plexus through N-type Ca^{2+} channels, but not through L-, T- or P/Q-type channels (Gomez et al 1996). In cultured guinea pig myenteric neurons, the Ca^{2+}

transient in soma following electrical stimulation to the extracellular fiber tract was blocked by tetrodotoxin and an N-type Ca^{2+} channel blocker, but not by L- or P/Q-type blockers, suggesting that neuronal presynaptic neurotransmitter release is mediated through N-type Ca^{2+} channels. N-type Ca^{2+} channels are inherently mechanosensitive (Calabrese et al. 2002). The N-type channel has not been cloned from GI smooth muscle or ICCs. The Ca^{2+} channels that are reported to be expressed in smooth muscle of the GI tract include L-type and T-type channels (Kraichely and Farrugia 2007). The L-type Ca^{2+} channel demonstrates mechanosensitivity when subjected to shear stress, positive and negative pressure, cell swelling and lateral membrane tension. The implications of a mechanosensitive L-type Ca^{2+} conductance in visceral smooth muscle are several, as both the contractile response and other functions of the GI smooth muscle that depend on Ca^{2+} as a signalling molecule can be directly regulated by mechanical forces on the cell, forces that can be generated by the smooth muscle cell itself or as a result of shear stress that occurs during adjacent contractile activity (Kraichely and Farrugia 2007).

T-type channel has not been irrefutably established in GI smooth muscle, but there is both molecular and electrophysiological evidence which suggests that T-type or T-like Ca^{2+} channels may be expressed in smooth muscle cells and/or ICC (Kraichely and Farrugia 2007). Although the T-type channel is an integral component of other mechanosensitive

systems, the channel itself does not appear to be inherently mechanosensitive (Shin et al. 2003).

- Non-selective cation (NSC) channels: The NSC channels typically are permeable to K^+ and Na^+, and to a lesser extent, divalent cations such as Ca^{2+} and Mg^{2+}. The flow of the ions through the non-selective channel then depends on the electrochemical gradient for these cations. Several types of NSC current have been described in GI smooth muscle and some appear to be mechanosensitive (Kraichely and Farrugia 2007). The TRP superfamily of non-selective channels has been identified in GI smooth muscle and ICC. The TRP channels, in general, have multiple gating mechanisms. Several TRP channels have demonstrated mechanosensitivity, including TRPA4 (Corey et al. 2004) and TRPC1 (Maroto et al. 2005). In the ENS of the mouse are functionally expressed TRPA1 on neurons that may also co-express TRPV1 (Penuelas et al. 2007).

- Sodium channels: several recent studies have been devoted to the identification of the voltage-gated sodium channels that are expressed in the ENS. These channels play a critical role in neurons, because they underlie the depolarizing phase of action potentials, which these neurons use to transmit information, and may contribute to subthreshold currents that influence action potential generation. The voltage-gated sodium channels differ between each other for sensitivity to tetrodotoxin (TTX):

$Na_v1.5$, $Na_v1.9$, and $Na_v1.8$ are resistant to TTX, whereas $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.6$, and $Na_v1.7$ are TTX sensitive, for tissue and cellular distribution, developmental regulation, and functional properties (Sage et al. 2007). In the guinea pig ENS, among the two neuronal voltage-gated sodium channels that are resistant to TTX ($Na_v1.9$ and $Na_v1.8$), only $Na_v1.9$ is expressed, with a location restricted to the sensory neurons (Rugiero et al. 2003**)**. On the other hand, among the neuronal voltage-gated sodium channels sensitive to TTX only $Na_v1.3$ and $Na_v1.7$ are expressed (Sage et al. 2007). Moreover, $Na_v1.9$ does not play a crucial role in the genesis of the action potential, so this role must be played by $Na_v1.7$ in many neurons and by $Na_v1.3$ plus $Na_v1.7$ in other myenteric neurons (Sage et al. 2007). A Na^+ current has been described in human and dog intestinal circular smooth muscle and ICC, and is mechanosensitive as demonstrated by increased peak inward current amplitude in response to membrane shear stress. Shear stress also alters the kinetics of this channel. Pharmacological blockade of the Na^+ current in intestinal muscle strips decreases slow wave frequency and hyperpolarizes smooth muscle.

- <u>Chloride channels:</u> The chloride family of ion channels is diverse. Chloride channels can be subclassified based on their gating mechanism, into chloride channels (voltage-gated), cystic fibrosis transmembrane conductance regulator (protein kinase/nucleotide-gated), calcium-activated chloride channels,

volume-regulated and ligand-gated channels [such as aminobutyric acid (GABA) channels]. Chloride channels described to date in GI smooth muscle and ICCs include ligand-gated, voltage-gated, volume-gated, Ca^{2+}-activated and G-protein-regulated channels. Opening of a smooth muscle or ICCs chloride channel generally allows Cl^- efflux and depolarization. However mechanosensitive chloride channels have not been described in GI smooth muscle and ICCs to date (Kraichely and Farrugia 2007).

Aim of the study

The aim of this study was to verify the presence of mechanosensitive neurons in the MP of the guinea pig ileum, to study their response to the application of a defined stimulus and to characterize these cells.

Identification of activity in any network, in particular in the gut, is experimentally very challenging as recordings from individual sensory cells are unlikely to reflect behaviour of the network. Since we understood the importance to have an overview on one entire ganglion of the MP to comprehend the complex interactions of the different neurons within the enteric network, we carried on our study using the Neuro Imaging technique. With this technique it was possible to record action potentials simultaneously in a large number of neurons with high spatial and temporal resolution and this allows us to understand the differential activation of enteric populations in response to a mechanical stimulation within the ganglion.

Identification of sensory neurons is of central importance to understand sensory transmission under normal conditions and in gut diseases associated with sensorimotor dysfunctions, such as Irritable Bowel Syndrome (IBS) or Inflammatory Bowel Disease (IBD). Many of the symptoms prominent in the functional GI disorders are indeed consistent with dysfunction of the sensory and/or motor apparatus of the digestive tract. Identification and characterization of sensory neurons will be an important step to identify novel targets that help to normalise sensory function.

Experimental part

Material and methods

Tissue preparations

For our experiments we used male guinea pigs “BFA-Bunt” and “Dunkin Hartley” (Charles River laboratories, Kisslegg, Germany; Harlan GmbH, Borchen, Germany). The experimental animals were kept under standardized conditions in airflow cabinets (Ehret Uniprotect, Emmendingen, Germany). The diet consisted of a standard diet for guinea pigs (Rohfaserpellets, Altromin, Germany). Water was available ad libidum. The animals had two weeks for acclimatisation. The daily rhythm was set by a timer to 14 hours of light (from 7 am to 9 pm).

Guinea pigs were killed by cervical dislocation followed by exsanguination. This method was approved by the local animal ethical committee and is according to the Germans law for animal protection and animal welfare guidelines. At slaughter the animals had an average weight of 395 ± 60 (mean ± standard deviation).

After killing the animals 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. The ileum was quickly removed with a pair of scissors with rounded tips (FST # 14010-15, Fine Science

Tools), to not damage the surrounding intestinal tissue. Ileum was placed in a petri dish (Greiner Bio-One, Germany), with Sylgard bottom (Sylgard 184, Down Cornig, Wiesbaden, Germany) and pinned with insect pins (FST # 26002-10, Fine Science Tools). Then ileum was opened along the mesenteric border and washed several times with aerated with Carbogen (5% CO_2 and 95% O_2) (Andel finger, Freising, Germany) equilibrated at pH 7.4. Krebs solution containing (in mM): 117 NaCl, 4.7 KCl, 1.2 $MgCl_2$ 6 H_2O, 1.2 NaH_2 PO_4, 25 $NaHCO_3$, 2.5 CaCl 2 H_2O, 11 glucose. During preparation the tissue was constantly perfused with a circulating pump (Ismatec ISM 827, Zurich, Switzerland) with Krebs solution gassed (95% O_2, 5% CO_2; pH = 7.40). The mucosa, the submucosa and the circular muscle layer were gently removed in order to obtain longitudinal muscle-myenteric plexus preparations. For the dissection of the tissue were used microscissors (FST # 14058-11, Fine Science Tools) and forceps (Dumostar # 5, Dumont, Switzerland). The preparation (5 x 10 mm) was pinned onto a silicone ring (Down Cornig, Midland, TX, USA) that was placed in a recording chamber with a 42-mm-diameter glass bottom (130-170 μm thickness, Sauer, Reutlingen, Germany) and continuously perfused with 37^0 C Krebs solution containing (in mM): 117 NaCl, 4.7 KCl, 1.2 $MgCl_2$ 6 H_2O, 1.2 NaH_2 PO_4, 20 $NaHCO_3$, 2.5 CaCl 2 H_2O, 11 glucose and, in some experiments, 1 μM Nifedipine to reduce muscle movements. The perfusing Krebs was circulated at a speed of 20-25 ml/min from a reservoir (500 ml) to the chamber. The plastic tubes (Tygon R3607) connecting the pump with the chamber

had a diameter of 2.79 mm. Temperature and pH of the Krebs solution were checked during the experiments.

In some preparations the circular muscle layer was kept intact in order to record contractions in intact mucosa-free tissue.

Pinning the preparations onto the silicon rings involved stretching the tissue. In order to adjust the degree of stretch in circular and longitudinal direction, we measured the resting size of unstrechted slack tissue before mounting it onto the silicon ring. After mounting the tissue the average amount of stretch 58% ± 20% (mean ± standard deviation) in the circular and 18% ± 8% in the longitudinal direction.

Multisite optical recording technique (MSORT) with voltage sensitive dye

To describe and analyze the neuronal population of the guinea pig ileum MP we utilize the Neuro Imaging technique together with voltage sensitive dye. We utilised a Multi-Site Optical Recording Technique (MSORT) to detect signals from a potentiometric dye (Di-8-ANEPPS) which is incorporated into the cell membrane. The activity of nerve cells can be measured by recording their membrane potential. Activated nerve cells fire action potentials. The dye that we injected in the membranes changes its fluorescence according to the membrane potential of the cell. These changes are recorded by CCD chips technology. Our set up is able to transforms the optical data in

electrical data. 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 main advantage of this technique is the fact that it is possible to record action potentials simultaneously in a large number of neurons with high temporal (1.6 kHz) spatial (up to 50 sensors on one neuron) resolution. This allows us to understand the differential activation of enteric populations in response to a mechanical stimulation within the ganglion.

Staining method

Individual ganglia were stained with the fluorescent voltage-sensitive dye Di-8-ANEPPS (1-(3-sulfanatopropyl)-4-[beta[2-(di-*n*-octylamino)-6-naphthyl]vinyl] pyridinium betaine) (D-3167 Molecular probes, Eugene, OR, USA) by local pressure application through a microejection pipette loaded with 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) (Fig. 1). The glass pipettes (Science products, Hofheim, Germany) were pulled with a Flaming/Brown micropipette puller (Sutter instrument Co., Novato, CA, USA). The glass pipettes were gently positioned inside an interganglionic fiber tract and pressure ejection pulses were used to apply the dye. These

pressure ejections were performed with the PicoSpritzer III (Parker Hannifin Co., Cleveland, OH, USA). The ejection pulses lasted between 9 and 15 s and the ejection volume was between 10 and 100 nl.

The staining was followed by a 5-10 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. The dye staining did not change the electrophysiological properties of the nerve cells (Neunlist et al. 1999).

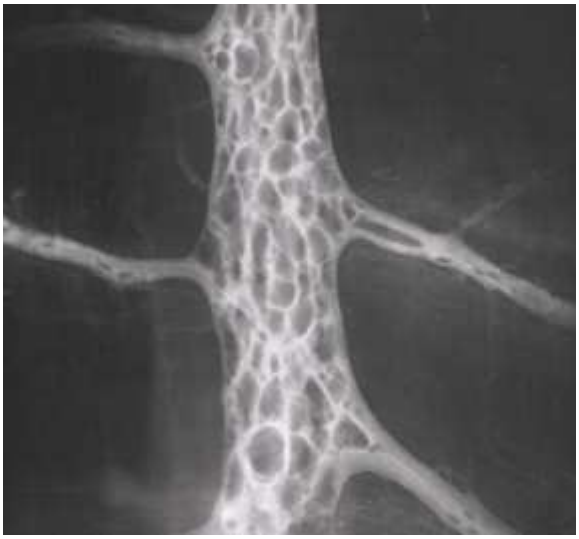


Fig 1. Myenteric ganglion stained woth the voltage sensitive dye Di-8-ANEPPS. Individual cells can be identified since the dye incorporates into the membrane revealing the outline of individual cell bodies (in white).

Optical recording method

The chamber containing the preparation was mounted onto an epifluorescence Olympus IX 71 microscope (Olympus, Hamburg, Germany) equipped with a 75-W xenon arc lamp which was connected to a stabilized power supply (Optosource, Cairn Research Ltd., Faversham, UK). Controlled illumination of the preparation was achieved by a software operated shutter (Uniblitz D122, Vincent Associates, New York, USA).

To reduce vibrational noise, lamp and shutter were separated from the microscope and the light was fed into the microscope with a quartz light guide and condensor optic (Cairn Research Ltd.). Di-8-ANEPPS-stained neurons were visualized with a ×40 or ×100 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). The fluorescent images were acquired and processed by the Neuroplex 8.3 software (RedShirt Imaging, Decatur, USA). This set up allowed us to measure relative changes in the fluorescence (ΔF/F), which is linearly related to changes in the membrane potential (Neunlist et al. 1999). The fluorescence changes are detected with a cooled charge-coupled device (CCD) camera made of 70 x 70 pixels (Redshirt Imaging). Optical signals were processed with a computer; frame rate was 1-2 kHz which enables the detection of action potentials.

With the 40x objective we detected fractional changes (ΔF/F = change in fluorescence divided by the resting light level) in the range of 0.05-

4%. With a 40x objective the spatial resolution of the CCD system was ≈24 μm^2 per pixel.

Individual cells can be identified since the dye incorporates into the membrane revealing the outline of individual cell bodies. The overlay of signals and ganglion image allowed the analysis of the responses from single neurons.

Duration of the acquisitions

Since any fluorescent dye recording will eventually lead to dye bleaching and/or phototoxicity illumination time was a crucial factor. On one hand it had to be kept to a minimum but had to be long enough to reveal representative responses of neurons to the mechanical stimuli. It turned out that recordings with durations of 1255 ms or 1800 ms for the mechanical stimulations and 628 ms for the electrical stimulations yielded reliable and reproducible responses. Nevertheless, in some ganglia it was possible to record for up to 5000 ms which was sufficient to record full responses to the mechanical stimuli.

Technique validation

This technique was validated by comparison with intracellular microelectrode recording (Neunlist et al. 1999). Simultaneous optical and intracellular recordings of neuronal activity were performed. Intracellularly measured potentials evoked by depolarizing current

pulses or synaptic activation always yielded timely synchronized optical signals which had a faster time course. Intracellular recordings of action potentials prior and after staining the ganglion by local dye-microejections revealed no detectable changes in excitability or in action potential characteristics (Neunlist et al. 1999). The fractional change in fluorescence (ΔF/F) was on average –0.26 ± 0.08% /100 mV using a 170-Hz low pass filter (n=4 neurons; guinea pig). With these filter settings, action potentials had ΔF/F of – 0.09 ± 0.06% (range from –0.24 to –0.02; n=10) in the guinea pig and of –0.03 ± 0.005% (range from –0.04 to –0.02; n=20) in the mouse. The peak-to-peak noise level of an action potential was 20 ± 14% (range: 6–54; n=10) of the action potential amplitude in the guinea pig and 33 ± 5% (range: 28–47; n=15) in the mouse. Under the staining and recording conditions used, these values of signal-to-noise ratio allowed the detection of sub threshold fast EPSPs with amplitudes as low as 10 mV (amplitude measurements based on simultaneous intracellular recordings). However, it is generally not possible to distinguish sub threshold fast EPSPs from action potentials by the amplitude of the optical signal (Neunlist et al. 1999). Each action potential evoked by depolarizing current pulses or by synaptic activation which has been recorded intracellularly had its optical correlate (Neunlist et al. 1999). The correspondence between simultaneous optical and intracellular recordings demonstrated that voltage-sensitive dye signals reports electrical activity with high fidelity, thus validating the use of MSORT to study activity in the ENS. It is noteworthy that the shapes of fast EPSPs and to a lesser extent those of action potentials differed

between intracellular and optical recordings; in most cases the optical signals had a faster time course. There are several possible explanations for this phenomenon. Firstly, it was shown that fast optical probes can monitor membrane potential changes with a higher time resolution than can microelectrodes. Secondly, it has to be considered that the optical recordings represent a compound signal to which dendritic, axonal as well as somal potential changes contribute. In contrast, the intracellular microelectrode records local potential changes and therefore detects only regional potential changes. In agreement, regional heterogeneity in action potential characteristics has been shown. In addition, the amplitude of the optical signal depends on the size of the recorded area, the illumination intensity and the density of dye binding (Neunlist et al. 1999).

Advantage and disadvantages of MSORT

The micro-electrode impalement technique is the classical electrophysiological technique to study the electrical and synaptic properties of neurons in the ENS. However, this technique, compared with the technique we use, yields relatively low experimental efficiency and do not allow the study of synaptic interactions between multiple neurons in the MP, since it only provide information at the single neuron level.

The first and main advantage of MSORT is, as previously told, the possibility to have simultaneous recordings of electrical activity from a large population of cells. Another advantage is the possibility to

record electrical activity in cells that would be too small to impale with a microelectrode, and obviously the fact that this is a non-invasive method if compared with the impalement technique that can induce somehow changes in the physiological properties of the cells.

There are also limitations in the use of the Neuro Imaging technique: the signal to noise ratio is crucial for the resolution of the electrical activity and depending on this small changes in the membrane potentials are not detected (Neunlist et al. 1999). In addiction is very difficult to obtain quantitative measurements of fast EPSP amplitudes and there are several possible source of noise which will negatively affect the signal-to-noise ratio.

The MSORT is a technique that allows detecting nerve activity in the ENS with high spatial and temporal resolutions and thereby to record actions potentials in all neurons of a given ganglion simultaneously (Neunlist et al. 1999; Schemann et al. 2005; Michel et al. 2005; Breunig et al. 2007).

Behaviour of ganglia in freely contracting tissues

In order to study the behaviour of dye labelled ganglia during muscle movement under almost physiological conditions, experiments were performed in intact mucosa-free stretched tissue that was allowed to freely contract. The movements of the tissue and the dye-labelled ganglia were recorded with a CCD camera (LU165M, Lumenera Co., Ottawa, ON, Canada) and the imaging software AMCap 9.10 (Noël

Danjou) and Infinity Capture 4.0.2 (Lumenera Co.) using ×10, ×20 or ×40 objectives. Thus, it was possible to study deformation at the ganglion and cell level which served two purposes: firstly, to demonstrate ganglion deformation during physiologically occurring contractions and, secondly, to compare the deformation with that occurring during the experimentally evoked mechanical stimulation.

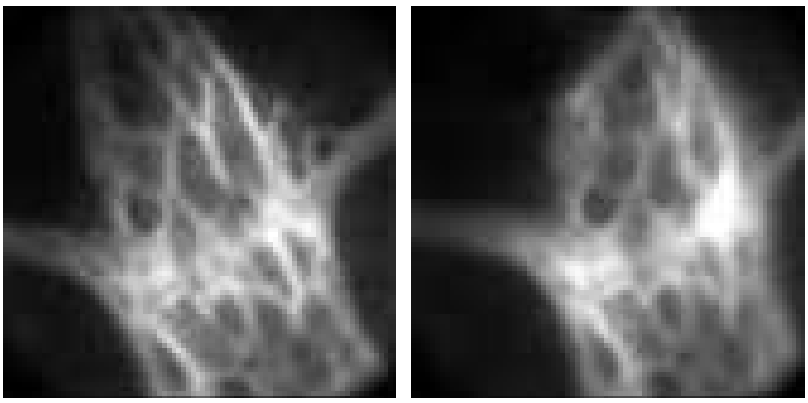
Different techniques for mechanical stimulation of ganglia and neurons

In order to investigate the relevance of different stimulus modalities, such as pressure, stress and/or strain, we used different mechanical stimulation protocols. Two techniques were applied to mimic the stress and strain during ganglion deformation observed in freely contracting tissue. Firstly, we used von Frey hairs to apply a defined force onto the ganglion. Secondly, we used intraganglionic injections of small volumes of Krebs solution. Both methods led to a ganglion deformation but the volume injection was much superior in producing reproducible deformations in all neurons in a given ganglion as well as in evoking most reliable and reproducible neuronal responses.

Von Frey hair technique

We used hairs from cat and horse to evoke a mechanical stimulation. These hairs were connected to a motorized stepper micromanipulator

(DC-3K, Märzhauser, Wetzlar, Germany) that permits remotely controlled displacement of the hairs along the 3 axes (x, y and z). We first positioned the tip of the hair just a few micrometers above the ganglia and used a piezo step controlled device to push the hair onto the ganglia's surface creating a mechanical stimulus. The mechanism of this micromanipulator is based on a conventional micrometer screw with a displacement up to 10 mm. The micrometer scale is visible and permits reading to 10 µm. The drive is powered by a flange-mounted DC motor with gearbox. The micrometer drive slider moves on a precision crossed-roller guide. A remote controller allowed us to control micrometric movements in the forward and backward directions. When von Frey hairs are pressed with their lever against a surface they exert a growing force onto this surface. As soon as the von Frey hair bends, this force remains constant even if the lever is further advanced. It is possible to calibrate this force. In our experiments we used hairs that product forces between 0.30 and 2.70 mN. With this method we could observe different degree of deformation in the ganglia (Fig. 2), but it was not possible to stimulate at the same time the entire ganglion and the cells that get more deformed were every time the ones nearest the site where the hair touched the surface of the ganglion. Moreover, with this technique, it was not possible to exactly reproduce the deformation because it was not possible to hit the exactly same spot on the ganglion surface twice. This was due to slight movements of the ganglion and the hair after retraction of the hair.



a. Time t= 0 ms **b**. Time t=1130 ms

Fig. 2. Example of deformation occurring during von Frey hair stimulation. In **a** the ganglion appear before the application of the stimulus. In **b** it is evident how the von Frey hair stimulation provoked a deformation in the cells of the ganglion.

Intraganglionic injections technique

To perform intraganglionic injections we principally applied the same method we used to stain the ganglia. We inserted a glass micropipette (Science products) into the fibre track. The micropipette was filled with oxygenated and buffered Krebs solution. Pressure ejection controlled by the Picospritzer III (Parker Hannifin Co.) injected reproducible volumes into the ganglia. We used stimulus parameters that mimic the time course of a contraction: injection times of 100-1000 ms with speed of 1-7.5 µl/s. As it is not possible to impale the fibre tract achieving a tight seal we used pressure ranging from 34 to

138 KPa. The final pressure adjustments were checked by visual inspection. In this way the solution was distributed in the ganglion along the intercellular spaces provoking a deformation in the entire ganglion (Fig. 3). We hypothesised that mechanosensitive neurons respond to this mechanical stimulation with an action potential discharge.

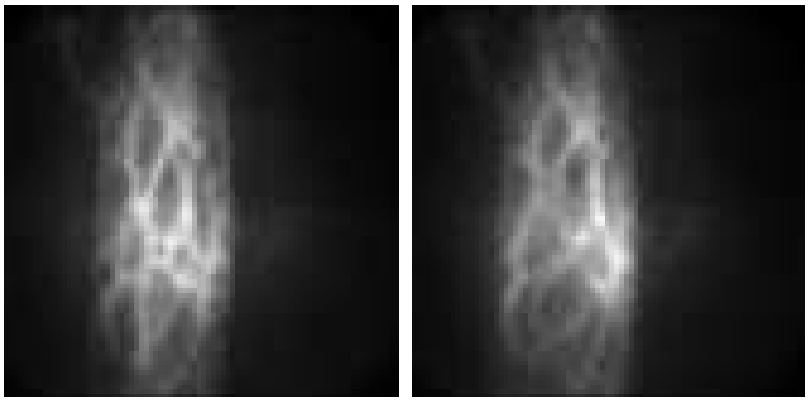
We usually used pressure pulse durations of 400 ms. In 11 ganglia from 7 guinea pigs we additionally applied 200, 600 and 800 ms pulses. The different pulses were applied in a random order. The device we used for our experiments allowed us only to set the pressure in the beginning part of the injector pump, thus we could not control the resulting pressure inside the ganglia. In 11 ganglia from 10 different guinea pigs we performed recording changing the injection pressure from 10 to 20 psi (69 to 138 KPa). With this technique we generally recorded using a duration time of 1255 ms.

Since with the pressure injection system we were not able to control the volume injected in the ganglia, we used also in separate experiments, a digital volume controlled injector (UltraMicroPump II, World precision instruments inc, Sarasota, FL, USA) for some of our experiments. This system consists of a microprocessor-based controller, Micro4, which provides an interface to a syringe pump. The following functions could be controlled: infusion or withdrawal volumes and rate of delivery. The syringe we used was a 1000 µl glass syringe (Innovative Labor System GmbH, Stützerbach, Germany) filled externally with Krebs solution and then inserted into the pump. Fluids injected or withdrawn were held entirely within the micro

syringe to maintain a low fluid dead volume. A rigid plastic tube (15 cm in length with 1 mm diameter) (KronLab, Sinsheim, Germany) connected the syringe with the glass pipette that was inserted in the ganglion fibre tract.

With the volume controlled injection technique we performed experiments in 7 ganglia from 3 guinea pigs in order to test a possible relationship between action potential discharge and injected volume. Experiments made with Fast Green (F7252, Sigma, Schnelldorf, Germany) labelled Krebs solution revealed that not all of the volume was injected into the ganglia but part of it leaked into the bath. This was due to the fact that it is not possible to achieve a tight seal at the site of the fibre tract impalement. Since we have never seen a response with volumes lower than 1000 nl, we decided to set the minimal volume to 1000 nl. We studied the same ganglion after application of 1000, 2000, 3000 and 4000 nl. Although it was not possible to exactly calculate the volume going into the ganglia, we suggest that we can at least make conclusions on the basis of relative changes since the volume ranges was applied to the same ganglion. With this technique we also performed experiments to investigate flow rate dependent neural responses. We tested 9 ganglia from 5 guinea pigs choosing a fixed volume of 1000 nl and 4 different flow rates: 1000, 2500, 5000 and 7500 nl/s resulting in injection durations of 1000, 400, 200, and 133 ms, respectively. The different flow rates values were applied in a random order. With this technique we generally recorded using a duration time of 1800 ms.

Unspecific effects evoked by the injected solution may be excluded as the composition, pH and osmolarity was identical to the solution perfusing the preparation.



a. Time t=0 ms **b**. Time t=720 ms

Fig. 3. Example of deformation occurring during intraganglionic injection. In **a** the ganglion appear before the application of the stimulus. In **b** it is evident how the stimulation provoked a deformation in the cells of the ganglion.

Reproducibility test

To be sure that the mechanically evoked responses were reproducible we performed two mechanical stimulations (pressure or volume injections into the ganglia) 15 min apart. For further analysis we considered only those neurons that fired action potentials to both

stimulations. Only a low percentage of neurons (1.3%) did not respond to a second stimulus.

Electrical stimulation of interganglionic fiber tracts

We used electrical stimulation of interganglionic fibre tracts to evoke fast EPSPs. This was achieved with a 20-25 μm-diameter Teflon-coated platinum electrode (Science Products, Hofheim, Germany) connected to a constant voltage stimulator (Hugo Sachs Elektronik, March-Hugstetten, Germany). We used rectangular pulses of 600 μsec duration and variable amplitude from 1 to 8 V. We performed these electrical stimulations for two reasons. Firstly, to verify viability of the neuronal network; secondly, we needed the fast EPSP recordings to verify drug actions on synaptic transmission.

Pharmacology

In this study we used various antagonists which were added to the Krebs solution perfusing the tissue; all the substances used are summarized in Table 1. We performed in all the different experiments one or two preliminary mechanical stimulation after that we perfused the drugs. After a variable time of perfusion (see Table 1), depending on the substance used, we performed another mechanical stimulation

and then, when it was possible, we washed out the drug used and we performed the last mechanical stimulation.

Immunohistochemistry

In order to define the neurochemical code of the mechanosensory neurons we performed immunohistochemistry on 11 ganglia from 6 guinea pigs. We performed these stainings in ganglia with 2-3 recordings only, because longer recordings periods would have compromised the staining. Although the final reason for this is unknown we assume that antigen retrieval is negatively affected by long exposure to fluorescence light. 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 (3 x 10 min) in phosphate buffer. Until further processing the samples were stored at 4º C in phosphate-buffered saline (PBS) containing 0.1% NaN_3. The samples were permeabilized in $NaHCO_3/Na_2CO_3$ (pH 8.6)/ NaN_3 (0.1%)/Glycerol (50%) for 30 min, (80%) for 30 min and (100%) for 120 min. Tissues were strongly agitated during permeabilization. Then the specimens were washed (3 x 10 min) in phosphate buffer. Whole mount preparations containing the MP were first incubated in PBS/NaN_3 (0.1%)/Horse serum (HS, 4%) for 1h at room temperature.

We used antibodies against Calb and NeuN because they have been described as reliable markers for AH/Dogiel type II neurons in the

guinea-pig ileum, those neurons that are supposed to act as sensory neurons in the ENS (Furness et al. 1988; Iyer et al. 1988; Furness et al. 1990b; Costa et al. 1996; Costa et al. 2001; Quinson et al. 2001; Brody et al. 2002; Costa et al.2002; Poole et al. 2002; Chiocchetti et al. 2003; Van Nassauw et al. 2005).

We used mouse anti NeuN (Ref. number MAB377, Chemicon, Limburg, Germany) and rabbit anti Calb (Ref. number AB 1778, Chemicon). The tissues were incubated in PBS/NaN_3/HS (4%) containing the primary antibodies at a diluition of 1: 500 for anti-NeuN and 1: 1000 for anti-Calb for 48h 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) and donkey anti mouse conjugated to Biotin (Ref number 715065151 Dianova). The final dilutions of secondary antibodies were 1: 200 (Cy2 conjugates), and 1: 50 (Biotin conjugates). Biotin-conjugated secondary antibodies were visualized using streptavidin conjugated with Aminomethylcoumarin-acetat (AMCA) (Ref number 016150084, Dianova) at a dilution of 1: 50. Finally, the specimens were washed in PBS, 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 (BX61W1, Olympus, Japan). Appropriate filters were used to visualize the Di-8-ANEPPS and the fluorophores separately. Pictures were acquired with a coloured video camera connected to computer and controlled by Scion image

software (Scion Corp, Frederick, MD, USA). Frame integration and contrast enhancement were employed for image processing.

Data analysis and statistic

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 we could analyze responses from single neurons. We thereby identified and analysed the number of ganglion cells in the field of view, the number of responding cells per ganglion and the number and the frequency of action potentials per cell. We also analysed the occurrence of the first action potential after the beginning of the stimulus, the duration of the spike discharge and the temporal occurrence of each action potential.

In the experiments with the drugs application we analyzed the difference in the electrical behavior of the mechanosensory neurons before and after the drug perfusion and, when feasible, after wash out.

For signal analysis we used Neuroplex 8.3 (RedShirt Imaging), Igor Pro 6.03 (Wavemetrics Inc, Lake Oswego, OR, USA), Microsoft Office Excel 2003 (Microsoft Corp., Redmont, WA, USA) and Image J 1.32j (Wayne Rasband, National Institute of Health, USA) software.

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 value when they are not normally distributed. To

test the differences in the frequency of action potential discharge between the 2 control groups of acquisitions we performed a t-test or a rank sum test when the data were not normally distributed. To test the differences in action potential frequency before and after drugs application we used one way analysis of variance on ranks or repeated measures analysis of variance on ranks. For all analysis the difference between two data groups was defined significant when P was ≤ 0.05.

To study response patterns of mechanosensitive neurons we constructed spatiotemporal plots of the neurons in every ganglion with Igor Pro software (Wavemetrics Inc) (Fig. 4).

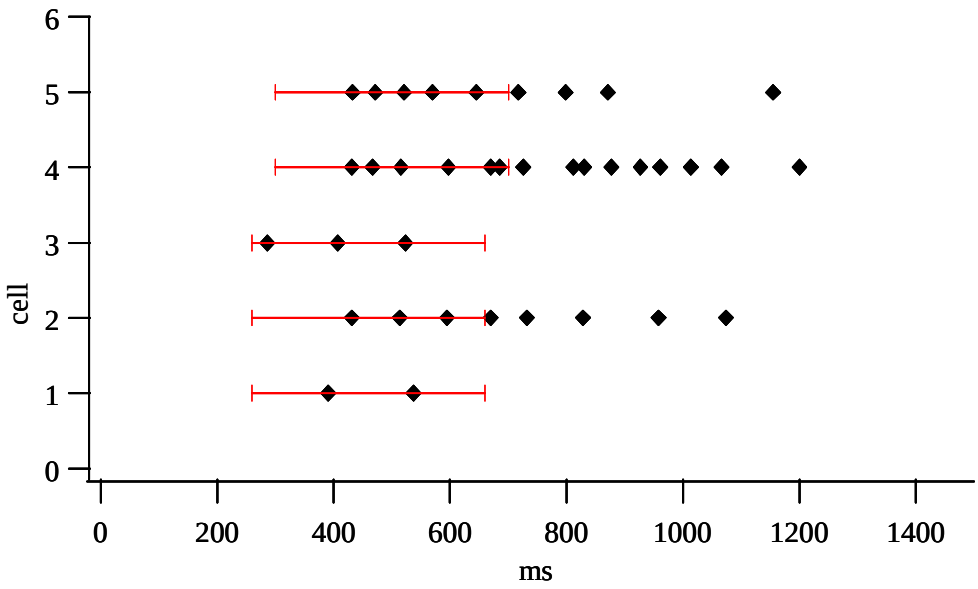
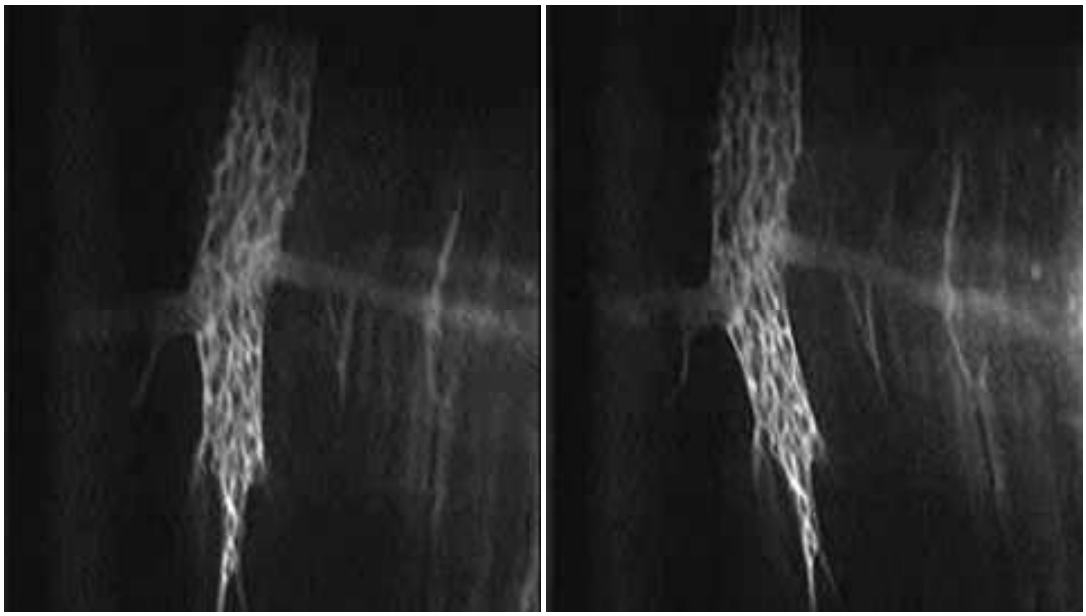


Fig. 4. Example of a spatiotemporal plot showing the action potential discharge of five neurons in a given ganglion. The Y axis indicates the numbered mechanosensitive neurons in the ganglion. The X axis shows the time axis. Each black diamonds represent an action potential. The red bars mark the beginning and end of the stimulus. The time of the beginning and end was deduced from the mechanical artefact evoked by the volume injection or the von Frey hair when it touched the tissue.

Results

Behaviour of ganglia in freely contracting tissues

The ganglion deformation was recorded in 30 ganglia in freely moving tissues containing both muscle layers in the absence of muscle paralysing drugs. The deformation of ganglia appeared to be stronger with motility of the longitudinal muscle compared to circular muscle activity. The contractions occurred randomly and each lasted for a maximum of 2 seconds. It was primarily the edges of the ganglia that were deformed during muscle activity. The movements of the ganglia were accompanied by deformation of individual neurons which changed their shape due to changes in the long and short axis dependent on the orientation of the cell within the ganglion (Fig. 5).



Time t=0 s Time t=42 s

Fig. 5. Different frames from a freely moving ganglion movie. It is noteworthy that the lower part of the ganglion during the physiological contraction twists on the right direction provoking a deformation in individual neurons.

Von Frey hair technique

With the von Frey hair technique we performed recordings in 52 ganglia from 16 guinea pigs (male, average of weight 370 ± 30 g). For all the experiments a recording time of 2000 ms was chosen. The mean number of nerve cells in the field of view with the 40x objective was 33 ± 9. In every ganglion tested we found from 0 to 20 cells responding to the mechanical stimulation. As explained later in this paragraph, we found a positive correlation between the increase in the number of responding cells and the stronger forces used. We counted a total number of 62 responding cells. The median number of responding cells per ganglion was 4 (16% of the total number of neurons). The percentage is related to the number of neurons in the field of view and is an underestimation of the proportion of responding neurons. As outlined below the von Frey hair is only deforming a small area of the ganglion. The analysis of only those neurons that get deformed by the stimulus revealed that a percentage of 20% responding to the von Frey hair stimulation. For each ganglion tested we did 2 to 5 different recordings.

Mechanosensory neurons, especially the ones situated near the stimulation site, showed a discharge of one or more action potentials. In order to understand if the response was directly related to the stimulus strength, we did experiments with different stiffness of the hair used: 0.30, 0.70, 1.00, 1.60, 2.00 and 2.70 mN.

In all the experiments performed we considered for the analysis only the neurons that appear deformed during the stimulation. Thus, the

values reported are always referred to the total number of deformed neurons in a given ganglion.

We did 26 recordings in 4 guinea pigs and 10 ganglia with 0.30 and 0.70 mN forces. The ganglia deformation was small and we could never see any electrical response in these neurons. With 1.00 mN stiffness we did 8 recordings in 4 ganglia from 3 guinea pigs, the deformation was stronger and a median value of 2.5 neurons per ganglion responded to the von Frey hair stimulation with a median action potential frequency of 1 Hz. All of them were located within the deformed area.

With 1.25 mN force we did 10 recordings in 5 ganglia from 2 guinea pigs. The degree of deformation increased again and a median value of 3.5 neurons per ganglion responded. The action potential frequency recorded was 1.5 Hz.

We made 14 recordings in 14 ganglia from 4 guinea pigs with a force of 1.60 mN and the median value of responding neurons increased to 5 with an action potential frequency of 1.5 Hz.

With 2.00 mN von Frey hair we did 14 recordings in 7 ganglia from 2 guinea pigs. The median value of responding neurons was 4. The action potential frequency was 1.5 Hz.

Finally, we performed 10 recordings in 6 ganglia from 2 guinea pigs with a force of 2.70 mN and the median of responding neurons was 5 with an action potential frequency of 2 Hz.

The progressive increase in the number of responding neurons and in the action potential frequency discharge is statistically significant (Fig. 6).

All these results are summarized in Table 2.

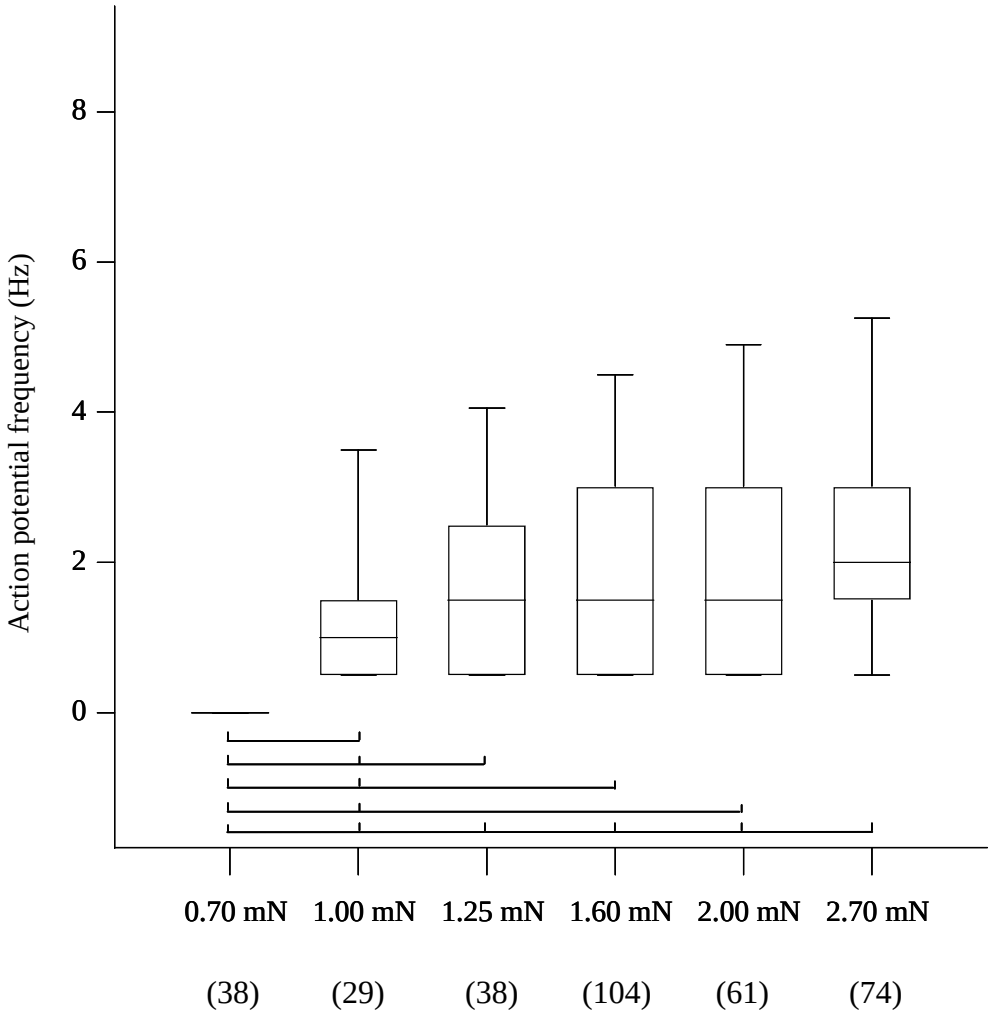


Fig. 6. Variation of the action potential frequency in the groups of data obtained with different forces of von Frey hair. The progressive increase in the action potential frequency is statistically significant. In parenthesis are indicated n numbers.

Intraganglionic injection technique

With this technique we used the two different protocols described in the method section: the pressure controlled injection and the volume controlled injection. While generally the experiments were performed with the first protocol, the volume controlled injection protocol was used in the experiments where we studied the relation between the injected volume/ rate of volume and the discharge pattern.

With the intraganglionic injection method we achieved first an immediate deformation of the entire ganglion and secondly a longer lasting deformation due to the injected volume. The injected volume is indeed only partially redistributed in the ganglion branches, so, even if the duration of the stimulus application was 400 ms, the deformation detected with visual inspection lasted a mean time of 160 ± 90 s. We measured this time considering the delay between the initial deformation and the recovery of the pre-stimulus ganglion shape. Thus, with this technique we could evaluate the response to the initial deformation and also to the long lasting deformation stimulus.

Deformation in response to intraganglionic pressure controlled injection was performed in 46 ganglia from 31 guinea pigs (male, average of weight 420 ± 89 g). We counted a total number of 352 mechanosensitive neurons. The mean number of nerve cells in the field of view with the 40x objective was 34 ± 10. In every ganglion tested we found from 1 to 21 (from 3% to 62%) responding cells with a median value of 7 (25 ± 11% of the total number of neurons). Among all the different experiments performed we found a large variability in the number of mechanosensitive neurones. This could be

due to the fact that the staining level of the membranes of the different cells in every ganglion was different, thus sometimes it was easier to detect the action potentials in one cell than in another one. The response of mechanosensory neurons consisted of an immediate rapidly adapting spike discharge. Indeed, it was noticed that the stimulus evoked action potential discharge primarily occurred during the initial phase of deformation (see example Fig. 7).

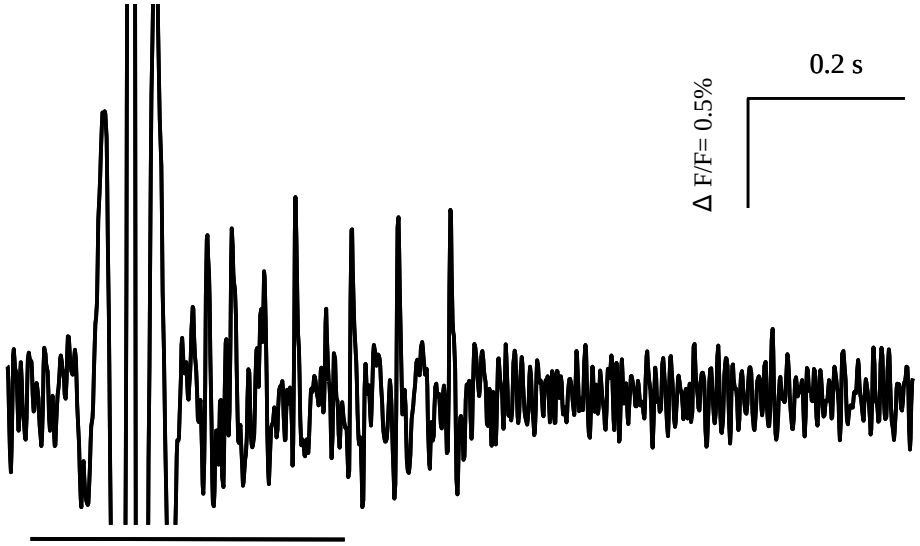


Fig. 7. Example of a trace recorded from a mechanosensory neuron. The first part of it shows the strong mechanical stimulus artefact due to the ganglion's movement. After that 7 action potentials are visible. The line underlining the ganglion shows the duration of the stimulus application. To notice the delay between the beginning of the line and the beginning of the deformation artefact (change in baseline fluorescence of the single cells).

The average time of occurrence of the first action potential was 182 ± 128 ms after the beginning of the stimulus. The beginning of the stimulus was considered for each cell the beginning of the artefact in the tracers, so it is based on the change in baseline fluorescence of the single cells and not on the real start of the injection. Measuring the latency between the beginning of the stimulus and the first action potential we often incurred in a methodological error. Mechanical stimuli will always dislocate ganglion cells. This dislocation will result in movement artefacts and consequently large signal changes. The sensors of the camera will detect changes in fluorescence whether they are evoked by genuine changes in membrane potential or movement of a dye labelled structures. Movement artefacts produced signals that were 10-100 times larger than signals evoked by action potentials. Therefore, it was not possible to selectively filter out those movement artefacts although they had a slower time course than spike evoked signals. As a result early spikes were missed because they disappear in the noise induced by the mechanical artefact. We could anyway measure the time of occurrence of the first action potential in some ganglia in which the stimulus did not produced such a big artefact, closely analysing the unfiltered traces. These neurons fired the first action potential after around 18-22 ms from the beginning of the stimulation. The average time of occurrence of the last action potential (in the cells in which we recorded more than 1 action potential) was 447 ± 231 ms after the beginning of the stimulus. The spike discharge lasted for 302 ± 231 ms and occurred only during the initial deformation phase.

Analysing the spatiotemporal plots (see example in Fig. 8) it was possible to better define the pattern of action potential discharge of mechanosensory neurons: the response appear to be a direct consequence of the initial deformation stimulus, is represented by a discharge of one or more action potentials and stop within a time of 1000 ms after the beginning of the stimulus. During sustained deformation no spike discharge was observed.

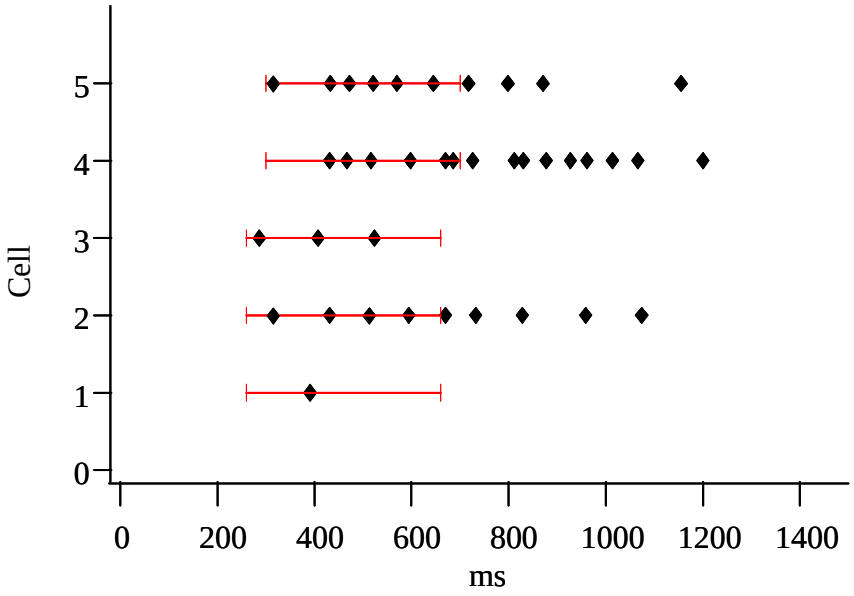


Fig. 8. Spatiotemporal plot showing the responses 5 mechanosensory neurons in a given ganglion. It is noteworthy that in all the cells the action potential discharge is over within 1000 ms after the beginning of the stimulation.

The median value of the action potential frequency in the responding neurons was 2.0 Hz. 107 neurons, which amount to 30% of responding neurons, fired one action potential only, all other neurons fired a volley of action potentials ranging from 2 to 28. In 6 ganglia recordings of up to 5000 ms after the injection were performed. All responding neurons fired action potentials within 1000 ms after the stimulus. Therefore, in all remaining experiments recording periods of 1255 or 1800 ms were used to minimise dye bleaching and phototoxicity.

In every experiment done we checked for reproducibility of the stimulus and analyzed the variation in the number of responding cells and in action potential frequency. We could verify that the responses of neurons to the mechanical stimulations were reproducible: only a percentage of 1.3% of neurons did not respond to the second control stimulation. In these experiments to verify the reproducibility of our results the median value of responding cell per ganglion was in both stimulation 7 and they, also in both stimulations, fired with an action potential frequency of 2.0 Hz (Fig. 9). In 5 ganglia it was possible to test reproducibility of 11 stimulations given every 15-20 min (last recording performed after 3 h 11 min). Neither the number of responding neurons (median value 5) nor the action potential frequency showed a significant variation.

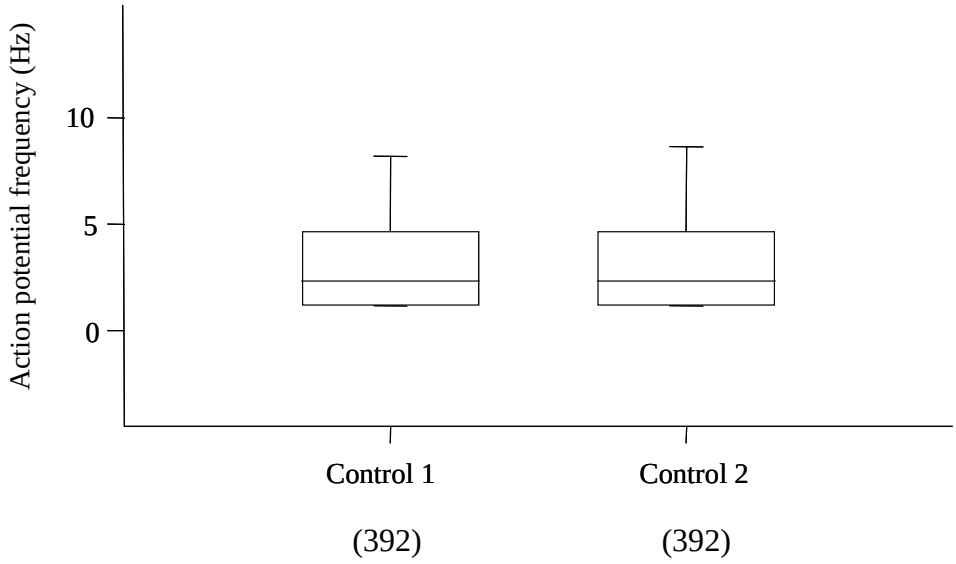


Fig. 9. Reproducibility test: analysis of the variation in the action potential frequency in the two control groups of data. A Mann-Whitney Rank Sum Test shows that between the two groups there is not a statistically significant difference. In parenthesis are indicated n number.

Experiments to characterize the stimulus modality

All the data are summarized in Table 3.

Relation between stimulus duration and discharge pattern

In 11 ganglia from 7 guinea pigs we performed experiments with different durations of the pressured controlled injections of 200, 400, 600 and 800 ms. We analysed 55 responding neurons. With increasing duration of the stimulus duration we observed a response in a median value of 4.5 neurons per ganglion for 200 ms pulses, and 6 neurons for 400, 600 and 800 ms pulses. The number of responding neurons showed a statistically significant increase between the 200 ms pulses stimulation and the other groups of data. The data about the action potential frequency showed also a significant increase from 200 ms to the other stimulation: the median values were 1.0, 2.8, 1.9 and 2.8 for the 200, 400, 600 and 800 ms pulses respectively (Fig. 10).

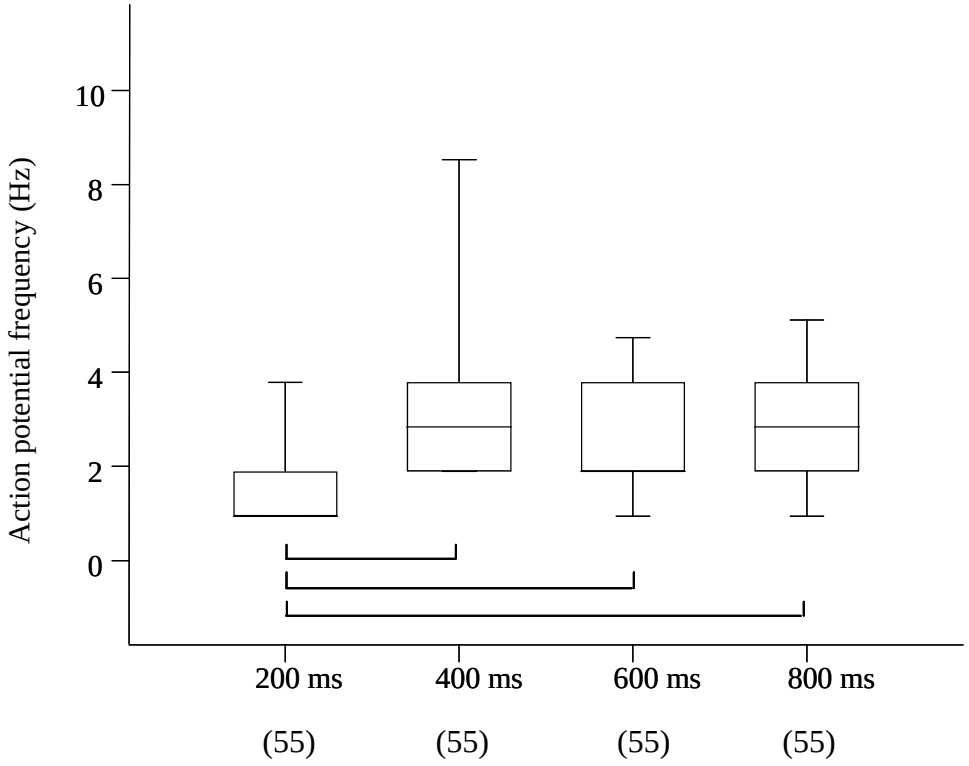


Fig. 10. Analysis of the four groups of data of the experiments with different duration of the stimulus application (200, 400, 600 and 800 ms). A Friedman Repeated Measures Analysis of Variance on Ranks Test and a Student-Newman-Keuls Test show that the increase in the action potential frequency from the 200 ms to the other stimulations is statistically significant. In parenthesis are indicated n numbers.

Relation between injection pressure and discharge pattern

In 10 ganglia from 9 guinea pigs 2 pressure injections with 10 psi and 20 psi (69 and 138 KPa) were performed. There was no significant

difference in number of responding cells per ganglion (median values of 5 at 69 KPa versus 3 at 138 KPa). Likewise, no significant differences in action potential discharge were observed (2.3 Hz at 69 KPa versus 3.5 Hz at 138 KPa) (Fig. 11).

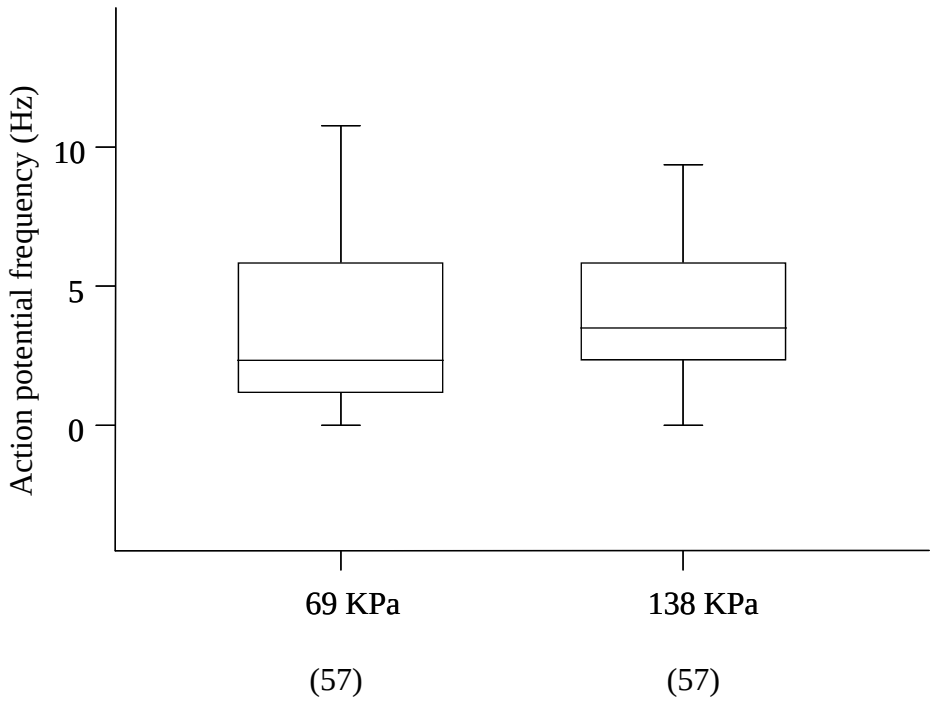


Fig. 11. Analysis of the two groups of data of the experiments with 69 KPa and 138 KPa pressure. Between the two groups there is no statistically significant difference. In parenthesis are indicated n numbers.

Relation between injected volume and discharge pattern

We performed 7 experiments (7 ganglia tested) in 3 guinea pigs changing in a random order the amount of volume injected into the ganglion. For these experiments we used the volume controlled injection protocol.

For each ganglion we recorded response to volumes of 1000, 2000, 3000 and 4000 nl. With this protocol the number of responding neurons remained constant and showed median value of 2 at 1000, and of 4 at 2000, 3000 and 4000 nl. The analysis of the action potential frequency within the different acquisitions revealed the following results. When we analyzed the data from all ganglia together, there was no significant difference in discharge rate. The median value was in each case of 0.6 Hz (Fig. 12). A more detailed analysis of the behaviour of each neuron revealed that in 5 out of ganglia there was a clear-cut increase in discharge pattern with increased volume injection. Moreover, in all the ganglia the discharge rate increased to a maximum value and dropped with higher volume injection.

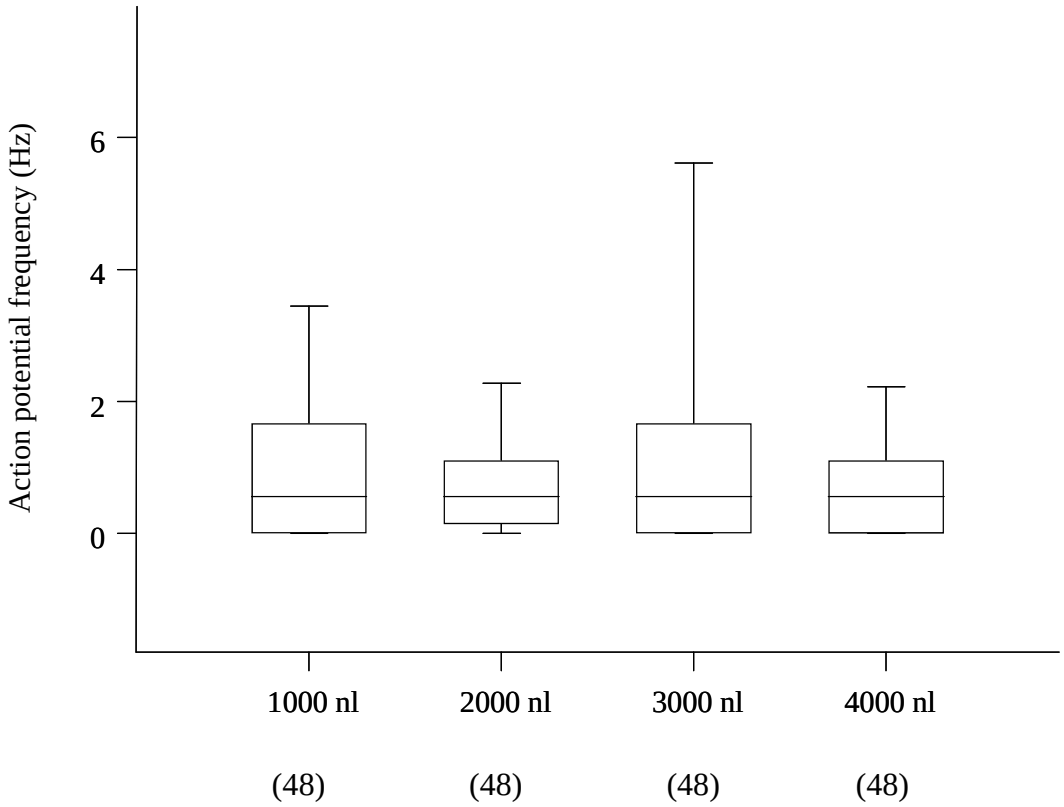


Fig. 12. Analysis of the four groups of data of the experiments with different volume of application (1000, 2000, 3000 and 4000 nl). A Kruskal-Wallis One Way Analysis of Variance on Ranks Test shows that there is not a statistically significant difference between the variations in the action potential frequency. In parenthesis are indicated n numbers.

Relation between rate of volume injection and discharge pattern

In 9 ganglia from 5 guinea pigs we tested 4 used flow rate of 1000, 2500, 5000 and 7500 nl/s with no change in the number of responding neurons per ganglion: 4, 6, 4 and 5 for 1000, 2500, 5000 and 7500 nl/s, respectively. Likewise, there were no significant differences in action potential discharge rate: 0.6 Hz for 1000 nl/s and 2500 nl/s, and 1.1 Hz for 5000 nl/s and 7500 nl/s (Fig. 13).

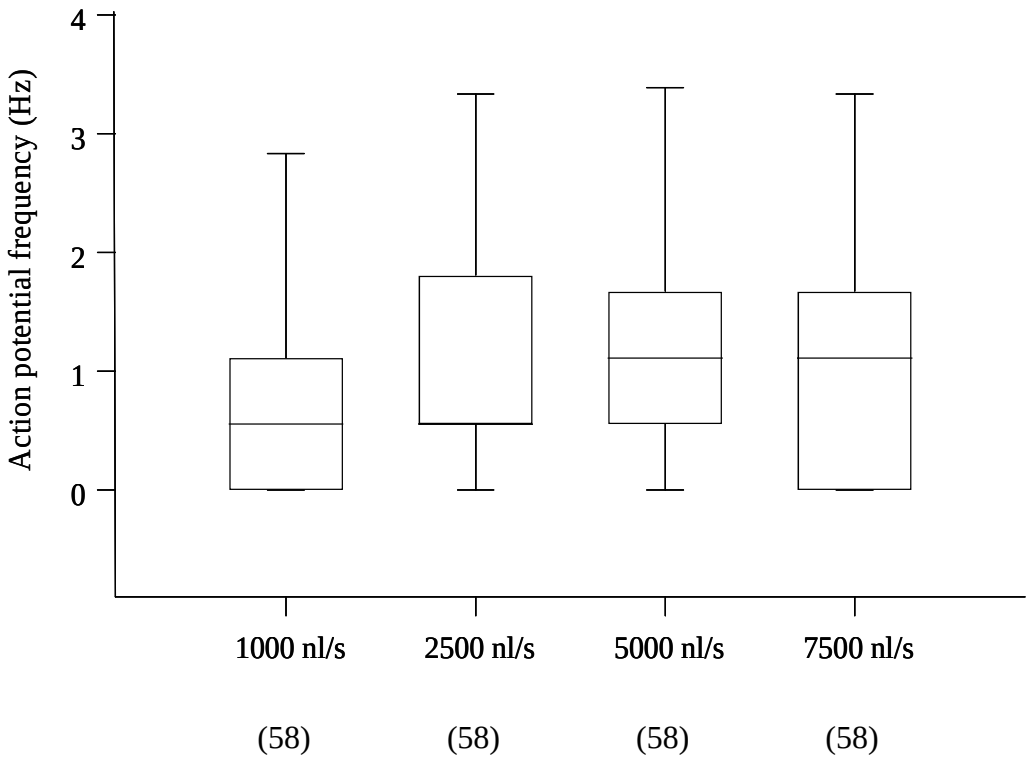


Fig. 13. Analysis of the four groups of data of the experiments with different flow rate of application (1000, 2500, 5000 and 7500 nl/s). A Kruskal-Wallis One Way Analysis of Variance on Ranks Test shows that there is not a statistically significant difference between the variations in the action potential frequency. In parenthesis are indicated n numbers.

Response to electrical stimulations of interganglionic fiber tracts

In every ganglion we performed electrical stimulations of interganglionic fiber tracts to record fast EPSPs. Without exceptions we recorded in all responding neurons an action potential followed by fast EPSPs (Fig. 14).

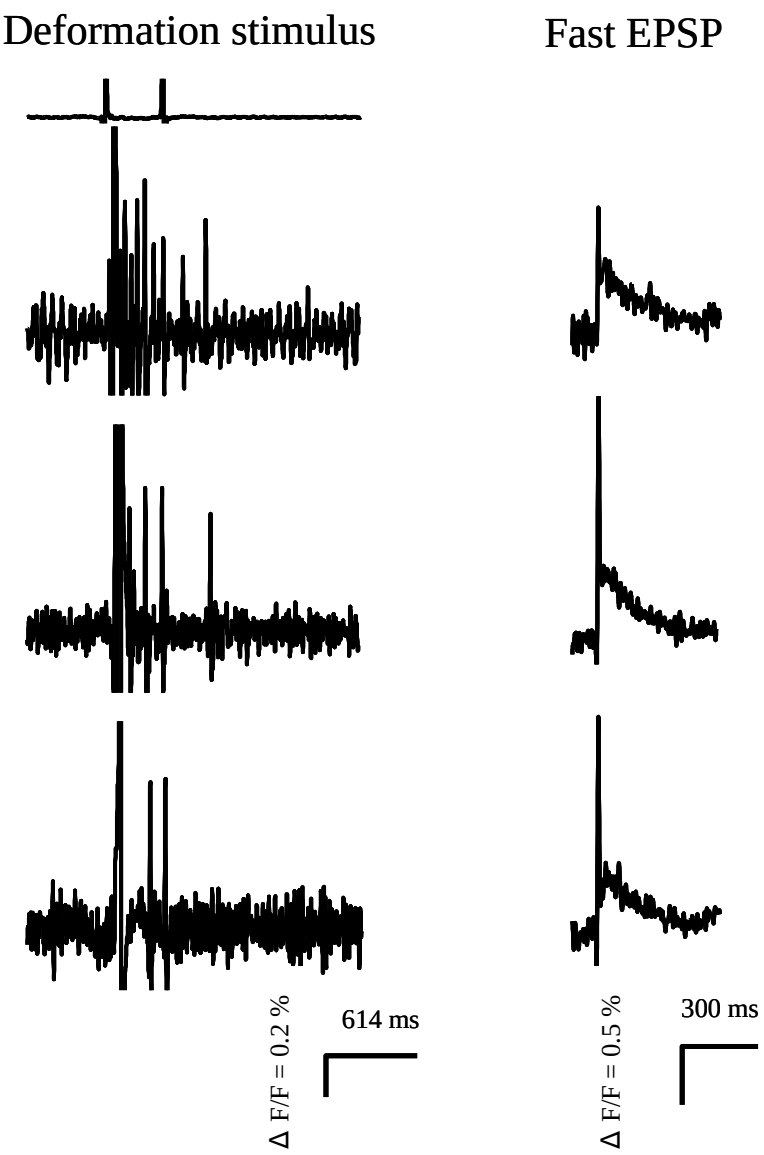


Fig. 14. Tracers from 3 different mechanosensory neurons comparing the response to the mechanical (left side) and to the electrical stimulation of interganglionic fiber tracts (right side). In these last ones the compound action potential is visible, followed by fast EPSPs.

The first action potential represents probably the compound of activity in a number of nerve fibers that project within interganglionic fiber tracts and cross through the ganglia because as previously reported (Schemann et al. 2002) most signals increased with the current strength of the stimulation pulse and also because they can be detected along nerve bundles. In 3 guinea pig 5 ganglia we measured the amplitude of the fast EPSPs before and after the mechanical stimulation, statistic analysis of the data showed not significant change confirming that the neuron electrical behaviour was not altered by the stimulus (Fig. 15).

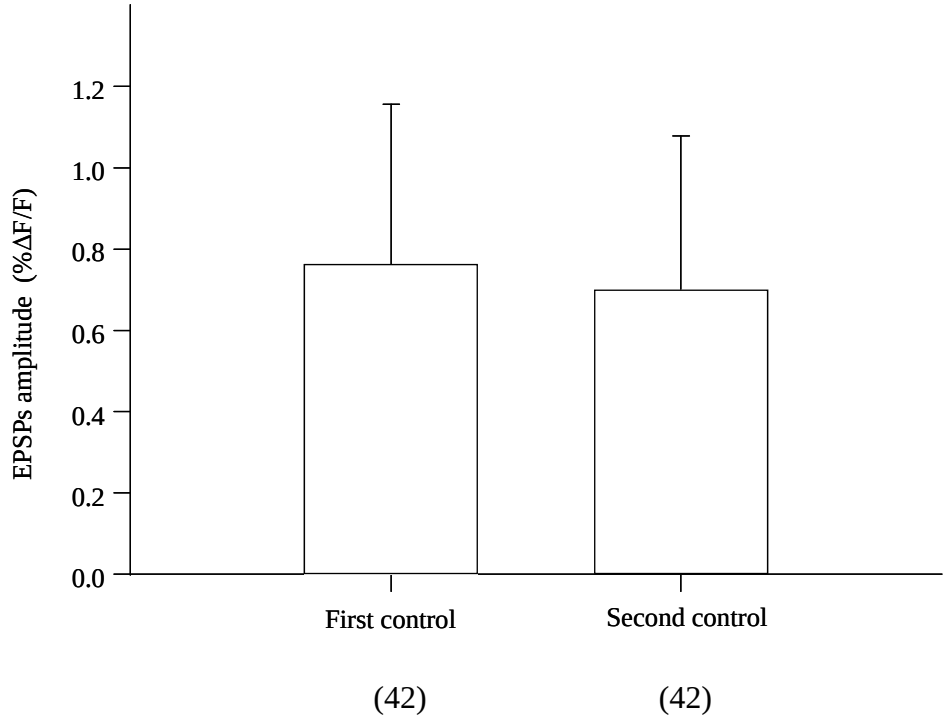


Fig. 15. Analysis of the two control groups of EPSPs stimulation. The first group of data was recorded at the beginning of the experiments, the second after the mechanical stimulation. A t test showed that there was not statistically significant difference between the 2 groups. In parenthesis are indicated n numbers.

All fast EPSPs were fully abolished after perfusion of 200 μM hexamethonium (10 ganglia from 9 guinea pigs) (Fig. 16e) or 0.02 μM ω-conotoxin GVIA (7 ganglia from 7 guinea pigs) (Fig. 16b). Fast EPSPs fully recovered after wash out of hexamethonium (Fig. 16f). As expected from previous studies and recent (Kerr and Yoshikami 1984; McCleskey et al. 1987; Feng et al. 2001; Motin and Adams 2008) the blockade with ω-conotoxin GVIA was irreversible (Fig. 16c).

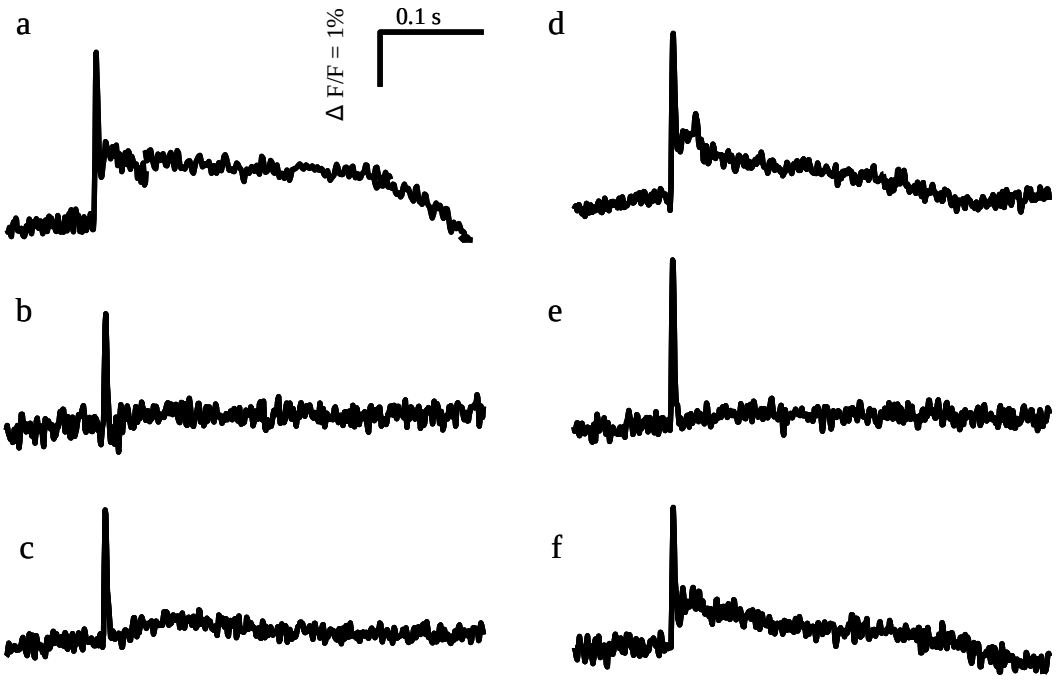


Fig. 16. Tracers from 2 different mechanosensory neurons showing the response to the electrical stimulation of interganglionic fiber tracts before, after the perfusion with ω-conotoxin on the left (a,b,c) and hexamethonium on the right (d,e,f) and after the washing out.

Pharmacology of mechanosensitivity

For all the pharmacological experiments the protocol of stimulation used was the intraganglionic pressure control injection, with a recording time of 1255 ms.

Effect of nifedipine

The movement of the tissue is a potential source for artefacts. In some experiments we used the L-type Ca^{2+} channel blocker nifedipine to prevent muscle movements. Thus, we performed some experiments in order to show that nifedipine did not affect the action potentials discharge of mechanosensitive neurons. We analysed 58 mechanosensitive neurons from 8 ganglia and 8 guinea pigs before and after the perfusion of the entire tissue with 1 µM nifedipine.

We performed two control stimulations before the perfusion and we often noticed an increase in the stimulation strength between the two stimulations, even if we did not change any parameter. Also analysing the artefact in the tracers it was clear that the second stimulation control and the one after nifedipine perfusion were stronger than the first one. This was due to the fact that without nifedipine the movement of the tissue was less controllable so the tip of the glass pipette, stuck in the ganglion, following the movements of contraction after the first injection, broke a bit more resulting in a stronger stimulation. In accord with this, we found a significant increase in the action potential frequency between the first stimulation and the others.

The median value of responding neurons remained unchanged and was 5 for the two stimulations before adding nifedipine and also 5 in the presence of nifedipine. The action potential frequency in the first control mechanical stimulation had a median value of 1.8 Hz, in the second was significantly increased at 3.9 Hz and, after adding nifedipine to the perfusion system remained elevated 4.9 Hz (Fig. 17). The significant difference revealed between the 2 control groups shows the variability of the response in conditions where the movement of the tissue is not strictly controlled.

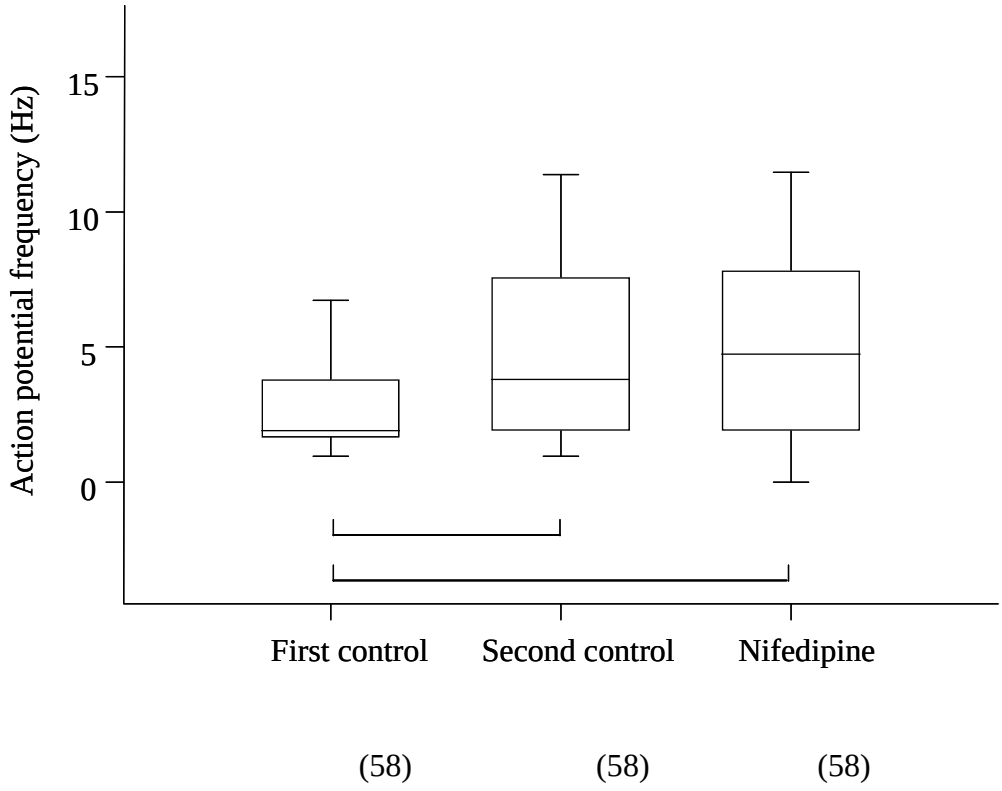


Fig. 17. Analysis of the mechanical stimulations before and after nifedipine perfusion. A Friedman Repeated Measures Analysis of Variance on Ranks Test shows a statistically significant difference. In parenthesis are indicated n numbers.

Effect of hexamethonium

In 10 ganglia from 9 guinea pigs we used 200 μM hexamethonium to block fast EPSPs. We analysed 46 mechanosensitive neurons. The median number of mechanosensitive neurons was 4 and remained unchanged in the presence of hexamethonium (4) and after 30 min washing out (3.5). The efficacy of hexamethonium was verified by the total block of fast EPSPs (Fig. 16d, e, f). The median value of the frequency of action potential discharge in response to stimulation was 3.5 Hz and did not change in the presence of hexamethonium (3.5 Hz), or after 30 min wash out (3.5) (Fig. 18).

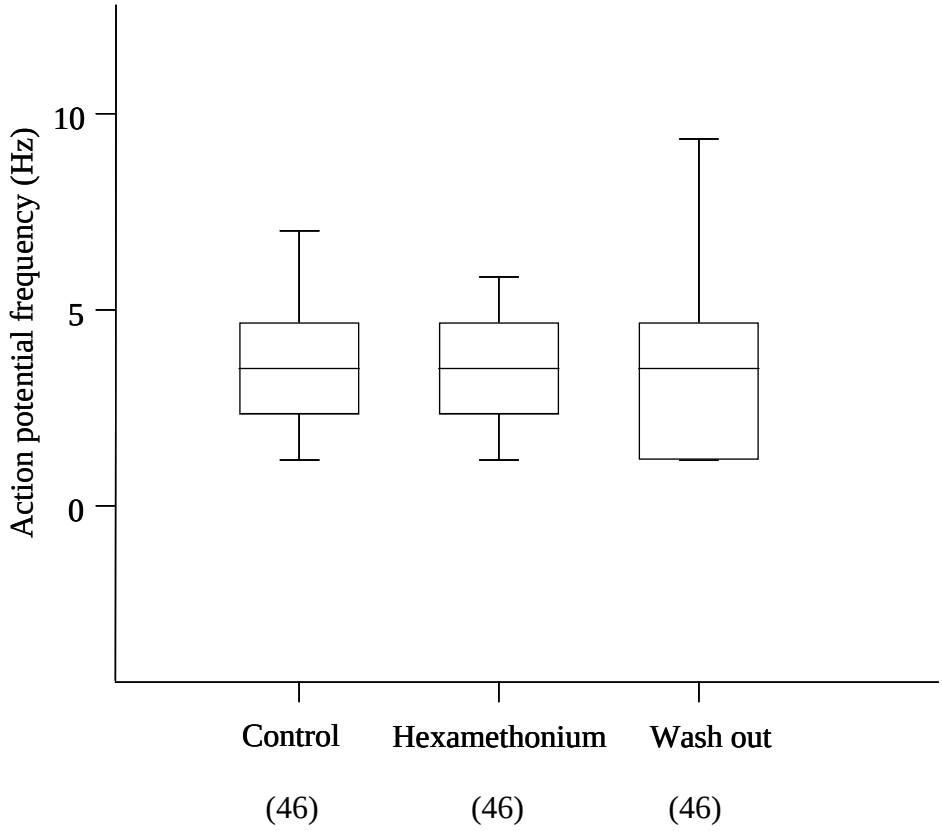


Fig. 18. Analysis of the experiments with hexamethonium perfusion. A Mann-Whitney Rank Sum Test shows that there is not statistically significant difference between the different groups. In parenthesis are indicated n numbers.

Effect of ω-conotoxin GVIA

ω-conotoxin GVIA is known like a potent and specific blocker of N-type calcium channels, it inhibits synaptic transmission in many system (Kerr and Yoshikami 1984; McCleskey et al. 1987; Feng et al. 2001; Motin and Adams 2008) and it is also reported to antagonize P2X receptors (Lalo et al. 2000).

In 7 ganglia from 7 guinea pigs we used 20 min perfusion with 0.02 µM ω-conotoxin GVIA to inhibit synaptic release. We analysed the response in 50 mechanosensitive neurons. All fast EPSPs were blocked in the presence of ω-conotoxin (Fig. 15a, b, c). The number of responding cells was not significantly changed in the presence of ω-conotoxin: 6 versus 5. Before perfusion of ω-conotoxin the median value of the action potential discharge was 3.5 Hz and was not significantly changed in the presence of ω-conotoxin (4.7 Hz) (Fig. 19).

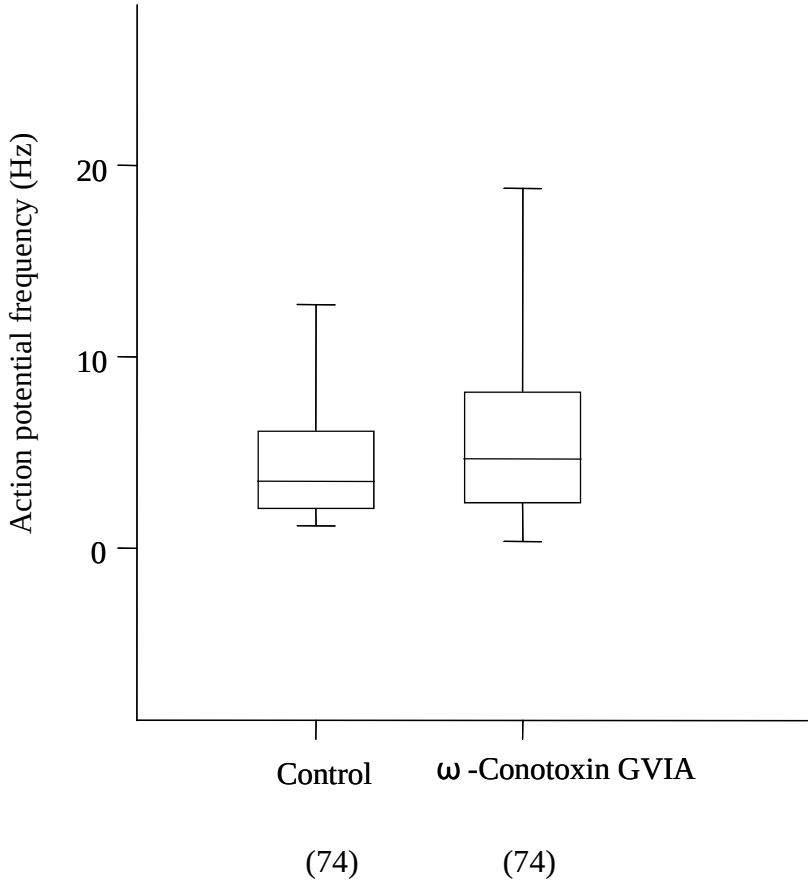


Fig. 19. Analysis of the two control groups of the experiments with ω-Conotoxin GVIA perfusion. A Mann-Whitney Rank Sum Test shows that there is not statistically significant difference between the different groups. In parenthesis are indicated n numbers.

Effect of capsaicin

We used long term perfusion of capsaicin to defunctionalise extrinsic primary afferents nerves. Acute capsaicin application activates TRPV1 receptors that are exclusively localised on extrinsic afferents and not on enteric neurons. Long term perfusion of capsaicin for more than 20 min, however, leads to a complete desensitization (Weber at al. 2001).

We analysed 51 responding cells from 6 ganglia from 6 guinea pigs. The number of mechanosensitive neurons did not significantly change after capsaicin perfusion (8 versus 7). The frequency of action potentials also remained stable after capsaicin perfusion (2.6 Hz versus 2.6 Hz) (Fig. 20).

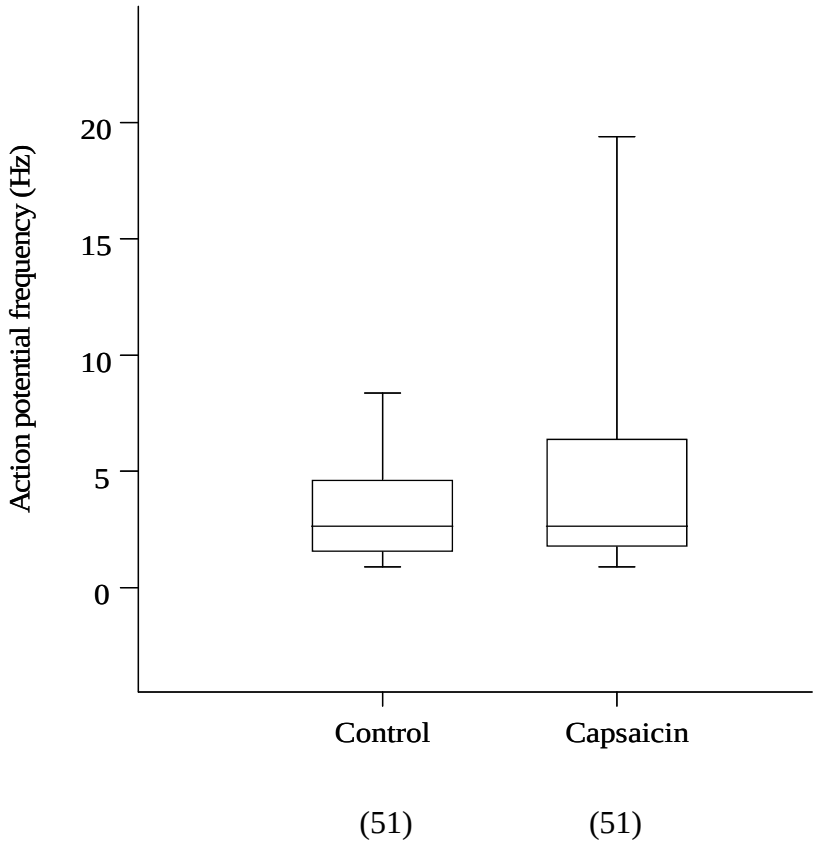


Fig. 20. Analysis of the experiments with capsaicin perfusion. A Friedman Repeated Measures Analysis of Variance on Ranks Test shows that there is not statistically significant difference between the two groups. In parenthesis are indicated n numbers.

Neurochemical coding of mechanosensory neurons

Immunohistochemical demonstration of Calb and NeuN was performed in 11 ganglia from 6 guinea pigs. These two markers were suggested to specifically label IPANs (Furness et al. 1990b; Costa et al. 1996; Brody et al. 2002; Costa et al. 2001, 2002; Poole et al. 2002; Van Nassauw et al. 2005).

The labelled ganglia contained a median value of 7 (19%) mechanosensitive neurons that responded with an action potential discharge frequency of 1.9 Hz. 56% of mechanosensitive neurons were immunoreactive for NeuN ($NeuN^+$) and 29% was immunoreactive for Calb ($Calb^+$). Double staining revealed that 29% of mechanosensitive neurons were immunoreactive for both NeuN and Calb ($NeuN^+/Calb^+$) whereas 27% of mechanosensory neurons were immunoreactive for NeuN only ($NeuN^+/Calb^-$) (Fig. 21; Fig. 22). The highest percentage of mechanosensitive neurons was neither Calb nor NeuN immunoreactive (44% $NeuN^-/Calb^-$) (Fig. 21; Fig. 22). All the data are summarized in Fig. 21.

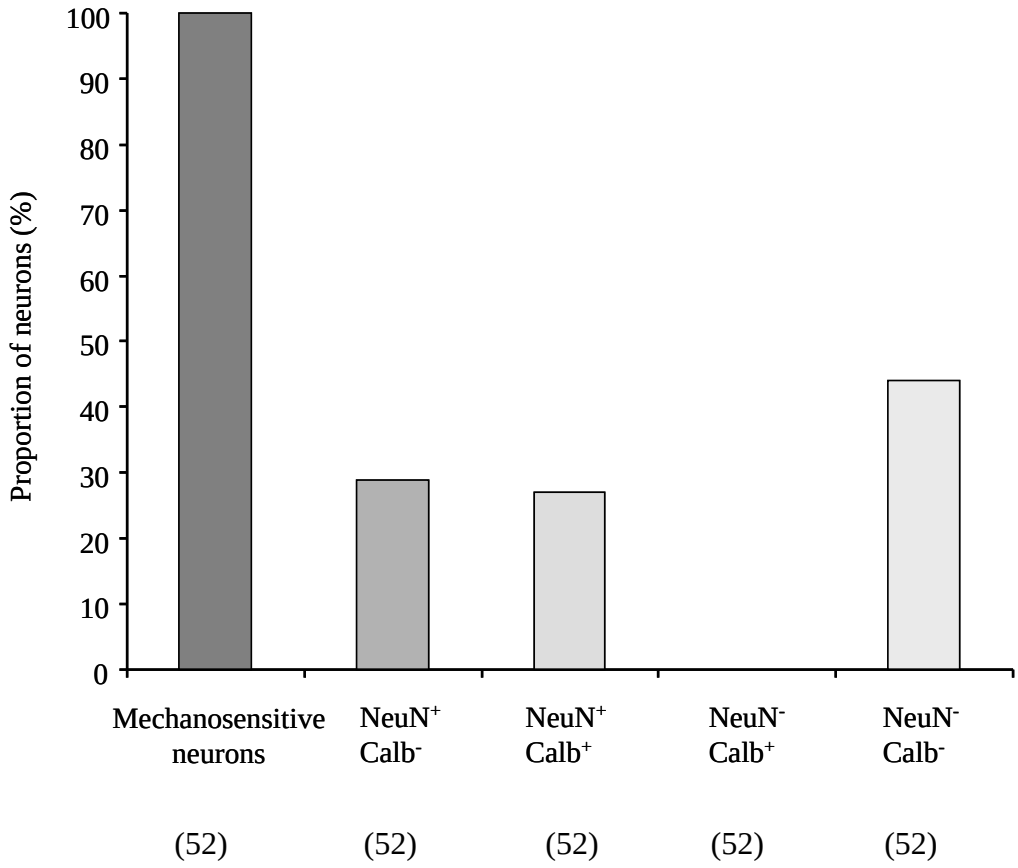


Fig. 21. Graph showing the proportion of mechanosensory neurons immunoreactive for the different neurochemical markers. It is noteworthy that 44% results negative for both these markers. In parenthesis the n numbers are indicated.

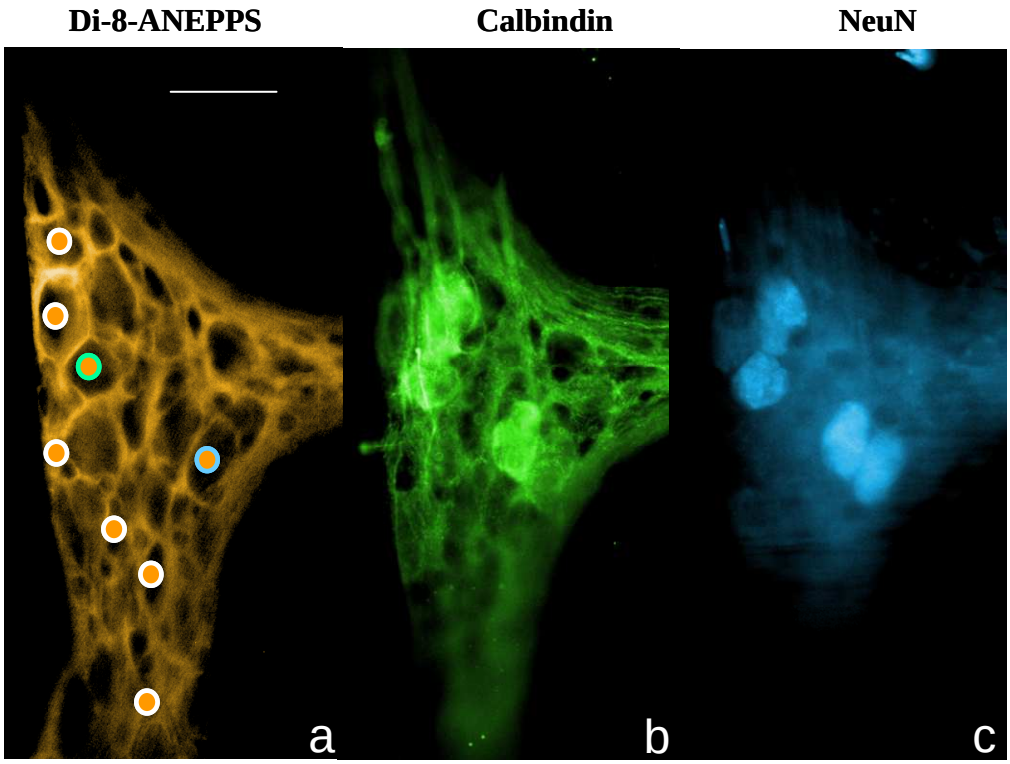


Fig. 22. Example of immunohistochemical labelling: in **a** the ganglion is stained with Di-8-ANEPPS, in **b** Calbindin staining is shown and in **c** NeuN staining. In **a** the cells marked with the orange dots are the mechanosensory neurons, the dot that is encircled with blue shows a cell immunoreactive for NeuN but not for Calb. The dot encircled in green shows a cell immunoreactive for Calb also positive for NeuN. The dots encircled in white represents mechanosensory neurons negative for both markers. Bar 50 µm.

Morphology of mechanosensory neurons

We observed among the responding neurons a great variety of cell morphologies. On one hand, we observed very big cells with a shape referable to Dogiel type II neurons, with large oval or round cell bodies and multiple processes departing from the soma. These cells were mostly immunoreactive for Calb. On the other hand, we also observed very tiny cells with angular outlines with a shape referable to Dogiel type I neurons.

Discussion

This study revealed three main results. Firstly, the guinea pig ileum myenteric plexus contains mechanosensitive neurons which respond to rapid changes in mechanical forces evoked by von Frey hair stimulation or intraganglionic volume injection with action potential discharge. Secondly, the response pattern suggests that myenteric neurons behave like rapidly adapting mechanosensors that respond to dynamic changes. Thirdly, the responses are a result of direct activation of neurons because synaptic blockade or defunctionalisation of capsaicin sensitive extrinsic afferents has no effect on mechanosensitivity.

The results indicate that myenteric neurons in the guinea pig ileum respond to mechanical stimuli that mimick those occurring during normal muscle motor activity. It is noteworthy that the mechanosensory neurons identified in this study respond to rapid changes in cell shape but not to sustained mechanical forces. The exact stimulus modality remains unknown but deformation must be an important stimulus. With the von Frey hair technique, indeed, the neurons situated near the stimulation site were most deformed and always responding. On the contrary, the neurons situated far away from the site of stimulation showed less deformation and rarely responded. From these results it is evident that responsiveness of mechanosensory neurons is related to the degree of deformation. The intraganglionic volume injection provoked a more reliable deformation of the entire ganglion and was not restricted to a small

ganglionic region as it was the case with the von Frey hair stimulation. Nevertheless, the volume was never equally distributed throughout the ganglion. Therefore, deformation varied within the ganglion and, depending on the location of the neurons, the degree of deformation was different. Thus, at the moment, it appears not possible to determine if mechanosensory neurons are a specialized cell population or if all the neurons are capable to respond to a mechanical stimulus. However, we hypothesize that the proportion of mechanosensory neurons found in this study (around 20%) is an underestimation. Technical limitations may have compromised reliable recordings from all neurons in a particular ganglion. These include low level of the signal to noise ratio or neurons that are not in the plane of focus.

The proportion of mechanosensory neurons identified in this study agrees with the percentage Dogiel type II/AH neurons (35%) which were suggested to be sensory neurons in the guinea pig MP of the ileum (Furness et al. 1990b). It also agree with the number of NeuN immunoreactive myenteric neurons (38%) (Costa et al. 2001; Costa et al. 2002). However 44% of the rapidly adapting mechanosensory neurons described in the present study were not Calb and NeuN immunoreactive. In addition, these cells vary in shape and thus belong to different morphological types of neurons. Thus, even if the proportion of mechanosensory neurons fit with the data in the literature, the population of rapidly adapting mechanosensory neurons has different characteristics compared to the mechanosensitive neurons that respond to sustained stretch.

The response of mechanosensory neurons to deformation consists of an immediate spike discharge that rapidly adapts. The latency between the stimulus application and the beginning of the response had a mean value of 182 ± 128 ms, but, as already discussed in the results section, the mechanical artefact made in most recordings impossible to identify early spike discharge as the action potentials disappeared in the artefact evoked signal. However, in some neurons we could record action potentials with latencies of 18-22 ms. This latency appears to be long compared to the studies performed on MSC, that indicates a time less than 5 ms to open MSC with a direct activation mechanism (Christensen and Carey 2007), or compared to the 6 ms latency for mechanotransduction in intraganglionic laminar endings of vagal tension receptors (Zagorodnyuk et al. 2003). We assume that the reason for the rather long delay between the start of the mechanical stimulus and the occurrence of the first spike is the unknown mechanical threshold for spike discharge. Although we use the beginning of the mechanical artefact as the start of the deformation, it remains unknown at what exact time the threshold has been reached. It also remains open whether other cells or non-neuronal structures are additionally involved in transducing the mechanical stimulus. Synaptic activation can be excluded as hexamethonium and ω-conotoxin, both of which block fast synaptic input, had no effect on the spike discharge. Unspecific activation of synapses by deformation is also unlikely because one would expect to see hexamethonium sensitive responses due to release of acetylcholine. This leaves glia or changes in the extracellular matrix as possible candidates that may be

involved in mechanotransduction. This however remains speculative as long as we have not unequivocally identified the channels or receptors involved in mechanotransduction in the MP.

Stretch sensitive mechanosensory neurons within the MP have been described by extracellular recording (Wood 1970; Wood 1974). In these experiments probing the tissue with the tip of a glass electrode was comparable to the mechanical stimulation we used. Moreover, the driver neurons respond to the stimulation with phasic spike discharge and this pattern is similar to the discharge pattern of our mechanosensory neurons (Wood 1970; Wood 1974).

The action potential discharge of the mechanosensory neurons never lasted more than 1000 ms after the beginning of the stimulus although the ganglion deformation lasted for 160 ± 90 s. This suggests that mechanosensory neurons behaved like rapidly accommodating neurons which respond to dynamic changes only. Kunze et al. (2000) also used a short lasting mechanical stimulus to record from AH neurons a pattern of action potential discharge very similar to the one we found; sensory neurons in fact responded with one or more action potentials and the discharge ceased before the stimulus was removed (Kunze et al. 2000). Thus, they also behaved like rapidly adapting neurons. The behavior of the rapidly adapting mechanosensory neurons appears to be perfectly suitable to respond to changes during phasic muscle activity. The rapid adaptation in the neuronal response may be very important in an environment like the gut where neurons have to rapidly integrate a variety of different stimuli in order to adjust their output and to appropriately modulate activity of the target tissue.

From the data obtained with the von Frey hair we can conclude that the threshold to evoke any response in mechanosensory myenteric neurons is around 1.0 mN. This value agrees with the threshold of activation of distal colon stretch sensitive extrinsic afferents which start to respond to mechanical stimulation between 0.9 and 2.0 mN (Lynn et al. 2003). Moreover, in the experiments with intraganglionic volume injection we found a significant difference in the percentage of responding neurons and in the action potentials frequency discharge between 200 ms pulses and longer applications. Increasing the duration from 400 to 800 ms did not change the number of responding neurons or their action potential frequency. This suggests that the threshold of putative MSC is not reached in all mechanosensory neurons with stimulation pulses of 200 ms.

The response to mechanical stimulation appears to be related to the stimulus strength. In the von Frey experiments we showed that mechanosensory neurons respond to increased forces with increased spike discharge. This is in agreement with the findings that showed that myenteric mechanosensory interneurons of the guinea pig colon (also identified with the von Frey hair technique) responded to gradual increases in the stimulus strength with increased action potential discharge (Smith et al. 2007). We can hypothesize that putative MSC are more sensitive with increased stimulus strength. This is a consequence of a larger force lowering the energy barrier to open the channel (Christensen and Corey 2007).

The response of mechanosensory neurons varied with variations of the injected volume and the flow rate. Increases in these parameters

initially led to a gradual increase in the response to reach a maximum at certain stimulus strength. Further increases caused a smaller response. The stimulus strength evoking the maximal spike discharge was variable between ganglia. This may be related to the different shapes of the ganglia and as a result the different volume distribution that will lead to different degrees of deformation. The decrease in spike discharge after exceeding certain stimulus strength may be a result of active inhibition. In an earlier study performed with a mechanical stimulation comparable to the one we used, results indicated that myenteric AH neurons are excited by mechanical distortion of their processes and are inhibited by mechanical force on the cell body: compression of the soma causes increased opening of potassium channels and thus has an inhibitory effect (Kunze et al. 2000). In our study we could not exactly locate the sites of mechanotrasduction as we very likely deformed both soma and neuritis.

The response of mechanosensory neurons to a mechanical stimulation is reproducible suggesting that the responses are physiological and not caused by mechanical damage to the neuron. This is also supported by the findings that we could stimulate and record from the same ganglia with no significant changes in the responses for up to 3 h 11 min. Other evidence that the ganglion was not damaged came from the immunohistochemical data which revealed normal structure of the ganglion and the neurons. Last but not least we found that fast EPSPs remain stable after mechanical stimulation.

The responses we evoked with mechanical stimulation were a direct action on neurons and not via synaptic transmission. Fast EPSPs are indeed the major form of communication between enteric neurons and are mainly mediated by ACh acting through nicotinic receptors. Data in the last 10 years have, however, suggested that a significant proportion of fast synaptic transmission is purinergic (Ren and Bertrand 2008). Thus, to more effectively block the synaptic transmission we performed mechanical stimulations of the ganglia after perfusion with ω-conotoxin GVIA, that is a persistent N-type Ca^{2+}-channels blocker and also an antagonist of the P2X receptors (Lalo et al. 2000). The electrical stimulations after the perfusion of the tissues with this toxin showed that the fast EPSPs were completely blocked. This means that total block of fast excitatory synaptic input did not affect responsiveness of the mechanosensory neurons. These data agree with previous findings that responses in mechanosensitive AH neurons were not diminished after synaptic blockade (Kunze et al. 2000).

Long term perfusion with the TRPV1 agonist capsaicin was performed to reveal involvement of extrinsic afferent neurons. Long term capsaicin treatment results in defunctionalisation of TRPV1 expressing extrinsic afferents (Weber at al. 2001). The data showed a non significant change in the number of responding cells as well in the action potential frequency. Thus, the response was not significantly affected by the perfusion with capsaicin. This allows us to say that the extrinsic afferent neurons are not significantly implied in the mechanical response but this response comes from the ENS neurons.

Our experiments also showed that action potential discharge in mechanosensory neurons was not affected by nifedipine. Therefore, we can conclude that muscle tone is not required for activation of mechanosensory neurons. This is in contrast with the findings showing that neuronal response to sustained stretch of the guinea pig ileum depends on opening of stretch activated channels in the muscle followed by muscle contraction that leads to mechanical activation of AH neurons (Kunze et al. 1999). This local muscle response seems to be crucial for the initiation of neural reflexes as it increases excitability in the neural circuits. However, the stimulus used in our experiments is different from the one used by Kunze et al. (1999), as they used sustained stretch while we used stimulations that resulted in fast developing neuronal deformation. It is noteworthy that brief probing of neurites elicited responses that were independent of muscle tone because they remained unchanged in the presence of isoprenaline or nicardipine (Kunze et al. 2000). Moreover, mechanosensory interneurons with S type electrophysiology also respond to mechanical stimulation in the presence of muscle blockers like nifedipine or papaverine (Spencer et al. 2002; Spencer et al. 2003; Spencer and Smith 2004). The conclusion from these studies would be that mechanosensory neurons in the gut have region specific properties and that their mechanosensitivity adapts to different environmental conditions. In addition, the MP contains mechanosensory neurons that respond to dynamic changes in cell shape as well as to sustained stretch. It is noteworthy that the rapidly adapting mechanosensory neurons identified in the present study did not respond to sustained

deformation. Although the neurons fired during the initial deformation they remained quiescent during the sustained phase of deformation.

Our data show that all mechanosensory neurons do receive fast EPSPs. In many studies it has been shown that AH neurones of the guinea pig small intestine rarely receive fast EPSPs (Iyer et al. 1988; Bornstein et al. 1991; Smith et al. 1992). However, others did regularly record fast EPSPs in myenteric AH neurons of the guinea pig (Grafe et al. 1979; Katayama et al. 1986; Tack and Wood 1992; Lomax et al. 1999; Tamura et al. 2001). Unequivocally AH neurons receive slow EPSPs (Wood 2008). The finding that rapidly adapting mechanosensory neurons receive fast EPSPs means that their activity can be highly modulated via synaptic transmission. Thus, there is a low stimulus fidelity which allows setting the gain in a sensory network. The modulation of the response to a stimulus via fast synaptic inputs allows the enteric circuits to rapidly accommodate to changes in the environment and thereby to determine gut behaviour (Blackshaw et al. 2007). It is likely that transmission within the enteric sensory networks is also modulated by extrinsic afferent neurons, enteric glial cells, enteroendocrine cells, immune cells, ICCs and muscle cells. Therefore, sensory neurons have to integrate all the inputs from varying sources. Our results support the concept of multifunctional enteric neurons which may fulfil sensory, integrative and motor functions. Mechanosensitivity is indeed a common feature of many enteric neurons belonging to different functional classes (physiological behaviour, neurochemical code, morphology). The idea of multifunctionality in the ENS can pave the way for novel concepts

on transmission of sensory modalities within the gut. Since nowadays a lot of GI disorders appear to be connected with sensorimotor dysfunctions, it is crucially important to further clarify the sensorimotor pathways.

Tables

Table 1. Table summarizing the different drugs used in the experiments with the respective concentrations and time of application. All these substances were added to the Krebs solution in the perfusion system.

Drugs	Company	Reference number	Concentration (µM)	Perfusion time (min)
Nifedipine	Sigma (Schnelldorf, Germany)	N-7634	1	20
Hexamethonium-bromide	Sigma	H-0979	200	30
ω-Conotoxin GVIA	Alomone labs (Jerusalem, Israel)	C-300	0.02	20
Capsaicin	Sigma	M-2028	10	30

Table 2. Table summarising the results obtained using different von Frey hair stiffness. The percentage values of the mechanosensory neurons are indicated as mean ± standard deviation. The action potential frequency is indicated as median value.

Force (mN)	**Guinea pigs**	**Ganglia**	**Mechanosensory neurons (% on deformed cells)**	**Action potential frequency (Hz)**
0.30	2	5	0%	/
0.70	2	5	0%	/
1.00	3	8	12 ± 8%	1.0
1.25	2	5	19 ± 11%	1.5
1.60	4	15	20 ± 12%	1.5
2.00	3	9	24 ± 15%	1.5
2.70	2	10	21 ± 13%	2.0

Table 3. Table summarizing all the data of the experiments made in order to define the stimulus modality. The first column indicate the experiment performed, the second one the percentage of mechanosensory neurons and the third one the median values of the action potential frequency.

Experiment	**% Mechanosensory neurons**	**AP frequency (Hz)**
Different duration (ms)		
200	16 ± 11%	1.0
400	22 ± 12%	2.8
600	22 ± 11%	1.9
800	21 ± 12%	2.8
Different pressure (KPa)		
69	16 ± 8%	2.3
138	10 ± 5%	3.5
Different volume (nl)		
1000	10 ± 8%	0.6
2000	13 ± 10%	0.6
3000	13 ± 7%	0.6
4000	11 ± 7%	0.6
Different flow rate (nl/s)		
1000	11 ± 12%	0.6
2500	14 ± 12 %	0.6
5000	13 ± 10%	1.1
7500	12 ± 8%	1.1

Table 4. Table summarizing the results of the pharmacological experiments. The first column indicate the experiment performed, the second one the percentage of mechanosensory neurons and the third one the median values of the action potential frequency.

Experiment	**% mechanosensory neurons**	**AP frequency (Hz)**
nifedipine		
• Control	13 ± 9%	3.9
• Perfusion	12 ± 10%	4.9
hexamethonium		
• Control	20 ± 8%	3.5
• Perfusion	20 ± 9%	3.5
ω-conotoxin GVIA		
• Control	18 ± 8%	3.5
• Perfusion	16 ± 5%	4.7
capsaicin		
• Control	23 ± 7%	2.6
• Perfusion	19 ± 5%	2.6

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