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Study of the role of substance P in the regulation of gastric motility and gastric mucosal integrity in rats

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DOCTORATE THESIS

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1. INTRODUCTION

1.1. Overview of the factors involved in the control of gastric functions

Gastric functions are regulated through a complex interacting network comprising several gut regulatory peptides, hormones, extrinsic afferent innervation, sympathetic and parasympathetic nerves and the enteric nervous system (ENS).

1.1.1. Local control of gastric functions: the ENS

The ENS is a part of the autonomic nervous systems (ANS) and its components form an integrated circuitry that controls and coordinates motility, blood flow and secretions in the gastrointestinal tract. The ENS is organized into an interconnected network of neurons and glial cells that are grouped into ganglia located in two major plexuses: the myenteric (Auerbach's) plexus and the submucosal (Meissner's) plexus [1]. The myenteric plexus is positioned between the longitudinal and circular muscle layers throughout the digestive tract, from the esophagus to the rectum. The submucosal plexus is positioned in the submucosa, being prominent only in the intestine. Indeed, the stomach almost completely lacks a ganglionated submucosal plexus and the myenteric plexus represents the only source of the intrinsic innervation of the muscle and the mucosa. ENS neurons can be classified according to their morphological, neurochemical, or functional properties [2]. Depending on t heir morphology,

neurons are distinguished into Dogiel type I to type VII and giant neurons. The function of neurons of the ENS depends on their chemical coding, which shows pronounced plasticity under pathophysiological conditions, as demonstrated in the margin of gastric ulcers and in atrophy following intestinal inactivity [3]. More than 30 neurotransmitters have been identified in the ENS, including either small molecules (e.g. norepinephrine and 5-HT), larger molecules (peptides) or gases (e.g. nitric oxide). Acetylcholine (Ach) represents the major excitatory transmitter of the ENS, whereas nitric oxide (NO) and vasoactive intestinal polypeptide (VIP) are the main inhibitory transmitters. According to their functions, enteric neurons are classified in sensory neurons, interneurons, motor neurons and vasomotor/secretomotor neurons. Intrinsic primary afferent sensory neurons (IPANs) are Dogiel Type II neurons innervating both mucosal and muscular layers of the stomach. They also synapse with each other forming self-reinforcing networks that issue outputs to interneurons, motor neurons, secretomotor neurons and vasodilator neurons [4]. IPANs include mucosal chemosensors, mucosal mechanosensor and muscular tension receptors, thus providing the ENS with the kind of sensory information that it requires for the autonomic control of gastric functions.

Myenteric interneurons are usually Dogiel type II neurons characterized by a single axon and a cell body with short lamellar of filamentous dendrites. They receive inputs from IPANs, extrinsic neurons, and from other interneurons and send synaptic outputs to other classes of enteric neurons. Interneurons

involved in motor reflexes project orally or anally and are designated as ascending or descending, respectively. The ascending interneurons are mainly cholinergic (therefore excitatory); they make synaptic contacts with other ascending interneurons, with excitatory motor neurons and with other classes of enteric neurons. The descending interneurons have a complex chemical coding including acetylcholine, NO, VIP, 5-HT and somatostatin.

Enteric motor neurons, which are S/Dogiel type 1 cells, comprise muscle motor neurons, secretomotor neurons and neurons innervating entero-endocrine cells, such as gastrin secreting endocrine cells of the stomach. Muscle motor neurons innervate the longitudinal and circular muscle and the muscularis mucosae throughout the stomach. They can be either excitatory or inhibitory and release transmitters that provoke muscle contractions or relaxation. The major mediator of the contractile response at the neuroeffector junction is acetylcholine acting at muscarinic receptors. Acetylcholine is probably released from more than one population of cholinergic myenteric neurons along with other transmitters including substance P (SP). For the inhibitory neurons, which account for much of the descending accommodating inhibitory reflexes, the transmitters are NO, VIP, ATP, and possibly pituitary adenylate cyclase-activating polypeptide (PACAP), gamma aminobutyric acid (GABA), neuropeptide Y and carbon monoxide. They constitute the non-adrenergic non-cholinergic (NANC) inhibitory gastric transmission. As for interneurons, the excitatory and inhibitory motor pathways within the

myenteric plexus are polarized into ascending and descending projections, respectively, in all gastric regions [5-7]. Sequential activation of polarized circuits in the stomach is responsible for aboral transport of luminal content and mediates relaxation below and contraction above a stimulus. Myenteric secretomotor and vasomotor neurons control secretory activity of epithelia cells and blood flow, respectively. IPANs (but also extrinsic afferent neurons) exert a direct control of secretomotor and vasomotor neurons by releasing several neurotransmitters [8].

1.1.2. Central control of gastric functions: the "brain-gut axis"

The ENS is connected to the central nervous system (CNS) by both extrinsic afferent and extrinsic efferent nerve fibers, which constitute the two-way communication pathway between the gut and the brain (the so called "brain-gut axis") [9].

The afferent component of brain-gut communication system convey to the CNS the information about processes and condition in the gut and participate in the organization of autonomic and neuroendocrine reflex circuits and in the maintenance of mucosal homeostasis. The extrinsic afferent innervation of the stomach is constituted by vagal and spinal primary sensory fibers originating from somata in the nodose and dorsal root ganglia, respectively [10-13]. Associated mostly with non-myelinated and some thinly myelinated axons (C and Aδ fibers), the extrinsic sensory nerve fibers supply gastric mucosa,

submucosa (particularly arterioles), muscle, myenteric plexus and serosa. With these projections and their sensory modalities, they can respond to changes of the chemical environment in the gastric lumen, interstitial space and vasculature and to mechanical distortion of the stomach wall (typically distension, but also contraction or relaxation of the muscle). Although the intrinsic and extrinsic afferent innervation of the stomach are distinct in terms of origin and functional implications, they share a number of characteristics. Both group of sensory fibers have a similar innervation territories in mucosa and muscle, are responsive to both chemical and mechanical stimuli and share neurochemical traits (see above). In contrast, only extrinsic afferents are sensitive to capsaicin, the pungent ingredient of red pepper, because of the expression of the transient receptor potential cation channel of vanilloid type 1 (TRPV1), on which capsaicin acts specifically [14, 15]. Opening of the non-selective cation channel in response to capsaicin leads to an influx of Na^+ and Ca^{2+}; as end effect, the membrane of the nerve ending is depolarized and gives rise to afferent signals [16, 17].

Vagal afferent fibers carry a large volume of information about the physiological status of the stomach directly to brainstem circuits regulating gastric functions. The central terminals of vagal afferent neurons enter the brainstem via the tractus solitarius and terminate within the nucleus tractus solitarii (NTS), using mainly glutamate as their neurotransmitter [18, 19]. NTS is a paired structure located in the dorsomedial medulla that, together with area

postrema (AP) and dorsal motor nucleus of the vagus (DMV), constitutes the dorsal vagal complex (DVC). In the NTS, second order neurons integrate the sensory information from vagal afferents with inputs from other CNS regions involved in the regulation of autonomic functions [20-22] and project the elaborated afferent information to the adjacent DMV, where are located parasympathetic preganglionic neurons that supply the vagal output to the stomach [19, 23]. The connection to the bodies of the DMV completes the so-called "vago-vagal reflex circuit", of primary importance for the regulation and coordination of several gastric functions. Second order NTS neurons project also to "higher" regions of the brain involved in the coordination of autonomic functions, including parabrachial nucleus, hypothalamic paraventricular nucleus (PVN), central nucleus of amygdala (CeA), bed nucleus of stria terminalis (BNST), ventral thalamus and insular cortex [24, 25]. Although NTS neurons have different biophysical and neurochemical properties, functional studies have determined that they primarily control the DMV through glutamatergic [18, 26], catecholaminergic [27, 28] and GABAergic [26] inputs. In general, GABA, acting on GABA-A receptors, mediates the inhibitory effects of the NTS on DMV neurons; conversely most of the excitation delivered to the DMV by the NTS is mediated by glutamate interacting with both NMDA and non-NMDA receptors [29, 30]. Catecholamines seem to be involved in both excitatory and inhibitory control of the DMV.

The spinal afferent fibers are involved in the neural regulation of gastric

reflexes and sensations (in particular in the communication of pain associated with visceral organs). These fibers reach the stomach mainly via splanchnic and mesenteric nerves passing through prevertebral ganglia and forming collateral synapses with sympathetic ganglion cells [31]. At central level, they terminate predominantly in distinct laminae of the dorsal spinal cord where they are organized in a segmental manner and distributed over several spinal segments [32, 33]. Typically, spinal afferents contain a variety of bioactive peptides, including calcitonin gene-related peptide (CGRP) and the tachykinins SP and neurokinin A (NKA) [34, 35]. The coexpression of CGRP and SP is characteristic of extrinsic afferent neurons, whereas intrinsic enteric neurons in the rat stomach do not coexpress these peptides. Although vagal afferent neurons also express CGRP and SP, spinal afferents represent the main extrinsic source of these neuropeptide in the rat stomach. Within the rat gastric wall, it is particularly the arterial and arteriolar system that receives a dense supply by spinal afferents expressing CGRP and SP [34-37]. In addition, some peptide-containing afferent fibers supply the myenteric plexus, the circular muscle layer and the gastric mucosa. These peptide-containing spinal afferents can release transmitters from their peripheral endings in response to a variety of stimuli; with this "efferent-like activity", they can regulate several gastric functions (including gastric mucosal blood flow, vascular permeability, acid secretion and motility) leading to an increased resistance of the gastric mucosa to injury and a facilitated repair of damaged tissue [10, 38]. From this

point of view, spinal afferent fibers represent a local neural emergency system that is not tonically active, but is called into operation in the face of pending injury to the stomach (see below).

The efferent fibers of the brain-gut signalling system provide the parasympathetic and sympathetic innervation that helps control and coordinate the different gut functions including secretions, motility patterns and circulation.

Sympathetic control of the stomach stems from cholinergic preganglionic neurons located in the intermediolateral column of the thoracic spinal cord (T5-T9 segments), which, running in the thoracic splanchnic nerves (in particular in the greater splanchnic nerves), impinge on catecholaminergic postganglionic neurons in the coeliac ganglia that provide the stomach with most of its sympathetic supply [39, 40]. Sympathetic preganglionic neurons give a tonic drive to prevertebral postganglionic neurons of the coeliac ganglia, which in turn exert a permanent control of the stomach. These ganglia also receive synaptic inputs of central and peripheral origin so that they function as a constitutive part of a more complex system of nervous regulation [39, 40]. Postganglionic sympathetic fibers from coeliac ganglia run through the coeliac plexus - which also receives some fibers from the vagus [41] - along the vascular supply of the stomach to innervate mainly myenteric neurons and blood vessels. The corresponding functions associated with these targets are regulation of motility and blood flow (particularly through the mucosa) [42].

Sympathetic regulation of gastric motility primarily involves inhibitory presynaptic modulation, via α_2 adrenoreceptors, of postganglionic cholinergic neurons in the myenteric plexus and of vagal cholinergic inputs to these neurons [43, 44]. In this way sympathetic outflow inhibits both local excitatory motor reflexes and extrinsic excitatory parasympathetic nervous activity. Norepinephrine from sympathetic fibers, acting on α_1 adrenoreceptors, can also have excitatory effects on enteric neurons. The stimulation of sympathetic nerves to the stomach elicits a characteristic blood flow response: a pronounced vasoconstriction that subsides within a few minutes to reach a steady state level of blood flow.

The stomach is highly dependent upon extrinsic parasympathetic (vagal) innervation and the gastric myenteric plexus essentially serves as a follower of vagal efferent inputs. As mentioned above, parasympathetic innervation of the stomach arises from vagal preganglionic neurons of the DMV [19, 45]. The vast majority of DMV neurons is cholinergic and activates cholinergic nicotinic receptors on postganglionic neurons within the stomach wall. Some DMV neurons also express immunoreactivity for nitric oxide synthase (NOS) and seem to have an inhibitory effect on gastric motility [46]. Parasympathetic preganglionic neurons innervating the stomach are site-specifically organized in the DMV: preganglionic neurons innervating the ventral corpus and antrum are chiefly located in the medial part of the left DMV, whereas those projecting to the dorsal corpus and antrum are predominantly observed in the

lateral and the medial part of the right DMV, respectively [47]. DMV neurons projecting to the stomach are remarkable in that they exhibit slow (1-2 Hz) spontaneous pacemaker-like activity (*in vivo* as well *in vitro*), the rate of which can be modulated by synaptic inputs [26, 48]. Gastric-projecting DMV neurons receive mainly glutamatergic, GABAergic and catecholaminergic inputs from the NTS. Several data have suggested that the firing rate of gastric-projecting DMV neurons is regulated by a tonic inhibitory GABAergic input arising from the NTS [49]. Therefore, factors modulating GABAergic inputs from the NTS to the DMV may have a significant impact on vagal control of the stomach. It has been documented that the ability of neurotransmitters and neuromodulators to affect tonic GABAergic connection between the NTS and the DMV depends upon the level of cAMP in the presynaptic GABAergic nerve terminals of the NTS. Under basal or resting conditions, glutamate released from vagal afferents dampens, by activating group II mGluRs, the level of cAMP in the NTS GABAergic terminals. As a consequence, receptors negatively coupled to adenylate cyclase are confined inside the synaptic terminal and neurotransmitters and neuromodulators cannot bind to them and affect GABA transmission [23]. When cAMP levels increases or the cAMP-PKA pathway is activated in the nerve terminal, the internalized receptors move rapidly and transiently to the membrane of the terminal, permitting other transmitters to bind to their receptors and modulate GABA release. Modulation of the NTS-DMV GABA transmission by

endogenous opioids acting on μ-opioid receptors is subjected to this type of regulation [50].

In the stomach, vagal efferents from DMV primarily innervate gastric myenteric neurons - that thus represents the parasympathetic postganglionic neurons - giving few branches to the submucosa or mucosa. Stimulation of vagal efferent fibers causes both inhibitory and excitatory effects in the stomach. Therefore, it is clear that both excitatory as well as inhibitory postganglionic neuroeffectors are released from enteric neurons in response to vagal inputs. The main excitatory postganglionic neurotransmitter is acetylcholine acting on muscarinic receptors in gastric smooth muscles, interstitial cells of Cajal (ICC) and parietal cells. Activation of this excitatory pathway enhances gastric motor activity and increases gastric acid secretion. The inhibitory postganglionic neurotransmitters are NO and vasoactive intestinal polypeptide (VIP) released by NANC myenteric neurons innervating gastric smooth muscles and ICC. Activation of this vagal NANC pathway produces a profound relaxation of the proximal stomach and depresses motility in the antrum [51, 52].

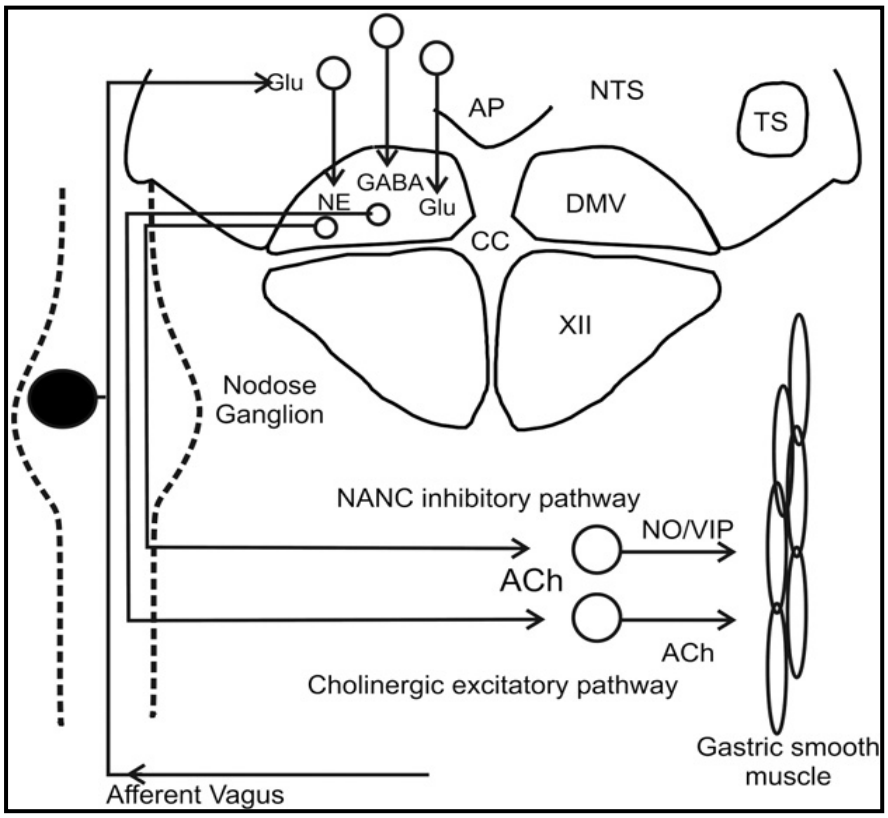


Fig. 1. Afferent and efferent vagal connection between the CNS and the stomach.

1.2. Gastric motor activity and its regulation by intrinsic and extrinsic pathways

The gastric wall comprises two layers of smooth muscles. An outer thin layer of cells arranged along the length of the stomach forms the longitudinal smooth muscle layer. A perpendicular, thicker, layer of cells immediately under the longitudinal muscle forms the circular smooth muscle layer. The smooth muscle cells of the stomach are connected by gap junctions and form an electrical syncytium. Therefore, electrical stimuli can spread between the cells through the gap junctions, causing parts of the muscle layer to act as one

single unit. The basic electrical rhythm in the stomach (as in the other parts of the gut) is fairly constant and characterized by slow waves, consisting of cyclic changes in the membrane potential due to activation and inactivation of different ion channels or pumps. These rhythmic electrical events may develop independently of neuronal activity and are responsible for rhythmic contractions of the muscles, either directly or indirectly by increasing the probability of an action potential. Electrical slow waves are initiated by interstitial cells of Cajal (ICCs) and spread passively to the smooth muscle cells. ICCs are mesenchymal cells typically situated between muscle cells or between myenteric neurons and muscle cells. They are coupled to each other and to muscle cells by gap junctions and act as pacemaker cells in the stomach walls [53].

1.2.1. Motility patterns of the stomach

Anatomically, the stomach is divided into fundus, corpus and antrum region, but with regard to its motor activity two parts can be distinguished: the proximal stomach, consisting of the fundus and the proximal part of the corpus, and the distal stomach, consisting of the distal part of the corpus and the antrum. The proximal stomach is characterized by tonic contractions but not by slow wave activity. The distal stomach, in contrast, exhibits slow wave activity, originating in a pacemaker region in the middle of corpus, and peristaltic contractions propagating towards the pylorus. Two different motor

patterns can be distinguished in the stomach: an interdigestive and a postprandial motor pattern. During the interdigestive phase, the proximal stomach muscle tone is high whereas the distal stomach is engaged in a recurrent contraction pattern known as the migrating myoelectrical complex (MMC). After food intake, the proximal stomach initially relaxes in response to swallowing to hold large amounts of food with limited increases in intraluminal pressure (receptive relaxation). When the food bolus reaches the stomach, gastric relaxation is maintained by another reflex triggered by the distension of the gastric wall. This second mechanism has been named "adaptive relaxation" or "gastric accommodation" and allows the stomach to serve as a reservoir of the ingested food. Then, a tonic contraction of the proximal stomach pushes the gastric content distally, whereas the distal stomach mixes and grinds the food by regular peristaltic contractions [54]. The coordinated tonic and peristaltic motor activity of the stomach generate a controlled flow of the gastric content to the duodenum. The subsequent gastric emptying depends on the coordination between the gastric motor activity and the contractile state of the pylorus sphincter and the proximal intestine.

1.2.2. Control of gastric motility patterns

The gastric motor activity is controlled by both the intrinsic and extrinsic innervation of the stomach. The ENS generates and propagates highly coordinated motor events such as peristalsis and triggers intrinsic ("short" or "intramural") motor reflexes, in which sensory information is transmitted within the ENS from IPANs to interneurons and then to effector neurons. Extrinsic nerves (sympathetic and parasympathetic) cooperate with ENS in modulating gastric motor programs and provide pathways for "long" or "extramural reflexes", i.e. reflex circuits involving neurons of the CNS [55] [Fig. 2].

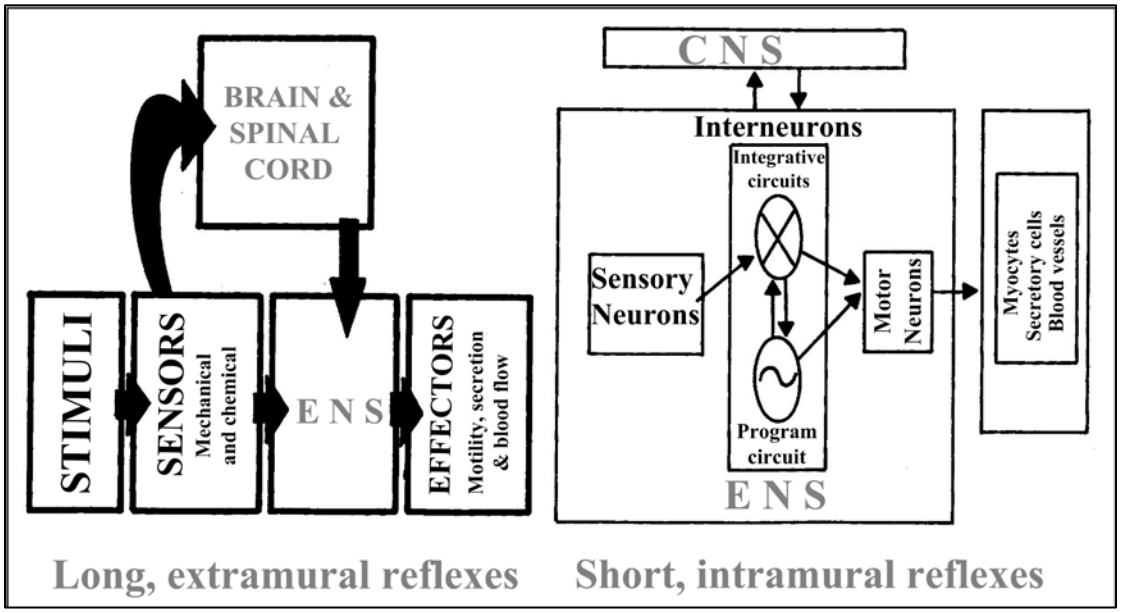


Fig. 2. Schematic diagram illustrating the cooperation between central and enteric nervous system in the control and coordination of gastric motor activity.

The high basal muscle tone of the proximal stomach during the interdigestive phase is partially due to the myoelectrical properties of the fundus: the resting

membrane potential in the fundic muscles is near or above the mechanical threshold. In addition, muscle tone in the proximal stomach is sustained by constant cholinergic input mediated by the vagal efferent fibers from the DMV [26]. The MMCs, which occur spontaneously during the interdigestive phase, are modulated and coordinated by both ENS and extrinsic innervation. The ENS is necessary to coordinate the propagation of MMCs, whereas extrinsic nerves modulate the frequency and the regularity of the MMC cycles.

The gastric receptive relaxation of the proximal stomach after food intake is a "vago-vagal reflex". The distension of the esophagus by ingested food is detected by low threshold mechanoreceptors on vagal afferents that, in contrast to the relatively high threshold for the activation of spinal afferents, respond to more physiological stimuli. Sensory pathways from the esophagus run to the central subnucleus of the NTS, which, in turn, connects to the efferent vagal pathways from the DMV [56]. The final element in the efferent pathway is represented by myenteric inhibitory motor neurons releasing NO and VIP [51, 57]. It seems that these neurotransmitters are co-released from the inhibitory motor neurons and are responsible for the different features of the NANC relaxation. NO would be responsible for the rapid beginning and the initial rapid development of the relaxation and VIP for the long duration of the relaxation. The effect is enforced by a simultaneous reflex that inhibits the excitatory cholinergic motor pathways to the stomach [56].

Adaptive relaxation in response to the distension of the gastric wall involves

both vagal and intramural (local) reflex pathways with NO as common final inhibitory transmitter [57, 58]. It has been demonstrated that stretch of the gastric wall activates not only vagal afferents but also capsaicin-sensitive (spinal) afferent sensory fibers. These latter release CGRP that, in turn, induce the release of NO from myenteric neurons (either directly or indirectly by acting on myenteric interneurons), which causes relaxation of circular muscle and hence of the fundus [59].

The peristaltic motor activity of the stomach is a combination of oral contractions and anal relaxations that allows the progression of the gastric content to the intestine. This motor activity is induced by the simultaneous activation of ascending excitatory motor pathways, which use acetylcholine as main neurotransmitter, and descending inhibitory motor pathways, using principally NO and VIP [55]. The intrinsic enteric innervation of the stomach is essential for the initiation and propagation of gastric peristaltic contractions, but extrinsic nerves coordinate these contractions via parasympathetic and sympathetic pathways. Preganglionic parasympathetic neurons in excitatory and inhibitory vagal pathways connect to enteric neurons acting on smooth muscle and sympathetic neurons exert an inhibitory effect by a direct action on enteric neurons or by inhibiting transmitter release from preganglionic parasympathetic fibers [60]. The reflex control of peristaltic activity of the stomach relay on feedback from intrinsic and extrinsic afferent innervation of the gastric wall, which monitors the prevailing conditions and triggers the

appropriate motor response in the gastric smooth muscle. Both excitatory and inhibitory reflexes occur, inducing an enhancement and a reduction of peristaltic activity, respectively.

1.3. Regulation of gastric mucosal integrity

The maintenance of gastric mucosal integrity depends on the fine balance between aggressive factors (e.g. pepsin, gastric acid, proteases, different chemicals and bacterial invasion) and defensive mechanisms (including the layer of mucus, the bicarbonate secretion, the mucosal microcirculation and the cell renewal). Therefore, gastric mucosal damage may occur when noxious factors "overwhelm" an intact mucosal defense or when the mucosal defensive mechanisms are impaired. From a therapeutic point of view, this means that besides the classical approach that involves the inhibition of gastric acid secretion by H_2 receptor antagonists and proton pump inhibitors as well as eradication of Helicobacter pylori by antibiotics, augmentation of endogenous defensive mechanisms may represents another possible approach of anti-ulcer therapy.

The surveillance system of the gastric mucosa involves barriers (mucus gel layer, epithelial cells) and different mechanisms that are coordinated by the ENS and the CNS, the endocrine system and the immune system. Moreover these systems are cooperating with each other in the regulation of defensive processes [61].

1.3.1. Structural and functional elements of the gastric mucosal defense

"Mucosal defense" is a term used to describe the various factors and components that permit the mucosa to resist to injury. In a healthy organism, mucosal defense is a dynamic process and is enhanced when irritants are present in the stomach. The various levels of mucosal defense can be viewed in a structural sense, starting at the lumen and moving into deeper levels of the tissue.

The mucus gel layer covering the mucosa constitutes the first line of gastric mucosal defense. The physiological functions of this mucus barrier are to impede the diffusion of gastric acid, bacteria and different macromolecules such as bacterial toxins to the epithelial cells [62, 63]. The mucus gel is secreted by apical expulsion from surface epithelial cells and contains HCO_3^-, which buffers gastric acidity and maintains a nearly neutral pH at the epithelial surface [64], and surfactant phospholipids with strong hydrophobic properties that make the surface impermeable to the luminal acid [65].

The next level of gastric mucosal defense is formed by a continuous layer of tightly connected surface epithelial cells that prevent back diffusion of acid and pepsin, secrete mucus and bicarbonate and generate prostaglandins (PGs) [66], heat shock proteins [67] and other protective substances. The epithelium has the ability to renovate itself continuously maintaining the structural integrity of the mucosa. A well-coordinated and controlled proliferation of progenitor

cells enables replacement of damaged or aged surface epithelial cells that are extruded into the lumen [68].

Gastric mucosal microcirculation is another essential element for maintaining gastric mucosal integrity, but also for healing of damaged mucosa [69]. Mucosal microcirculation is constituted by a dense network of capillaries underlying the surface epithelium that, at the base of surface epithelial cells, converge into collecting venules [70]. In addition to supplying nutrients and oxygen to the epithelium, gastric microcirculation also removes, dilutes and neutralizes noxious chemicals that diffuse into the mucosa from the lumen. It also plays a critical role in the disposal of H^+ ions back-diffused from the lumen or parietal cells to the mucosa [71] and facilitates the delivery of bicarbonate to the epithelium. When the epithelium is damaged, the microcirculation also contributes to create a microenvironment over the site of injury conducive for repair.

Mucosal blood flow is modulated by several endogenous substances. The endothelial cells lining the microvessels generate potent vasodilators such as NO and prostacyclin (PGI_2) [68], which maintain viability of vessels and prevent platelet and leukocyte adherence to the microvascular endothelium, thus preventing compromise of the microcirculation. Mucosal blood flow is also regulated by extrinsic innervation of the stomach. Vagal efferents from DMV are able to stimulate the release of NO by a cholinergic mechanism [72]; they also stimulate the gastric production of PGs [73, 74] - such as PGE_2 - that

contribute to increase and maintain gastric mucosal blood flow (GMBF)[75]. Capsaicin-sensitive primary afferent fibers innervating gastric mucosa and submucosal vessels release, in response to luminal aggressive factors or to acid back-diffusion, CGRP that, in turn, induces a hyperemic response mainly by stimulation of NO production, but also by a direct action on vascular smooth muscle cells [76, 77].

The mucosal immune system is a further component of the gastrointestinal surveillance system. The gut possesses a highly specialized immune system that contains organized and non-organized cellular elements [78], including antigen-sampling M cells, lymphocytes and immune-associated cells such as macrophages, eosinophils, neutrophils and mast cells. In addition, many epithelial cells are able to secrete chemokines (e.g., interleukin-8) and thus to recruit immune cells [79]. The GI immune system is called into operation whenever the mucosa is affected by microbial infection, allergen exposure, inflammation or other types of injury. The activation of immune cells induces the release of cytokines, PGs, leukotrienes, bradykinin, histamine, 5-HT and proteases that can either acutely excite sensory nerve fibres or alter their sensitivity in the long term [80].

1.3.2. Mediators of gastric mucosal defense

Several local mediators are involved in the regulation of the physiological defensive mechanisms mediating the resistance of the gastric mucosa to injury. These include PGs, gaseous mediators (NO and hydrogen sulfide) and neuropeptides (CGRP).

Continuous generation of PGE_2 and PGI_2 by the gastric mucosa is crucial for the maintenance of mucosal integrity and protection against ulcerogenic and necrotizing agents [81]. Almost all of the mucosal defense mechanisms are stimulated and/or facilitated by PGs. They inhibit gastric acid secretion; stimulate mucus, bicarbonate and surfactant phospholipids production (increasing the mucosal hydrophobicity); enhance the mucosal content of sulfhydryl compounds (reduced glutathione) that are able of binding reactive free oxygen radicals; maintain and increase GMBF; inhibit platelet and leukocyte adhesion to vascular epithelium; accelerate epithelial restitution and mucosal healing. In addition, PGs inhibit mast cell activation reducing the release of inflammatory mediators (such as histamine, tumor necrosis factor-α, and platelet-activating factor) that have been suggested to contribute to the generation of mucosal injury in certain situations [81, 82].

NO is considered to be another essential mediator of the mucosal defensive mechanisms [83]. As mentioned above, NO has been shown to participate in the regulation of gastric mucosal microcirculation [84] and to mediate the vasodilator effect of CGRP [76]. Besides the regulation of GMBF, NO

participate in gastric mucosal defense also by stimulation of mucus and bicarbonate secretion, inhibition of gastric acid secretion, modulation of the activity of mucosal immunocytes (e.g., mast cells and macrophages), reduction of leukocyte-endothelial adhesive interactions, and acceleration of mucosal damage healing [85]. NO has proven to be the primary NANC neurotransmitter in the GI tract [86]. Not surprisingly, therefore, inhibition of NOS results in disturbance of gastric blood flow, motility and secretion. NO also contributes to mucosal protection through its cytotoxic properties, a primary defense against ingested bacteria and parasites [87]. In the stomach, suppression of NO synthesis renders the mucosa more susceptible to injury [83], whereas administration of NO donors can protect the stomach from injury [88]. However, when NO donors have been given in higher doses, extensive mucosal injury has been observed, suggesting that while physiological formation of NO plays a role in maintaining mucosal integrity, inappropriate release of NO can lead to mucosal injury [89]. This could be due to a direct cytotoxic action of NO or to the formation of oxidant metabolites like peroxynitrite [90].

NO seems to be involved in the mucosal protective effect of several anti-ulcer agents, like carbenoxolone [91], sucralfate [92] and aluminum-containing antacids [93], and in the mucosal protective process of experimental gastroprotective agents, including capsaicin [94], opioids [95], pentagastrin and cholecystokinin-8 [96].

Interestingly, the actions of NO overlap considerably with those of PGs. It has been observed that simultaneous suppression of both PGs and NO synthesis leads to a synergistic increase in mucosal susceptibility to injury and the gastric injury that can be induced by suppression of gastric PGs synthesis is prevented by administration of NO donors. These data suggest a close interaction between NO and PGs in the maintenance of mucosal integrity, that seems to be confirmed by the finding that these mediators can regulate the synthesis of each other [83, 97, 98].

Hydrogen sulfide is a further endogenously generated compound that exerts a strong mucosal protective action similar to NO; it reduces tumor necrosis factor α (TNF-α) expression, decreases leukocyte adherence to vascular endothelium, and inhibits NSAID-induced gastric mucosal injury [99, 100].

CGRP from capsaicin-sensitive primary afferent fibers innervating mucosa and submucosal vessels is one of the most important mediators of gastroduodenal defense. This neuropeptide plays its gastroprotective effect primarily through an increase of GMBF [101, 102]. As mentioned above, the hyperemic effect of CGRP is mediated by NO, although the peptide can also acts directly on vascular smooth muscle [76]. CGRP - through activation of $CGRP_1$ receptors - is also able to inhibit gastric acid secretion [103].

This antisecretory effect involves the release of somatostatin and inhibition of gastrin and acetylcholine release [104] and may contribute to its mucosal protective effect. Different reports have suggested that CGRP is involved not

only in the prevention of gastric mucosal damage, but also in facilitation of gastric ulcer healing [101, 105]. This action may be due to the ability of the peptide to enhance angiogenesis *in vivo* [101].

The pivotal role of CGRP released from peripheral terminals of visceral afferent fibers in gastric mucosal defense has been confirmed by several pharmacological studies. Low doses of capsaicin or TRPV1 agonists have clearly shown to exert gastroprotection via the stimulation of sensory nerves and the local release of CGRP [16, 106]. In addition, close arterial infusion of CGRP to the stomach - a route of administration that closely resembles the local release of the peptide in response to the stimulation of capsaicin-sensitive afferent fibers - significantly reduces gross mucosal damage caused by ethanol and aspirin [107]. The gastroprotective effect of intragastric capsaicin is abolished by systemic administration of the human C fragment of CGRP (hCGRP 8-37) [108], a CGRP antagonist, and of monoclonal antibodies to CGRP [109]. The same agents have been also reported to enhance gastric mucosal lesions induced, in rat, by ethanol [109, 110]. Recent studies, performed using CGRP-knockout mice, have strongly confirmed these findings: the protective action of capsaicin against ethanol-induced lesions is completely abolished in CGRP $^{-/-}$ mice [101].

Different reports have suggested that CGRP is involved not only in the prevention of gastric mucosal damage, but also in facilitation of gastric ulcer

healing [101, 105]. This action may be due to the ability of the peptide to enhance angiogenesis *in vivo* [101].

CGRP-mediated protective action may be regulated by endogenous PGs. It is widely known that pain sensation is enhanced by PGE_2 and PGI_2 by sensitizing the sensory nerves [111], with PGI_2 being more potent that PGE_2 in this sensitizing action [112]. It has been reported that inhibition of PG synthesis results in a reduction of the mucosal protective effect of capsaicin [113] and that endogenous PGI_2 facilitate the release of CGRP from capsaicin-sensitive afferent fibers and gastric mucosal protection against ethanol [114, 115].

1.3.3. CNS and gastric mucosal integrity

Besides the structural and functional elements of gastric mucosal defense and the local release of protective mediators, the CNS also plays an important role in the maintenance of gastric mucosal integrity.

As mentioned previously, DVC and vagus nerve have a pivotal role in the regulation of gastric functions, including acid secretion, motor activity and mucosal defense [19]. Within the DVC, NTS receives afferent sensory information from the stomach, carried by vagal afferent fibers originating from nodose ganglion, and DMV sends preganglionic vagal efferent fibers innervating myenteric neurons in the gastric wall.

Disruption of gastric mucosal barrier by aggressive chemicals such as ethanol induces the back-diffusion of gastric acid from the lumen into the mucosa. The

surge of acid intruding the lamina propria stimulates spinal afferent fibers innervating the stomach (through activation of proton-sensitive TRPV1 ion channels), which induce a rapid increase of GMBF and initiate other defensive mechanisms by local release of CGRP [38]. In parallel, acid challenge of the mucosa activates vagal afferent fibers that communicate the pending injury to the NTS (Fig. 3), as demonstrated by expression in this area of messenger RNA (mRNA) for the immediate early gene c-fos [116] - which reflects neuronal excitation and is hence widely used to visualize central neurons that receive a message from the periphery [117]. Glutamate (via NMDA and AMPA receptors), but also SP and NKA (via NK1 and NK2 receptors) are involved in the communication of afferent information to NTS neurons [118].

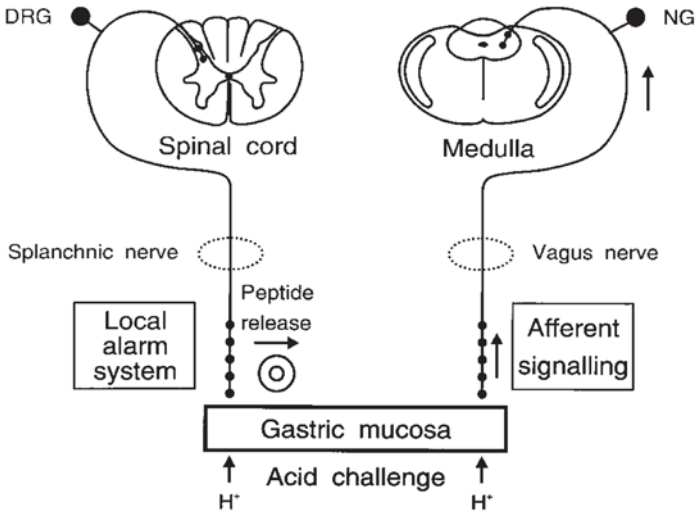


Fig. 3. Neural circuits activated by acid back-diffusion into the gastric mucosa.

The vagal afferent input from the acid-threatened stomach is further relayed to hypothalamic and limbic areas of the rat brain (lateral parabrachial nucleus,

thalamic and hypothalamic paraventricular nuclei, supraoptic nucleus, subfornical organ, CeA and mediolateral habenula), but not to insular cortex (the major cerebral representation area of visceral input) [25]; therefore, vagal afferent signalling of gastric challenge does not give rise to perception of pain, but evokes autonomic, endocrine, affective and behavioral reactions.

After its central processing, the afferent information is sent to the DMV leading to a modification of the activity of vagal efferent pathways to the stomach. Biochemical and pharmacological studies have shown that activation of these pathways stimulates, in the stomach, the release of PGs and NO [72, 119, 120] and the effector function of capsaicin-sensitive afferent fibers containing CGRP [121]. The exact mechanism by which central vagal activation stimulates spinal afferents is still not well defined. PGs are probably involved in the sensitization of these fibers [115]. Alternatively, acetylcholine from vagal efferents could activate directly spinal afferents (an ability already demonstrated in the rat skin) [122]; furthermore vagal activation is able to induce, through activation of muscarinic receptors, the release of histamine and serotonin that are known to evoke sensory C-fiber excitation [123].

Several neuropeptides (e.g. TRH, neuropeptide Y, adrenomedullin and opioid peptides) have been demonstrated to induce a gastroprotective effect, after central administration, through the activation of this vagal cholinergic pathway and then through this NO/PGs/CGRP mechanism [121, 124-126].

Beside the NTS and DMV, other central structures are also implicated in the

regulation of gastric mucosal integrity mainly through descending neuronal projection to the DVC. They include two hypothalamic formations - the lateral hypothalamus (LH) and the PVN - but also the CeA and caudal raphe nuclei, namely the raphe pallidus (Rpa) and the raphe obscurus (Rob).

Anatomical data revealed that LH projects directly to NTS and DMV and receives ascending information from the NTS. LH is able to modulate the activity of DVC neurons involved in the regulation of gastric functions; the stimulation of LH induces predominantly inhibitory effects on NTS neurons and excitatory effects on DMV neurons [127]. LH lesions have been reported to induce gastric mucosal erosion through a mechanism involving a decrease of mucosal barrier function, an increase of gastric acid secretion and gastric hypermotility [128, 129]. Vagotomy or anticholinergic agents protect against LH-induced gastric erosions suggesting the involvement of vagus nerve [130].

Also PVN has direct connections with the DVC and exert an inhibitory influence on NTS neurons, regulating their responsiveness to incoming vagal information, and an excitatory influence on DMV neurons, modulating the vagal output to the stomach [20]. Electrical stimulation of PVN causes gastric mucosal damage [131] and exacerbates stress-induced ulceration [20]. A reduction of GMBF and an increase of gastric motor activity are supposed to be involved in the detrimental effects of PVN stimulation, which are reduced by vagotomy and electrolytic lesion of PVN.

The caudal raphe nuclei - namely Rpa and Rob - represent a significant source of inputs to NTS and DMV through direct projections [132]. Electrical or chemical stimulation of Rpa or Rob enhances gastric motility [133, 134] and secretion of acid, pepsin and PGs [135, 136] by a vagal cholinergic pathway, but is also able to induce an alteration of gastric mucosal resistance to lesion formation. For example, microinjection of kainic acid into the Rob or Rpa causes gastric erosions in fasted rats [137]. On the other hand, kainic acid microinjected into the Rpa at a subthreshold acid secretory dose induces gastric cytoprotection against ethanol injury [138].

Anterograde tract-tracing studies have revealed that efferent fibers from CeA terminate in both the NTS and the DMV, in regions that are involved in the regulation of gastric functions [139]. Electrical stimulation of the CeA modifies the activity of NTS and DMV neurons and enhances c-Fos expression in the NTS [21, 140] inducing vagal-dependent changes in gastric acid secretion, gastric motility and gastric mucosal resistance. In particular, stimulation of some areas of the CeA increases gastric acid secretion and motility and produces gastric erosion, whereas stimulation of other areas inhibits gastric motility and acid secretion [141]. Both excitatory and inhibitory changes were blocked by vagotomy.

1.3.4. Gastric motility - gastric mucosal integrity

Several experimental data have suggested the existence of a close relationship between gastric motility and maintenance of gastric mucosal integrity. All the most important peripheral mediators of gastric mucosal defense - namely NO, CGRP and PGs - are capable of modifying gastric motor activity, an ability that has been supposed to participate in their protective action.

As mentioned, NO represents, together with VIP, the major mediator of the NANC inhibitory motor transmission in the rat stomach. Acid challenge of gastric mucosa causes the activation of a subpopulation of myenteric nitrergic inhibitory motor neurons through a mechanism involving extrinsic capsaicin-sensitive afferent fibers and nicotinic cholinergic transmission [142, 143]. The activation of these nitrergic neurons may represent a physiologic response aimed at protecting the mucosa towards luminal noxae by inhibition of motor activity.

PGs have been reported to influence the gastric smooth muscle contractility and to have a complex effect on gastric motility [144, 145]. In the distal stomach endogenous PGs decrease the amplitude of contractions and the ability of the muscles to respond to excitatory stimuli. In the proximal stomach PGs have an opposite role: they promote tonic contraction [144]. Mucosal endogenous PG deficiency induced by indomethacin has been shown to be associated with gastric hypermotility, which seems to be an important factor in the

pathogenesis of gastric mucosal lesions produced by this non-steroidal anti-inflammatory drug (see below) [146]. However, local deficiency of PGs cannot induce, by itself, the formation of severe gastric damage in the absence of other risk factors such as intraluminal acid, chemical ablation of capsaicin-sensitive afferent fibers or stimulated gastric motility [147, 148].

Endogenous CGRP from spinal afferent fibers seems to have an inhibitory effect on gastric motility, which can contribute to the protective effect of intragastric capsaicin against ethanol-induced gastric mucosal damage [149]. In addition, it has been demonstrated that exogenously administered CGRP can inhibit gastric motility and emptying by a direct action on gastric smooth muscle through receptors linked with cAMP [150].

However, in spite of the intensive research, it is still not clear how alterations in gastric motor activity correlate with gastric ulcer formation. Both increased and decreased motility have been proposed to contribute to gastric mucosal damage.

Gastric contractions characterized by high amplitudes may induce microvascular disturbances in specific sites of the mucosa probably by abnormal compression of the gastric wall, thereby leading to insufficient mucosal blood flow, increased vascular permeability and cellular damage [151, 152]. Furthermore, it is known that contraction of gastric circular smooth muscle leads to the appearance of mucosal folds that has been implicated in the pathogenesis of several ulcer models, including ethanol-induced lesions [153].

Therefore, inhibition of gastric motility may lead to an attenuation of microvascular disturbances due to gastric hypermotility and to flattening of the mucosal folds, resulting in a reduction of mucosal vulnerability to irritants and of severity of damage.

As mentioned above, stimulated gastric motor activity, due to the gastric mucosal deficiency of PGs, seems to be an important factor in the pathogenesis of indomethacin-induced gastric mucosal damage [154]. The ability of different substances - such as amylin - to prevent mucosal lesions induced by indomethacin has been attributed to their inhibitory effect on enhanced gastric motility response observed in this ulcer model [155]. Furthermore, under PG-deficient conditions induced by pretreatment with indomethacin, prokinetic drugs (that alone have no effects on gastric mucosal integrity) are able to induce gastric mucosal damage in the rat at doses that enhance gastric motility and emptying but not at doses that expedite gastric emptying only [147]. 2-deoxy-D-glucose (2-DG), which enhances the gastric motility by a central vagal stimulation, causes by itself non-hemorrhagic lesions on gastric mucosa, but these lesions become hemorrhagic under PG-deficient conditions induced by a low dose of indomethacin [148].

An increased gastric motility seems to be associated also with gastric mucosal lesions induced by cold-restraint stress [151, 156]; suppression of this gastric hypermotility inhibits the stress-induced lesion formation.

Ethanol has been reported to exert a complex action on gastric motility. In the

canine stomach, ethanol induces an inhibition of antral phasic motor activity [157], which can account for the inhibition of gastric emptying observed in several experimental studies, and a stimulation of corpus tonic motor activity [158]. In the guinea pig stomach, ethanol induces only a contractile response in both longitudinal and circular muscle [159], which may contribute to mucosal necrosis and subsequent ulceration.

Exogenously administered PGs (in particular those acting on EP_1 receptors) inhibit gastric motor activity at the doses that significantly reduce the severity of gastric mucosal injury caused by ethanol [160, 161], suggesting a close relationship between the inhibited gastric motility and the cytoprotective action of PGs in this ulcer model.

An inhibition of gastric motility seems to be also involved in the protective action of intragastric capsaicin against ethanol [149]. The inhibition of gastric motility induced by capsaicin at gastroprotective doses is impaired by indomethacin pretreatment, desensitization of capsaicin-sensitive afferent neurons or CGRP antagonists [149, 162], suggesting the involvement of endogenous PGs and CGRP released from spinal afferents innervating the stomach.

In contrast with these reports, other experiments have suggested that also inhibition of gastric motility and delaying of gastric emptying may play an important role in the pathogenic mechanism of gastric ulcer formation, probably through a prolongation of the contact between ulcerogenic substance

and gastric walls. For example, the protective effect of the prokinetic drug metoclopramide against aspirin-induced gastric mucosal damage is thought to be mediated, at least in part, by acceleration of gastric emptying [163]. Furthermore, the delay of gastric emptying caused by large doses of morphine has been proposed to aggravate the ethanol-induced gastric lesions [164].

However, the involvement of altered gastric motility in the pathogenesis of gastric mucosal damage has been questioned by some experimental data. Gutierrez-Cabano [165] has found that gastric contractile activity is unlikely to play a major role in the development or prevention of gastric lesions induced by necrotizing agents such as 96% ethanol. Likewise, hypermotility is unlikely to serve as a major factor in stress ulceration, and the smooth muscle relaxing effect of atropine and verapamil may contribute only partly to their anti-ulcer effect [166]. Moreover, inhibition of gastric motor activity seems to not contribute to the protective effect of clonidine against ethanol-induced gastric mucosal damage [167].

1.4. Substance P and regulation of gastric functions

The undecapeptide SP (Fig. 4) is the most widely known representative of a family of small biologically active peptides, the tachykinins, which consist of a large number of mammalian and non-mammalian members characterized by a common and strongly evolutionarily conserved carboxy-

terminal amidated amino acid region, Phe-X-Gly-Leu-Met-NH_2 (where X is an aromatic or hydrophobic residue) [168].

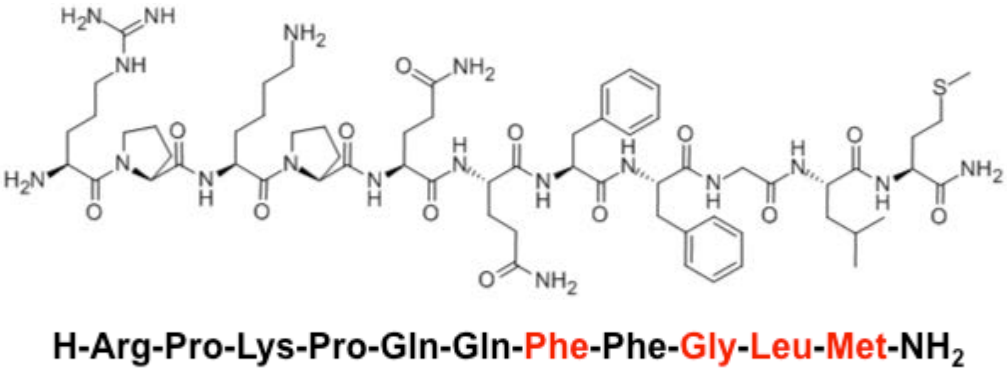


Fig 4. Primary structure of substance P. The C-terminal sequence common to all the tachykinins is showed in red.

Besides SP, mammalian tachykinins include NKA, neurokinin B (NKB) and two elongated forms of NKA, neuropeptide K (NPK) and neuropeptide γ (NPγ) [169, 170]. Recently (17 years after the isolation of NKA and NKB), other tachykinin peptides, hemokinin-1 (HK-1), endokinin-1 (EK-1), endokinin A (EKA) and endokinin B (EKB), were identified in rodents and humans [171, 172]. Being considered of potential importance for understanding and therapy of human disease, tachykinins have become one of the largest investigated groups of neuropeptides and, despite the long period over which these substances have been studied, new informations on their functions continue to emerge. Ever since SP has been discovered to occur in the intestine and to contract gastrointestinal smooth muscle [173], the implication of tachykinins in

the regulation of gastrointestinal functions has been one of the most extensively studied areas of tachykinin research.

1.4.1. Molecular biology of substance P and tachykinin receptors

SP is encoded by the tachykinin precursor 1 (TAC1) gene (originally known as preprotachykinin (PPT)-A or PPT-I gene) that codifies also for NKA and its two elongated forms, NPK and NPγ. Transcription of TAC1 gene generates a pre-mRNA that could be spliced giving rise to four different mRNA isoforms (α, β, γ, and δ) that differ in their exon combinations [174]. SP can be produced from all the mRNA isoforms, whereas NKA production is confined to the α and γ TAC1 mRNA (Fig. 5). This means that SP can be expressed alone, but NKA is always produced along with SP.

Translation of the mature mRNA from TAC1 gene generates a large polypeptide, designated as prepropeptide, that consists of a signal peptide, one or several copies of the neuropeptide and one or more spacer parts. The signal peptide is located at the N-terminal and allows the forming peptide to attach to and pass into the endoplasmic reticulum during synthesis; it is then rapidly cleaved off, after polypeptide synthesis, to allow the formation of the propeptide. This one is transported to the Golgi apparatus where the spacer parts are split off by proteases called convertases. After the cleavage of the propeptide, the final peptide is amidated at C-terminal by peptidyl-Gly-α-amidating monoxygenase, that use glycine as amide donor. SP is then packed

into storage vesicles budding off from the Golgi apparatus and is axonally transported from the perikaryon (were its synthesis is confined) to the nerve terminals for final enzymatic processing. From the nerve terminal the peptide, stored in large dense core vesicles, is released by a Ca^{2+}-dependent exocytosis process [175].

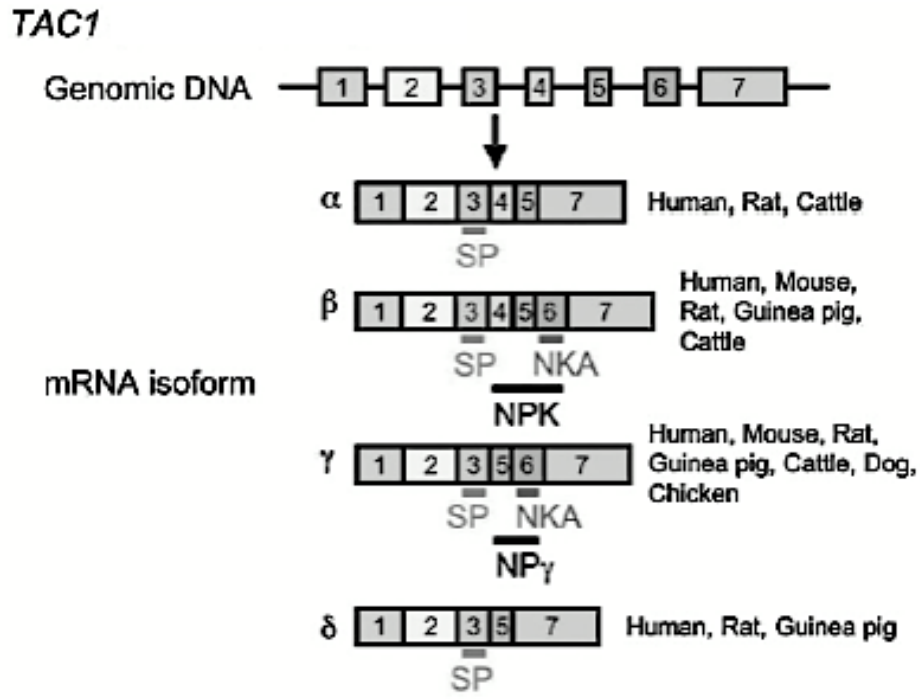


Fig. 5. Splices variants of TAC1 gene. Exons are showed as boxes. The positions of the predicted tachykinin peptides (SP, NKA, NPK, and NPγ) are indicated by underlining.

As with other peptide transmitters, the biological effects of synaptically released SP are terminated by enzymatic degradation, since neurons lack an active uptake mechanism for intact SP. Although several enzymes capable of hydrolyzing the peptide have been described, the cleavage of SP is carried out

mainly by angiotensin-converting enzyme (ACE), neutral endopeptidase (endopeptidase 24.11) (NEP) and substance P endopeptidase (SPE) [176]. ACE is a membrane-bound zinc metallopeptidase cleaving SP at Phe^{8}-Gly^{9} and Gly^{9}-Leu^{10} as the major cleaving sites. ACE also acts as a peptidyl dipeptidase, cleaving dipeptides from the remaining N-terminal fragment, and is thus able to generate the fragment (1-7) of SP [177]. NEP primarily hydrolyzes SP at the Gln^{6}-Phe^{7}, Phe^{7}-Phe^{8} and Gly^{9}-Leu^{10} bonds [178, 179]. SPE is a metalloenzyme highly selective for SP. This endopeptidase hydrolyzes SP mainly within and at the carboxylic side of the double-Phe bond thus releasing SP (1-7) and SP (1-8) fragments from the parent peptide. Other tachykinins lacking the double - Phe residues, such as NKA and NKB, are almost unaffected by this enzyme. SP is also cleaved, between the Pro^{4}-Gln^{5} residues, by the post-proline cleaving enzyme (PPCE) or prolyl endopeptidase [180]. Post proline dipeptidyl aminopeptidase (DPP-IV) successively removes the dipeptides Arg^{1}-Pro^{2} and Lys^{3}-Pro^{4} from the undecapeptide [181]. This enzyme is probably responsible for the degradation of SP in the blood circulation [182].

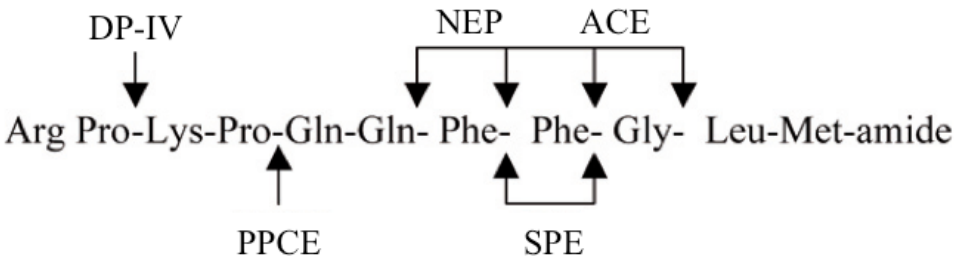


Fig. 6. Schematic representation of enzymatic degradation of Substance P.

The biological actions of SP are mediated by tachykinin (neurokinin: NK) receptors that belong to family 1 (rhodopsin-like) of G protein-coupled receptors. Tachykinin receptors have been first shown to be coupled to a Gq-protein and then to induce the activation of phospholipase C_β (PLC_β) - followed by production of 1,4,5-inositol triphosphate (IP3) and elevation of intracellular Ca^{2+} as second messengers - and of phospholipase A2 - followed by an increase in arachidonic acid mobilization. Afterward, it has also been revealed that production of another second messenger, cAMP, was stimulated by tachykinin receptors coupled to Gs-protein.

Currently three different tachykinin receptors, termed NK1, NK2 and NK3, have been identified. They are codified by three distinct genes: TACR1, encoding for NK1 receptor, TACR2, encoding for NK2 receptor, and TACR3, encoding for NK3 receptor. SP exhibits preferential binding to NK1 receptor, whereas NKA and NKB bind preferentially to NK2 and NK3 receptors, respectively. The rank order of potency for the NK1 receptor is SP > NKA > NKB, while it is NKA > NKB > SP for the NK2 receptor and NKB > NKA > SP for the NK3 receptor [183] (Table 1).

Tachykinin receptor	Affinity	Encoding gene
NK1 (neurokinin-1; SPR/ substance P receptor/)	SP>NKA>NKB	*TACR 1*
NK2 (neurokinin-2)	NKA>NKB>SP	*TACR2*
NK3 (neurokinin-3)	NKB>NKA>SP	*TACR3*

Table 1. Tachykinin preference for receptors.

However, endogenous tachykinins are not highly selective for any given receptor, and all can act as full agonists on all three receptors under certain conditions such as receptor availability or at high peptide concentrations. For this reason SP activates not only NK1 receptors, but also NK2 and NK3 receptors in a number of tissues [183]. The participation of NK2 and NK3 receptors, together with NK1 receptor, to the effects of SP has been confirmed in different experimental studies. For example, it has been observed that both NK1 and NK2 receptors are involved in the excitatory effect of SP on DMV neurons projecting to the stomach [184] and in the cardiovascular and behavioral effects induced by intracerebroventricular (i.c.v.) injection of the peptide [185], whereas all the three tachykinin receptors seem to be involved in the postsynaptic action of SP on PAG neurons [186].

1.4.2. Distribution of substance P and tachykinin receptors in the stomach and in the central nervous areas involved in the regulation of gastric functions

SP is highly abundant in the gastrointestinal tract, in which represents a neurotransmitter and a neuromodulator of primary importance. The main source of SP in the stomach is represented by intrinsic enteric neurons of the myenteric plexus, in which SP is extensively colocalized with choline acetyltransferase (ChAT) [2, 4]. In contrast, SP does not coexist with VIP and NO synthase in the same myenteric neurons [7, 187]. Thorough analysis has

shown that SP is expressed by several classes of myenteric neurons including excitatory motor neurons and interneurons. Myenteric neurons expressing SP have been found to innervate the longitudinal muscle, circular muscle and muscularis mucosae of the murine, rat, guinea-pig and canine stomach [188-190].

In the enteric nervous system of the rat GI tract, γ isoform of TAC1 gene accounts for as much as 80-90% of the tachykinin encoding mRNA. This fact implies that in most (if not all) myenteric neurons of the stomach SP coexist with NKA. Extrinsic spinal afferents (capsaicin-sensitive) also significantly contribute to the SP content of the stomach. Characteristically many spinal afferents containing SP co-express CGRP, a combination of peptides that is not found in the ENS. The main targets of these spinal afferents are gastric blood vessels and mucosa. In contrast, less than 10% of extrinsic vagal afferents innervating the rat, mouse and guinea pig stomach express SP, making a relatively small contribution to the SP content of the stomach [34, 191]. The rest of SP content is contributed by enterochromaffin and immune cells of the gastric mucosa [192].

In the stomach, tachykinin NK1, NK2 and NK3 receptors are widely distributed. NK1 receptors have been detected on myenteric neurons, including interneurons, NOS-immunoreactive (IR) inhibitory motor neurons and ChAT-IR excitatory motor neurons. Furthermore, NK1 receptors have been found on intrinsic and extrinsic nerve fibers throughout the stomach, smooth muscle cells of the circular muscle layer and vascular endothelial cells

[193]. Likewise, immune cells involved in mucosal defense, such as enterocytes, eosinophils, mucosal mononuclear cells (e.g., lymphocytes) and mast cells, can express NK1 receptors. These locations are congruent with a role of gastric NK1 receptors in regulating neuronal excitability, release of neurotransmitters, motility, vascular permeability, blood flow and inflammatory processes. In addition, the presence of NK1 receptors has been also demonstrated on gastric chief cells, where they seem to be involved in pepsinogen secretion [194]. Double-staining experiments have demonstrated that a majority of NK1 receptor expressing nerve fibers in the circular and longitudinal muscle layers and a minority of NK1 receptor expressing nerve fibers in the myenteric plexus contain SP. The observation that SP is co-localized with NK1 receptors raises the possibility that an autocrine regulatory feedback mechanism exists to control the release of SP in the stomach. It is worthy of note that in Wistar rat stomach a high percentage of SP-NK1 co-expressing fibers also express CGRP, suggesting an interaction between SP and the regulation of extrinsic spinal afferent fibers innervating the stomach [193].

NK2 receptors are typically expressed by the circular muscle layer of the stomach [195, 196], while NK3 receptors are largely confined to myenteric neurons [197]. In addition, NK3 receptors have been localized on muscle cells of the stomach, although there is little pharmacological evidence that these receptors play a functional role (Table 2). However, the density of NK3

receptors in the stomach seems to be very low compared with those of the other two tachykinin receptors.

Tachykinin receptor	Gastric localization
NK1	• myenteric neurons (interneurons, excitatory and inhibitory motor neurons) • intrinsic and extrinsic nerve fibers • circular muscle layer • vascular endothelial cells • immune cells
NK2	• circular smooth muscle layer
NK3	• myenteric neurons (interneurons, excitatory and inhibitory motor neurons) • smooth muscle cells (?)

Table 2. Distribution of tachykinin receptors in the rat stomach.

SP and tachykinin receptors are also highly expressed in those areas of CNS - including DVC, hypothalamic PVN and LH - that play a prominent role in the central regulation of gastric functions like acid secretion, motility and mucosal defense.

SP-like IR has been detected in cell bodies and central axons of vagal sensory neurons of the nodose ganglion, where SP is often co-expressed with glutamate, CGRP and other neuropeptides [198]. These SP-containing vagal afferents are involved in conveying sensory information from the stomach to the NTS [199, 200]. Indeed, *in vivo* microdialysis studies have shown that SP is

released in the NTS following peripheral afferent stimulation [201]. The SP content of the nodose ganglion is reduced by 58% in capsaicin treated rats, suggesting that at least a portion of the vagal afferent cell bodies of the nodose ganglion and their projections to the brainstem are capsaicin sensitive [202].

SP-IR fibers have been observed throughout both the NTS and the DMV [203]. In particular, a higher density of SP-containing terminals has been observed in the medial and dorsolateral subnuclei of NTS, while they have been sparsely found in the subnucleus gelatinosus (also termed parvocellular subdivision) and subnucleus centralis. Unilateral nodose ganglionectomy is known to reduce SP-IR in the NTS [204], in agreement with the idea that a majority of the peptide arises from primary vagal afferents. SP, along with serotonin and TRH, is contained within projections from the raphe nuclei (Rob and Rpa) to the NTS and DMV [132]. These projections may represent an important pathway in the medullary regulation of vagal activity to the stomach. A large amount of SP has also been found in CeA [205], LH and hypothalamic PVN [206], which have important connections with the DVC. In addition, some SP-containing neurons are intrinsic to the NTS [207] and may participate in the connection between NTS and DMV [208].

Tachykinin receptor subtypes (NK1, NK2 and NK3) are all expressed in the DVC [209, 210]. In the NTS, the NK1 receptor is mainly located in the medial portion but, surprisingly, not in the subnucleus gelatinosus, in which there is a high number of gastric vagal afferents synapsing directly onto the dendrites of

gastric vagal efferents extending into this subnucleus from the DMV [211]. The pattern of SP staining observed in the NTS almost completely overlaps with the pattern of NK1 receptor-IR staining. Utilizing intracellular recordings, it has been observed that NK1 receptor activation in the NTS results in a direct postsynaptic depolarization of principal neurons by a PKC-dependent mechanism [210], but also in a stimulation of the release of glutamate and GABA. These data suggest that NK1 receptors in the rat NTS are located at pre- and post-synaptic sites on both excitatory and inhibitory interneurons [212]. Several reports have confirmed the involvement of medullary NK1 receptors in the transmission of the afferent information from the stomach to the NTS. For example, it has been observed that the c-Fos expression induced in the NTS by acid challenge of the gastric mucosa is reduced by a triple combination of an NK1, an NK2 and an NMDA receptor antagonist, suggesting that glutamate acting via NMDA receptors and tachykinins acting via NK1 and NK2 receptors cooperate in the transmission of gastric mucosal acid challenge to the NTS [118]. In addition, stimulation of NK1 receptors is able to induce by itself the expression of the c-Fos protein in NTS and in many other nuclei including AP, hypothalamic PVN and CeA [213].

Besides NK1 receptors, also NK3 receptors are highly expressed in the NTS neurons, although they are never co-expressed in the same neurons [214].

In the DMV, NK1 receptors are expressed on the plasma membrane of soma and dendrites (but never on axon terminals and axons) of preganglionic vagal

efferent neurons innervating the stomach [215]. Most NK1 receptor-IR neurons are found to be located in the lateral half of the DMV, in close proximity to, but separate from, nitrergic neurons [216]. The presence of NK1 receptors on efferent vagal neurons is consistent with the dense innervation of the DMV by SP-containing fibers [217]. NK1 receptor is most likely to be synthesized by preganglionic neurons, since ipsilateral vagotomy almost completely abolishes the stain for the receptor, as well as SP binding in the DMV [218]. Together, these data imply that SP is intimately involved in controlling vagal output to the stomach via NK1 receptors on preganglionic motor neurons of the DMV.

Efferent vagal neurons of the DMV innervating the stomach highly express also NK3 receptors. Ultrastructural examination has shown that NK3 receptor-IR (similarly to NK1 receptor-IR) is principally located at non-synaptic membrane of somatic and dendritic profiles [219]. The existence of NK3 receptor-expressing neurons well correlates with the presence of its preferential endogenous agonist, NKB, in the DMV [220]. NK3 receptors are rarely co-expressed with NK1 receptors in DMV neurons. The majority of NK1 positive neurons are ChAT positive whereas the NK3 positive neurons are ChAT negative [221]. Part of the NK3 non-cholinergic cells presumably corresponds to GABAergic interneurons. Cholinergic neurons immunoreactive for NK3 only, or for both NK3 and NK1, probably represent also neurons projecting to stomach [222].

Although autoradiographic studies have not pointed out the presence of NK2 receptors in the DMV [209], it has been pharmacologically confirmed. *In vitro* studies have demonstrated that SP is able to induce depolarization and then activation of DMV neurons projecting to the gastric fundus, corpus, antrum/pylorus and duodenum by activation of NK1 and NK2 receptors [223]. Furthermore, SP acts at NK1 and NK2 receptors located presynaptically within the DVC to increase synaptic transmission to gastrointestinal-projecting DMV neurons [184]. In addition, NKA - the endogenous ligand for NK2 receptor - has also been found in numerous fibers and axon terminals within the DVC [224].

This distribution of SP-IR and tachykinin receptors is compatible with a putative role of the peptide in the central regulation of vagal activity that is of primary importance in the coordination of gastric motor activity, in the control of gastric acid secretion, in the activation of emesis circuits and in the activation of peripheral mechanisms responsible for maintaining the integrity of gastric mucosa.

1.4.3. Role of substance P in the regulation of gastric motility

Local effects on gastric motility:

SP and, in general, tachykinins, influence gastrointestinal motor activity in a complex manner. In contrast to the intestine, where SP has mostly excitatory motor effects and represents the main mediator of the NANC excitatory motor

transmission [225], the effects of the peptide on gastric motility and its role in the different motor programs of the stomach (i.e. gastric emptying) have not yet been fully clarified. With regard to gastric motility, both excitatory and inhibitory effects of SP have been documented in different experimental models. Systemic administration of SP to anaesthetized rats has been reported to cause an atropine/TTX-sensitive contraction of the stomach, which appears to be mediated by both muscular NK2 receptors and neuronal NK1 receptors [226]. In another *in vivo* experiment, a selective NK1 receptor agonist has been resulted markedly less effective than SP in contracting rat stomach, indicating that NK1 receptors contribute only partially to the gastric contraction induced by SP [227]. A stimulation of gastric motility *in vivo* has been observed also in dogs and cats [228, 229]. However, in cats a more complex effect of SP on gastric motility has been described: after systemic administration, an initial distension of the stomach is followed by a sustained contraction (frequently accompanied by phasic contractions) and a late distension phase [230]. At lower doses distention is the dominant effect with a sustained contraction-late distention response appearing as the dose increases. The inhibitory component of the effect of SP is, at least in part, vagally mediated, whereas the mechanism of the subsequent contraction-late distension may reside locally in the gut.

SP has manifested a contractile effect also *in vitro* on both longitudinal and circular muscle strips from the rat stomach and pylorus [231, 232]. In the rat antrum this effect is probably mediated by myenteric cholinergic neurons and

the release of 5-HT acting via 5-HT_2 receptors [231], whereas, in the rat fundus, it appears to be purely myogenic since it is not affected by TTX and atropine [232]. SP has shown a contractile effect on gastric muscle strips from several other species including guinea-pig, dog and cat [233-235].

Besides these excitatory effects, inhibitory effects of SP on *in vitro* preparations have been documented as well. For example, in circular muscle strips from guinea-pig stomach, SP and the NK1 receptor selective agonist, SP methyl ester, induce a VIP/NO-mediated NANC relaxation that is converted into contraction after treatment of the strips with TTX [236]. In the same preparation, a selective NK3 receptor agonist also induces a motor inhibitory effect, whereas a selective NK2 agonist elicits a TTX/atropine-sensitive contractile effect.

In a mouse-isolated stomach preparation, a biphasic effect of SP has been observed: a contraction followed by a relaxation [237]. In this case, the contractile response induced by SP seems mediated by NK2 receptors located on gastric smooth muscle cells, while NK1 receptors localized on nitrergic inhibitory myenteric neurons probably elicit the subsequent relaxant effect. In addition, SP has been demonstrated to indirectly inhibit neuronal acetylcholine release from myenteric neurons of the canine antrum through the release of VIP and PGE_2 [238].

In the light of these published data, the inhibitory effects of SP on gastric motility seem to be due mainly to the stimulation of inhibitory neural pathways and/or to prejunctional interruption of excitatory transmitters relay.

Conflicting results have been obtained also with regard to the effect of the peptide on gastric emptying. In fact, SP has been found to increase the rate of gastric emptying [239, 240], to decrease the rate [241], or to have no effects [242]. These differences between experimental data may be explained by the way SP has been administered and in view of the complex nature of gastric emptying. It has to be considered that, when given systemically, SP can affect the intrinsic nerves in the stomach, the extrinsic nerves and the musculature, it may stimulate the secretion of hormones affecting stomach emptying and it may possibly have additional effects. Therefore, the overall effect of the peptide on stomach emptying is the result of several, frequently opposing effects. However, the finding that a SP antagonist inhibits the gastrointestinal transit [243] supports a role for endogenous SP in the physiologic regulation of gastric emptying.

Central effects on gastric motility:

Besides having local gastric motor effects, SP seems to be also involved in the central regulation of gastric motor activity. As mentioned above, *in vitro* electrophysiological studies have demonstrated that SP directly activates, acting at postsynaptic NK1 and NK2, DMV neurons projecting to the stomach. The peptide is also able, by acting at presynaptic NK1 and NK2

receptors in DVC, to increase synaptic transmission to gastro-projecting DMV neurons [184, 223].

In vivo, mostly inhibitory effects of SP on gastric motility after central administration have been reported. For example, microinjection of SP in the dorsomedial NTS of anesthetized rats elicits a dose-dependent decrease in tonic intragastric pressure and an inhibition of gastric phasic activity [244]. The direct administration of SP in the DMV has also been demonstrated to induce a reduction of intragastric pressure associated with an inhibition of antral motility [208]. At this level, the gastric motor inhibition induced by SP is reduced by an NK1 receptor antagonist and completely abolished by both vagotomy and hexamethonium. These results have suggested that SP acts on NK1 receptors located on preganglionic cholinergic vagal neurons of DMV to induce a gastric relaxation that, in the stomach, is mediated by myenteric NANC inhibitory motor neurons. In the same study, microinjection of SP in the nucleus ambiguus (nAmb) significantly increases the intragastric pressure [208], but the mechanism of this central stimulatory effect has not been clarified.

Similarly to NTS and DMV, microinjection of SP in the nRob of rats has been demonstrated to induce a reduction of intragastric pressure [245, 246]; this effect is most likely mediated through the action of the peptide on neurons of nRob projecting to the DMV, which ultimately induces the inhibition of gastric motor activity [132]. The bilateral microinjection of L-NAME into the DVC significantly reduces the gastric inhibitory effect of SP microinjected in the

nRob, suggesting that it could depend on the release of NO in the DMV [247]. Gastric relaxation evoked by SP in the nRob is reduced by systemic administration of atropine, while the NOS inhibitor L-NAME abolishes the residual response. Therefore, it is possible to speculate that cholinergic vagal pathway and NANC myenteric inhibitory motor neurons are involved in the observed effect of the peptide [248].

Beside the inhibition of gastric motility, an inhibition of gastric emptying has also been documented after central administration of SP [249].

1.4.4. Role of substance P in the regulation of gastric mucosal integrity

It is well documented that capsaicin-sensitive afferent fibers innervating the stomach can exert, in certain situations, a local efferent function by the release of neuropeptides from their peripheral endings [38]. In these fibers tachykinins (SP and NKA) are characteristically co-expressed with CGRP, which has been proved to have a prominent role in the maintenance of gastric mucosal integrity [101]. Besides CGRP, NKA and its analogues have also been shown to induce gastroprotection after peripheral administration in both acid-dependent and acid-independent (ethanol-induced) gastric ulcer models [250, 251]. The gastroprotective effect of NKA depends on intact capsaicin-sensitive primary afferent fibers in the stomach and is mediated by the local release of CGRP and NO [252]. NK2 receptor seems to be the only tachykinin receptor subtype involved, since exclusively NK2 receptor

antagonists counteract the observed gastroprotective effect. In addition, the NK2 receptor blockade has been reported to attenuate the mucosal protective effect against ethanol induced by capsaicin, but not its hyperemic response [252]. These findings support the hypothesis that endogenous NKA may contribute to the physiological maintenance of gastric mucosal homeostasis and that increase of GMBF is not the exclusive mechanism of primary afferent nerve-mediated protection.

NK2 receptor agonists have manifested a gastroprotective effect also after central administration in an acid-dependent ulcer model [253]; in this case the antiulcer action seems to be associated with an inhibitory effect on gastric acid secretion.

In contrast to NKA, SP given peripherally either does not affect ethanol-induced gastric mucosal lesions [250, 252], or even aggravates them. Several studies have documented that intraperitoneal (i.p.) or intravenous (i.v.) administration of SP in rats significantly exacerbates gastric mucosal damage induced by intragastric ethanol and this exacerbation is effectively blocked by a SP-antagonist [254-256]. In addition, the exposure of the rat stomach to ethanol induces a significant and prolonged increase in the endogenous SP levels, suggesting that the peptide is released both during and after the gastric mucosal challenge [254]. These results, together with the observation that the antagonism or deletion of the NK1 receptors markedly reduces by itself ethanol-induced gastric lesions [254, 255], have suggested a role for the

endogenous peptide in the pathogenesis of ethanol-induced gastric ulcer. Various mechanisms have been proposed to mediate this detrimental action of SP, including reduction of GMBF, stimulation of mucosal mast cell degranulation, and formation of reactive oxygen species (ROS).

Several lines of evidence have indicated that exogenous SP reduces the GMBF by a direct action on the gastric vascular system and counteract the increased GMBF in response to capsaicin [257]. Furthermore, SP has been proven to inhibit the gastric hyperemic response to acid back diffusion induced by acidified ethanol and this effect is accompanied by an aggravation of the mucosal hemorrhagic lesions [258].

Intravital microscopic studies have demonstrated that ethanol-induced rat gastric mucosal injury is initially attributable to congestion of the mucosal blood flow, caused by constriction of the collecting venules of the gastric mucosa consequent to the release of leukotriene (LT) C4 from mucosal type mast cells (5-lipoxygenase activity has been localized only in mucosal type mast cells in the rat gastric mucosa) [259]. This observation is consistent with another study showing that topical administration of 40% ethanol causes degranulation of mast cells in the rat stomach [260]. The constriction of the collecting venules in the gastric mucosa after the exposure to ethanol is attenuated by the NK1 receptor antagonist spantide [261]. Moreover, the mast cell stabilizer ketotifen has been reported to prevent the aggravation of ethanol-induced gastric mucosal damage produced by exogenous SP [255].

Therefore, since SP induces degranulation in mast cells [262], it is possible to hypotize that degranulation of mucosal type mast cells by SP released from the sensory nerve terminals may be one of the factors responsible for ethanol-induced gastric mucosal damage.

More recently, it has been demonstrated that ethanol activates (by itself, or in combination with back-diffused acid) TRPV1 located on capsaicin-sensitive afferent fibers innervating the stomach, stimulating the neurosecretion of SP and CGRP [256]. Desensitization of primary afferent fibers with capsaicin, removal of extracellular Ca^{2+}, and TRPV1 antagonist capsazepine prevent the release of neuropeptides. SP, in turn, acting on NK1 receptors on superficial gastric epithelial cells, is able to induce the generation of cytotoxic reactive oxygen species (ROS), responsible for the generation of gastric damage (Fig. 7).

However, none of these proposals mechanisms has been satisfactorily demonstrated and the real mechanism of SP-mediated gastric injury is still unknown.

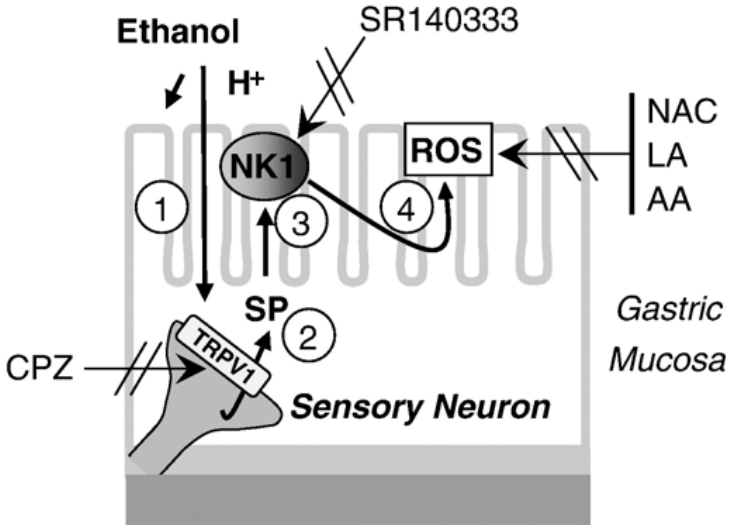


Fig. 7. Proposed mechanism by which SP c ontributes to the ethanol-induced gastric hemorrhagic lesion. Ethanol after a still undetermined initial (1) action, by itself, or in combination with backdiffused acid stimulates TRPV1 to release SP (2) that, by activation of epithelial NK1 receptors (3), generates cytotoxic reactive oxygen species (ROS) (4). The inhibitory effects of capsazepine (CPZ), the NK1 receptor antagonist, SR140333, and the ROS scavengers, N-acetylcysteine (NAC), lipolic acid (LA), and ascorbic acid (AA) on their respective targets are also reported.

Much less information is available about the central effect of SP on the formation of gastric ulcers. To our best knowledge only one paper deals with the central effect of SP on gastric ulcer formation, in which it was tested in an acid-dependent ulcer model [263]. SP injected intracisternally inhibits the development of cold-restraint stress-induced gastric ulcers, but a precise analysis of the effect has been not achieved in this study. However, this data - together with the demonstration of the presence of the peptide and its receptors in the central areas involved in mucosal homeostasis - suggests that SP (similarly to numerous other neuropeptides, like TRH, adrenomedullin or

amylin) [264] may be involved in the maintenance of gastric mucosal integrity at central level.

1.4.5. Interaction between substance P and endogenous opioid system in the maintenance of gastric mucosal integrity

The involvement of SP in the central maintenance of gastric mucosal integrity is strengthened also by the fact that there is a close interaction between SP and the endogenous opioid system, which has been demonstrated to have, at supraspinal level, a pivotal role in the regulation of mucosal barrier functions. In particular, it has been observed that δ- and μ-opioid receptor agonists inhibit, after central administration, gastric mucosal damage induced by acidified ethanol [126, 265, 266]. A significant reduction of the protective effect of opioid peptides has been observed following acute vagotomy, indicating that the DVC is likely to be involved in conveying the central effect of opioids to the periphery. This assumption is in good correlation with the findings that μ- and δ-opioid receptors are present in the brainstem, in NTS and vagal efferent neurons of DMV [267-270], together with neurons expressing endogenous opioid receptor ligands, like endomorphin-1 and endomorphin-2 (EM-1 and EM-2) - selective for μ-opioid receptor - and enkephalins - acting on both μ- and δ-opioid receptors [270-272].

In addition, in the periphery, the inhibition of both NO and PG synthesis also reduces the central mucosal protective effect of opioids [126], suggesting the involvement of local gastric release of NO and PGs.

With regard to the interaction between SP and endogenous opioid system, SP has been demonstrated to have an important and specific role in mediating the motivational properties of opiates [273] and in the opiate withdrawal response [274, 275]. Moreover, SP markedly potentiates the antinociceptive effect of intrathecally administered morphine in rats [276] and the supraspinal antinociceptive effect of SP is inhibited by the opioid antagonist naloxone and blocked by Met-enkephalin antiserum [277, 278]. Thus, it has been proposed that supraspinal administration of SP secondarily releases endogenous opioid peptides both in the brainstem and spinal cord, thereby producing an opioid-dependent analgesia. This proposed mechanism has been confirmed by experimental data demonstrating evoked release of endogenous opioids by SP in different brain areas [279-281].

The release of endogenous opioids has been demonstrated to mediate the central gastroprotective effect of several compounds, including clonidine [282], nociceptin, nocistatin [283] and cannabinoids [284]. Therefore, taking into account the ability of SP to release endogenous opioids and the co-localization of SP and tachykinin receptors with endogenous opioid peptides and opioid receptors in the DVC [285, 286], it is reasonable to hypotize that an interaction

between SP and endogenous opioid system may occur also in the central regulation of gastric mucosal integrity.

2. AIMS OF THE STUDY

Since the discovery of SP in dry extracts from both intestine and brain [173] - clearly showing that focus could be on the nervous system and foreseeing, in a way, the later so much discussed "brain-gut axis" concept - the SP system has received attention as a putative important target for drug therapy of human diseases.

As discussed in the introduction, SP and tachykinin receptors are widely expressed in the gastrointestinal tract [225] and in the areas of CNS that have been demonstrated to play a prominent role in the control of gastrointestinal functions including motility, secretion and mucosal homeostasis [212, 215, 222]. With regard to the stomach, both inhibitory and facilitatory effects of the peptide on motility have been documented depending on the route of administration, doses and experimental conditions [287]. Furthermore, while the effects of peripherally injected SP in different experimental ulcer models have been extensively studied [250, 254, 256], almost no data are available about the central effects of the peptide on the development of gastric mucosal damage. Therefore, despite the intensive research, the real role of SP in the regulation of gastric motility and in the maintenance of gastric mucosal integrity remains to be fully clarified.

In the light of these data, the overall aim of this thesis was to clarify the effects of SP on gastric motility and to characterize for the first time the effect of the

peptide in an acid-independent ulcer model in rats (in which the protective effect of a compound is unrelated to its effect on gastric acid secretion and refers to its ability to improve gastric mucosal defense). Moreover, studies of the receptorial systems and peripheral factors mediating the observed effects of SP were carried out in order to improve the understanding of the mechanisms involved in the regulation of gastric functions, providing new potential therapeutical strategies for the treatment of motility dysfunctions and ulcer.

For this purpose, we investigated:

1) the effect of SP on basal gastric motility after both central and peripheral administration and which peripheral factors and receptors are involved by using specific antagonists,

2) to characterize the effects of centrally administered SP in ethanol-induced ulcer model and the tachykinin receptor subtypes involved,

3) which peripheral factors (NO, CGRP, PGs, gastric cholinergic transmission) are involved in mediating the central gastroprotective effect of SP,

4) whether there may be a correlation between the alteration of gastric motility induced by SP and gastric mucosal protective processes, and

5) whether an interaction between SP and endogenous opioid system occurs in the regulation of gastric mucosal integrity.

3. MATERIALS AND METHODS

3.1. Animals

For all experiments male Wistar rats weighting 140-170 g (gastric ulcer) and 250-400 g (gastric motility) were used. The rats were deprived of food 24 h before experimentation with free access to tap water. Animals were housed under a standard 12 h l ight-dark cycle, in a temperature controlled room (22 ± 2°C) in wire mesh bottom cages to prevent coprophagy.

All procedures conformed to the European Convention for the protection of vertebrate animals used for experimental and other scientific purpose. The study was approved by the Animal Ethic Committee of Semmelweis University, Budapest (permission number: 22.1/606/001/2010). The animals were humanely killed before removing stomachs for determination of gastric mucosal damage and after studying gastric motor activity.

3.2. *In vivo* measurement of gastric motor activity

The gastric motility was determined by the previously described rubber balloon-method [288]. After 24 h food deprivation animals were anesthetized with urethane (1.25 g/kg i.p.), a tracheal cannula was inserted to ensure a clear airway and femoral vein was cannulated with a polyethylene tube for i.v. administration of the drugs. A miniature rubber balloon (approximately 10 mm - 30 mm) created from thin latex rubber connected with plastic tubing was

leaned into the stomach via mouth. The balloon was filled with 2 ml of warm (~37°C) saline to set the basal intragastric pressure to approximately 10 ± 0.5 cmH_2O. The exact location of the balloon was verified after each experiment. The distal end of tubing was connected to a pressure transducer and to a PowerLab Instrument with a Chart 5 program (ADInstruments, Bella Vista, Australia) to monitor the intragastric balloon pressure. A 15-30 min equilibrium period was registered before every experiment. When the gastric motor activity became stable, the test compounds were injected into the femoral vein. Alternatively, for studying the motor effects of centrally administered SP, the peptide was given intracerebroventricularly in a volume of 10 µl within 5 minutes by using a CMA/100 microinjection pump. For i.c.v. injection guide cannulas (Bilaney Consultants, Düsseldorf, Germany) were implanted under pentobarbital anesthesia (35 mg/kg i.p.) with stereotaxic surgery (Stoelting, Illinois, USA) 5 days before the analysis of motility. Coordinates for the guide cannulas relative to bregma are as follows: posterior 0.8 mm; lateral 1.6 mm; ventral 4.5 mm [289]. The guide cannulas were fixed with dental cement (Adhesor Cement, Spofa Dental, Jičín, Czech Republic). The site of the injection was verified after each experiment.

For analysis of gastric motor activity two parameters were determined: mean intragastric pressure (gastric tone) and mean amplitude of phasic contractions. The mean intragastric pressure, which correlates well with fundic tonic activity [290], was calculated from the bottom points of phasic pressure wave [291].

The mean amplitude of phasic contractions, which correlates with the antral contractions superimposed on tonic pressure, was calculated from the amplitude of each contraction [292]. Both parameters were determined from 5 min segments, before and after the injection of the substances [293], and expressed in cm H_2O. Values were expressed in percentage of the basal (pre-injection) values.

In the experiments in which SP was injected intravenously three parameters were determined: lowest intragastric pressure, mean intragastric pressure and mean amplitude of phasic contractions.

3.3. Gastric mucosal damage induced by acidified ethanol

In order to study gastroprotection, gastric lesions were produced by acidified ethanol. In this way, an acid-independent ulcer model was obtained. After 24 hours food deprivation, 0.5 ml of acidified ethanol (98% ethanol in 200 mmol/ml HCl) was given to the animals orally by using a stainless feeding tube. One hour later, the animals were euthanized by overdose of ether, the stomach were excised, opened along the greater curvature, rinsed with saline and examined for lesions. Total number of mucosal lesions was assessed in blinded manner by calculation of the ulcer index (U.I.) based on a 0-4 scoring system described by Gyires [294]. Briefly, in case of small petechies and hemorrhages 1 score was given, whereas 2, 3, and 4 mm long lesions received 2, 3, and 4 score, respectively. The ulcer index was then calculated as

the total number of lesions multiplied by the respective severity factor. The percentual inhibition of mucosal damage was calculated as follows:

$$100 \times \left(1 - \frac{U.I.in\ treated\ group}{U.I.in\ control\ group}\right)$$

SP was given either i.c.v. 10 min before the ethanol challenge in a volume of 10 μl, or intravenously (i.v., via the tail vein) in a volume of 0.5 ml/100 g 15 min before the administration of ethanol, as described previously [282]. The i.c.v. injection to the lateral ventricle was performed according to Noble et al. in conscious rats [295]. Briefly, animals were gently fixed and injections were made with microsyringe bearing 27 gauge needle with stops at 4 mm from the needle tip at point 1.5 mm caudal and 1.5 mm lateral from bregma. Antagonists were given either together with the agonists (if both were injected i.c.v.) or 15 and 60 min (i.v. and oral administration, respectively) before injecting the agonists. Only in the case of β-funaltrexamine, the compound was injected i.c.v. 1 hour before the i.c.v. injection of SP.

3.4. Bilateral cervical vagotomy

To investigate whether vagus nerve is involved in the gastric motor effects of SP, the cervical section of the vagus nerves was exposed, under ether anesthesia, on either side of the neck of the animals and cut off approximately 30 min before the administration of SP.

3.5. Radioimmunoassay determination

For determination of gastric mucosal level of CGRP and somatostatin the rats were euthanized, the stomachs were removed and gastric mucosa was separated on cooled plate. Gastric mucosa was then weighed and put in 1 ml cold distilled water, sonicated and stored at -80 ºC till the determination.

CGRP and somatostatin concentrations were determined by radioimmunoassay (RIA) described previously [296, 297]. For the specific RIA determinations, the antisera (CGRP: C1012; somatostatin: 775/7) were raised in rabbits or, in the case of somatostatin, in sheeps immunized with synthetic peptides conjugated to thyroglobulin by glutaraldehyde. The tracers were mono-^{125}I-labeled peptides prepared by Németh et al. [298]. Synthetic peptides were used as RIA standards ranging from 0 to 1000 fmol/ml (somatostatin RIA) and from 0 to 100 fmol/ml (CGRP RIA). Detection limits of the assays were 2 fmol/ml (somatostatin) and 0.2 fmol/ml (CGRP). These techniques have proved to be specific, sensitive and valid for the measurement of neuropeptides in pharmacological research. Peptide concentrations were calculated as the measured amount of peptide per wet tissue weight, expressed as fmol/mg.

3.6. Materials

The following substances were used: the non-selective opioid receptor antagonist naloxone hydrochloride, the selective μ-opioid receptor antagonist β-funaltrexamine hydrochloride, the selective δ-opioid receptor antagonist naltrindole hydrochloride, the selective κ-opioid receptor antagonist nor-Binaltorphimine dihydrochloride (norBNI), the NO synthase inhibitors N^G-nitro-L-arginine (L-NNA) and N^G-nitro-L-arginine methyl ester (L-NAME), the non-steroidal antiinflammatory drug indomethacin, the cholinergic muscarinic receptor antagonist atropine sulphate (all purchased from Sigma Chemical Co., St. Louis, USA), substance P (Ascent Scientific, Bristol, UK), the NK1 receptor antagonist (2S,3S)-3-[[3,5-bis(trifluoromethyl)phenyl]methoxy]-2-phenylpiperidine (L-733,060) hydrochloride, the NK2 receptor antagonist 5-fluoro-3-[2-[4-methoxy-4-[[(R)-phenylsulphinyl]methyl]-1-piperidinyl]ethyl]-1H-indole (GR 159897), and the NK3- receptor antagonist 3-methyl-2-phenyl-N-[(1S)-1-phenylpropyl]-4-quinolinecarboxamide (SB 222200) (all purchased from Tocris Biosciences, Bristol, UK).

All drugs were dissolved in saline, with the exception of indomethacin - which was suspended in 1% methylcellulose - and the NK2 and NK3 receptor antagonists (GR 159897 and SB 222200) - which were dissolved in dimethyl sulfoxide (DMSO) and then diluted with saline. Control animals received the drug solvents only. For the receptorial analysis, the doses of antagonists were

selected based partly on previous experiments of my research group, partly on the literature data (Table 3).

No data have been published to our knowledge on the i.c.v. dose of L-733,060 and GR 159897 in rats, however a wide dose range (from picomolar to micromolar) [299-301] were used in the case of other routes of administration. The applied i.c.v. doses in the present study (1 nmol for L-733,060 and 0.5 nmol for GR 159897) were based partly on our preliminary results, partly on the estimated affinity of these ligands for the NK1 and NK2 receptors, respectively, derived from *in vitro* studies [302, 303]. The EM-2 antiserum was produced by István Barna (HAS), and its properties have been described previously in detail [304]. The antiserum was used at a 20-fold final dilution. The same dilution of non-reactive rabbit serum (NRS) was used as control.

Antagonist	Route of administration	Applied dose	Reference
Atropine	i.v.	1 mg/kg	*Gyires et al., 2000* [282]
β-funaltrexamine	i.c.v.	20 nmol/rat	*Zádori et al., 2008* [283]
GR 159897	i.v.	0.5 nmol/rat	-
Indomethacin	p. os.	20 mg/kg	*Gyires and Rónai, 2001* [126]
L-733,060	i.v. i.c.v.	11 μmol/kg 1 nmol/rat	*Seabrook et al., 1996* [302] -
L-NAME	i.v.	37 μmol/kg	*Kaneko et al., 1998* [125] *Saperas et al., 1995*[305]
L-NNA	i.v.	3 mg/kg	*Gyires and Rónai, 2001* [126]
Naloxone	i.c.v.	27 nmol/rat	*Zádori et al., 2008* [283]
Naltrindole	i.c.v.	5 nmol/rat	*Zádori et al., 2008* [283]
Nor-Binaltorphimine	i.c.v.	14 nmol/rat	*Zádori et al., 2008* [283]
SB 222200	i.c.v.	1 nmol/rat	*Haley and Flynn, 2007* [306]

Table 3. The list of antagonists and their doses.

3.7. Statistical analysis

Statistical analysis of the data was evaluated by means of analysis of variance (ANOVA) followed by Newmann-Keuls post hoc test for multiple comparisons. In the case of motility experiments, the pre- and post-injection values were compared with paired Student's t-test. A probability value of less than 0.005 was considered statistically significant.

4. RESULTS

4.1. GASTRIC MOTILITY - Experiments on urethane-anesthetized rats by using intragastric balloon

4.1.1. Effect of centrally administered substance P on basal gastric motor activity

In this set of experiments, two characteristic components of gastric motility, namely intragastric pressure (fundic tone) and amplitude of phasic contractions (antral phasic motor activity), were studied. Fig. 8 shows that i.c.v. administration of SP in doses between 0.74 and 740 pmol/rat did not significantly influence the rat basal gastric motor activity. However, at the highest dose (740 pmol/rat i.c.v.), the peptide slightly reduced the amplitude of contractions (24% inhibition), but this reduction was not statistically significant ($p=0.14$, pre- vs post-injection values, paired t-test).

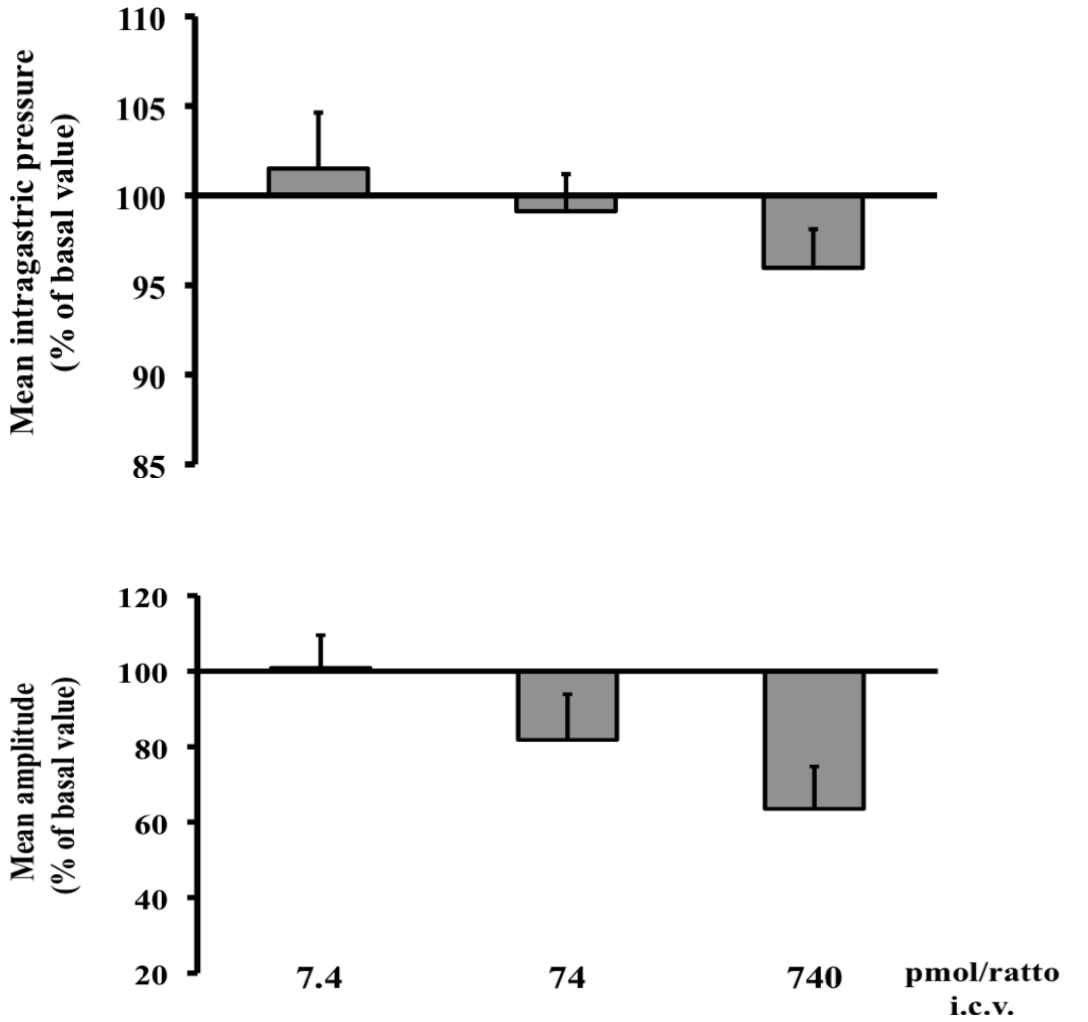


Fig. 8. The effect of intracerebroventricularly (i.c.v.) injected SP (7.4-740 pmol) on basal gastric motor activity of rats. The basal intragastric pressure was set to 10 cmH_2O and SP was injected in a volume of 10 µl within 5 min after recording the basal motility for 15-30 min. Every parameter was determined from 5 min segments, before and after the injection of SP. All the experimental values were expressed as a percentage of the basal value (100%). Each column represents mean ± S.E.M., the number of animals was 5 per group.

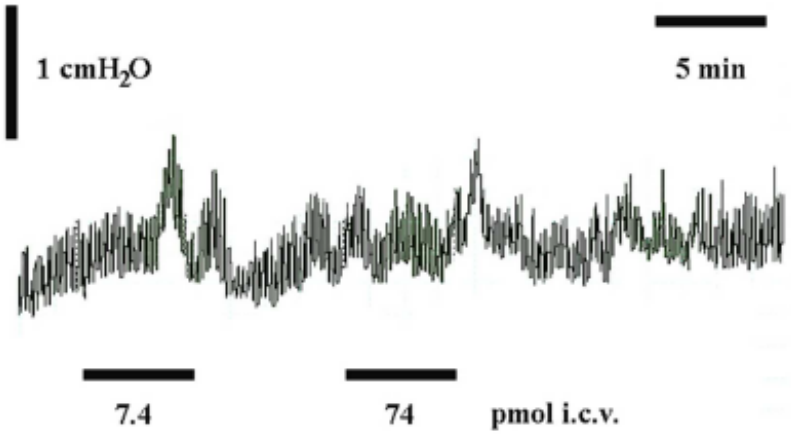


Fig. 9. Representative gastric contractility trace illustrating the effect of i.c.v. injected SP (7.4 and 74 pmol) in rats.

4.1.2. Effect of peripherally administered substance P on b asal gastric motor activity

Intravenous administration of SP in a low dose range (0.0074-7.4 nmol/kg) induced a biphasic effect on basal gastric motility: a transient relaxation followed by an increase in gastric tone and amplitude of phasic contractions. The initial distention phase occurred immediately after the injection of SP and its amplitude increased with the dose of the peptide. The amplitude of the contractile phase that followed the initial distention also varied in a dose-dependent manner, resulting more pronounced for the doses of 0.074 (mean IP 109±3% and mean amplitude of phasic contractions 150±13% of basal value) and 0.74 nmol/kg (mean IP 104±2% and mean amplitude of phasic contractions 158±10% of basal value). The overall effect of low doses of SP is characterized by a short duration and in few minutes the

motility returned to the basal level. In a higher dose-range (74-740 nmol/kg i.v.) only a relaxant effect of the peptide was observed (Fig. 10).

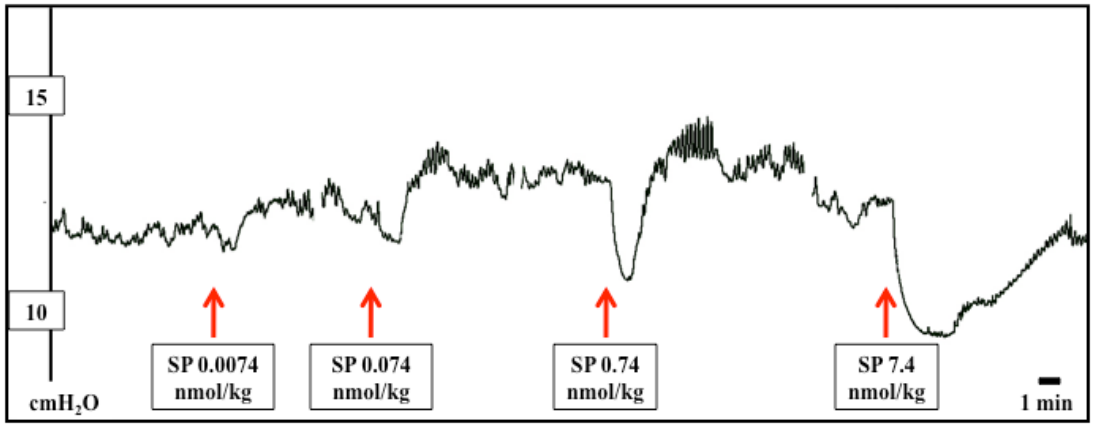


Fig. 10. Representative chart recording showing the effect of i.v. injected SP (0.0074-7.4 nmol/kg) on basal gastric motility of rats.

Since SP i.v. induced a biphasic effect, in this experimental series three parameters were evaluated: the lowest point of intragastric pressure in the first phase, the mean intragastric pressure and the mean amplitude of contractions in the second phase (Fig 11).

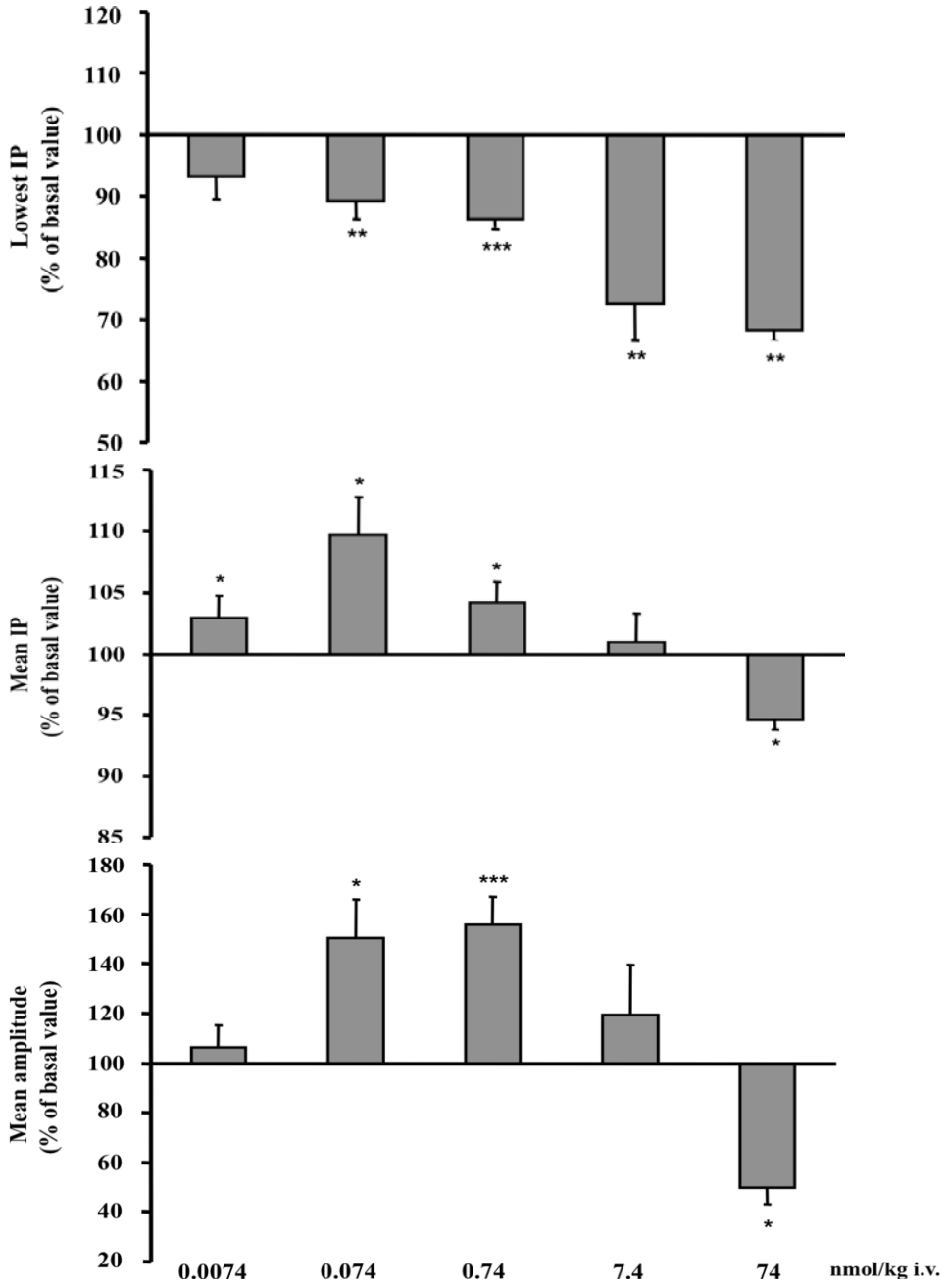


Fig. 11. The effect of intravenously administered SP (0.0074-74 nmol/kg) on basal gastric motility of anesthetized rats. The basal intragastric pressure was set to 10 cmH_2O and SP was injected i.v. after recording the basal motility for 15-30 min. Every parameter was determined from 5 min segments, before and after the injection of SP. All the experimental values were expressed as a percentage of the basal (preinjection) value (100%). Each column represents mean ± S.E.M.. ***P<0.001, **P<0.01, *P<0.05. (ANOVA, Newman-Keuls post hoc test, compared with the respective control group).

4.1.3. Analysis of the peripheral factors involved in the gastric motor effects induced by intravenous administration of substance P

In this experimental series we investigated the factors (receptors and mediators) involved, at peripheral level, in the gastric motor effects induced by i.v. administration of SP.

a) The effect of NK1 receptor blockage on the gastric motor action of intravenous substance P

In order to confirm the receptorial mechanism of the peripheral gastric motor action induced by SP (0.074 and 0.74 nmol/kg i.v.), the NK1 receptor antagonist L-733,060 was used. In our experiments, pretreatment with L-733,060 (11 μmol/kg i.v.) significantly inhibited the biphasic effect of the lowest dose of SP (0.074 nmol/kg), whereas induced only a slight reduction of both the inhibitory and excitatory effect of the highest dose of the peptide (0.74 nmol/kg) (Fig. 12 and Table 4).

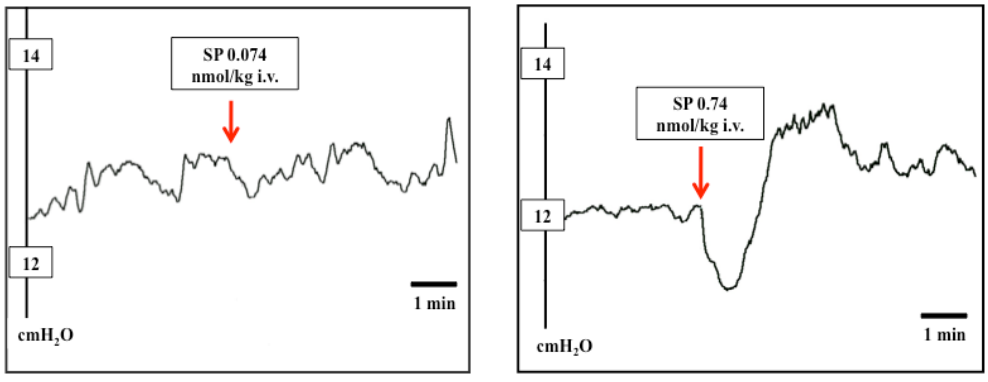


Fig. 12. Representative chart recording showing the effect of L-733,060 (11 μmol/kg i.v.) on gastric motor action of SP (0.074 and 0.74 nmol/kg i.v.). The arrow indicates the moment of injection of SP. The NK1 antagonist was injected immediately before SP.

Treatment	Lowest IP (%)	Mean IP (%)	Mean Amplitude (%)
SP 0.074 nmol/kg	**89 ± 3 (6) ****	**110 ± 3 (6) ***	**150 ± 13 (7) ***
SP 0.74 nmol/kg	**87 ± 1 (10) *****	**104 ± 1 (10) ***	**151 ± 9 (8) *****
L-733,060	**99 ± 1 (3) #**	**101 ± 1 (3) #**	**107 ± 1 (3) #**
	93 ± 2 (3) *	**104 ± 3 (3)**	**129 ± 3 (3) ***

Table 4. The effect of L-733,060 on the gastric motor action of i.v. SP. Numbers in brackets indicate the number of animals studied. ***P<0.001, **P<0.01, *P<0.05, compared with the respective control group; #P<0.05, compared to the respective SP-treated group (ANOVA, Newman-Keuls post hoc test).

b) The effect of cholinergic muscarinic blockage and vagotomy on the gastric motor action of intravenous substance P

In this set of experiments, the cholinergic muscarinic antagonist atropine (1.4 μmol/kg i.v.), which by itself induced a depression of gastric motility, significantly reduced both the two phases of the effect of SP (0.074 and 0.74 nmol/kg i.v.), although it had a stronger effect on the second stimulatory phase. Bilateral cervical vagotomy inhibited mainly the second phase of the effect of the peptide, leaving almost unaffected the first inhibitory phase (Table 5).

Treatment	Lowest IP (%)	Mean IP (%)	Mean Amplitude (%)
SP 0.074 nmol/kg	**89 ± 3 (6) ****	**110 ± 3 (6) ***	**150 ± 13 (7) ***
SP 0.74 nmol/kg	**87 ± 1 (10) *****	**104 ± 1 (10) ***	**151 ± 9 (8) *****
Atropine	**99 ± 1 (4) $^{\#}$**	**101 ± 0.1 (4) $^{\# *}$**	**83 ± 5 (4) $^{\# *}$**
	96 ± 2 (3) $^{\#}$	**102 ± 1 (3)**	**83 ± 4 (3) $^{\#\# **}$**
Vagotomy	**95 ± 1 (4) ****	**99 ± 1 (4) $^{\#}$**	**89 ± 4 (3) $^{\#}$**
	86 ± 1 (7) ***	**99 ± 1 (7) $^{\#}$**	**89 ± 3 (5) $^{\#\#\# *}$**

Table 5. The effect of atropine and vagotomy on the gastric motor action of i.v. SP. Numbers in brackets indicate the number of animals studied. ***P<0.001, **P<0.01, *P<0.05, compared with the respective control group; #P<0.05, ##P<0.01, ###P<0.001, compared to the respective SP-treated group (ANOVA, Newman-Keuls post hoc test).

c) The effect of intravenous naloxone on the biphasic gastric motor action of substance P

In the subsequent set of experiments we studied whether endogenous opioid system is involved in the peripheral effect of SP on gastric motility. The i.v. administration of the non-selective opioid receptor antagonist naloxone in a dose of 11 μmol/kg did not influence the gastric motor effect of SP (both first and second phase) (Table 6).

Treatment	Lowest IP (%)	Mean IP (%)	Mean Amplitude (%)
SP 0.074 nmol/kg	**89 ± 3 (6) ****	**110 ± 3 (6) ***	**150 ± 13 (7) ***
SP 0.74 nmol/kg	**87 ± 1 (10) *****	**104 ± 1 (10) ***	**151 ± 9 (8) *****
Naloxone	**93 ± 1 (4) ***	**104 ± 2 (4)**	**130 ± 11 (3) ***
	82 ± 3 (3) *	**107 ± 3 (3)**	**145 ± 1 (2) *****

Table 6. The effect of naloxone on the gastric motor action of i.v. SP. Numbers in brackets indicate the number of animals studied. ***P<0.001, **P<0.01, *P<0.05, compared with the respective control group (ANOVA, Newman-Keuls post hoc test).

d) The effect of inhibition of nitric oxide synthesis on the biphasic gastric motor action of substance P

To determine whether NO (the most important NANC transmitter of ENS) is involved in the peripheral gastric motor effect of SP, we used the NOS inhibitor L-NAME. In this experiments, the i.v. administration of L-NAME in a dose of 37 μmol/kg i.v. slightly reduced the first inhibitory phase of the motor effect of SP and significantly potentiated the stimulation of gastric phasic contractions induced by the peptide (especially that induced by the dose of 0.74 nmol/kg i.v.) (Table 7).

Treatment	Lowest IP (%)	Mean IP (%)	Mean Amplitude (%)
SP 0.074 nmol/kg	**89 ± 3 (6) ****	**110 ± 3 (6) ***	**150 ± 13 (7) ***
SP 0.74 nmol/kg	**87 ± 1 (10) *****	**104 ± 1 (10) ***	**151 ± 9 (8) *****
L-NAME	**96 ± 1 (6) $^{\#***}$**	**105 ± 1 (6) ****	**183 ± 15 (5) *****
	90 ± 1 (6) ***	**105 ± 3 (6)**	**236 ± 12 (4) $^{\# ***}$**

Table 7. The effect of L-NAME on the gastric motor action of i.v. SP. Numbers in brackets indicate the number of animals studied. ***P<0.001, **P<0.01, *P<0.05, compared with the respective control group; #P<0.05, (ANOVA, Newman-Keuls post hoc test).

The following diagrams summarize all the results obtained in this experimental series (Fig. 14):

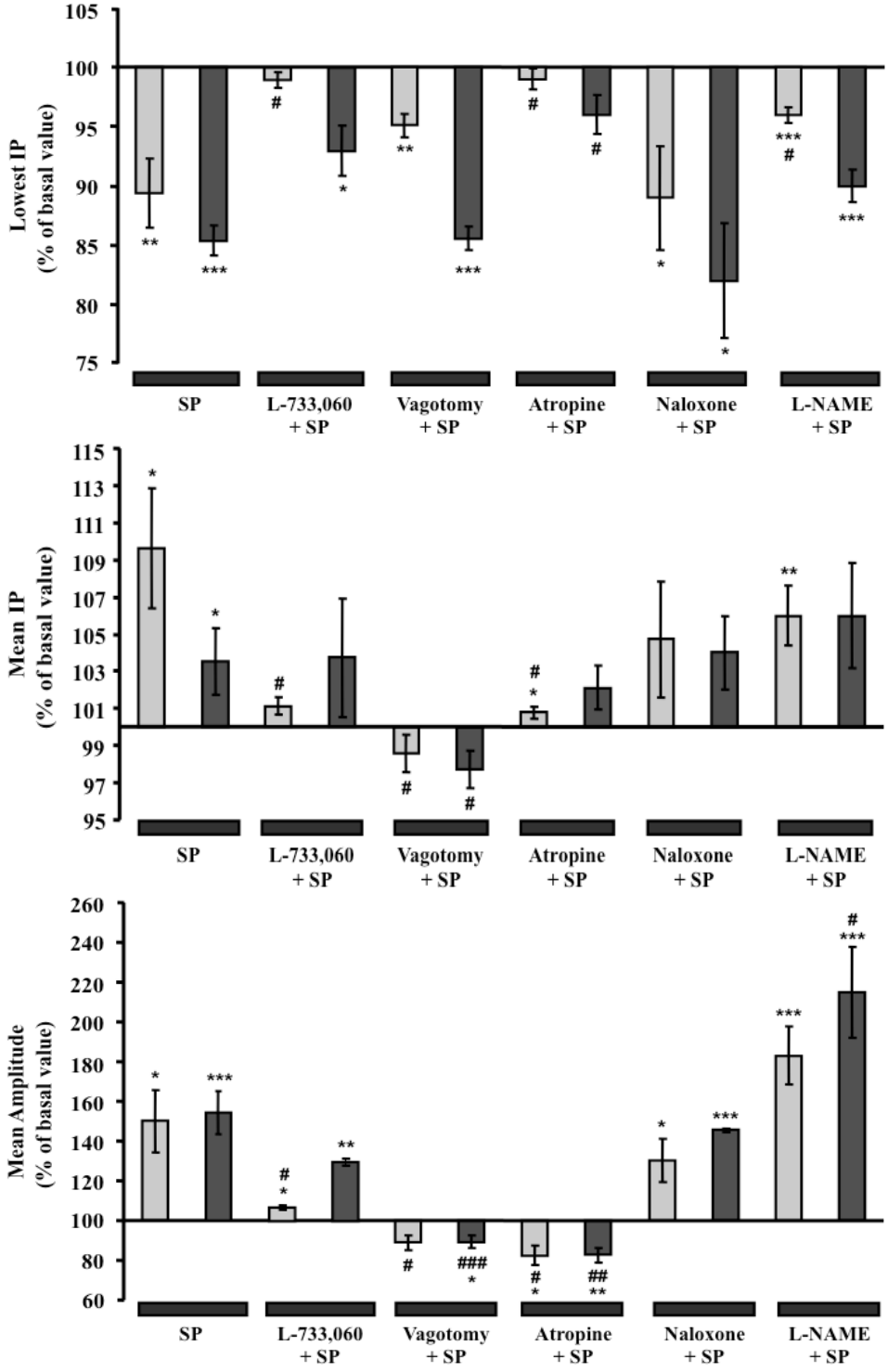


Fig. 14. The effect of L-733,060 (11 µmol/kg i.v.), bilateral cervical vagotomy, atropine (1.4 µmol/kg i.v.), naloxone (11 µmol/kg i.v.) and L-NAME (37 µmol/kg i.v.) on the peripheral gastric motor action of SP (0.074 and 0.74 nmol/kg i.v.). All the experimental values were expressed as a p ercentage of the basal (preinjection) value (100%). Each column represents mean ± S.E.M.. ***P<0.001, **P<0.01, *P<0.05, compared with the respective control group; #P<0.05, ##P<0.01, ###P<0.001, compared to the respective SP-treated group (ANOVA, Newman-Keuls post hoc test).

4.2. GASTROPROTECTION - Experiments on acidified ethanol-induced gastric ulcer

4.2.1. Effects of centrally and peripherally administered substance P on the ethanol-induced lesion formation

Oral injection of acidified ethanol induces deep, multiple longitudinal hemorrhagic lesions on the gastric mucosa (Fig. 15, ulcer index: 97±7, n=10). In our experiments, acidified ethanol was given orally 10 min after the i.c.v. administration of SP and 15 min after the i.v. injection of SP.

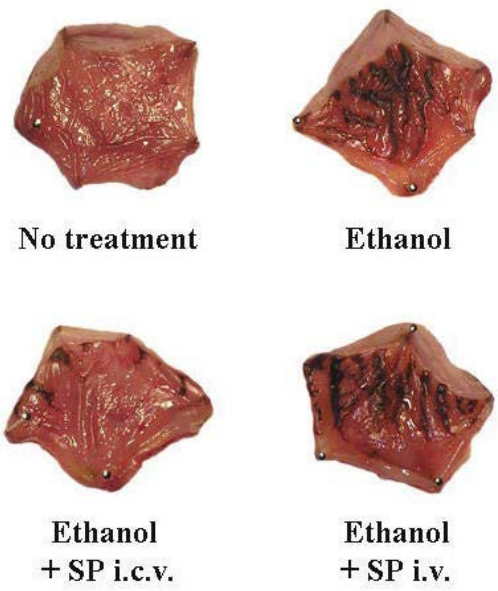


Fig. 15. Macroscopic picture of the acidified ethanol-induced gastric mucosal damage in rats treated either with saline, or with SP i.c.v. (18.5 pmol/rat) or i.v. (0.74 nmol/kg). The dark, livid areas represent the hemorrhagic ulcerous parts of the mucosa.

SP, when injected i.c.v. in doses between 4.6 and 148 pmol/rat, significantly inhibited the ethanol-induced lesion formation (Fig. 16). The maximal inhibition was reached after the doses of 9.3 and 18.5 pmol/rat (89.8% and 75.8% inhibition, respectively) and the latter dose was chosen for further experiments. At higher dose range (74 and 148 pmol/rat), however, the gastroprotective effect of SP declined (33.0% and 29.4 inhibition, respectively).

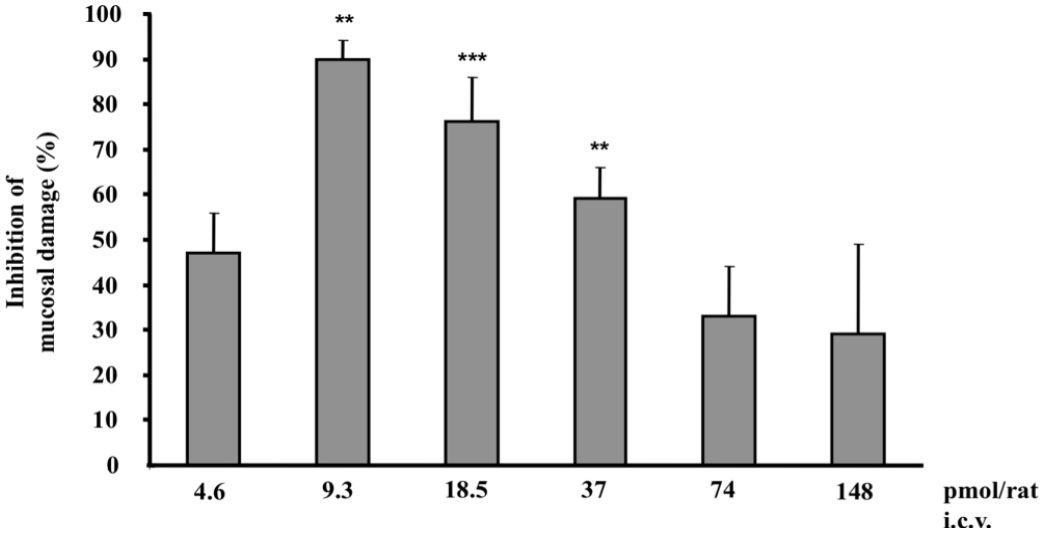


Fig. 16. The inhibitory effect of i.c.v. injected SP (4.6 - 148 pmol/rat) on ethanol-induced gastric mucosal injury in rats. SP was injected i.c.v. 10 min before the ethanol challenge. Each column represents mean ± S.E.M., the number of animals was 5 per group. **P<0.01, ***P<0.001 (ANOVA, Newman-Keuls post hoc test, compared with the respective control group).

In contrast, SP injected intravenously in the doses of 0.37-7.4 nmol/kg (0.5-10 μg/kg) failed to inhibit the development of gastric mucosal lesions. The two lowest doses (0.37 and 0.74 nmol/kg) resulted in a slight increase of ulcer

index (24.4% and 27.4%, respectively), although the difference was not statistically significant (Fig. 17).

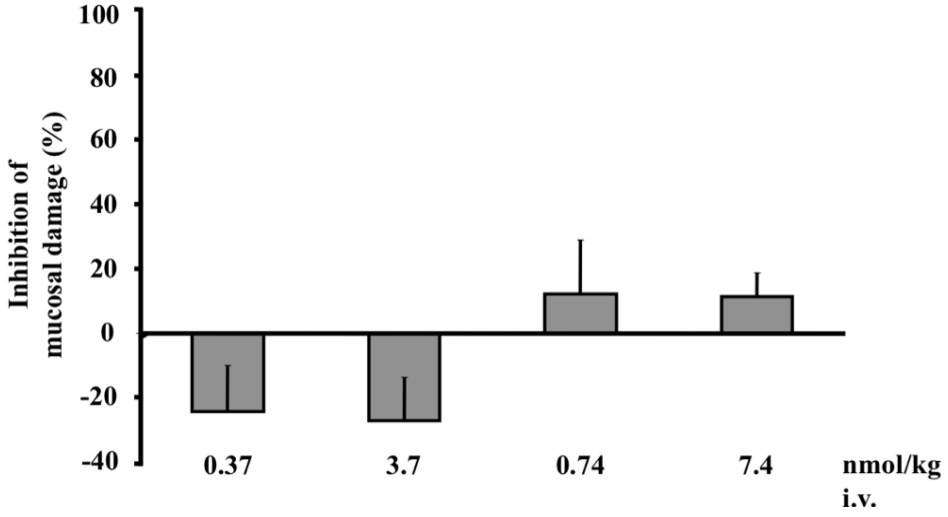


Fig. 17. Effect of i.v. administered SP (0.37-7.4 nmol/kg) on ethanol-induced gastric mucosal damage in rats. SP was injected i.v. 15 min before the oral administration of acidified ethanol. Each column represents mean ± S.E.M., the number of animals was 5 per group.

4.2.2. Effect of centrally administered tachykinin receptor antagonists on the supraspinal gastric mucosal protective action of substance P

In the next set of experiments we examined the effects of i.c.v. injected L-733,060 (1 nmol/rat), GR 159897 (0.5 nmol/rat) and SB 222200 (1 nmol/rat) - potent and selective non-peptide antagonist of the NK1, NK2 and NK3 receptor, respectively - on the gastroprotective action induced by centrally administered SP. SP was injected i.c.v. in a dose of 18.5 pmol/rat, 10 min before the ethanol challenge either alone, on in combination with the different antagonists. As Fig. 18 (A, B and C) demonstrates, all three

antagonists, that alone did not modify the ethanol-induced mucosal damage, reversed the effect of SP.

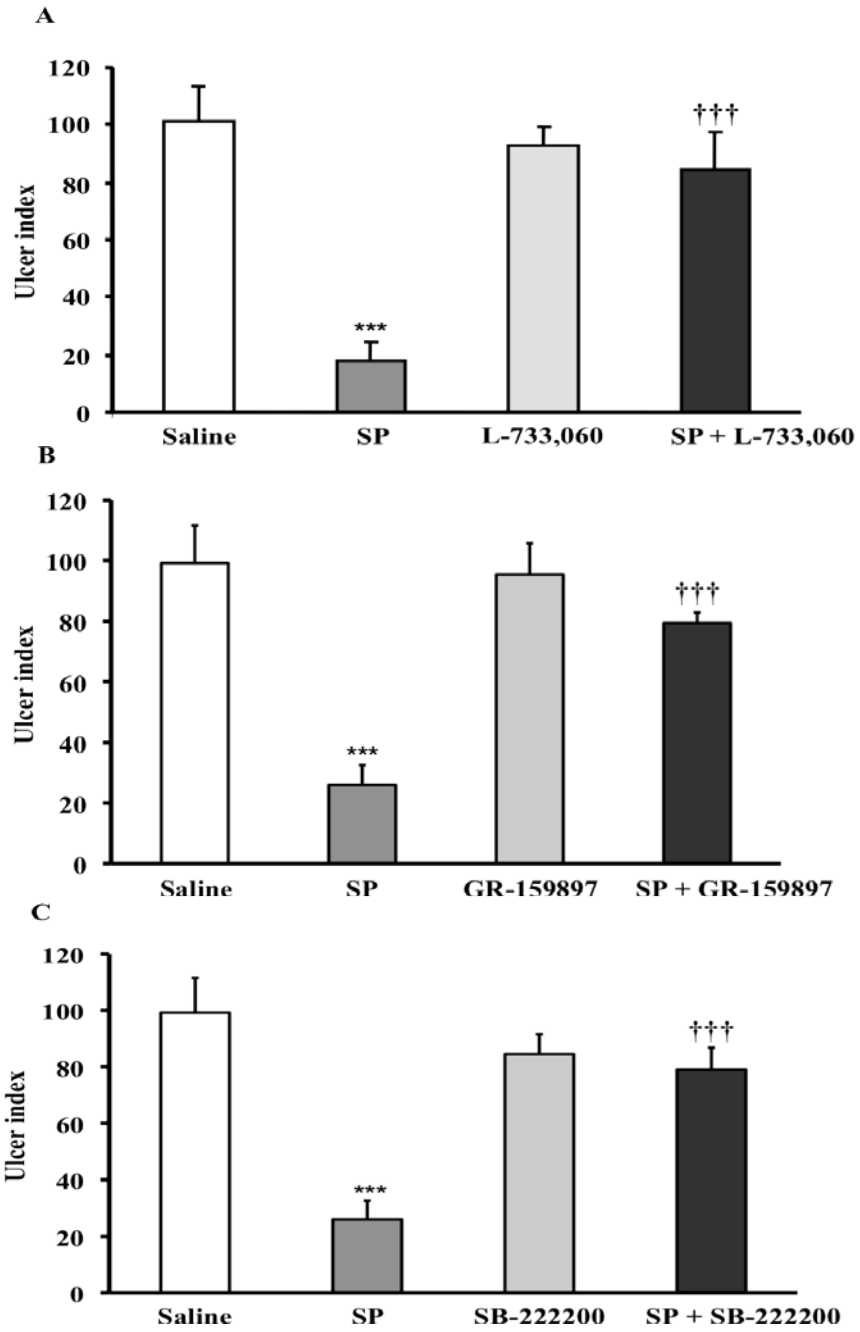


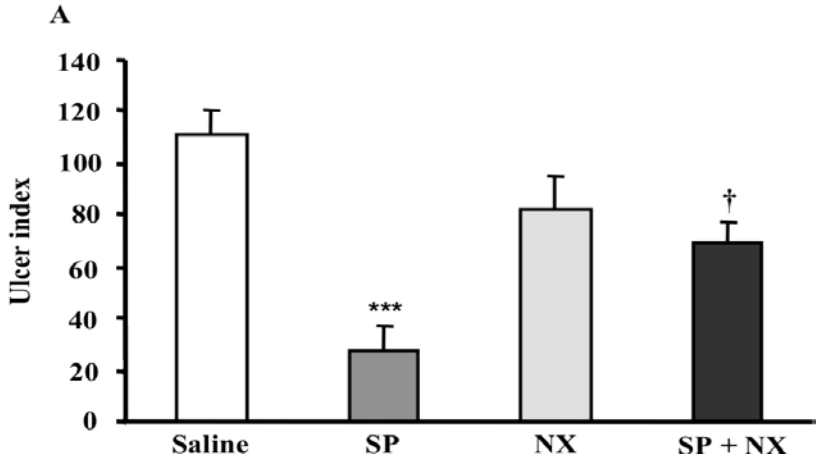
Fig. 18. The effect of L-733,060 (1 nmol/rat i.c.v.) [A], GR-159897 (0.5 nmol/rat i.c.v.) [B] and SB-222200 (1 nmol/rat i.c.v.) [C] on the central gastroprotection induced by SP (18.5 pmol/rat i.c.v.). Each column represents mean S.E.M., n = 5. ***P<0.001 compared with vehicle-treated group (column 1); †††P<0.001 compared with SP-treated group (column 2) (ANOVA, Newman-Keuls post hoc test).

4.2.3. Analysis of the role of endogenous opioid system in the gastroprotective effect of substance P

In this set of experiments we studied whether there was an interaction, at supraspinal level, between SP and endogenous opioid system in the maintenance of gastric mucosal integrity.

a) Effect of centrally administered opioid receptor antagonists on t he supraspinal gastric mucosal protective action of substance P

As Fig. 19A shows, i.c.v. injection of the non-selective opioid receptor antagonist naloxone (27 nmol/rat) induced, by itself, a slight, non-significant reduction of ethanol-induced gastric mucosal lesions (ulcer indices: 110 ± 10 vs 83 ± 13, n = 5). When naloxone was injected i.c.v. together with SP (18.5 pmol/rat i.c.v.), it significantly reduced the central gastroprotection induced by the peptide. Similar results were obtained with the selective μ-opioid receptor antagonist β-funaltrexamine (20 nmol/rat i.c.v.) (Fig. 19B). In contrast, neither the selective δ-opioid receptor antagonist naltrindole (5 nmol/rat i.c.v.), nor the selective κ-opioid receptor antagonist norbinaltorphimine (norBNI, 14 nmol/rat) inhibited the central gastroprotective effect of SP (Fig. 19C and 19D).



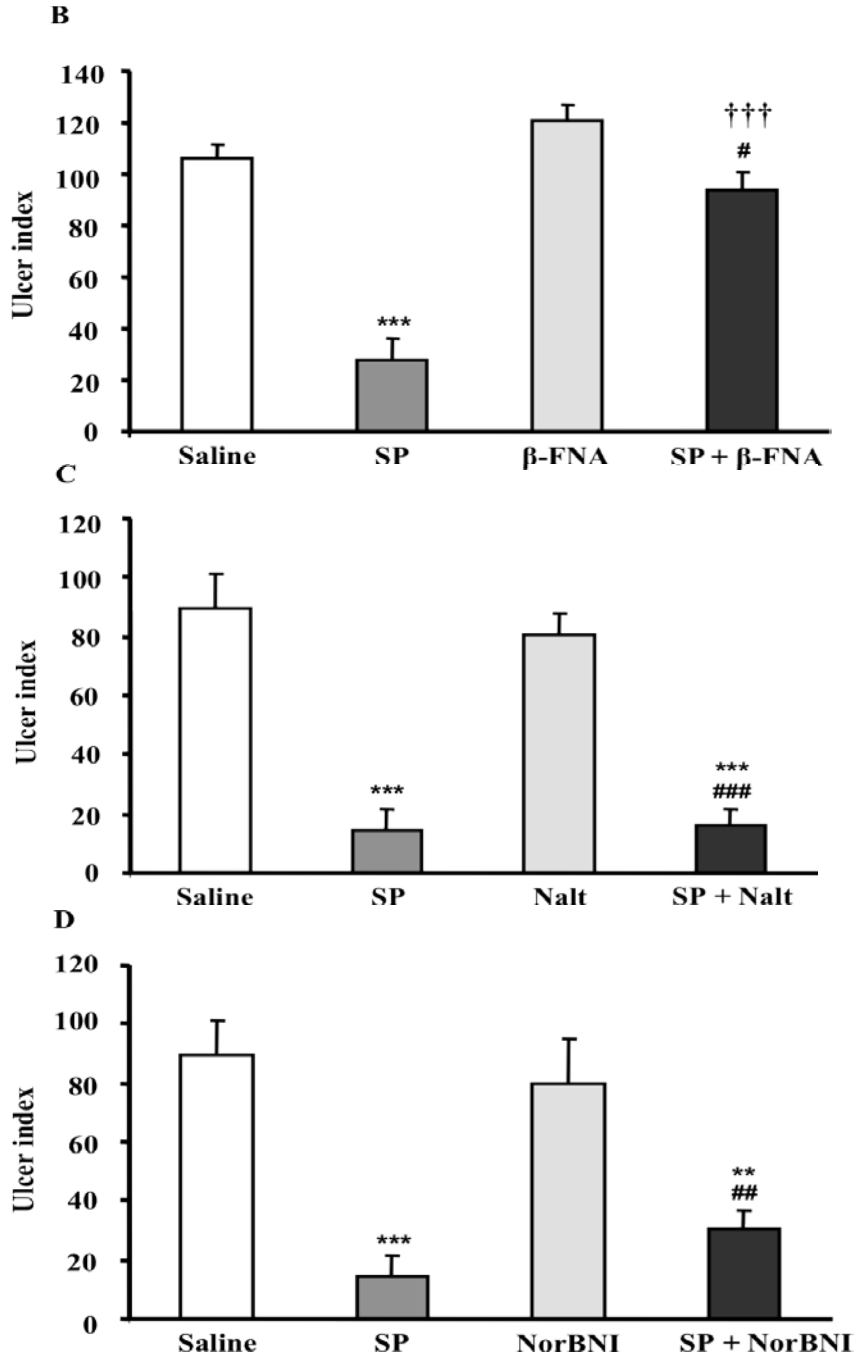


Fig. 19. The effect of naloxone (NX, 27 nmol/rat i.c.v.) [A], β-funaltrexamine (β-FNA, 20 nmol/rat i.c.v.) [B], naltrindole (Nalt, 5 nmol/rat i.c.v.) [C] and norbinaltorphimine (norBNI, 14 nmol/rat i.c.v.) [D] on the gastroprotective effect of SP (18.5 pmol/rat i.c.v.). SP was injected 10 min before acidified ethanol challenge either alone, or in combination with the antagonist. Each column represents mean ± S.E.M., n = 5. **P<0.01, ***P<0.001 compared with saline-treated group (column 1); †P<0.05, †††P<0.001 compared with SP-treated group (column 2), #P<0.05, ##P<0.01, ###P<0.001 compared with antagonist-treated group (column 3) (ANOVA, Newman-Keuls post hoc test).

b) Effect of endomorphin-2 antiserum on the central gastroprotective action of substance P

Since the results obtained with opioid receptor antagonists strongly suggested the involvement of μ-opioid receptors in the protective effect of SP, in the next set of experiments the interaction between EM-2 (endogenous ligand of μ-opioid receptor) and SP was analyzed. SP was injected 10 min before the ethanol challenge together with the non-reactive rabbit serum (NRS), or with endomorphin-2 antiserum (EM-2 AS). Antiserum against EM-2 co-injected i.c.v. with SP (18.5 pmol/rat) completely reversed the protective effect of the latter compound. Neither EM-2 antiserum injected alone, nor the non-reactive rabbit serum used as control had any effect on the ulcer index (Fig. 20).

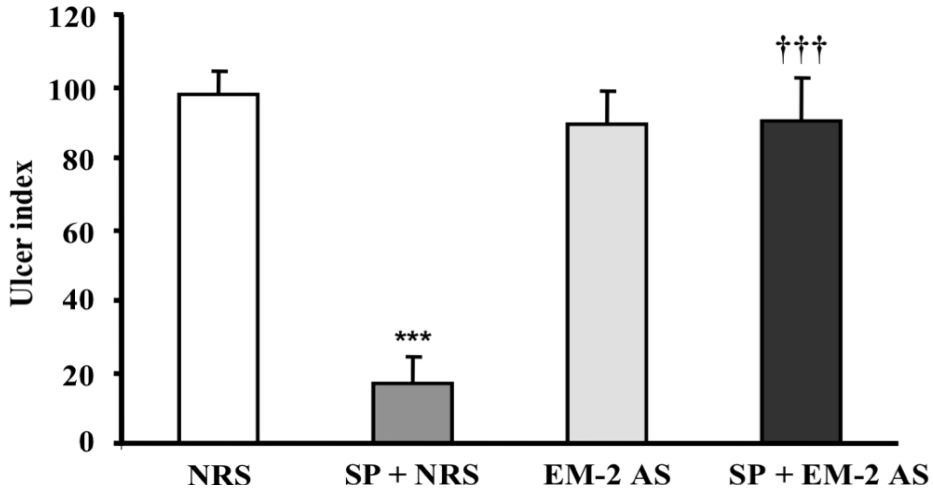


Fig. 20. The effect of EM-2 AS on the gastroprotective effect of SP (18.5 pmol/rat i.c.v.). SP was injected 10 min before the ethanol challenge together with the non-reactive rabbit serum (NRS), or with EM-2 AS. Each column represents mean S.E.M., n = 5. ***P<0.001 compared with NRS-treated group (column 1); †††P<0.001 compared with SP+NRS-treated group (column 2) (ANOVA, Newman-Keuls post hoc test).

4.2.4. Analysis of the peripheral factors mediating the central gastroprotective effect of substance P

a) Effect of peripheral administration of atropine, N^G-nitro-L-arginine (L-NNA) and indomethacin on the central gastroprotection induced by substance P

In our experiments we observed that inhibition of muscarinic acetylcholine receptors by atropine (1 mg/kg i.v., Fig. 21A), blockade of prostaglandin synthesis by i ndomethacin (20 mg/kg p.os, Fig. 21B) and administration of the NO-synthase inhibitor L-NNA (3 mg/kg i.v., Fig. 21C) all reversed the protective effect of centrally injected SP (18.5 pmol/rat i.c.v.). In these experiments SP was injected 10 min before the ethanol challenge, whereas the antagonists were given 15 min (i.v.) or 60 min (p.os) before the administration of SP.

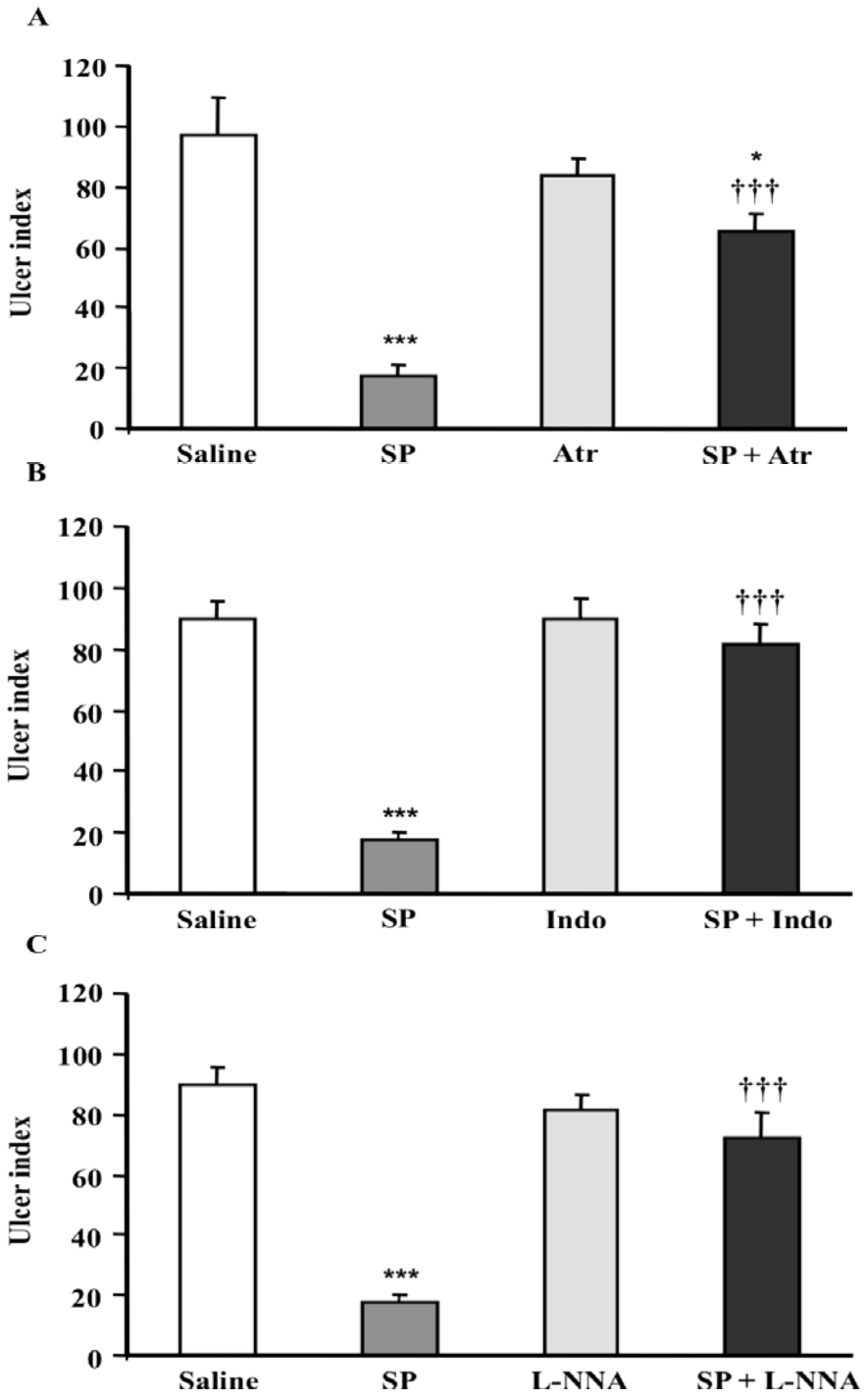


Fig. 21. The effect of atropine (Atr, 1 mg/kg i.v.) [A], indomethacin (Indo, 20 mg/kg p.os) [B], and N^G-nitro-L-arginine (L-NNA, 3 mg/kg i.v.) [C] on the gastroprotective action of SP (18.5 pmol/rat i.c.v.). SP was injected 10 min before the ethanol challenge, the antagonists were given 15 min (i.v.) or 60 min (p.os) before the administration of SP. Each column represents mean S.E.M., n = 5. *P<0.05, ***P<0.001 compared with vehicle-treated group (column 1); †††P<0.001 compared with SP-treated group (column 2) (ANOVA, Newman-Keuls post hoc test).

b) Effects of centrally and peripherally administered substance P on gastric mucosal levels of CGRP and somatostatin

Acidified ethanol given orally dramatically decreased the gastric mucosal concentration of both CGRP and somatostatin measured by radioimmunoassay. Centrally injected SP in a gastroprotective dose (18.5 pmol/rat i.c.v.) almost completely reversed the effect of ethanol on the CGRP level (Fig. 22A), and caused a slight, non significant elevation of the somatostatin concentration (Fig. 22B). In contrast, i.v. administered SP (0.74 nmol/kg) failed to influence the ethanol-induced reduction of mucosal CGRP and somatostatin.

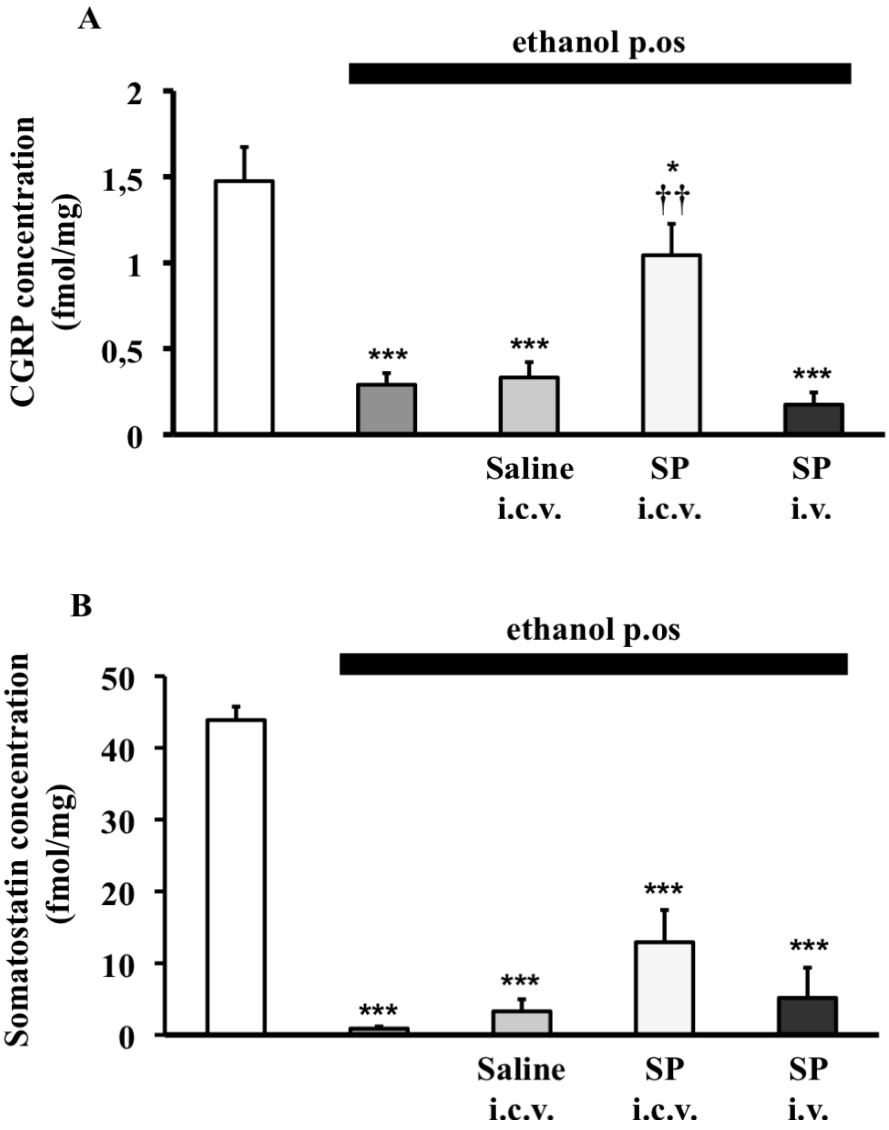


Fig. 22. The effect of SP on the mucosal CGRP (Fig. 16A) and somatostatin (Fig. 16B) concentration. CGRP and somatostatin concentrations were determined by radioimmunoassay (RIA). Each column represents mean S.E.M., n = 5. *P <0.05, ***P<0.001 compared with control group (no ethanol treatment, column 1); ††P<0.01 compared with ethanol+saline i.c.v.-treated group (column 3) (ANOVA, Newman-Keuls post hoc test).

5. DISCUSSION

SP and, in general, tachykinins, are important messenger molecules of enteric neurons [225] and supraspinal neurons located in the central areas that have a key role in the control of gastric motility [203]. Regulation of gastric motor activity by tachykinins is an area that has been studied most intensively ever since SP was discovered to occur in the gut; however, beside the wealth of information that has become available through the use of molecular biological, immunocytochemical, physiological and pharmacological techniques, the physiological and pathophysiological implications of SP in gastric motility have not been completely understood. Therefore, the first purpose of our experimental series was to clarify the effect of exogenously administered SP on rat basal gastric motility by using the rubber balloon method described previously.

In different *in vivo* experiments on anesthetized rats, systemic administration of SP has been reported to induce mainly a stimulation of basal gastric motility [226, 227]. In contrast with these results, in the present study i.v. injection of SP in a low dose-range induced a biphasic motor response characterized by an initial distention followed by a sustained contraction accompanied by stimulated superimposed phasic contractions. Both two phases of the SP response resulted dose-related in amplitude. The initial distention, which occurred immediately after the injection of the peptide, increased with the

dose of SP, whereas the subsequent stimulation of gastric motility was maximal with the doses of 0.074 and 0.74 nmol/kg, the dose-response curve for this last effect thus resulting bell-shaped. The short duration of the effect of SP probably refers to the short half-life of the peptide after systemic administration. In fact, analysis by high-performance liquid chromatography of plasma extracts following intravenous infusion of SP into anaesthetized rats showed that the peptide was cleared from the circulation within 1-2 minutes [182]. SP is also rapidly metabolized by membrane-bound peptidases (probably neutral endopeptidase and ACE) located in the stomach wall [307].

The selective NK1 receptor antagonist L-733,060 reduced both the two phases of the effect of SP. However, the inhibition of the effect was not complete (especially for the highest most effective dose of the peptide) raising the involvement of other potential mechanisms and receptors in SP gastric motor action. Several lines of evidence have suggested a pivotal role of muscular NK2 receptors in the contractile effect of SP on the rat stomach [232, 308, 309]. On the other hand, NK3 receptors seem to be involved in the relaxation of guinea-pig stomach induced by SP [236]. Therefore, further studies are needed to clarify, whether the other two tachykinin receptor subtypes are involved in the observed gastric motor effect of peripherally administered SP.

The inhibitory effect of SP on gastric motility could be due to the stimulation of inhibitory neural pathways to the muscle or to the interruption of cholinergic excitatory neurotransmission.

NO represents, together with VIP, the major inhibitory transmitter involved in the gastric relaxation processes and seems to have an important role in the regulation of gastric motility [310]. In the rat stomach, relaxation induced by the stimulation of NANC nerves is significantly antagonized by the NO biosynthesis inhibitor N^G-nitro-L-arginine (L-NNA) or N^G-nitro-L-arginine methyl ester (L-NAME) [311, 312]. In addition, exogenously applied NO produces, in the rat gastric fundus, a relaxation that mimics that induced by NANC stimulation [311]. A colocalization between NK1 receptors and NO synthase has been observed in enteric neurons of the guinea-pig gut [313]. Furthermore, activation of NK1 and NK3 receptors has been reported to relax the circular muscle of the guinea-pig stomach through the stimulation of inhibitory motor neurons and the subsequent release of NO [236]. In our experiments, the inhibition of nitric oxide biosynthesis by L -NAME only slightly reduced the initial distention induced by low doses of SP. Therefore, NO seems to have a marginal role in the gastric inhibitory motor effect the peptide. Noteworthy, L-NAME potentiated the stimulation of gastric phasic contractions induced by SP, suggesting, in accordance with the reports of Gustafsson [314] and Takahashi [51], a neuromodulatory role for NO on excitatory neurotransmission.

SP has been demonstrated to inhibit, in the canine gastric antrum, electrically induced release of acetylcholine [238]. Presynaptic NK1 receptors seem to be responsible for this inhibitory effect of the peptide on excitatory cholinergic

transmission [238, 315], although the participation of neuronal NK3 receptors cannot be excluded [316]. Therefore, since NO is not important in mediating the inhibitory action of SP observed in our experimental conditions, this effect could be due to the inhibition of acetylcholine release induced by SP through the activation of prejunctional tachykinin receptors.

In *in vivo* experiments on anesthetized rats and cats, systemic administration of SP produced an atropine-sensitive contractile effect [226, 229, 230, 317], suggesting the involvement of a cholinergic muscarinic pathway in the stimulatory motor effect of the peptide. In our experiments, pretreatment with atropine - that by itself depressed basal gastric motility - reduced not only the stimulation but also the initial inhibition of gastric motility induced by low doses of SP. A similar atropine-sensitive inhibitory effect of SP has been documented by Fox et al. [318] on the canine intestine *in vivo*. While the inhibition of the second stimulatory phase by atropine could be easily explained on the basis of the documented ability of SP to induce the release of acetylcholine in the mammalian gastrointestinal tract [225], the reason of the inhibition of the first inhibitory phase is not clear. It might be speculated that the inhibitory effect of SP on the excitatory transmission is measurable only with an intact cholinergic transmission. If the cholinergic system is inhibited, the basal contractions and gastric tone are depressed and no further inhibition by SP can be observed.

As mentioned in the introduction, the stomach is highly dependent upon extrinsic vagal innervation. Vagal efferent fibers originating from DMV control gastric functions through excitatory and inhibitory pathways. The excitatory pathway involves preganglionic cholinergic neurons and postganglionic cholinergic neurons innervating gastric smooth musculature, ICC and parietal cells. Two inhibitory vagal pathways seemed to exist, one consisting of preganglionic cholinergic neurons synapsing, via nicotinic receptors, onto postganglionic NANC neurons [51] and the other involving nitrergic vagal preganglionic neurons that innervate the gastrointestinal tract [46]. Tachykinin receptors - namely NK1 and NK3 receptors - have been detected on extrinsic nerve fibers innervating the stomach including vagal efferent fibers [193, 197]. In an *in vivo* study in cats, systemic administration of SP induced a triphasic effect characterized by an initial distension followed by a sustained stimulation and a late distention phase [230]. Cervical bilateral vagotomy has been documented to inhibit the initial distension phase, without having any effect on SP-induced contraction. In our study, cervical bilateral vagotomy reduced mainly the stimulatory action of SP on gastric motility, leaving almost unaffected the first inhibitory phase. This result has suggested that a component of the SP response is vagally mediated.

In the last step, the involvement of endogenous opioid system in the peripheral gastric motor effect of SP was analyzed. The effect of opioids on gastric emptying and gastric motility has intensively been studied. Opioids has been

reported to inhibit both basal and stimulated gastric motility and strongly increase the pyloric contractions [319-322]. Our present experiments failed to demonstrate an interaction between SP and opioid system in the regulation of gastric motility, since the non-selective opioid receptor antagonist naloxone did not influence the motor effect of the peptide.

In conclusion, our results indicate that peripherally injected SP induces a biphasic effect on gastric motility, which is, at least partly, mediated by NK1 receptors. Vagus nerve and cholinergic muscarinic transmission seem to be also involved in the effect of the peptide, although their precise role remains to be fully clarified. NO has a marginal role in the inhibitory phase of the effect of SP on gastric motility, whereas endogenous opioid system seems to be not involved.

The other main purpose of our experimental work was to analyze the potential role of SP in gastric mucosal defense and to clarify the receptors and mechanisms that may be involved in it. Maintenance of gastric mucosal integrity depends on several factors, e.g. mucosal microcirculation, mucosal barrier, production of gastric mucus, and mucosal protective elements. The distribution pattern of SP and tachykinin receptors in the CNS areas that have a pivotal role in the regulation of gastric mucosal homeostasis [203, 210], together with the findings of Hernandez et al [263], who documented a central gastroprotective effect of SP in an acid-dependent ulcer model, have suggested

the involvement of the peptide in the maintenance of mucosal integrity at supraspinal level.

We found that centrally administered SP significantly reduced the ethanol-induced gastric mucosal lesions in a low dose range (9.3-37 pmol i.c.v.), while at higher doses the gastroprotective effect diminished. Therefore, the dose-response curve for SP-induced central gastroprotection resulted bell-shaped. With this property SP joins to several other neuropeptides proved to be gastroprotective after central administration, e.g. amylin, peptide YY, adrenomedullin, TRH and the opioid peptides [264]. It might be speculated that SP in high doses may act not only in the central nervous system, but also in the periphery, activating mechanisms that counteract its protective action, e.g. reduced mucosal blood flow, mast cell degranulation or formation of reactive oxygen species [255-257]. A similar bell-shaped dose-response curve has been observed for other SP-induced effects as well, like supraspinal antinociception [323], improvement of learning and memory [324] or in the case of its anxiolytic-like effect [325]. It is also worthy of note that the bell-shaped dose-response relationship is not a unique property of SP, but it has also been described for the central gastroprotective effect of several other neuropeptides, such as nociceptin, nocistatin, β-endorphin or somatostatin [263, 282, 283].

As mentioned in the introduction, in contrast with the centrally induced effect, SP given i.v., i.p. or s.c. either failed to reduce ethanol-induced gastric

mucosal damage [250], or aggravated it [255, 256]. In our experimental model, i.v. injected SP had no significant effect on the ethanol-induced lesions.

The main aim of the subsequent set of experiments was to clarify which central and peripheral mechanisms may be responsible for the mucosal protective effect of SP and to determine if changes of gastric motility might have any role in the gastroprotective action induced by SP.

First we analyzed the involvement of the central tachykinin receptors. Since SP has the major affinity for the NK1 receptor, we supposed that this tachykinin receptor subtype had the pivotal role in mediating the SP-induced mucosal protection. However, our results suggest that besides NK1 receptors, NK2 and NK3 receptors may also mediate the centrally-induced gastroprotective effect of SP, since both L-733,060, GR-159897 and SB-222200 - antagonists of the NK1, NK2 and NK3 receptors, respectively - caused complete inhibition. These results are in agreement with the findings that demonstrated the presence of all three tachykinin receptors in the DVC [211, 216], a key region in the central modulation of GI functions, and with the ability of SP to act as an agonist at multiple tachykinin receptor subtypes [183].

The involvement of NK2 and NK3 receptors in the central effect of SP is unexpected, but not unprecedented. For example, it was observed that both NK1 and NK2 receptors are involved in the excitatory effect of SP on DMV neurons projecting to the stomach [184] and in the cardiovascular and behavioral effects induced by i.c.v. injection of the peptide [185], whereas both NK2 and

NK3 receptors seem to mediate the inhibitory effect of SP on pancreatic ductal bicarbonate secretion [326]. Moreover, all three tachykinin receptors mediate the postsynaptic action of SP on rat periaqueductal grey neurons *in vitro* [186]. The ability of central NK2 receptors to regulate gastric acid secretion and mucosal integrity is also supported by the findings of Improta [253], who found that the selective NK2 receptor agonist Ala^5NKA(4-10) reduced, after i.c.v. injection, the secretion of gastric acid and the formation of mucosal lesions in an acid-dependent ulcer model (pylorus ligation) in rats. However, since the affinity of SP for NK2 and NK3 receptors is not so high as to justify the complete inhibition of the gastroprotective effect by the NK2 and NK3 antagonists, it can be also raised that the peptide, by activating NK1 receptors, may release other endogenous tachykinins that, in turn, activate NK2 and NK3 receptors. The rat DVC is enriched with axon terminals containing NKA and NKB [327], and the activation of NK1 receptors in the NTS has been shown to increase the release of glutamate [212], which, in turn, can lead to the release of neurokinins [327]. However, further studies are needed to clarify whether the release of NKA and NKB is involved in the gastroprotective effect of SP.

As mentioned in the introduction, endogenous opioid system has been demonstrated to have, at supraspinal level, a very important role in the maintenance of gastric mucosal integrity [328]. Central administration of δ- and μ-opioid receptor selective agonists has been shown to induce a gastroprotective effect in both acid-dependent and independent ulcer models

[126, 265, 329]. Moreover, endogenous opioids are likely to mediate the gastroprotective effect of several compounds, like clonidine [282], nociceptin, nocistatin [283] and cannabinoids [284]. Several lines of evidence have suggested the existence of a close interaction between SP and opioid system. Well documented is also the ability of SP to release endogenous opioids [277, 330, 331]. Therefore, the question was raised whether interaction between endogenous opioid system and SP occurs also in gastroprotection.

In accordance with the above data, in our experiments the mucosal protective effect of SP was significantly reduced by pretreatment with naloxone, suggesting that the release of endogenous opioids is indeed one potential link in the chain of events resulting in gastroprotection. It is noteworthy that in the study of Kream [331] the potential opioid releasing effect of SP could be observed at exactly the same dose-range (10-100 pmol) that induced gastroprotection in our experiments (9.3-74 pmol). Since the selective μ-opioid receptor antagonist β-funaltrexamine also antagonized the effect of SP, while the δ- and κ-opioid receptor antagonist naltrindole and norbinaltorphimine, respectively, failed to affect it, we raised the hypothesis that SP may release endogenous μ-opioid receptor selective ligands that mediate the gastroprotective effect. The only endogenous opioids characterized by high selectivity for μ-opioid receptors are endomorphins (EM-1 and EM-2) [332]. Endomorphins are widely distributed in the CNS - also in several hypothalamic nuclei and in the NTS [271] - and, in a pilot experiment,

they inhibited the ethanol-induced ulcer formation after i.c.v. injection in a low nanomolar dose-range [333]. Our experiments focused on the potential role of EM-2, because it is more abundant in the lower brain stem compared to EM-1 [271], and it has been shown to be co-localized with SP in the NTS [285]. As our present results demonstrate, pretreatment with EM-2 antiserum resulted in inhibition of the protective effect of SP, suggesting that, among the endogenous opioids, EM-2 is likely to have an essential role in the SP-induced action.

Gastric motility may represent one of the factors affecting the integrity of the mucosa. Gastric contractions characterized by high amplitudes may induce microvascular disturbances in specific sites of the mucosa probably by abnormal compression of the gastric wall, thereby leading to increased vascular permeability and cellular damage [147, 151, 154, 334]. In contrast, inhibition of gastric motility may result in flattening of the mucosal foldings and decrease the mucosal vulnerability to irritants. The inhibition of gastric motor activity may contribute to the gastroprotective effect of amylin [155], dopamine or capsaicin [149, 335]. Since direct injection of SP (135-405 pmol) into the DMV [208] or into the NTS [244] has been reported to inhibit gastric motility, we investigated whether a modification of gastric motor activity induced by centrally injected SP may play a role in its gastroprotective effect. We found that i.c.v. injection of SP in the gastroprotective dose-range (7.4 and 74 pmol) did not induce any significant action on gastric motility. Furthermore, it

caused even at 10 times higher dose (740 pmol) only a moderate reduction of gastric contractions. Thus, it is unlikely that inhibition of gastric motility would have any relevance in the gastroprotective effect of SP. Our result is in good correlation with the findings of Improta & Broccardo [249], who observed that SP given i.c.v. inhibited only moderately the gastric emptying even in a high dose (6 nmol).

In the last step, we investigated how the SP-induced protective effect is conveyed to the periphery. Several neuropeptides - e.g. adrenomedullin, thyrotropin-releasing hormone or peptide YY - have been shown to exert, after central administration, a gastroprotective action by a vagal-dependent mechanism [121, 124, 125]. Namely, activation of vagal efferents has been demonstrated to induce, through a cholinergic muscarinic pathway, gastric mucosal prostaglandin and NO production as well as activation of "efferent function" of capsaicin-sensitive afferent fibers expressing CGRP [264, 336]. As our results demonstrate, blockade of muscarinic cholinergic receptors by peripheral injection of atropine, inhibition of gastric prostaglandin synthesis by oral administration of indomethacin and intravenous injection of the NO synthase inhibitor L-NNA all abolished the mucosal protective effect of SP.

CGRP, as mentioned above, has a major role in the maintenance of gastric mucosal integrity mainly via increasing mucosal blood flow both directly, by acting on vascular smooth muscle, and indirectly, by releasing NO [76] and somatostatin [337]. In accordance with the findings of Evangelista et al. [338], we

observed that the mucosal content of CGRP was dramatically decreased after oral administration of acidified ethanol. As our results show, i.c.v., but not i.v. injected SP almost completely reversed the detrimental effect of acidified ethanol on gastric mucosal CGRP content. The mucosal somatostatin level similarly to that of CGRP dramatically decreased after the ethanol challenge. However, SP failed to reverse the reduced somatostatin level, suggesting that somatostatin may not be involved or has only a minor role in the CGRP-mediated gastric mucosal protective action.

In summary, the present results indicate that SP given i.c.v. in picomolar dose-range exerts a powerful gastroprotective action that is likely mediated by the endogenous opioid system and EM-2. Vagus nerve may convey the centrally initiated protection induced by SP to the periphery, where both PGs, NO and CGRP are involved in mediating this effect.

The following figure (Fig. 23) shows a hypothetical scheme illustrating the receptors and mediators involved in the central gastroprotective action of SP.

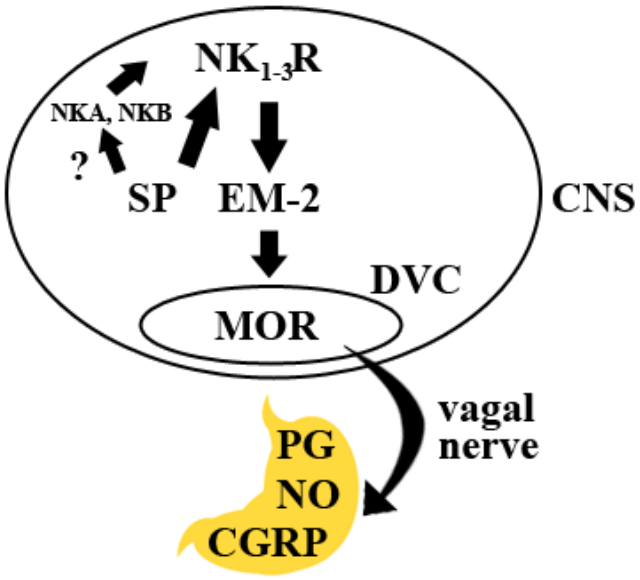


Fig. 23. Hypothetical model of the central gastroprotective action of SP. In this model, SP activates neurokinin NK1-3 receptors either directly, or via releasing NKA and NKB. The activation of tachykinin receptors leads to elevation of the endomorphin-2 (EM-2) level, which increases the mucosal concentration of peripheral protective factors (PGs, NO and CGRP) via a vagal-dependent mechanism.

6. CONCLUSIONS

Our present study indicates that peripherally injected SP induces, in a low dose-range, a biphasic effect on gastric motility characterized by a transient relaxation followed by an increase in gastric tone and amplitude of phasic contractions. Based on our results it can be concluded that:

- the gastric motor effect of peripherally injected SP is mediated, at least in part, by tachykinin NK1 receptors;
- NO has a marginal role in the inhibitory component of the motor effect of SP;
- vagus nerve and cholinergic muscarinic transmission seem to be involved in the observed effect of the peptide, whereas endogenous opioid system does not have any role.

In our experimental work the involvement of SP in the regulation of gastric mucosal integrity at supraspinal level has also been demonstrated. SP, in a picomolar dose-range, has been shown able to induce a gastroprotective effect after i.c.v. administration. Our results have suggested that:

- both NK1, NK2 and NK3 receptors are involved in the central gastroprotective effect of SP;

- mucosal protective processes activated at central level by SP may involve endogenous opioids acting on μ receptors, namely endomorphins;
- vagus nerve conveys the centrally initiated protection induced by SP to the periphery, where PGs, NO and CGRP are involved in mediating this effect;
- no correlation is likely to exist between the gastroprotective effect of SP and the inhibition of gastric motor activity, since the gastroprotective doses of the peptide do not induce any modification of gastric motility.

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