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Abstract: The aim of the present study was to analyze the potential role of substance P (SP) in gastric mucosal defense and to clarify the receptors and mechanisms, that may be involved in it. Gastric ulceration was induced by oral administration of acidified ethanol in male Wistar rats. Mucosal levels of calcitonin gene-related peptide (CGRP) and somatostatin were determined by radioimmunoassay. For analysis of gastric motor activity the rubber balloon method was used. We found that central (intracerebroventricular) injection of SP (9.3 - 74 pmol) dose-dependently inhibited the formation of ethanol-induced ulcers, while intravenously injected SP (0.37-7.4 nmol/kg) had no effect. The mucosal protective effect of SP was inhibited by pretreatment with neurokinin 1-, neurokinin 2-, neurokinin 3- and μ-opioid receptor antagonists, while δ- and κ-opioid receptor antagonists had no effect. Endomorphin-2 antiserum also antagonized the SP-induced mucosal protection. In the gastroprotective dose range SP failed to influence the gastric motor activity. Inhibition of muscarinic cholinergic receptors, or the synthesis of nitric oxide or prostaglandins significantly reduced the effect of SP. In addition, centrally injected SP reversed the ethanol-induced reduction of gastric mucosal CGRP content. It can be concluded, that SP may induce gastric mucosal protection initiated centrally. Its protective effect is likely to be mediated by endomorphin-2, and vagal nerve may convey the centrally initiated protection to the periphery, where both prostaglandins, nitric oxide and CGRP are involved in mediating this effect.

Substance P induces gastric mucosal protection at supraspinal level via increasing the level of endomorphin-2 in rats

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Short title: SP induces EM-2-mediated gastroprotection

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Abstract

The aim of the present study was to analyze the potential role of substance P (SP) in gastric mucosal defense and to clarify the receptors and mechanisms, that may be involved in it. Gastric ulceration was induced by oral administration of acidified ethanol in male Wistar rats. Mucosal levels of calcitonin gene-related peptide (CGRP) and somatostatin were determined by radioimmunoassay. For analysis of gastric motor activity the rubber balloon method was used. We found that central (intracerebroventricular) injection of SP (9.3 - 74 pmol) dose-dependently inhibited the formation of ethanol-induced ulcers, while intravenously injected SP (0.37-7.4 nmol/kg) had no effect. The mucosal protective effect of SP was inhibited by pretreatment with neurokinin 1-, neurokinin 2-, neurokinin 3- and μ-opioid receptor antagonists, while δ- and κ-opioid receptor antagonists had no effect. Endomorphin-2 antiserum also antagonized the SP-induced mucosal protection. In the gastroprotective dose range SP failed to influence the gastric motor activity. Inhibition of muscarinic cholinergic receptors, or the synthesis of nitric oxide or prostaglandins significantly reduced the effect of SP. In addition, centrally injected SP reversed the ethanol-induced reduction of gastric mucosal CGRP content. It can be concluded, that SP may induce gastric mucosal protection initiated centrally. Its protective effect is likely to be mediated by endomorphin-2, and vagal nerve may convey the centrally initiated protection to the periphery, where both prostaglandins, nitric oxide and CGRP are involved in mediating this effect.

Key words: calcitonin gene-related peptide, endomorphin, gastric ulcer, substance P, tachykinins

1. Introduction

Substance P (SP) is an undecapeptide belonging to the family of tachykinins, that is characterized by a common carboxy-terminal amino acid sequence (Phe-X-Gly-Leu-Met-NH_2, where X is an aromatic or hydrophobic residue). Substance P is encoded by the tachykinin precursor 1 (TAC1) gene, originally known as preprotachykinin (PPT)-A or PPT-I and represents the major endogenous ligand for the neurokinin receptor 1 (NK1), though it possesses appreciable affinity also for the other two tachykinin receptor subtypes, NK2 and NK3 (Harrison and Geppetti, 2001; Pantaleo et al., 2010).

SP is highly abundant in the gastrointestinal (GI) tract, and acts both as a neurotransmitter and neuromodulator. The major source of SP is the intrinsic enteric neurons, but a smaller amount is contained in capsaicin-sensitive extrinsic afferents as well, where it is co-expressed with neurokinin A (NKA) and calcitonin gene-related peptide (CGRP) (Holzer and Holzer-Petsche, 1997). CGRP has a prominent role in the maintenance of gastric mucosal integrity (Holzer, 2007) and NKA and its analogues have also been shown to induce gastroprotection after peripheral administration (Evangelista et al., 1989; Stroff et al., 1996). In contrast, SP given peripherally either did not affect ethanol-induced mucosal lesions (Evangelista et al., 1989; Stroff et al., 1996), or even aggravated it, probably through a mechanism involving reduced mucosal blood flow, mast cell degranulation and formation of reactive oxygen species (Gazzieri et al., 2007; Karmeli et al., 1991).

Besides the periphery, gastric mucosal defense can also be induced by central mechanisms (for reviews see Gyires, 2004; Taché, 2012). Dorsal vagal complex (DVC) and vagal nerve were shown to play a crucial role in conveying the centrally initiated gastroprotective effect to the periphery (Kato et al., 1994; Yang et al., 1999). Moreover, hypothalamic paraventricular nucleus and lateral hypothalamus also take part in the central control of gastric functions, like acid secretion, motility and gastric mucosal defense (Grijalva

and Novin, 1990). SP is widely distributed in the central nervous system (Mantyh, 2002), and high density of both SP and NK1 receptors were shown in the above-mentioned areas (Dixon et al., 1998; Larsen, 1992; Lewis and Travagli, 2001, Haller, 2012), suggesting that SP besides its widespread role in mediation/modulation of central mechanisms, may play a role in the regulation of GI functions. Indeed, direct injection of SP into the dorsal motor nucleus of the vagus (DMV) (Krowicki and Hornby, 2000) or into the nucleus of the solitary tract (NTS) (Spencer and Talman, 1986) was shown to inhibit gastric motor activity and activation of NK1 receptors in the DVC reduced the medullary TRH-induced stimulation of gastric acid secretion (Yang and Taché, 1997). However, very limited data are available on the role of SP in central regulation of gastric mucosal integrity. To our best knowledge only one paper has been published on the central effect of SP on gastric ulcer formation, demonstrating that SP injected intracisternally inhibited the development of gastric ulcer formation in an acid-dependent ulcer model, the cold-restraint stress-induced gastric ulceration, but the mechanism of the gastroprotective effect has not been clarified (Hernandez et al., 1983).

Therefore the present study aimed to investigate:

1) the effect of centrally injected SP on an acid-independent ulcer model (to exclude the potential role of inhibition of gastric acid secretion in the gastroprotective action), and to clarify the receptors that may mediate this effect,

2) the peripheral, mucosal factors (altered motility, release of local mediators or neuropeptides) that may contribute to the gastroprotective effect of SP.

2. Materials and methods

2.1. Animals.

For all experiments male Wistar rats were used. The rats were deprived of food for 24 h with free access to tap water. They were housed in wire mesh bottom cages to prevent

coprophagy. The rats were kept on a 12 h light-dark cycle and under conditions of controlled temperature.

All procedures conformed to the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. The study was approved by the Animal Ethics Committee of Semmelweis University, Budapest (permission number: 22.1/606/001/2010).

2.2. Gastric mucosal damage induced by acidified ethanol.

For ethanol ulcer experiments 140-170 g rats were used. After 24 h food deprivation the animals were given orally 0.5 ml acidified ethanol (98 ml absolute ethanol + 2 ml concentrated HCl). One hour later the animals were euthanized, the stomachs 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 previously (Gyires, 1990). The ulcer index was calculated as the total number of lesions multiplied by the respective severity factor. The percentage inhibition of mucosal damage was calculated as follows:

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

SP was injected either intracerebroventricularly (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 (Gyires et al., 2000). 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 agonist.

2.3. In vivo measurement of gastric motor activity.

For in vivo measurement of gastric motility the rubber balloon method was used (LeFebvre et al., 1992). Briefly, after 24 h food deprivation 250-400 g male Wistar rats were anesthetized with urethane (1.25 g/kg i.p.), a tracheal cannula was inserted to ensure a clear

airway and an intragastric balloon created from thin latex rubber connected with plastic tubing was introduced into the stomach via mouth. The balloon was filled with 2 ml warm water (37°C) to set the basal intragastric pressure to 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 experiments. SP was given i.c.v. in a volume of 10 µl within 5 min, injected with 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 (Paxinos and Watson, 1986). 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. The gastric tone, which correlates well with fundic activity (Ferreira Jr. et al., 2002), was calculated from the bottom points of phasic pressure wave. The mean amplitude of phasic contractions, which correlates with the antral contractions superimposed on tonic pressure, was calculated from the amplitude of each contraction. Both parameters were determined from 5 min segments, before and after the injection of SP (Zádori et al., 2007). Values were expressed in percentage of the basal (pre-injection) values.

2.4. Radioimmunoassay determinations.

For determination of gastric mucosal level of CGRP and somatostatin the rats were euthanized, the stomachs were removed and gastric mucosa was separated on a cooled plate.

It was 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 (Németh et al., 1996, 1998). For the specific RIA assays the antisera (CGRP: C1012; somatostatin: 775/7) were raised in rabbit or in case of somatostatin in sheep immunized with synthetic peptides conjugated to thyroglobulin by glutaraldehyde. The tracers were mono-125l-labelled peptides prepared by Németh et al. (2002). 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.

2.5. Materials.

The following drugs were used: naloxone hydrochloride, naltrindole hydrochloride, nor-Binaltorphimine dihydrochloride (norBNI), N^G-nitro-L-arginine (L-NNA), indomethacin, atropine sulphate (all purchased from Sigma Chemical Co., St. Louis, USA), substance P (Ascent Scientific, Bristol, UK), β-funaltrexamine hydrochloride, L-733,060 hydrochloride ((2S,3S)-3-[[3,5-bis(Trifluoromethyl)phenyl]methoxy]-2-phenylpiperidine hydrochloride), GR 159897 (5-Fluoro-3-[2-[4-methoxy-4-[[(R)-phenylsulphinyl]methyl]-1-piperidinyl]ethyl]-1H-indole), SB 222200 (3-Methyl-2-phenyl-N-[(1S)-1-phenylpropyl]-4-quinoline-carboxamide) (all from Tocris Bioscience, Bristol, UK) and EM-2 antiserum (produced by István Barna, Hungarian Academy of Sciences).

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 DMSO and then diluted with saline. Control animals received the drug solvents.

For the receptorial analysis the doses of antagonists were selected based partly on our previous experiments, party on the literature data (Table 1).

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: Pacharinsak et al., 2008; Rittner et al., 2007; Walsh et al., 1995) was used in the case of other routes of administration. The applied 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 (Beresford et al., 1995; Seabrook et al., 1996).

The properties of EM-2 antiserum have been described previously in detail (Szemenyei et al., 2008). The antiserum was used at a 20-fold final dilution. The same dilution of non-reactive rabbit serum (NRS) was used as control.

2.6. Statistical analysis.

Statistical analysis of the data was evaluated by means of analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons. In the case of motility experiments the pre- and postinjection values were compared with paired Student's t-test. A probability value of less than 0.05 was considered statistically significant.

3. Results

3.1. The effect of SP on the ethanol-induced gastric mucosal damage.

Oral administration of acidified ethanol induces deep, multiple longitudinal hemorrhagic lesions on the gastric mucosa (Fig. 1, ulcer index: 97 ± 7, n=10). SP, when injected i.c.v., significantly decreased the formation of ethanol induced gastric mucosal

lesions in the doses of 4.6-148 pmol/rat (Fig. 1 and 2A). The maximal inhibition was induced by 9.3 and 18.5 pmol (89.8 and 75.8 %, respectively), and the latter dose was chosen for further experiments. At higher dose range (74 and 148 pmol), however, the gastroprotective effect of SP declined (33.0 and 29.4 % inhibition).

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), however, the difference was not statistically significant (Fig. 1 and 2B).

3.2. The effect of SP on the gastric motor activity.

SP given i.c.v. in the gastroprotective dose range did not influence the gastric motor activity (Table 2). At the highest dose (740 pmol) it reduced slightly the amplitude of contractions (24 % inhibition), but it was not statistically significant ($p=0.14$, pre- vs postinjection values, paired t-test).

3.3. The effect of tachykinin receptor antagonists on the gastroprotective effect of SP.

The effect of i.c.v. injected L-733,060 (1 nmol/rat), GR 159897 (0.5 nmol/rat) and SB 222200 (1 nmol/rat), potent, selective non-peptide antagonists of the NK1, NK2 and NK3 receptors, respectively, was studied on the protective effect of SP (18.5 pmol/rat, i.c.v.). As Fig. 3 demonstrates, all three antagonists reversed the effect of SP. Alone they did not modify the ethanol-induced mucosal damage.

3.4. Analysis of the role of opioid system in the gastroprotective effect of SP.

I.c.v. injection of the non-selective opioid receptor antagonist naloxone (27 nmol/rat) induced a slight, non-significant reduction of mucosal ulcers (ulcer indices: 110 ± 10 vs 83 ± 13, n=5). When it was injected together with SP (18.5 pmol/rat), the SP-induced gastroprotection significantly decreased (Fig. 4A). Similar results were obtained with the selective μ-opioid receptor antagonist β-funaltrexamine (20 nmol/rat, Fig. 4B). In contrast,

neither the selective δ-opioid receptor antagonist naltrindole (5 nmol/rat), nor the selective κ-opioid receptor antagonist norbinaltorphimine (norBNI, 14 nmol/rat) inhibited the central gastroprotective effect of SP (Fig. 4C and 4D).

Since these results strongly suggest 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 receptors) and SP was analyzed. Antiserum against EM-2 was co-injected with SP, which completely reversed the protective effect of the latter compound. EM-2 antiserum injected alone had no effect on the ulcer index, neither the non-reactive rabbit serum, which was used as control (Fig. 4E).

3.5. Analysis of the peripheral factors mediating the central gastroprotective effect of SP.

Blockade of muscarinic cholinergic receptors by atropine (1 mg/kg i.v., Fig. 5A), the prostaglandin synthesis by indomethacin (20 mg/kg p.os., Fig. 5B) and the nitric oxide synthesis by L-NNA (3 mg/kg i.v., Fig. 5C) all significantly reduced the mucosal protective effect of SP.

CGRP and somatostatin levels of the gastric mucosa were also measured with radioimmunoassay. Acidified ethanol given orally dramatically decreased the mucosal concentration of both CGRP and somatostatin. Centrally injected SP (18.5 pmol/rat) almost completely reversed the effect of ethanol on the CGRP level (Fig. 6A), and caused a slight elevation of the somatostatin concentration as well, although this effect was not significant (Fig. 6B). In contrast, i.v. administered SP (0.74 nmol/kg) failed to influence the ethanol-induced reduction of mucosal CGRP and somatostatin.

4. Discussion

It was first demonstrated that centrally (i.c.v.) injected substance P induces gastroprotective effect and inhibits the direct necrotizing action of acidified ethanol. We

found that SP was effective only in lower dose range (9.3 - 37 pmol i.c.v.), while at higher doses the gastroprotective effect diminished. It might be speculated that SP in high dose may act not only in the central nervous system, but also in the periphery, and induces such mechanisms which counteract its protective action, e.g. reduced mucosal blood flow, mast cell degranulation or formation of reactive oxygen species (Gazzieri et al., 2007; Karmeli et al., 1991; Yokotani and Fujiwara, 1985). Similar bell-shaped dose-response curve has been observed at other SP-induced effects as well, like in supraspinal antinociception (Frederickson et al., 1978), in improvement of learning and memory (Kertes et al., 2009) or in the case of its anxiolytic-like effect (Hasenöhrl et al., 1998). It is also worthy of note that the bell-shaped dose response relationship is not a unique property of SP, it has also been described for several other neuropeptides, for example for nociceptin, nocistatin, β-endorphin or somatostatin (Gyires et al., 2000; Hernandez et al., 1983; Zádori et al., 2008).

As mentioned above, in contrast with the centrally induced effect, SP given i.v., i.p. or s.c. either failed to reduce ethanol-induced mucosal damage (Evangelista et al., 1989), or aggravated it (Gazzieri et al., 2007; Karmeli et al., 1991). In our experimental model i.v. injected SP had no significant effect on the ethanol-induced lesions.

Although the terms “gastric cytoprotection” and “gastroprotection” are used since decades (Robert et al., 1979; Rainsford and Whitehouse, 1980; Szabó et al., 1981), and refer to the ability of various compounds to prevent the formation of gastric ulcers without influencing gastric acid secretion, the exact mechanism of this gastroprotective effect is still not clear, though several peripheral and central factors have already been reported to be involved in it (Mózsik et al., 2011). The main purpose of this study was to clarify, which mechanisms may be responsible for the mucosal protective effect of SP. First we analyzed the involvement of the central NK receptors. Since SP is the ligand of NK1 receptors primarily, we supposed that the SP-induced mucosal protection is mediated by the NK1 receptors.

However, our results suggest that besides NK1 receptors, NK2 and NK3 receptors may also mediate the effect. These results are in agreement with the findings that demonstrate the presence of all three tachykinin receptors in the DVC (Dixon et al., 1998; Mazzone and Geraghty, 2000), a key region in the central modulation of GI functions. Although the involvement of NK2 and/or NK3 receptors in the central effect of SP is unexpected, it is not unprecedented. For example, it was observed that both NK1 and NK2 receptors are involved in the excitatory effect of SP on neurons of the DMV projecting to the stomach (Lewis and Travagli, 2001) and both NK2 and NK3 receptors seem to mediate the inhibitory effect of SP on pancreatic ductal bicarbonate secretion (Kemény et al., 2011). Moreover, all three tachykinin receptors mediate the postsynaptic action of SP on rat periaqueductal grey neurons in vitro (Drew et al., 2005).

The ability of central NK2 receptors to regulate gastric acid secretion and mucosal integrity is also supported by the findings of Improta et al. (1997), who found that the selective NK2 agonist Ala^5NKA(4-10) injected i.c.v. reduced the secretion of gastric acid and the formation of mucosal lesions in an acid-dependent ulcer model (pylorus ligation) in rats.

It also can be raised that SP by activating NK1 receptors may release other endogenous tachykinins, that activate NK2 and NK3 receptors. The DVC in rats is enriched with axon terminals containing NKA and NKB (Colin et al., 2002), and the activation of NK1 receptors in the NTS has been shown to increase the release of glutamate (Bailey et al., 2004), which, in turn can lead to the release of neurokinins (Colin et al., 2002). However, further studies are needed to clarify, whether the release of NKA and NKB is involved in the gastroprotective effect of SP.

Correlation between gastric motor activity and gastric mucosal protection has been described, namely, 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 (Guidobono et al., 1998), dopamine or capsaicin (Takeuchi et al., 1988, 1991). Direct injection of SP (135 - 405 pmol) into the DMV (Krowicki and Hornby, 2000) or into the NTS (Spencer and Talman, 1986) has been reported to inhibit gastric motor activity, therefore we investigated, whether the effect of SP on gastric motility 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 and Broccardo (1990), who found that SP given i.c.v. inhibited only moderately the gastric emptying even in a high dose (6 nmol).

Centrally administered SP has been reported to inhibit feeding behaviour in both rats (Dib, 1999) and recently in chicks (Tachibana et al., 2010). Several orexigenic and anorexigenic gut hormones (e.g. amylin, peptide YY, ghrelin, leptin, cholecystokinin) have been shown to inhibit gastric mucosal ulceration in both acid-dependent and independent ulcer models (reviewed by Gyires, 2004; Taché, 2012). Though, it can be speculated that the altered feeding status and gastrointestinal motility is related to their mucosal protective effect physiologically, it is not likely that the effect of SP on feeding behaviour plays a role in its gastroprotective effect. Especially as the anorexigenic effect of SP could be observed only at higher (nanomolar) dose range, while the gastroprotective action could be induced by picomolar doses.

The possible role of the endogenous opioids has also been analyzed in the central gastroprotective action of SP, because several lines of evidence suggest that SP may be able to release endogenous opioids (Kream et al., 1993; Naranjo et al., 1986; Parenti et al., 2012),

and opioids have been shown to induce gastroprotection by central mechanism in much lower doses than those required to induce antinociceptive effect (Gyires et al., 2013; Gyires and Rónai, 2001; Al-Khrasani et al., 2012). Moreover, endogenous opioids are likely to mediate the gastroprotective effect of several compounds, like clonidine (Gyires et al., 2000), nociceptin, nocistatin (Zádori et al., 2008) and cannabinoids (Shujaa et al., 2009).

In accordance with the above data, 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 et al. (1993) 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).

Because the 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 the highly selective μ-opioid receptor agonist endomorphins (Zadina et al., 1997), that mediate the gastroprotective effect. Endomorphins are widely distributed in the CNS, also in several hypothalamic nuclei and in the NTS (Martin-Schild et al., 1999). Their co-localization with SP and CGRP in primary sensory neurons and different brain regions is supposed to provide a complex control of the transmission of nociceptive and autonomic informations (Greenwell et al., 2007), which is also supported by the findings showing that the binding of endomorphins to μ-opioid receptors may be influenced by TRPV1 receptors (Wollemann et al., 2013).

Our experiments focused on the potential role of EM-2, because it is more abundant in the lower brain stem compared to EM-1 (Martin-Schild et al., 1999), and it has been shown to be co-localized with SP in the NTS (Greenwell et al., 2007). 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.

In the last step we investigated, how the SP-induced protective effect is conveyed to the periphery. Several neuropeptides have been shown to exert gastroprotective action after central administration, e.g. adrenomedullin, thyrotropin-releasing hormone or peptide YY in a vagal dependent mechanism (Kaneko et al., 1998; Kato et al., 1994; Yang et al., 1999). Namely, dual effects of the vagal nerve have been described; activation of vagal efferents beside stimulation of gastric acid secretion, may induce gastric mucosal prostaglandin and NO production as well as activation of efferent function of capsaicin-sensitive afferent fibers containing CGRP (Gyires, 2004; Taché, 2012).

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.

Moreover, the mucosal content of CGRP was dramatically decreased after oral administration of acidified ethanol in agreement with the findings of Evangelista et al. (1993). CGRP, as mentioned above, has a major role in the maintenance of gastric mucosal integrity via increasing mucosal blood flow partly directly by acting on vascular smooth muscle, partly indirectly, by releasing NO (Holzer et al., 1993) and somatostatin (Bunnett et al., 1990). As our result 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 in the gastric mucosa suggesting that somatostatin may not be involved or has only minor role in the CGRP-mediated gastric mucosal protective action.

5. Conclusions

In summary, the present results indicate that SP given i.c.v. in picomolar dose-range exerts a strong gastroprotective action, which is mediated by all the three (NK1, NK2 and NK3) neurokinin receptors, and - at least partly - by the endogenous opioid system and EM-2. This centrally initiated effect is conveyed to the periphery by a vagal-dependent pathway, and in the gastric mucosa CGRP, prostaglandins and NO may mediate the mucosal defense.

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Figure captions

Fig. 1.

Macroscopic picture of the acidified ethanol-induced gastric mucosal damage in rats treated either with saline, or with substance P i.c.v. (18.5 pmol/rat) or i.v. (0.74 nmol/kg). Acidified ethanol was given orally 10 min after the i.c.v. injection. The dark, livid areas represent the hemorrhagic ulcerous part of the mucosa.

Fig. 2.

The inhibitory effect of substance P on ethanol-induced gastric mucosal injury in rats. SP was injected either intracerebroventricularly (i.c.v., 4.6-148 pmol/rat, Fig. 2A) or intravenously (i.v., 0.37-7.4 nmol/kg, Fig. 2B) 10 and 15 min before the ethanol challenge, respectively. Each column represents mean ± S.E.M., the number of animals was 5 per group. *P<0.05, **P<0.01; ***P<0.001 (ANOVA, Newman-Keuls post hoc test, compared with the respective control group).

Fig. 3.

The effect of L-733,060 (L-733, 1 nmol/rat i.c.v., Fig. 3A), GR 159897 (GR, 0.5 nmol/rat i.c.v., Fig. 3B) and SB 222200 (SB, 1 nmol/rat i.c.v., Fig. 3C) on the gastroprotective effect of substance P (SP, 18.5 pmol/rat i.c.v.). SP was injected 10 min before the ethanol challenge either alone, or in combination with the different antagonists.
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).

Fig. 4.

The effect of naloxone (NX, 27 nmol/rat i.c.v., Fig. 4A), β-funaltrexamine (β-FNA, 20 nmol/rat, Fig. 4B), naltrindole (Nalt, 5 nmol/rat i.c.v., Fig. 4C), norbinaltorphimine (norBNI, 14 nmol/rat i.c.v., Fig. 4D) and endomorphin-2 antiserum (EM-2 AS, Fig. 4E) on the gastroprotective effect of substance P (SP, 18.5 pmol/rat i.c.v.). SP was injected 10 min before the ethanol challenge either alone (dissolved in saline or in non-reactive rabbit serum (NRS)), or in combination with the antagonists (opioid receptor antagonists or endomorphin-2 antiserum).

Each column represents mean ± S.E.M., n=5; **P<0.01, ***P<0.001 compared with saline- (or NRS-) 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).

Fig. 5.

The effect of atropine (Atr, 1 mg/kg i.v., Fig. 5A), indomethacin (Indo, 20 mg kg p.os, Fig. 5B), and N^{G}-nitro-L-arginine (L-NNA, 3 mg/kg i.v., 5C) on the gastroprotective effect of substance P (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).

Fig. 6.

The effect of substance P (SP) on the mucosal CGRP (Fig. 6A) and somatostatin concentration (Fig. 6B). SP was injected either intracerebroventricularly (i.c.v., 18.5 pmol/rat)

or intravenously (i.v., 0.74 nmol/kg). 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).

***Highlights**

1. Substance P via acting on central NK1-3 receptors induces gastroprotection.
2. Endomorphin-2 is likely to play a role in the SP-induced gastroprotective effect.
3. The centrally induced effect is mediated by the vagal nerve to the periphery.
4. In the periphery prostaglandins, nitric oxide and CGRP mediate the effect.
5. Alterations in gastric motor activity do not contribute to the gastroprotection.

Figure1

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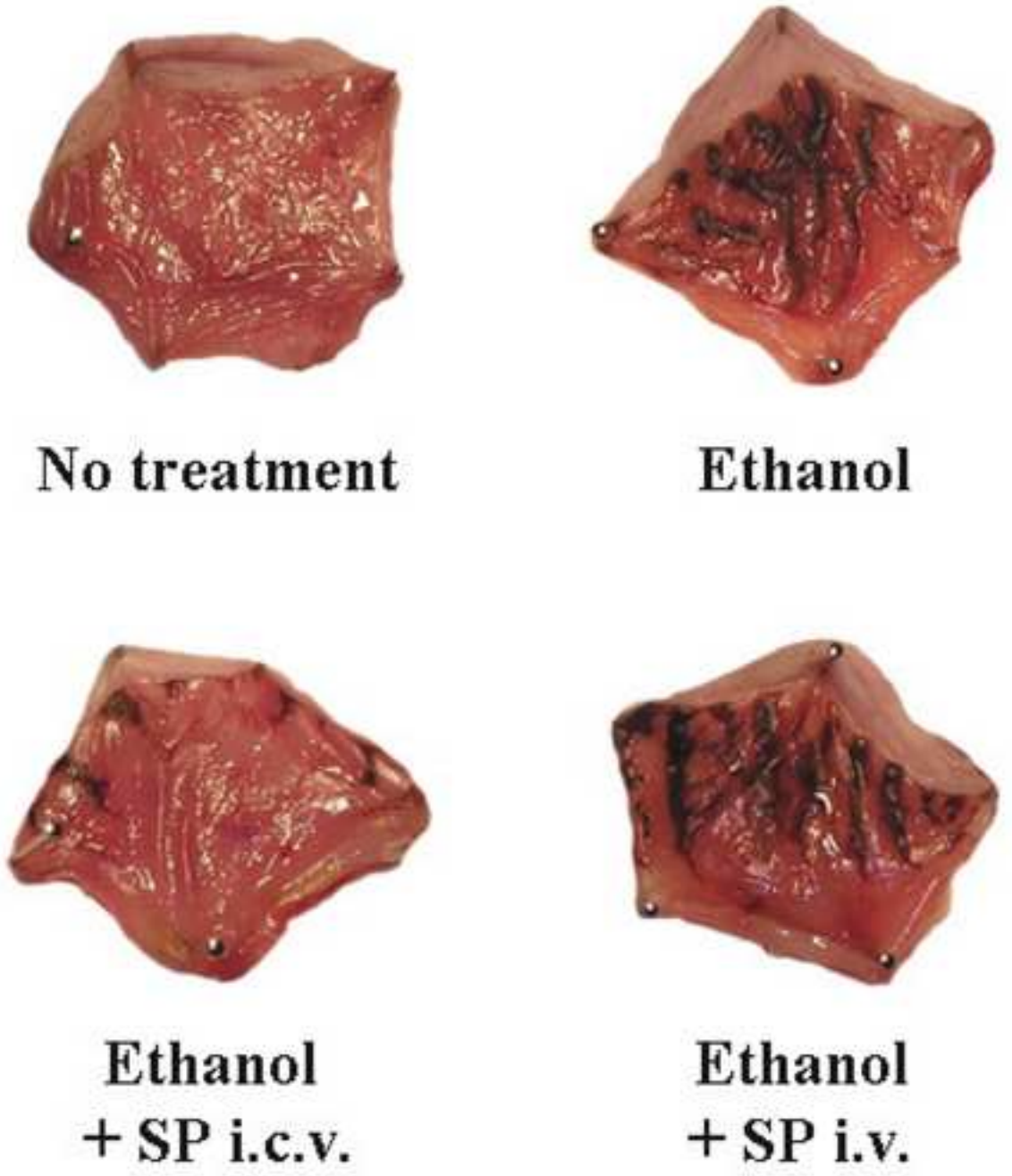

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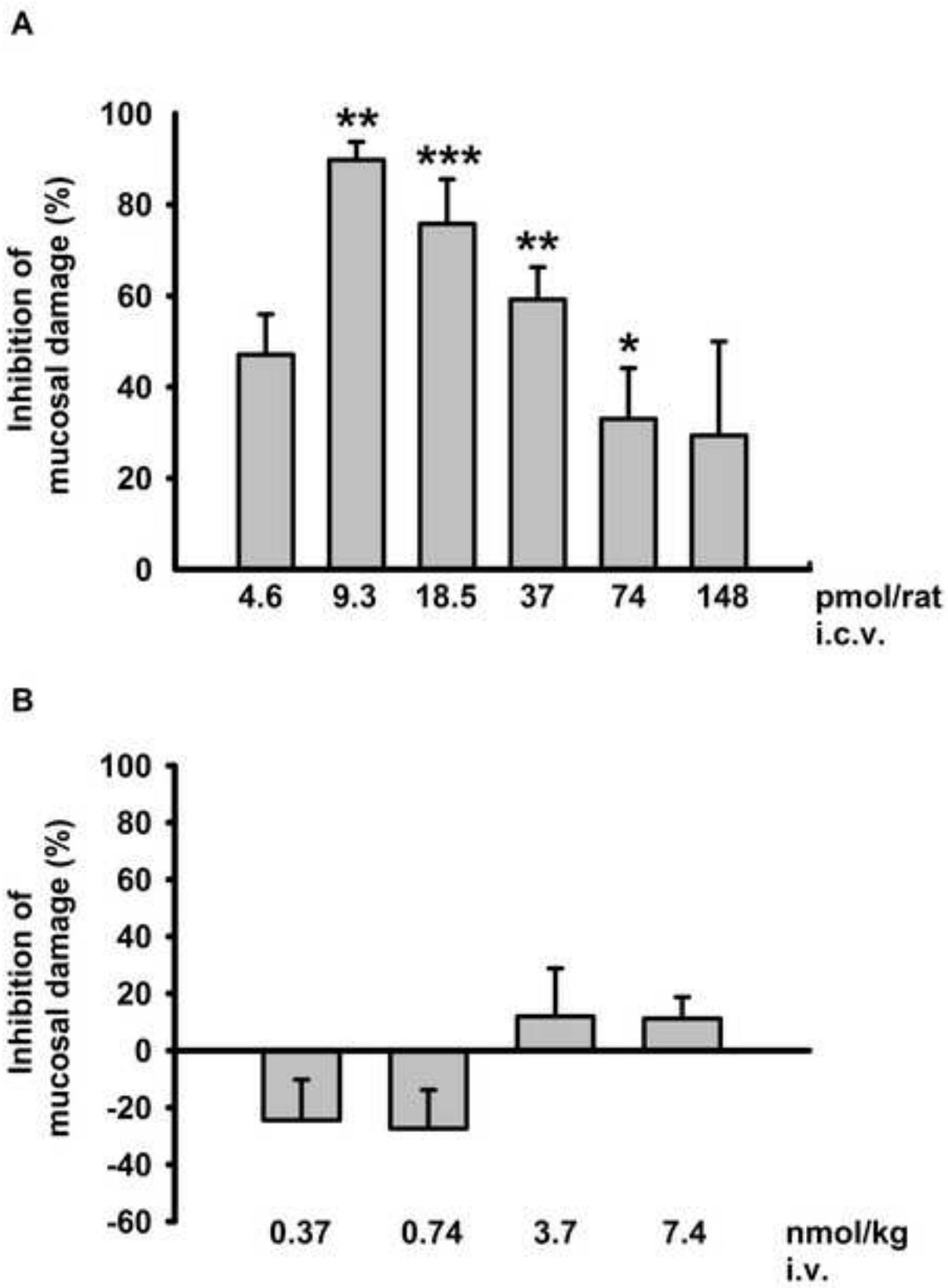

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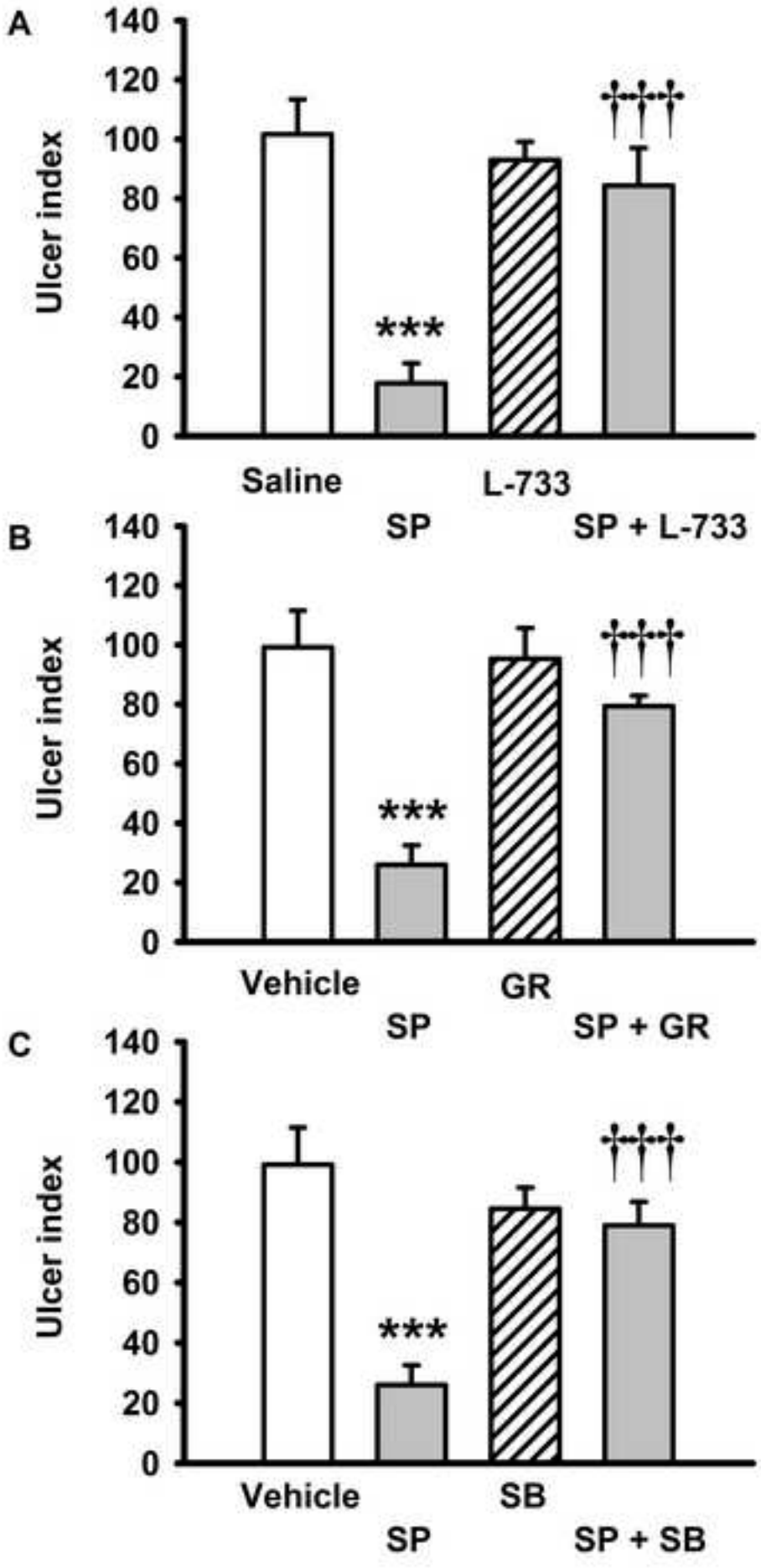

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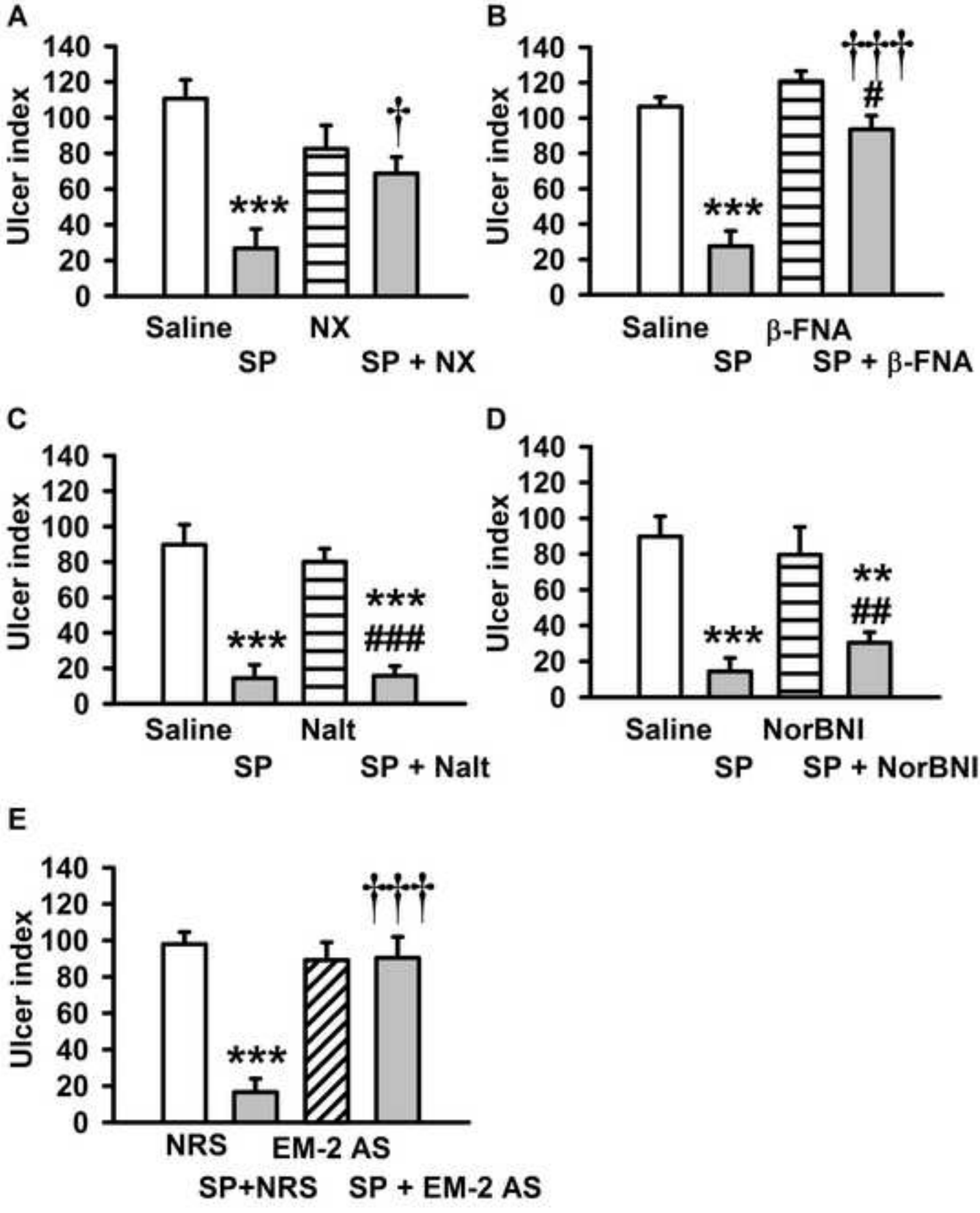

Figure 5

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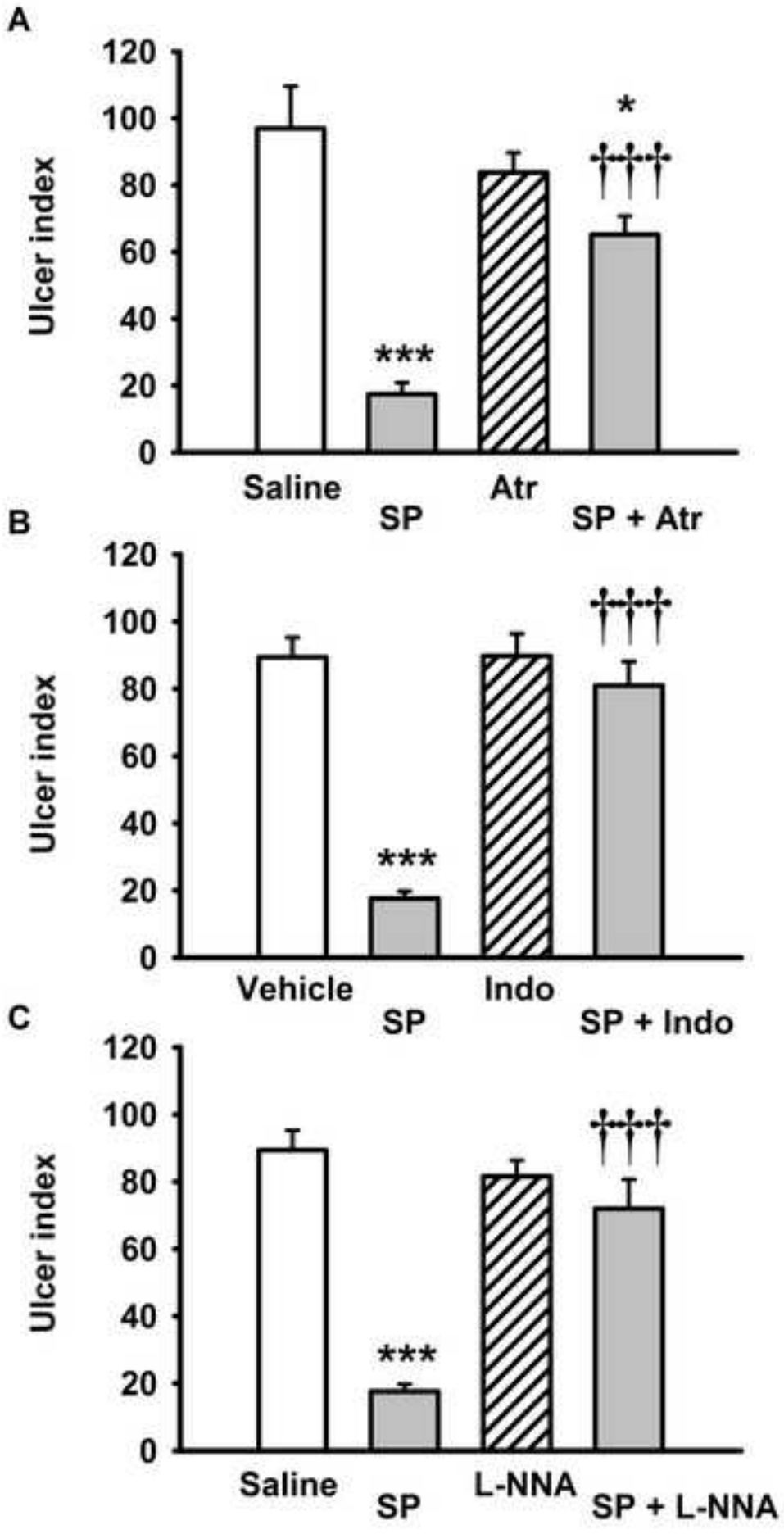

Figure 6

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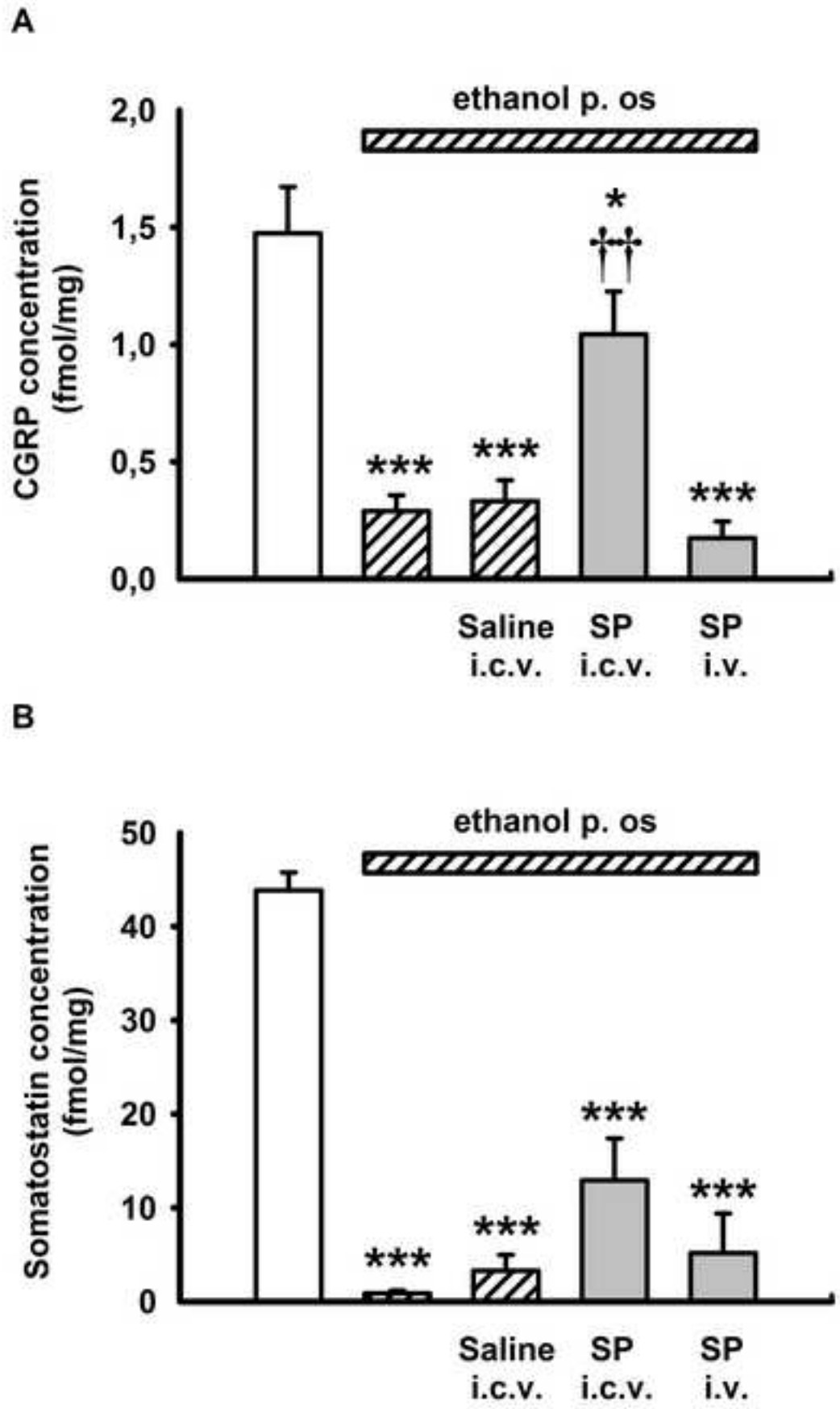

Table 1.

The list of antagonists and their doses.

Antagonist	Route of administration	Applied dose	Reference
Atropine	i.v.	1 mg/kg	Gyires *et al.* 2000
β-funaltrexamine	i.c.v.	20 nmol/rat	Zádori *et al.* 2008
GR 159897	i.c.v.	0.5 nmol/rat	-
Indomethacin	p. os	20 mg/kg	Gyires & Rónai 2001
L-733,060	i.c.v.	1 nmol/rat	-
L-NNA	i.v.	3 mg/kg	Gyires & Rónai 2001
Naloxone	i.c.v.	27 nmol/rat	Zádori *et al.* 2008
Naltrindole	i.c.v.	5 nmol/rat	Zádori *et al.* 2008
Nor-Binaltorphimine	i.c.v.	14 nmol/rat	Zádori *et al.* 2008
SB 222200	i.c.v.	1 nmol/rat	Haley & Flynn 2007

Table 2.

The effect of SP given intracerebroventricularly on the gastric motor activity.

Values indicate the percentage of the baseline (preinjection) value.

Treatment	**Gastric tone (%)**	**Mean Amplitude of contractions (%)**	**n**
Saline i.c.v.	100 ± 1	99 ± 17	5
SP 7.4 pmol i.c.v.	100 ± 1	98 ± 9	5
SP 74 pmol i.c.v.	102 ± 3	99 ± 10	6
SP 740 pmol i.c.v.	98 ± 2	76 ± 10	5