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Evidence for the gastric cytoprotective effect of centrally injected agmatine

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Short title: Agmatine induces vagally-mediated gastroprotection

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1. Introduction.

Agmatine, an endogenous aminoguanidine, has long been known in lower life forms as a metabolic intermediate in polyamine synthesis, but its biosynthesis in mammalian tissues has been recognized only in 1994 (Li et al., 1994). It is present in low (pico- and nanomolar) concentrations in many organs (Raasch et al., 1995), and originates from arginine by decarboxylation, although a significant portion is probably absorbed from the gastrointestinal (GI) tract as well (Li et al., 1994; Molderings et al., 2003).

It was originally described as an endogenous ligand of the imidazoline I_1 receptors, but subsequent studies revealed that agmatine has much wider actions. It is a neuromodulator and co-transmitter, which is capable to interact with multiple molecular targets, including several receptors (e.g. imidazoline I_1 and I_2, alpha$_2$-adrenergic, nicotinic Ach, $5HT_3$ or NMDA), ion channels (voltage-gated Ca^{2+} channels, ATP-sensitive K^+ channels) or enzymes (e.g. all isoforms of nitric oxide synthases) (recently reviewed by Molderings and Haenisch, 2012; Piletz et al., 2013). Furthermore, it possesses cytoprotective action by scavenging free radicals and protecting mitochondrial functions (Arndt et al., 2009). Accordingly, over the past two decades numerous effects, including neuro-, nephro- and cardioprotection have been attributed to agmatine and ample evidence has accumulated that it might have beneficial effect in the treatment of various diseases, like neuropsychiatric disorders, hypertension or diabetes mellitus (Molderings and Haenisch, 2012; Moretti et al., 2014; Piletz et al., 2013).

Both agmatine and imidazoline binding sites are localized in the GI tract (Houi et al., 1987; Molderings et al., 1999b; Raasch et al., 1995), and various studies have been conducted to assess whether agmatine has a role in the regulation of GI functions. However, there is still controversy over the effects of agmatine on the gastric mucosal integrity. Early investigations reported that it aggravates stress- and ethanol-induced gastric mucosal damage probably by acting on imidazoline receptors (Glavin et al., 1995; Utkan et al., 2000), but a recent study

demonstrated that sub-chronic oral administration of agmatine in high dose (about 100 mg/kg) is safe, and does not lead to gastric mucosal damage (Gilad and Gilad, 2013). Moreover, Al Masri and El Eter (2012) found that agmatine has protective effect against ischemia reperfusion injury and it has also been raised that the presence of agmatine (a strong base) in the mucosa of the stomach may enhance mucosal defense against gastric acid (Steer, 2009). Furthermore, agmatine is able to interact with alpha$_2$-adrenergic receptors (Li et al., 1994; Piletz et al., 1995) and with the endogenous opioid system (Wu et al., 2008), which both have been implicated in mucosal protection (Gyires and Rónai, 2001; Gyires et al., 2000).

Many lines of evidence indicate that besides peripheral factors also central nervous system (CNS) has significant influence on the development of gastric erosions. Numerous neuropeptides induce gastroprotection after central administration (for reviews see Gyires, 2012; Tache, 2012), which is mediated (in most cases) by a common peripheral effector pathway, namely the activation of vagal efferents and the consequent release of prostaglandins, nitric oxide (NO) and CGRP (originating from the capsaicin sensitive primary afferent nerves).

Beside the dorsal vagal complex (DVC) the hypothalamus has also significant impact on GI functions and gastric mucosal defense (Gyires et al., 2013; Tache, 2012). It is of interest that agmatine is localized in both the DVC and hypothalamus (Otake et al., 1998), and the highest activity of both arginine decarboxylase and agmatinase (the enzymes responsible for the synthesis and degradation of agmatine) was found in the hypothalamus (Iyo et al., 2006; Sastre et al., 1996). These findings imply that agmatine, besides its role in the etiopathogenesis of various CNS disorders (Moretti et al., 2014; Uzbay, 2012) may also be involved in the central regulation of gastric mucosal integrity.

Therefore, the present study aimed to analyze the potential gastroprotective effect of centrally and peripherally injected agmatine on gastric mucosal defense and to investigate the

involvement of imidazoline I_1 receptors, alpha$_2$-adrenergic receptors and the endogenous opioid system in the agmatine-induced action. In addition, we aimed to identify, which peripheral factors (e.g. release of local mediators or alterations in gastric motility) are involved in the gastroprotective effect of agmatine.

2. Materials and methods

2.1. Animals.

For all experiments male Wistar rats were used. The animals were housed in a temperature- and humidity-controlled room at a 12-h light/dark cycle under conditions of animal housing and experimentation according to ethical guidelines issued by the Ethical Board of Semmelweis University, based on EC Directive 86/609/EEC.

After one week habituation rats were randomly divided into the experimental groups (5-8 rats/group). All procedures conformed to the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes, and all efforts were made to minimize the suffering of animals. The experiments were approved by the Animal Ethics Committee of Semmelweis University, Budapest (permission number: 22.1/606/001/2010).

2.2. Experimental procedures

2.2.1. Gastric mucosal damage induced by acidified ethanol

Gastric mucosal lesions were induced by acidified ethanol (98 ml absolute ethanol + 2 ml concentrated HCl), which was given intragastrically in a volume of 0.5 ml/rat by an oral gavage using a stainless steel cannula. The experiments were performed on young 6 weeks old male Wistar rats (140-170 g), because our former study revealed that gastric mucosal susceptibility to ethanol and the gastroprotective effect of opioid peptides, capsaicin and prostaglandin E_2 is age-related, and mucosal defensive processes are more efficient in 6-8 weeks old rats than in elder ones (Gyires and Barna, 2002). Before the experiments rats were

deprived of food for 24 h with free access to tap water. 60 min after the injection of ethanol the animals were sacrificed, 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 lesion index based on a 0-4 scoring system described previously (Gyires, 1990). The lesion index was calculated as the total number of lesions multiplied by the respective severity factor.

In order to determine the effect of agmatine on ethanol-induced mucosal damage, in four consecutive experiments a total of 80 rats were randomly divided into 16 groups (5 rats/group) and agmatine was injected either into the lateral brain ventricle (intracerebroventricularly, i.c.v.) 10 min before the ethanol challenge in a volume of 10 μl, or intraperitoneally (i.p.) in a volume of 0.5 ml/100 g 20 min before the administration of ethanol, as described previously (Gyires et al., 2000), in the following doses: 0.044, 0.22, 0.88, 1.76, 4.4, 44 and 220 nmol/rat i.c.v. and 0.001, 0.005, 0.02, 0.1, 1, 10 and 50 mg/kg i.p.

To compare the effect of the applied doses and to establish the dose-response relationships, results were expressed as the percentage of the lesion indices of the respective control groups.

In another experiment agmatine was injected directly into the lateral hypothalamus (LH), in order to analyze the role of this nucleus in the gastroprotective action. 15 rats were anesthetized with pentobarbital (35 mg/kg i.p.), and guide cannulas (Bilaney Consultants, Düsseldorf, Germany) were implanted with stereotaxic surgery (Stoelting, IL, USA) and fixed with dental cement (Adhesor Cement, Spofa Dental, Jiˇcín, Czech Republic). After 3 days recovery, rats were randomly divided into 2 groups (7-8 rats/group), and either saline or agmatine (0.88 nmol/rat) was injected into the LH in a volume of 1 μl, 10 min before the ethanol challenge. For the injection the following coordinates were used (relative to bregma): posterior 1.8 mm; lateral 2.0 mm; ventral 8 mm (Paxinos and Watson, 1986). The site of

injection was subsequently confirmed histologically, and only the animals with appropriately placed injection sites were used for data analysis.

In additional experiments various antagonists were combined with agmatine. They were given either i.c.v. (together with i.c.v. injected agmatine, in a total volume of 10 μl), or intravenously (i.v.), subcutaneosly (s.c.) or orally (15 min, 20 min or 60 min before the i.c.v. injection of agmatine, respectively, in a volume of 0.5 ml/100 g) (Gyires et al., 2000; Gyires et al., 2014). The applied doses of drugs were selected based partly on our preliminary results, partly on the literature data.

2.2. Bilateral cervical vagotomy

Under pentobarbital anesthesia (35 mg/kg i.p.), the cervical section of vagal nerves was exposed and bilateral cervical vagotomy was performed. Vagotomy slowed the respiration phase and enlarged the magnitude of respiration. Sham operated control rats had their vagus similarly exposed but the vagal trunks were not sectioned. The incisions were closed and all animals were allowed 3 h recovery from operation (Gyires et al., 2014).

2.2.3. Measurement of gastric mucosal level of CGRP and somatostatin

Rats were euthanized, their 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-^{125}I-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.

2.2.4. In vivo measurement of gastric motor activity

Gastric motility was measured in anesthetized rats with the rubber balloon method (Zádori and Gyires, 2013; Zádori et al., 2007). Briefly, after 24 h food deprivation 200-300 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 40-60 min equilibrium period was registered before every experiment. Agmatine 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 were implanted with stereotaxic surgery, and the following coordinates were used (relative to bregma): posterior 0.8 mm; lateral 1.6 mm; ventral 4.5 mm (Paxinos and Watson, 1986). The site of 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, 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 agmatine. Values were expressed in percentage of the basal (pre-injection) values.

2.3. Materials

Agmatine sulfate, idazoxan hydrochloride, yohimbine hydrochloride, efaroxan hydrochloride, naloxone hydrochloride, naltrindole hydrochloride, nor-Binaltorphimine dihydrochloride (norBNI), N^G-nitro-L-arginine (L-NNA) and indomethacin were all purchased from Sigma Chemical Co. (St. Louis, USA).

AGN 192403 ((±)-2-endo-Amino-3-exo-isopropylbicyclo [2.2.1] heptane hydrochloride) and β-funaltrexamine hydrochloride were ordered from Tocris Bioscience (Bristol, UK).

All drugs were dissolved in saline, with the exception of indomethacin, which was suspended in 1% methylcellulose. Control animals received the drug solvents.

2.4. Statistical analysis.

Statistical analysis of the data was performed either with Student's *t*-test (two treatment groups), or with one-way ANOVA followed by Newman-Keuls post-hoc test. 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 centrally and peripherally injected agmatine on the ethanol-induced mucosal damage.

Acidified ethanol given orally induced severe mucosal damage (lesion index: 92.3 ± 8.4, n=20), which was significantly attenuated by i.c.v. injected agmatine (Fig. 1, Table 1). The maximal protection was achieved at a dose of 0.88 nmol/rat (lesion index: 35.8 ± 9.5, which is 27 % of that of the respective control group: 132.4 ± 13.7, n=5/group). The dose-response curve of agmatine proved to be U-shaped, because at higher doses (44 and 220 nmol/rat) the protective effect declined (lesion indices were 45 and 68.5 % of the control groups).

In contrast to the i.c.v. administration, agmatine given i.p. had only minor effect on the development of mucosal lesions (Fig. 1, Table 1). A slight, but statictically significant reduction of mucosal damage was observed at three doses (0.005, 0.02 and 0.1 mg/kg, in which groups the lesion indices were 75, 65 and 72 % of the control groups, respectively).

When agmatine (0.88 nmol/rat) was injected directly into the LH, it induced only a partial protective effect: it inhibited almost completely the formation of mucosal lesions in two of five rats, but had minor or no effect in the other three animals (Fig. 2). The mean lesion index of the control group did not differ significantly from that of the agmatine-treated group (105.5 ± 7.7 vs 80.8 ± 22.3, $p<0.37$).

3.2. The involvement of central imidazoline receptors and alpha$_2$-adrenoceptors in the mucosal protective action.

The gastroprotective effect of i.c.v. injected agmatine was completely inhibited by the non-selective alpha$_2$-adrenoceptor and imidazoline I_1 receptor antagonists efaroxan and idazoxan (4 nmol/rat and 160 nmol/rat, respectively), and partially antagonized by the alpha$_2$-adrenoceptor antagonist yohimbine (50 nmol/rat) (Figs. 3A-3C). These antagonists alone did not influence the ethanol-induced mucosal damage.

In contrast, the highly selective I_1 receptor ligand (putative antagonist) AGN 192403, besides reducing the effect of agmatine (lesion indices: 10.4 ± 6.0 vs 59.3 ± 18.8, $p<0.05$), also elicited a dose-dependent protection per se (0.52 - 52 nmol/rat i.c.v.). The maximal effect was achieved at the highest tested dose (lesion index was 40 % of the control group) (Figs. 3D and 3E).

3.3. The interaction of agmatine with the endogenous opioid system.

Pretreatment with naloxone (a non-selective opioid receptor antagonist, 1 mg/kg s.c.) reversed the protective action of centrally injected agmatine (Fig. 4A), therefore in the next step subtype-selective antagonists were used. Only naltrindole (a selective δ-opioid receptor

antagonist, 0.5 nmol/rat i.c.v.) could inhibit the effect of agmatine, neither the selective μ-opioid receptor antagonist β-funaltrexamine (20 nmol/rat i.c.v.), nor the κ-opioid receptor antagonist norBNI (14 nmol/rat i.c.v.) had any significant action (Fig. 4B-4D).

3.4. The involvement of peripheral factors in the central gastroprotective effect of agmatine.

The mucosal protective effect of agmatine was abolished by bilateral cervical vagotomy, and by pretreatment with the prostaglandin synthesis inhibitor indomethacin (20 mg/kg p.os.) and the NO synthase inhibitor L-NNA (3 mg/kg i.v.) (Fig. 5A-5C). Neither vagotomy, nor any of the antagonists changed significantly the ethanol-induced damage per se.

The levels of CGRP and somatostatin in the gastric mucosa were also measured with RIA. As Figs. 5D and 5E show, their mucosal levels were markedly reduced by acidified ethanol in the control groups, but not in the agmatine-treated animals.

Finally, the effect of centrally injected agmatine on the gastric motor activity was evaluated. Agmatine in the tested, gastroprotective dose range (0.44 - 440 nmol/rat) did not influence significantly the tonic gastric pressure or the amplitude of phasic contractions (Figs. 6A and 6B).

4. Discussion.

Over the past years several studies demonstrated the cytoprotective property of agmatine in different tissues, such as in the brain, heart, retina and kidneys (Molderings and Haenisch, 2012; Piletz et al., 2013), but its effect on the gastric mucosa has remained obscure. Glavin et al. (1995) reported that agmatine, given both i.p. and i.c.v. increased gastric acid and pepsin secretion, decreased gastric glandular mucus levels and worsened stress-induced gastric mucosal injury and similar results (aggravation of ulcers by agmatine given i.p.) were found five years later by Utkan et al. (2000) in the ethanol-ulcer model. These results,

together with the findings that Helicobacter pylori (H. pylori) can produce agmatine (Molderings et al., 1999a), and agmatine can induce histamine-release from the enterochromaffin-like cells (Molderings et al., 1999c) led to the assumption that agmatine may be an important factor in the pathogenesis of peptic ulcers.

Other findings, however, suggest that agmatine is rather protective than deleterious in the stomach. For instance, it was recently reported that agmatine given i.p. significantly decreased gastric mucosal injury in the ischemia reperfusion model, probably by acting on the PI3K/Akt pathway (Al Masri and El Eter, 2012). Furthermore, both indirect and direct evidence suggest that agmatine can induce the release of endogenous opioids, like β-endorphin (Chang et al., 2010; Zomkowski et al., 2005), which has been shown to increase mucosal protective processes by activating μ- and δ-opioid receptors in the DVC (Gyires et al., 2000).

Our results provide further evidence for the protective role of agmatine. We found that i.c.v. injection of agmatine inhibited markedly, dose-dependently the development of mucosal lesions evoked by acidified ethanol. Because ethanol-induced damage is independent from gastric acid secretion and caused mainly by microvascular impairment (Oates and Hakkinen, 1988), the effect of agmatine is likely to result from its ability to recruit protective factors (see below) and enhance mucosal defense. It is of importance that agmatine was effective in pico- and nanomolar doses, which corresponds to its brain concentration in rats (Raasch et al., 1995; Zhu et al., 2008). This suggests that agmatine is able to modulate gastric mucosal integrity under physiological conditions.

Interestingly, the effect of agmatine diminished at higher doses, that is the dose-response curve proved to be U-shaped. Similar biphasic (or hormetic) effect of agmatine has been observed by other groups as well (Aricioglu and Altunbas, 2003; Lavinsky et al., 2003; Utkan et al., 2000), and also in the case of central gastroprotection induced by numerous

neuropeptides (Gyires and Zádori, 2014). The underlying mechanism of this phenomenon remains to be elucidated, but it can be speculated that agmatine in high dose activates (or inhibits) such receptors that counteract its protective action. NMDA receptor might be such a candidate, because it mediates mucosal protection (Namiki et al., 1993), but also binds agmatine with relatively low affinity, which thereafter inhibits it (Boronat et al., 1998; Gibson et al., 2002; Olmos et al., 1999). Another possibility is that agmatine in higher doses increases gastric acid secretion (Glavin et al., 1995), which aggravates the direct damaging effect of ethanol and attenuates the gastroprotective action.

Several brain nuclei take part in the centrally induced gastroprotection, such as various hypothalamic areas, the centromedial amygdala, the raphe pallidus and the DVC (Gyires, 2005; Tache, 2012). In this study we analyzed the role of LH, which has long been implicated in the central regulation of mucosal integrity (Grijalva et al., 1980) and contains agmatinergic neurons as well (Otake et al., 1998). However, in contrast to the i.c.v. injection, where agmatine induced protection in all rats, in the case of intraLH injection we found protection only in 40% of the rats, and the mean lesion index did not differ significantly from that of the control group. Thus, we could neither confirm nor rule out the involvement of LH in the protective effect of agmatine, and further studies are needed to clarify it, and the exact site of action.

In the case of systemic administration agmatine failed to remarkably influence the development of mucosal lesions at higher doses (1 - 50 mg/kg). This result differs from the findings of other groups, who reported an aggravatory effect of agmatine at similar dose range (0.5 - 20 mg/kg) (Glavin et al., 1995; Utkan et al., 2000). This apparent contradiction may result from methodological differences, because in these studies ulcers were evoked either by stress or by 50 % ethanol, and the mucosal damage is not as severe as in our model. Therefore, it is possible that agmatine in this dose range aggravates gastric injury, for example

due to increased acid secretion (Glavin et al., 1995), but this effect is not apparent in our model. We found, however, that at lower dose range (0.005 - 0.1 mg/kg) agmatine induced a mild, but statistically significant reduction of mucosal damage. Thus, the peripheral dose-response curve (similarly to the central one) has a biphasic character, and because agmatine can cross the blood-brain barrier (Piletz et al., 2003), it can be speculated, that the observed slight protection results from the entry of agmatine into the CNS.

In further experiments we aimed to identify, which receptors and mediators may play a role in the central gastroprotective effect of agmatine. Among the several possible molecular targets, where agmatine may act (Piletz et al., 2013), our study focused on the imidazoline I_1 receptors and alpha$_2$-adrenoceptors, because their mixed agonists (clonidine and rilmenidine) have already been shown to enhance gastric mucosal defense (Gyires et al., 2000; Gyires et al., 2007). As our results demonstrate, co-injection of efaroxan or idazoxan with agmatine completely inhibited the protective effect, while yohimbine, a selective alpha$_2$-adrenoceptor antagonist reduced it only partially. This indicates that both receptors may be involved in the agmatine-induced protection. To obtain further evidence for the role of I_1 receptors, we combined agmatine with AGN 192403, a highly selective putative antagonist of the I_1 receptors (Munk et al., 1996). As expected, it reduced the effect of agmatine significantly, but it also exerted dose-dependent protective effect per se. This was somewhat surprising, because in the majority of studies AGN 192403 either did not have any effect (e.g. Munk et al., 1996; Sastre-Coll et al., 1999; Zádori et al., 2013) or it behaved as a neutral antagonist (Mukaddam-Daher et al., 2006; Raasch et al., 2003). Thus, our results not only provide evidence for the involvement of I_1 receptors in the centrally induced gastroprotection, but also raise the possibility that AGN 192403 may have partial agonistic effect on these receptors.

There is ample evidence that agmatine closely interacts with the endogenous opioid system (Wu et al., 2008) and it was reported that opioid receptors participate in the

antidepressant-like and antinociceptive effects of agmatine (Santos et al., 2005; Zomkowski et al., 2005). The results presented herein show that activation of δ-opioid receptors is one link in the chain of events leading to gastroprotection, because beside naloxone also naltrindole reversed the effect of agmatine. This is in line with previous findings indicating that opioids, far below the analgesic dose range, are able to induce central gastroprotection (Al-Khrasani et al., 2012; Gyires and Rónai, 2001; Szentirmay et al., 2013) and that δ-opioid receptors play pivotal role both in the central and peripheral regulation of mucosal defense (Gyires and Rónai, 2001; Gyires et al., 1997). On the other hand, the inability of β-funaltrexamine and norBNI to influence the effect of agmatine indicate that μ- and κ-opioid receptors probably do not take part in it, in contrast to that of various other gastroprotective agents, such as substance P (Brancati et al., 2013), the endocannabinoid anandamide (Shujaa et al., 2009) or nociceptin and nocistatin (Zádori et al., 2008).

Because agmatine does not bind to opioid receptors (Bradley and Headley, 1997), the activation of δ-opioid receptors might be due to the release of endogenous opioids (as also proposed by (Zomkowski et al., 2005). This assumption is supported by the findings that agmatine can release β-endorphin in the adrenal gland of rats (Chang et al., 2010). Enkephalins are considered to be the endogenous ligands of δ-opioid receptors (Hughes et al., 1975), but it remains to be established, whether or not they are involved in the protective action of agmatine.

In the final step we analyzed how the central effect is conveyed to the periphery and which peripheral factors are involved in it. Our results suggest that activation of vagal efferent fibers and subsequent mucosal release of NO and prostaglandins are likely to play a role in the gastroprotective action of agmatine, since both cervical vagotomy and pretreatment with indomethacin or L-NNA abolished it. Similarly, vagally-mediated, NO- and prostaglandin-dependent gastroprotection has been described in the case of numerous neuropeptides, such as

adrenomedullin (Kaneko et al., 1998), thyrotropin-releasing hormone (Kato et al., 1995), opioids (Gyires and Rónai, 2001), nesfatin-1 (Szlachcic et al., 2013) or substance P (Brancati et al., 2013). NO and prostaglandins increase mucosal blood flow, stimulate mucus, bicarbonate and phospholipid secretion, and accelerate epithelial restitution and mucosal healing, therefore represent crucial factors for the maintenance of mucosal integrity (Gyires, 2005; Laine et al., 2008). Although agmatine can stimulate NO synthesis directly by activating eNOS (Mun et al., 2010), it is unlikely that it has relevance in the present case, because such a direct effect would induce mucosal protection even after vagotomy.

It is noteworthy, that in the ischemia reperfusion model high dose of agmatine (100 mg/kg i.p.) induced mucosal protection by directly inhibiting iNOS and the excessive production of NO and reactive nitrogen species (Al Masri and El Eter, 2012).

CGRP, released from the sensory afferent nerve fibers, has also a pivotal role in the maintenance of mucosal integrity. It increases mucosal microcirculation, stimulates mucin synthesis and inhibits inflammatory reactions partly directly, partly by releasing NO, prostacyclin and somatostatin (Gyires, 2005; Holzer, 2007). Somatostatin has been shown to effectively prevent ethanol- and indomethacin-induced gastric mucosal damage by reducing the elevated level of substance P, vasoactive intestinal peptide and leukotriens (Karmeli et al., 1994). As our results demonstrate, acidified ethanol given orally markedly decreased the mucosal levels of both CGRP and somatostatin, which was almost completely reversed by i.c.v. injection of agmatine. These findings suggest that the protective action of agmatine against ethanol is - at least partly – relies on its ability to release CGRP and somatostatin.

Because gastric mucosal protection may correlate with gastric motor activity (Guidobono et al., 1998; Takeuchi et al., 1991), we analyzed whether agmatine, given at the gastroprotective dose-range, has any significant effect on it. We found that neither the basal tone, nor the phasic antral contractions were influenced by centrally injected agmatine, which

is in line with previous findings showing that neither agmatine nor imidazoline receptors have relevant effect on GI motility, and the inhibitory effect of mixed alpha$_2$-adrenergic/imidazoline receptor agonists on GI motor activity is mainly mediated by alpha$_{2A}$-adrenergic receptors (Blandizzi et al., 1991; Liu and Coupar, 1997; Colucci et al., 1998; Zádori et al., 2013). Thus, changes in gastric motility are not likely to contribute to the inhibitory effect of agmatine on ulcer development.

5. Conclusions.

In summary, the present results indicate that agmatine given centrally induces gastric cytoprotection and potently inhibits the necrotizing action of acidified ethanol. This effect is mediated by various receptors in the CNS (imidazoline I_1 receptors, alpha$_2$-adrenoceptors, δ-opioid receptors), which induce the release of different mucosal protective factors, such as NO, prostaglandins, CGRP and somatostatin by a vagal-dependent mechanism.

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Figure captions

Fig. 1.

The effect of agmatine on ethanol-induced gastric mucosal injury in rats. Agmatine was injected either intracerebroventricularly (i.c.v., 0.044 - 220 nmol/rat, filled circles) or intraperitoneally (i.p., 0.001 - 50 mg/kg, empty circles) 10 and 20 min before the ethanol challenge, respectively. To compare the central and peripheral potencies of agmatine, i.p. doses are expressed in nmol/rat units similarly to the i.c.v. ones (calculated with 150 g body weight of rats). Each circle represents the mean lesion index (± S.E.M.) of 5 rats. *P<0.05, **P<0.01; ***P<0.001 (ANOVA, Newman-Keuls post hoc test, compared with the lesion index of the respective control group).

Fig. 2.

The effect of agmatine on ethanol-induced gastric mucosal injury, when injected into the lateral hypothalamus. Each diamond represents the lesion index of an individual rat, treated either with saline (SAL, empty diamonds) or with agmatine (AGM, 0.88 nmol/rat, filled diamonds). Circles represent the mean lesion indices (± S.E.M.). Student's *t*-test did not indicate statistically significant change.

Fig. 3.

Panels 3A-3D: The effect of efaroxan (EFA, 4 nmol/rat i.c.v., Fig. 3A), idazoxan (IDA, 160 nmol/rat i.c.v., Fig. 3B), yohimbine (YOH, 50 nmol/rat i.c.v., Fig. 3C) and AGN 192403 (AGN, 0.52 nmol/rat i.c.v., Fig. 3D) on the gastroprotective effect of agmatine (AGM, 0.88 nmol/rat i.c.v.). Agmatine was injected 10 min before the ethanol challenge either alone, or in combination with the antagonists. Each column represents mean ± S.E.M., n=5/group; **P<0.01, ***P<0.001 compared with saline-treated group (column 1); $^{\#}$P<0.05,

$^{\#\#}P<0.01$ compared with agmatine-treated group (column 2); $^{+}P<0.05$ compared with antagonist-treated group (column 3) (ANOVA, Newman-Keuls post hoc test).

Panel 3E: The effect of AGN 192403 on ethanol-induced gastric mucosal injury in rats. AGN 192403 was injected intracerebroventricularly (0.52 - 52.7 nmol/rat, i.c.v.) 10 min before the ethanol challenge. Each circle represents the mean lesion index (± S.E.M.) of 5 rats. ***P<0.001 (ANOVA, Newman-Keuls post hoc test, compared with the lesion index of the control group).

Fig. 4.

The effect of naloxone (NX, 1 mg/kg s.c., Fig. 4A), β-funaltrexamine (β-FNA, 20 nmol/rat i.c.v., Fig. 4B), naltrindole (NALT, 0.5 nmol/rat i.c.v., Fig. 4C) and nor-Binaltorphimine (NOR, 14 nmol/rat i.c.v., Fig. 4D) on the gastroprotective effect of agmatine (AGM, 0.88 nmol/rat i.c.v.). Agmatine was injected 10 min before the ethanol challenge. I.c.v. injected antagonists were given together with agmatine, while naloxone was given s.c. 20 min prior to it. Each column represents mean ± S.E.M., n=5/group; **P<0.01, ***P<0.001 compared with saline-treated group (column 1); $^{\#\#\#}P<0.001$ compared with agmatine-treated group (column 2), $^{+++}P<0.001$ compared with antagonist-treated group (column 3) (ANOVA, Newman-Keuls post hoc test).

Fig. 5.

Panels 5A-5C: The effect of bilateral cervical vagotomy (VAG, Fig. 5A), N^{G}-nitro-L-arginine (LNNA, 3 mg/kg i.v., 5B) and indomethacin (INDO, 20 mg/kg p.os, Fig. 5C) on the gastroprotective effect of agmatine (AGM, 0.88 nmol/rat i.c.v.). Agmatine was injected 10 min before the ethanol challenge. N^{G}-nitro-L-arginine and indomethacin were given 15 and 60 min before agmatine, respectively. Bilateral cervical vagotomy was performed under

pentobarbital anesthesia 3 h before the experiment. Each column represents mean ± S.E.M., n=5/group; ***P<0.001 compared with vehicle-treated group (column 1); $^{###}$P<0.001 compared with agmatine-treated group (column 2) (ANOVA, Newman-Keuls post hoc test).

Panels 5D-5E: The effect of agmatine (AGM, 0.88 nmol/rat i.c.v.) on the mucosal CGRP (Fig. 5D) and somatostatin content (Fig. 5E). Each column represents mean ± S.E.M., n=5/group; *P<0.05, **P<0.01, ***P<0.001 compared with absolute control group (no ethanol treatment, column 1); $^{#}$P<0.05, $^{##}$P<0.01 compared with ethanol + no treatment /saline i.c.v.-treated groups (columns 2 and 3) (ANOVA, Newman-Keuls post hoc test).

Fig. 6.

Panel A: The effect of agmatine (AGM, 0.44 - 440 nmol/rat i.c.v.) on the amplitudes of phasic antral contractions and gastric tone in anesthetized rats. Each column represents mean ± S.E.M., n=4-7/group. Paired Student's *t*-test did not indicate statistically significant difference between the pre- and postinjection values.

Panel B: A representative gastric contractility trace illustrating the effect of agmatine (AGM, 0.44 nmol i.c.v.) on the gastric motor activity in the anesthetized rat. Agmatine was injected within 5 min, with a microinjection pump.

***Highlights (for review)**

- Central injection of agmatine induces gastric cytoprotection
- The protective effect depends on the integrity of the vagal nerve
- The effect is mediated by I_1 receptors, alpha$_2$-adrenoceptors and δ-opioid receptors
- NO, prostaglandins, CGRP and somatostatin are involved in the mucosal protection

Figure1

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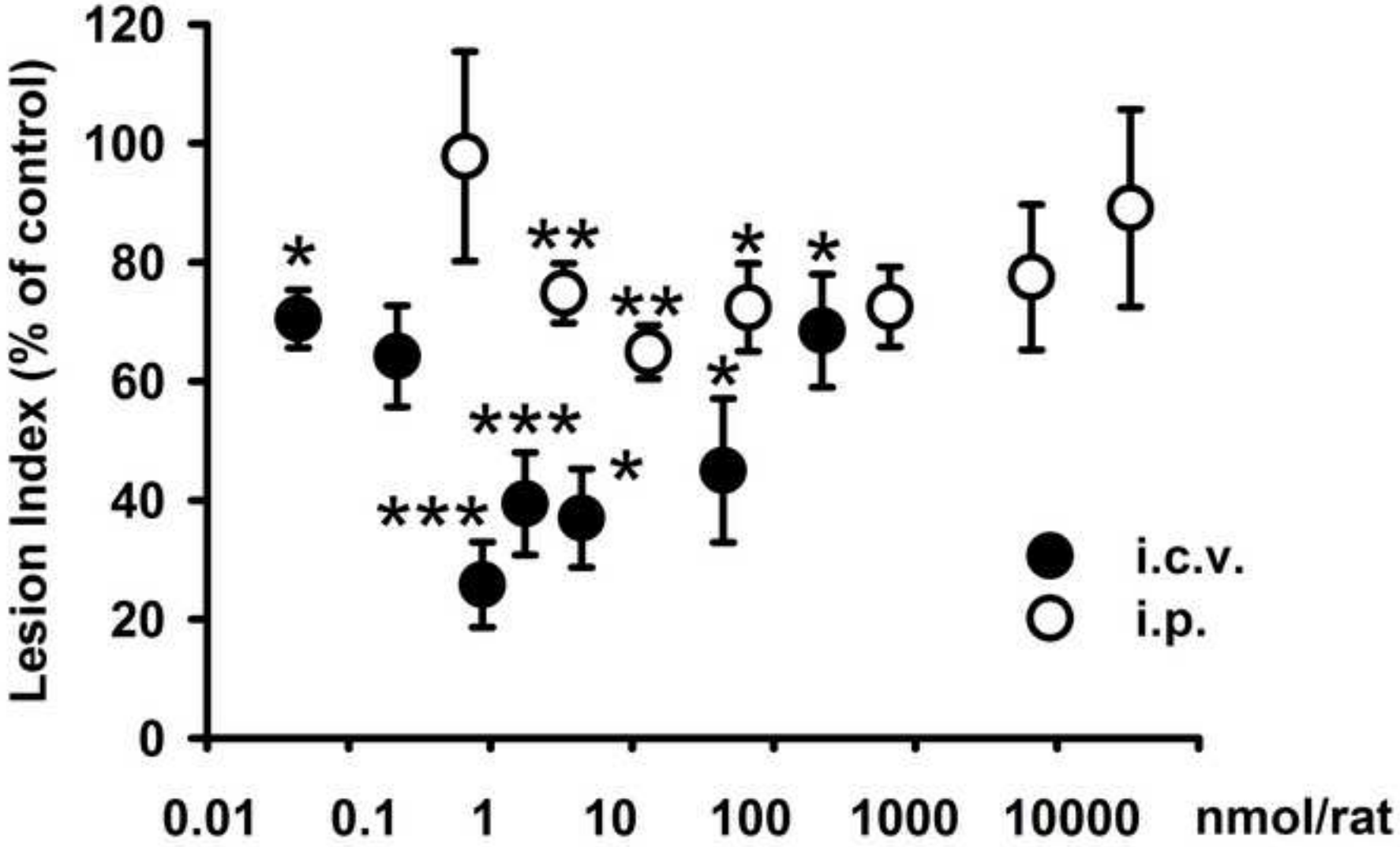

Figure2
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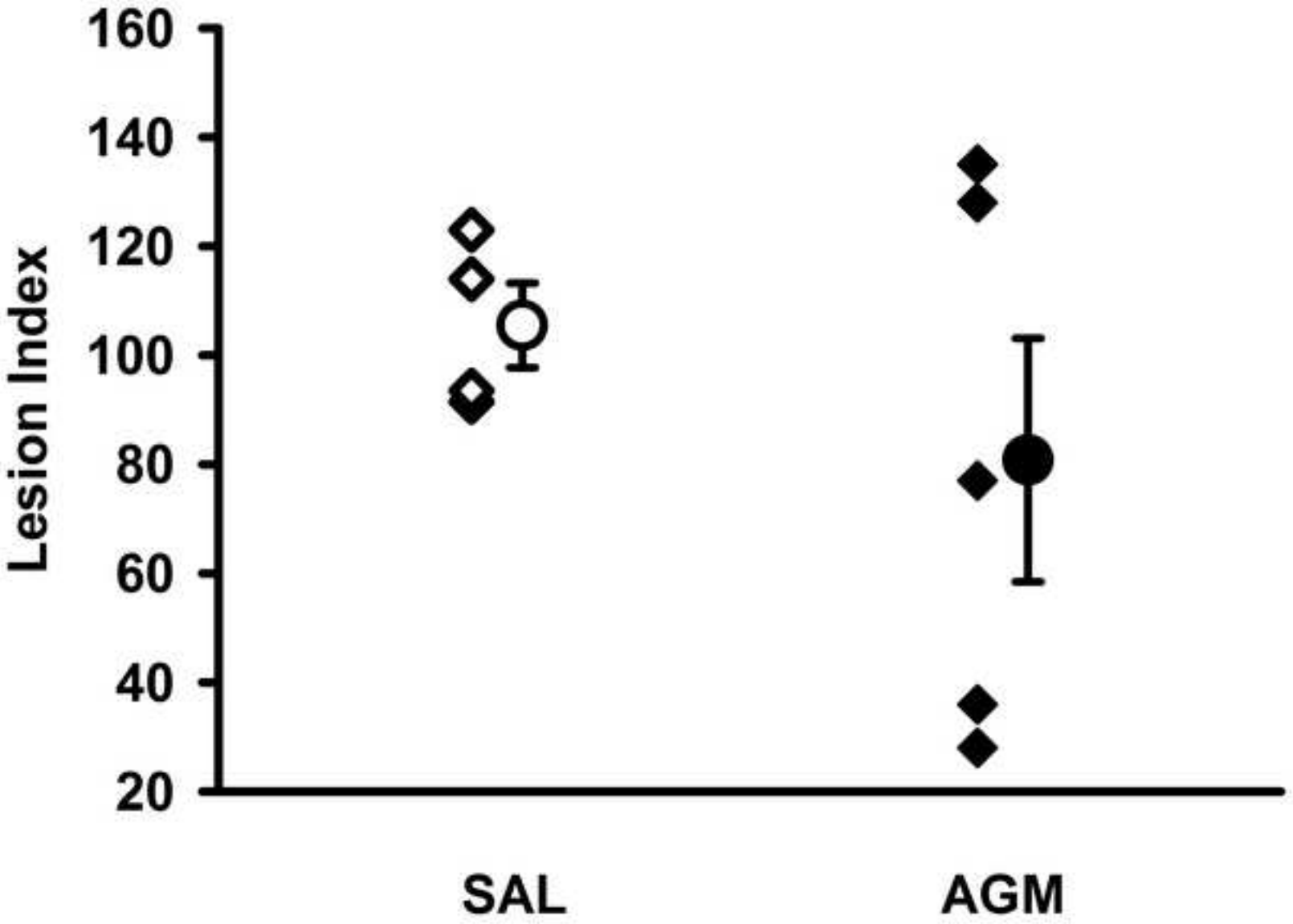

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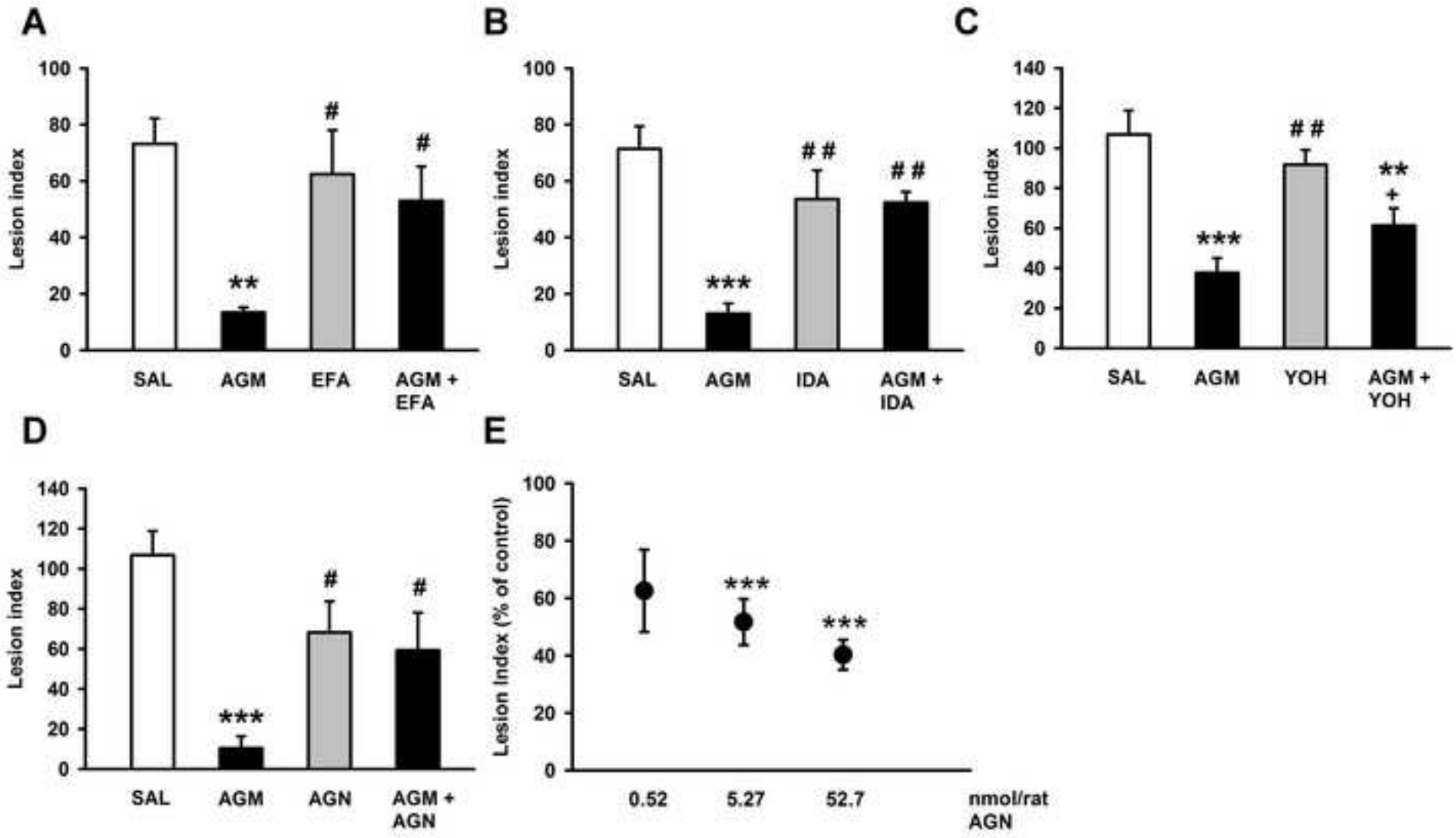

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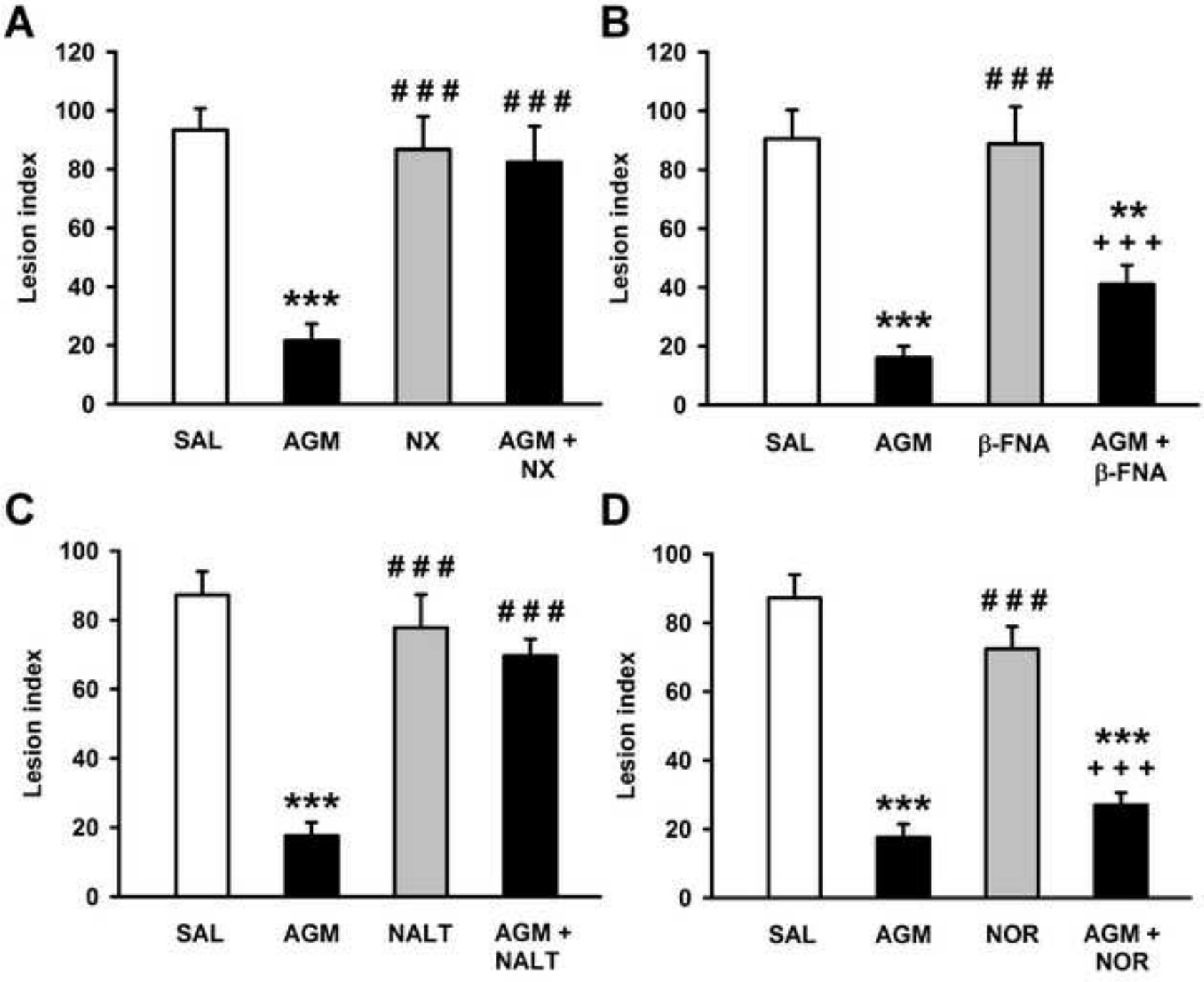

Figure5

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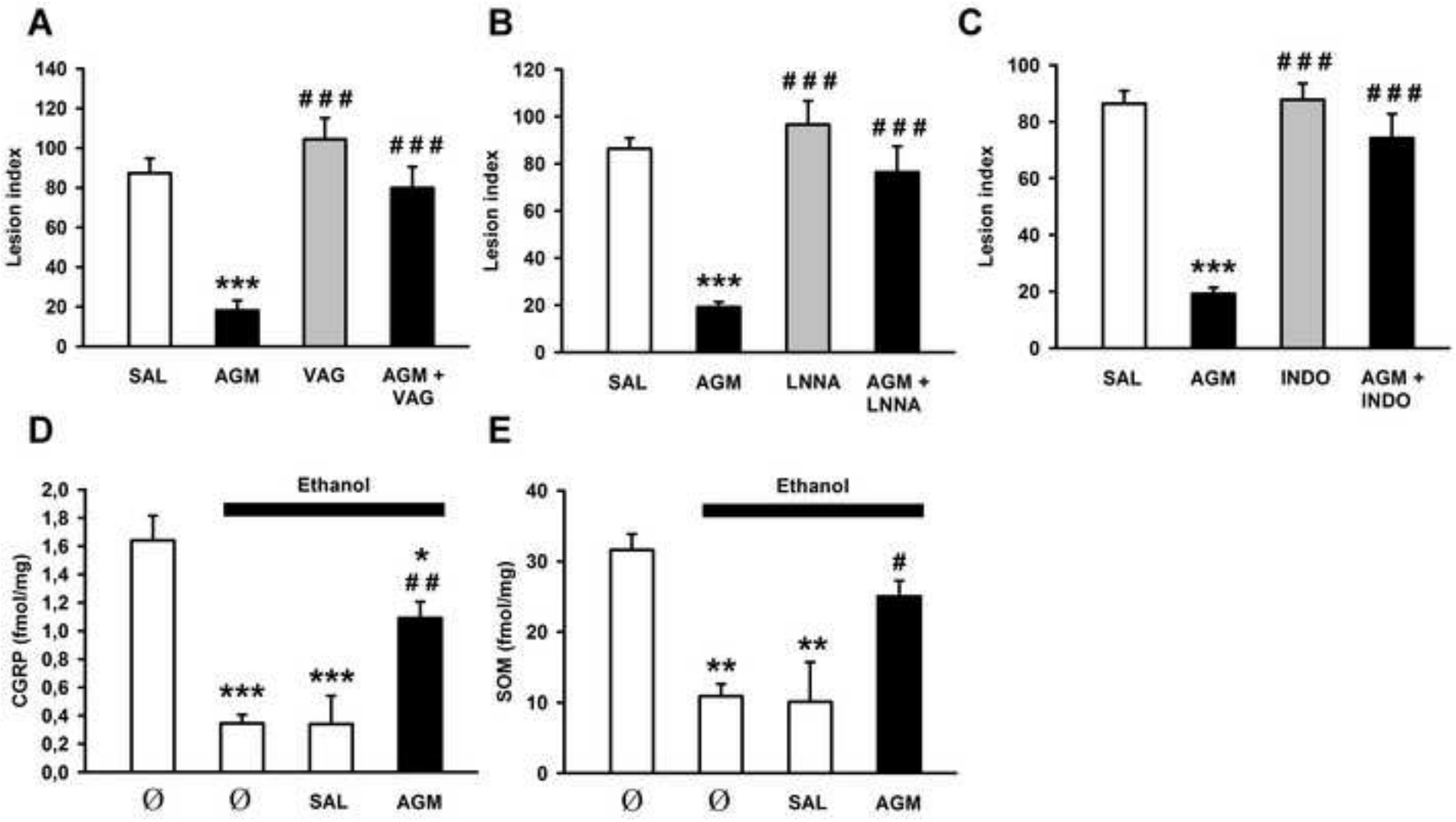

Figure6

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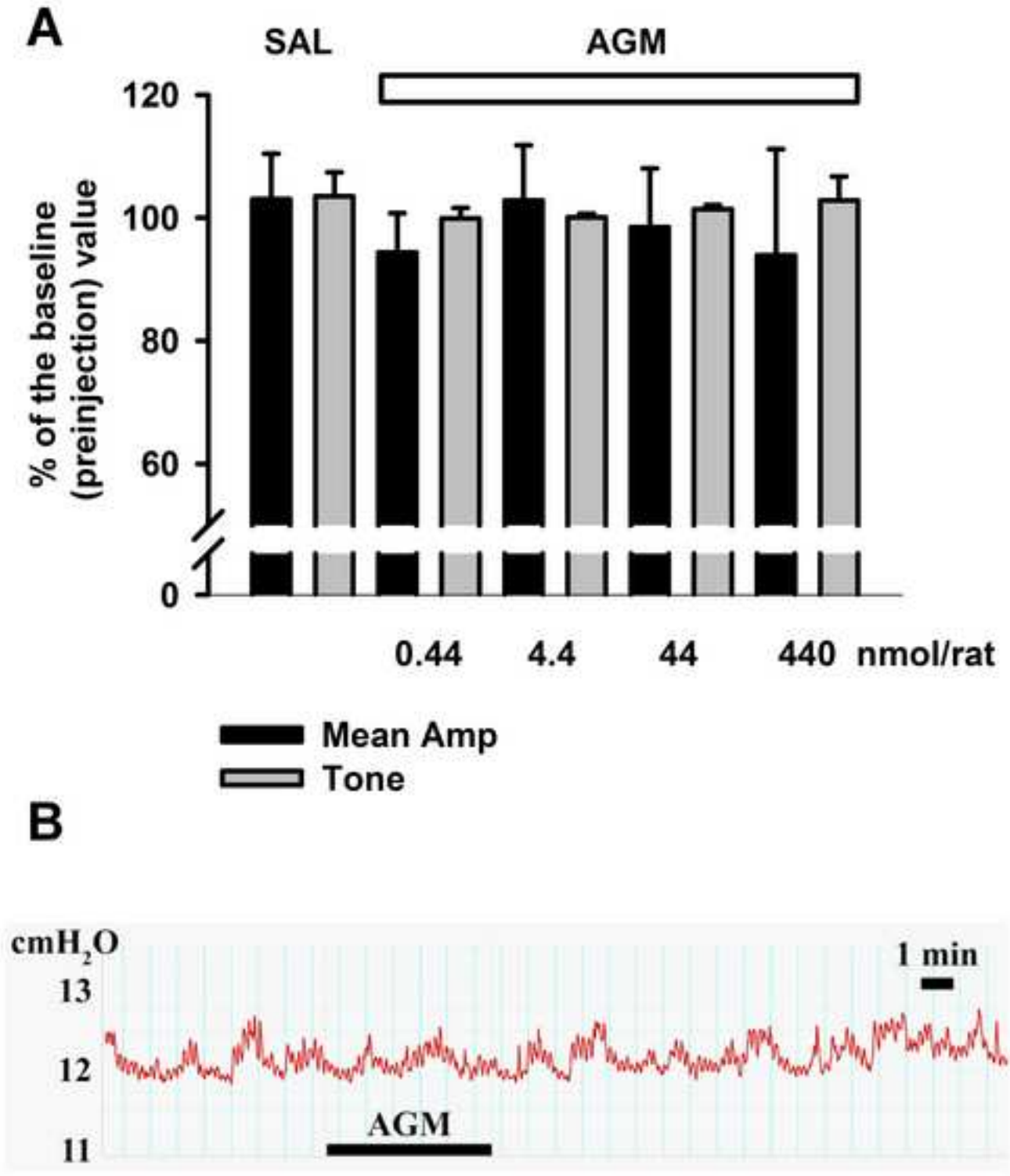

Table 1.

I.c.v.			I.p.		
Dose of AGM (nmol/rat)	Lesion index (% of control, mean ± S.E.M.)	Significance	Dose of AGM (mg/kg)	Lesion index (% of control, mean ± S.E.M.)	Significance
0.044	70.5 ± 4.8	$p < 0.05$	0.001	97.8 ± 17.6	ns
0.22	64.2 ± 8.5	ns	0.005	74.8 ± 5.0	$p < 0.01$
0.88	25.8 ± 7.2	$p < 0.001$	0.02	64.9 ± 4.5	$p < 0.01$
1.76	39.4 ± 8.6	$p < 0.001$	0.1	72.4 ± 7.4	$p < 0.05$
4.4	37.0 ± 8.3	$p < 0.05$	1	72.5 ± 6.7	ns
44	45.0 ± 12.1	$p < 0.05$	10	77.5 ± 12.2	ns
220	68.5 ± 9.5	$p < 0.05$	50	89.1 ± 16.6	ns

The effect of agmatine on ethanol-induced gastric mucosal injury in rats. The number of animals was 5 per group, for statistical analysis ANOVA was used, followed by Newman-Keuls post hoc test. Abbreviations: AGM: agmatine, ns: not significant.