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# Gut region-specific rearrangement of the cellular and subcellular compartments of nitric oxide synthase isoforms after chronic ethanol consumption in rats

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**Summary.** We recently provided evidence of cell-type-specific differences in the subcellular distributions of the three nitric oxide synthase (NOS) isoforms in the myenteric neurons, enteric smooth muscle cells and the capillary endothelium of the rat duodenum. We hypothesized that the presence of three NOS isoforms in the same type of cells with differences in subcellular compartmentalization might reflect a functional plasticity. Therefore, investigation of the possible rearrangement of cellular and subcellular NOS compartments in different gut segments following chronic ethanol treatment was the aim of this study.

Rats were randomly divided into two groups and received water or 20% ethanol solution, preceded by short periods of adaptation with 10% and 15% ethanol. After 8 weeks, segments of duodenum, ileum and colon of the control and the alcohol-treated rats were processed for post-embedding immunohistochemistry and RT-PCR.

The quantitative differences in the numbers of gold particles indicative of the different NOSs and their relative mRNA levels between the two experimental groups varied greatly, depending on the gut segment, and also on the cellular and subcellular compartments investigated. The chronic ethanol administration had the opposite effect on the quantitative distribution of the neuronal and endothelial NOS labelling gold particles in the different cellular compartments and resulted in subcellular rearrangement of NOS labels along the gastrointestinal tract.

The intestinal region-specific rearrangement of the cellular and subcellular NOS compartments may possibly result in functional plasticity and help to maintain the optimum NO level under pathological conditions.

**Key words:** Chronic ethanol consumption, Nitric oxide synthase, Gut region specificity, Cellular localization

## Introduction

Chronic alcohol consumption in man (Barboriak and Meade, 1970; Jian et al., 1986; Addolorato et al., 1997) and in experimental animals (Willson et al., 1990; Izbéki et al., 2001; Bagyánszki et al., 2010) disturbs normal gastrointestinal (GI) motor function leading to motility disturbances (Papa et al., 1998; Bode and Bode, 2003). An increasing body of evidence is available to suggest that these motor disturbances are caused by the effects of ethanol on the nitric oxide (NO) system of relaxation in the gut (Krecsmarik et al., 2006; Wang et al., 2007; Bagyánszki et al., 2011).

It is well documented that the major inhibitory non-

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**Abbreviations.** eNOS, Endothelial nitric oxide synthase; GI, Gastrointestinal; iNOS, Inducible nitric oxide synthase; NANC, Non-adrenergic, non-cholinergic; nNOS, Neuronal nitric oxide synthase; NO, Nitric oxide; NOS, Nitric oxide synthase; OsO4, Osmium tetroxide; RT-PCR, Reverse transcription-polymerase chain reaction

adrenergic, non-cholinergic (NANC) neurotransmitter in the enteric nervous system is endogenous NO (Bult et al., 1990; Moncada et al., 1991; Lefebvre, 1995; Mizuta et al., 1999). NO is synthesized by the neuronal (n), endothelial (e) and inducible (i) nitric oxide synthases (NOSs) (Bredt et al., 1991), all of which have been identified in the myenteric neurons in different species (Valentine et al., 1996; Vannucchi et al., 2002; Qu et al., 2008; Talapka et al., 2011), suggesting that the myenteric neurons constitutively express all three NOSs. Accumulating evidence indicates that augmented NO synthesis may contribute to a number of GI motility disorders associated with alcohol abuse due to the up-regulation of iNOS expression. Overproduction of NO and enhanced iNOS expression was demonstrated in intestinal inflammation and in the intestinal ischemia-reperfusion injury (Barocelli et al., 2006; Lundberg et al., 2006). Ethanol-induced up-regulation of iNOS and increased NO release was also demonstrated in an in vitro study on rat colonic muscle strips (Wang et al., 2010). Additionally, pharmacological evidence that selective iNOS inhibitors reversed the inhibitory effect of ethanol (Tang et al., 2009; Wang et al., 2010) while the non-selective NOS inhibitor only partly blocked it (Wang et al., 2007) also underline the importance of iNOS in the development of ethanol-induced GI motility disturbances. Measuring the momentaneous NO synthesis in the myenteric neurons of the mouse jejunal wall, we demonstrated a markedly increased basal NO synthesis in response to chronic ethanol consumption. The increased NO synthesis was accompanied by an up-regulation of iNOS expression and down-regulation of nNOS expression (Bagyánszki et al., 2011).

Besides myenteric neurons, the presence of nNOS, eNOS and iNOS immunoreactivity has been revealed in the enteric smooth muscle cells and in the endothelium of the capillaries running in the vicinity of the myenteric plexus of the rat duodenum, with a characteristic cell type-specific distribution. In the capillary endothelium, nNOS, eNOS or iNOS-labelling gold particles are localized almost exclusively on the vesicle membranes or within the vesicles. The bulk of the nNOS in the enteric muscle cells or the eNOS in the nerve terminals is accumulated in the mitochondria. The gold particles labelling the nNOS in the myenteric neuropils or the eNOS in the enteric smooth muscle cells are dispersed above the structureless cytoplasm, while the iNOS is evenly distributed in the enteric muscle cells and also in the myenteric neurons of the rat duodenum (Talapka et al., 2011). In accord with a recent publication (Giaroni et al., 2013), we therefore hypothesized that, the presence of the three NOS isoforms with similar functions in the same type of cells, the gut wall is able to adapt to different pathological conditions through the rearrangement of the subcellular NOS compartments.

Hence, the aim of this study was to investigate the possible rearrangement of the cellular and subcellular NOS compartments in three gut segments following chronic ethanol treatment. The quantitative features and the subcellular distributions of all three NOSs in the myenteric ganglia, intestinal smooth muscle cells and capillary endothelium were investigated by means of post-embedding immunohistochemistry, while the relative levels of NOS mRNAs were measured in tissue samples through the reverse transcription-polymerase chain reaction (RT-PCR).

## Materials and methods

### *Animals*

In all experiments, the principles of laboratory animal care (NIH Publication No. 85–23, revised 1985), approved by the Committee for Animal Research Studies at the University of Szeged, were strictly followed. Adult male Wistar rats weighing 250-300 g, kept on standard laboratory chow (Bioplan Kft., Isaszeg, Hungary), were randomly divided into two groups. One group (n=7) received 20% (v/v) ethanol solution for drinking purposes *ad libitum* for 6 weeks, following 1 week adaptation periods with 10% and then 15% ethanol (Krecsmarik et al., 2006). Sex- and age-matched controls (n=7) received normal drinking water during the experimental period. Fluid consumption was recorded daily. The average daily alcohol intake relative to the average weight of the rats during the study period was 13.58±1.74 g ethanol/kg body weight.

### *Tissue preparation and post-embedding immunohistochemistry*

After 8 weeks, the rats were killed by cervical dislocation under chloral hydrate anaesthesia (375 mg/kg i. p.), and tissue samples were taken from the duodenum (1 cm distal to the pylorus), the ileum (1 cm proximal to the ileo–caecal junction), and the middle of the transverse colon of the control and the alcohol-treated rats. The dissected intestinal segments were processed for post-embedding immunohistochemistry as described earlier (Talapka et al., 2011). Briefly, small pieces of the gut segments were fixed overnight at 4°C in 2% paraformaldehyde and 2% glutaraldehyde solution, and then further fixed for 1 h in 1% $OsO_4$. After rinsing in buffer and dehydrating in increasing ethanol concentrations and acetone, they were embedded in Epon (Electron Microscopy Sciences, Hatfield, PA, USA). The Epon blocks were used to prepare semithin (0.7 $\mu$m) sections, to select the area of interest, and also ultrathin (70 nm) sections, which were mounted on Formvar-coated nickel grids and processed for immunogold labelling. Ultrathin sections from each block were incubated overnight in the primary antibodies (Table 1), followed by protein A-gold-conjugated anti-mouse (18 nm gold particles; Jackson ImmunoResearch, West Grove, PA, USA; final dilution 1:20) or anti-rabbit (18 nm gold particles; Jackson ImmunoResearch, West Grove, PA, USA; final dilution 1:20) secondary antibodies. The specificity of the

immunoreaction was assessed in all cases by omitting the primary antibodies from the labelling protocol and incubating the sections only in the protein A-gold-conjugated secondary antibodies. Sections were counterstained with uranyl acetate (Merck, Darmstadt, Germany) and lead citrate (Merck, Darmstadt, Germany), and were examined and photographed with a Philips CM 10 transmission electron microscope equipped with a MEGAVIEW II camera. The quantitative features and the subcellular distributions of the gold particles labelling the different NOSs were determined in the myenteric ganglia, the endothelium of the capillaries in the vicinity of these ganglia and the intestinal smooth muscle cells (Fig. 1). Counting was performed on digital photographs at a magnification of 34,000x with the AnalySIS 3.2 program (Soft Imaging System GmbH, Münster, Germany). Pictures of five ganglia, the entire endothelial profile of five well-oriented capillaries, and 25 electron micrographs of smooth muscle cells per intestinal segment per condition were used. The intensity of the labelling was expressed as the total number of gold particles per unit area.

### *RNA extraction, reverse transcription and PCR amplification*

For the molecular experiments, the gut segments were cut along the mesentery and pinched flat. The layers of mucosa and submucosa were removed, and the residual material was snap-frozen in liquid nitrogen and stored at -80°C until use. 50 mg of intestinal samples were homogenized in RNA Bee reagent (Tel-Test, Inc., Friendswood, TX, USA) and total RNA was prepared according to the procedure suggested by the manufacturer. To determine rat nNOS-, eNOS- and iNOS-specific mRNAs, first-strand cDNA was synthesized by using 3 μg total RNA as template, 200 pmol of each dNTP (Thermo Scientific), 200 U Maxima H Minus Reverse Transcriptase, (Thermo Scientific) and 500 pmol random hexamer primers (Sigma) in a final volume of 20 μL, and incubated for 10 min at 37°C, followed by 1 h at 52°C. 0.5 μL reverse transcription product was added to 12.5 μL DreamTaq Green PCR Master Mix 2x (Thermo Scientific). Amplification was performed in a PTC 200 Peltier Thermal Cycler (MJ Research, Watertown, MA, USA). The number of amplification cycles during which PCR product formation was limited by the template concentration was determined in pilot experiments: for ß-actin mRNA, used as internal reference, 23 cycles, for nNOS and eNOS 40, and for iNOS 45 cycles. The sequences of primers were derived from the GenBank entry NM_052799.1 for nNOS (forward: 5'gaaggagcgggtcagcaagcc3' and reverse: 5'ggggctgggtcacccggatgg3'), NM_021838.2 for eNOS (forward: 5'ctacgctggccgggagact3' and reverse: 5'ggcataccagcgcaggccaa3'), NM_012611.3 for iNOS (forward: 5'caacaacgtggagaaagcccc3' and reverse: 5'ccctgggtcctctggtcaaact3'), and NM_031144 for ß-actin (forward: 5'gcaagagaggtatcctgacc3' and reverse: 5'ccctcgtagatgggcacagt3').

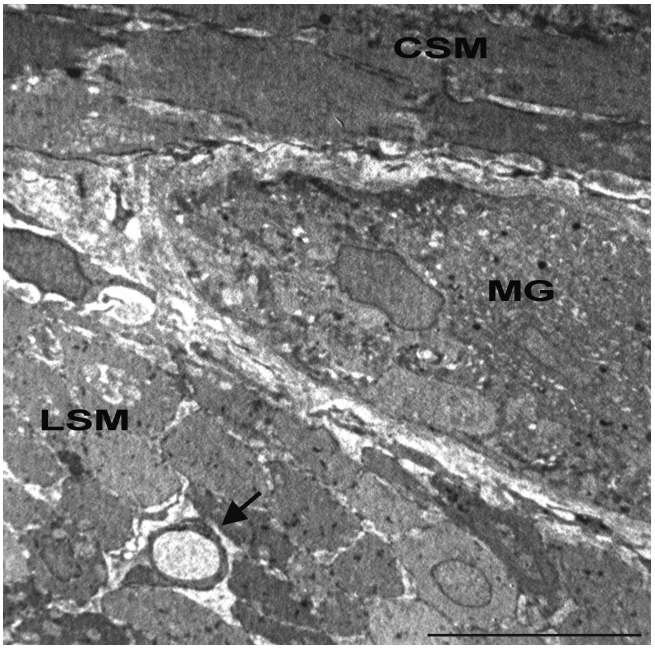


**Fig. 1.** Representative electron micrograph of the investigated cellular elements in the duodenum of a control rat. The quantification and subcellular localization of gold particles labelling the NOS isoforms were determined in the myenteric ganglia (MG), the endothelium of the intestinal capillaries (arrow) adjacent to these ganglia and the enteric smooth muscle cells. CSM: circular smooth muscle layer, LSM: longitudinal smooth muscle layer. Bar: 10 $\mu$m.

**Table 1.** Primary antibodies and working dilutions applied in the experiments.

| Primary antibody | Host | Final dilution | Product code | Company |
|---|---|---|---|---|
| anti-nNOS (NOS I) nitric oxide synthase, brain (bNOS) (251-270) polyclonal IgG fraction | rabbit | 1:150 | N7155 | Sigma-Aldrich (St. Louis, MO, USA) |
| anti-eNOS (NOS III) 3/eNOS/NOS Type III monoclonal IgG1 | mouse | 1:150 | 610297 | BD Transduction LaboratoriesTM (Lexington, KY, USA) |
| anti-iNOS (NOS II) NOS2 (N-20): sc-651 polyclonal IgG | rabbit | 1:50 | sc-651 | Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) |

Images of ethidium bromide-stained agarose gels were digitized with a GDS 7500 Gel Documentation System and analysed with GelBase/GelBlotTM Pro Gel Analysis Software (Ultra Violet Products Ltd., Upland, CA, USA). The levels of the different NOS mRNAs were expressed relative to the level of ß-actin (nNOS or eNOS or iNOS/ß-actin). To increase reliability, the measurements of the different NOSs and ß-actin mRNA levels were performed in triplicate for each sample.

### *Statistical analysis*

Statistical analysis was performed with one-way ANOVA and the Newman–Keuls test. All analyses were carried out with GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA, USA) and MedCalc Statistical Software version 9.4.2.0 (MedCalc Software, Mariakerke, Belgium). A probability level of $p<0.05$ was set as the level of statistical significance. All data were expressed as means±SE.

## Results

### *Quantitative distribution of nNOS-labelling gold particles in the three gut segments of the control and ethanol-treated rats*

Under normal metabolic conditions, the numbers of gold particles labelling nNOS were significantly higher in the myenteric ganglia of duodenum ($p<0.001$) and the colon ($p<0.001$) than in the ileum. Similar differences were observed also in the capillary endothelium ($p<0.01$ compare to ileum) and the intestinal smooth muscle cells ($p<0.001$ between ileum and colon) (Fig. 2A-C, significances under normal metabolic conditions are not indicated). After chronic alcohol consumption, pronounced decreases in the numbers of nNOS-labelling gold particles were demonstrated in all three cell types in the duodenum and the colon. The largest decrease, of more than 50%, was observed in the duodenal ganglia ($p<0.001$). In contrast, sharp increases in the numbers of nNOS-labelling gold particles were demonstrated in the ileum of the ethanol-drinking rats as compared with the controls (3.04±0.15 vs. 4.48±0.24 in the ganglia, 3.81±0.19 vs. 4.74±0.31 in the muscle cells, and 3.50±0.37 vs. 6.92±0.84 in the endothelium) (Fig. 2A-C). The relative level of nNOS mRNA in the control and the alcohol-treated rats was the same in the duodenum and ileum (Fig. 2D), but displayed a robust increase in the colon (Fig. 2D, 3).

Concerning the subcellular distribution of the nNOS-labelling gold particles, there were no visible differences between the various gut segments or between the control and the ethanol-treated groups. However, while the majority of the gold particles within the neuropil were grouped above the structureless cytoplasm, in the perikarya groups of nNOS-labelling gold particles were predominantly seen within the swollen cisternae of the endoplasmic reticulum and/or attached to the plasma

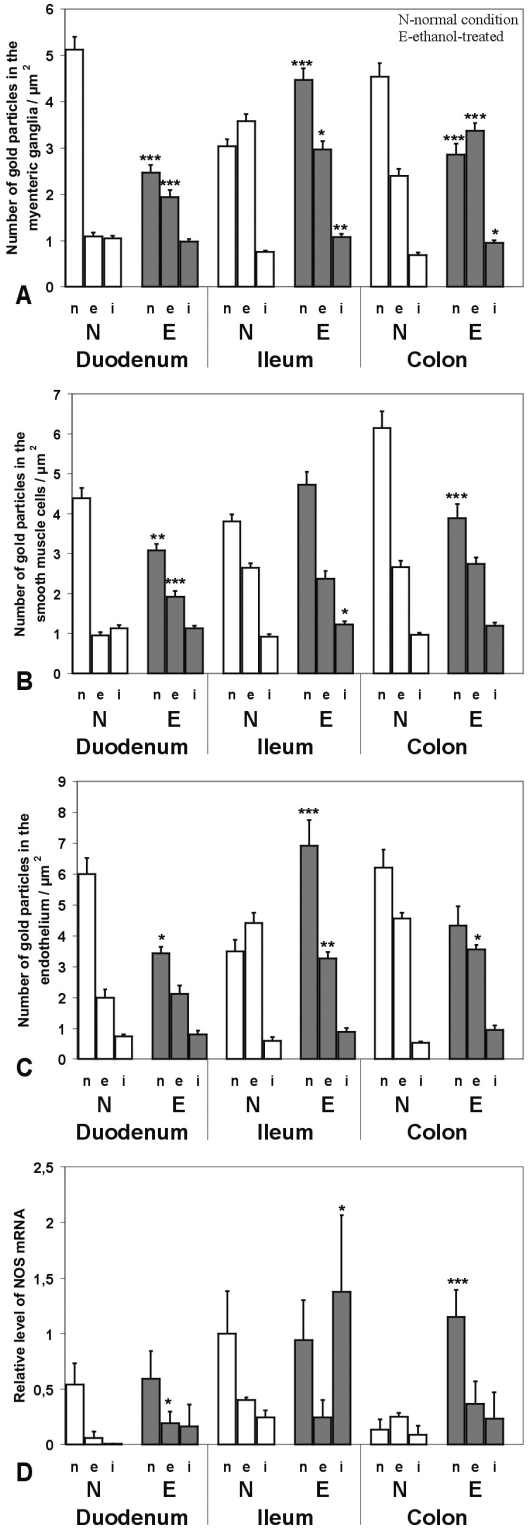


**Fig. 2.** Quantitative evaluation of the numbers of gold particles labelling nNOS (n), eNOS (e) and iNOS (i) in the myenteric ganglia **(A)**, enteric muscle cells **(B)** and capillary endothelium **(C)** and the relative levels of NOS mRNAs in tissue homogenates **(D)** from different gut segments of control and ethanol-treated rats. Data were expressed as means±SE. Significant differences were solely indicated between ethanol-treated and control groups (*p<0.05; **p<0.01; ***p<0.001).

membranes. The grouping of gold particles was most pronounced in the ganglionic neuropil of the ileum and colon (Fig. 4). Within the intestinal smooth muscle cells, nNOS-labelling gold particles accumulated above the mitochondria. In both the control and the ethanol-treated rats, 50% of all the mitochondria contained groups of gold particles (not shown). Gold particles labelling nNOS were always localized to the vesicle or plasma membranes or the interior of the vesicles above the capillary endothelium in all segments of both the control and the alcohol-treated rats (Fig. 5).

*Quantitative distribution of eNOS-labelling gold particles in the three gut segments of the control and ethanol-treated rats*

There were well-pronounced cell type-specific differences in the quantitative distribution of eNOS-labelling gold particles. In the control animals, the number of particles was always low in the duodenal compartments, but high in the ileum ($p<0.001$) and colon ($p<0.001$) (Fig. 2A-C, significances under normal metabolic conditions are not indicated). The quantitative changes related to chronic ethanol consumption also displayed strict gut segment and cell type specificity. The number of eNOS-labelling gold particles after ethanol consumption was higher in the duodenal ganglia and smooth muscle cells (1.09±0.08 vs. 1.94±0.15 and 0.96±0.08 vs. 1.93±0.14, respectively), but unchanged in the endothelium (1.99±0.25 vs. 2.12±0.27), while in the colon it was increased in the ganglia (2.40±0.16 vs. 3.37±0.17), decreased in the endothelium (4.56±0.18 vs. 3.56±0.14) and unchanged in the smooth muscle cells (2.67±0.15 vs. 2.74±0.17). In the ileum, it was decreased

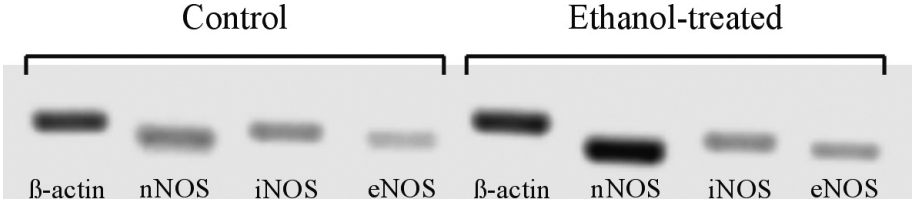


**Fig. 3.** Representative gel images of the nNOS, iNOS, and eNOS mRNA-specific PCR products prepared from tissue homogenates of the colon of control and ethanol-treated rats. ß-actin was used as an internal control. The tissue homogenates from the colon of ethanol-treated rats were characterized by a robust up-regulation of nNOS mRNA, while there were no changes in the level of eNOS and iNOS mRNA between the control and the ethanol-treated rats.

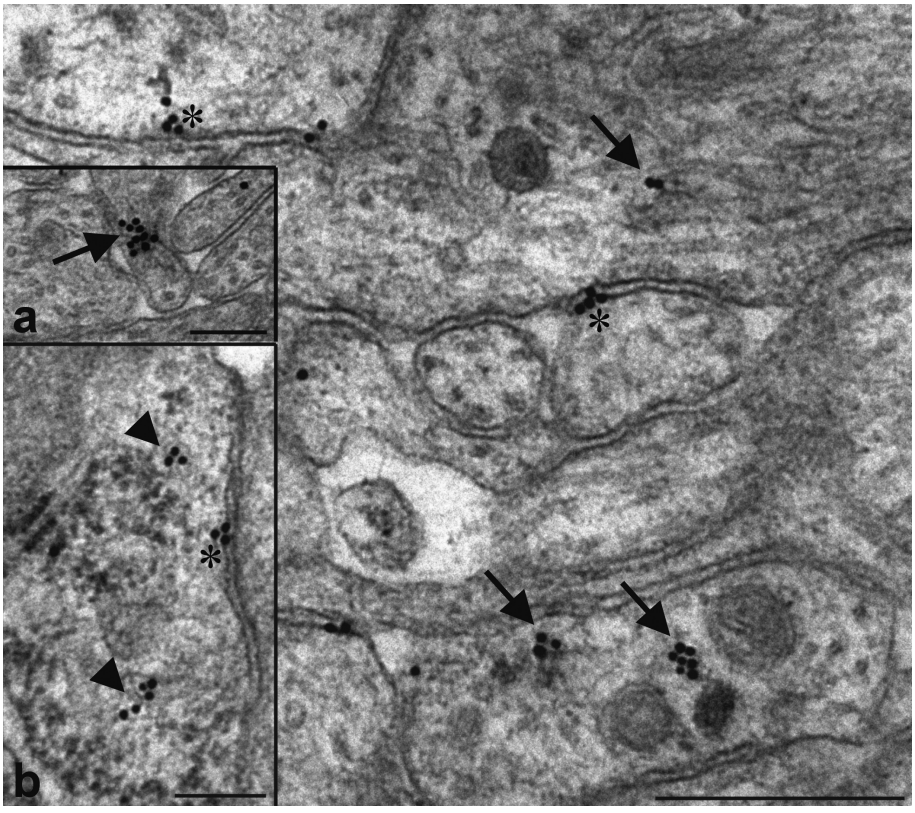


**Fig. 4.** Representative electron micrographs of a portion of the ganglionic neuropil of the myenteric plexus in the duodenum and the ileum and a small detail of a perikaryon from the duodenum of a control rat after post-embedding immunohistochemistry, using a nNOS-specific antibody. The majority of the 18 nm gold particles labelling nNOS were grouped above the structureless cytoplasm in the nerve terminals (arrows). The grouping of the gold particles was more pronounced in the ileal neuropil (**insert a**, arrow). In the perikarya, groups of nNOS-labelling particles were predominantly seen within the swollen cisternae of the endoplasmic reticulum (**insert b**, arrowheads). At several locations within the myenteric plexus, groups of gold particles tended to attach to the plasma membrane (asterisks). Bars: 500 nm; inserts, 200 nm.

in the ganglia and the capillary endothelium, but unchanged in the smooth muscle cells (Fig. 2A-C). With the exception of the duodenum, where the relative level of eNOS mRNA was increased after chronic ethanol consumption, there were no changes in either the control or the ethanol-treated rats (Figs. 2D, 3).

Most of the eNOS-labelling gold particles in the myenteric neurons and the enteric muscle cells were dispersed above the structureless cytoplasm or bound to the plasma membrane of neuronal projections. In the duodenal muscle cells of the controls, barely 10% of the local mitochondria contained eNOS particles. After chronic ethanol administration, eNOS was observed in more than 60% of the mitochondria (Fig. 6). Whereas close to 70% of the mitochondria in the ileum contained eNOS-labelling particles in the myenteric ganglia of the controls, while only 40% of mitochondria were labelled here following ethanol administration (not shown). The gold particles labelling eNOS in the endothelium were always localized to vesicle membranes or the interior of vesicles in all segments of both the control and the alcohol-treated rats (not shown).

*Quantitative distribution of iNOS-labelling gold particles in the three gut segments of the control and ethanol-treated rats*

The baseline numbers of iNOS-labelling gold particles were lower than those for nNOS and eNOS in all three gut segments and for all cell types. However, quantitative changes related to chronic ethanol consumption were documented in the ileal and colonic myenteric ganglia (0.75±0.04 vs. 1.08±0.07 and 0.69±0.05 vs. 0.95±0.06; respectively) and also in the ileal muscle cells (0.92±0.06 vs. 1.24±0.07). A significant increase in the relative level of iNOS mRNA was demonstrated in the ileum (Fig. 2).

iNOS was evenly distributed above the myenteric ganglia and the muscle cell cytoplasm with a limited

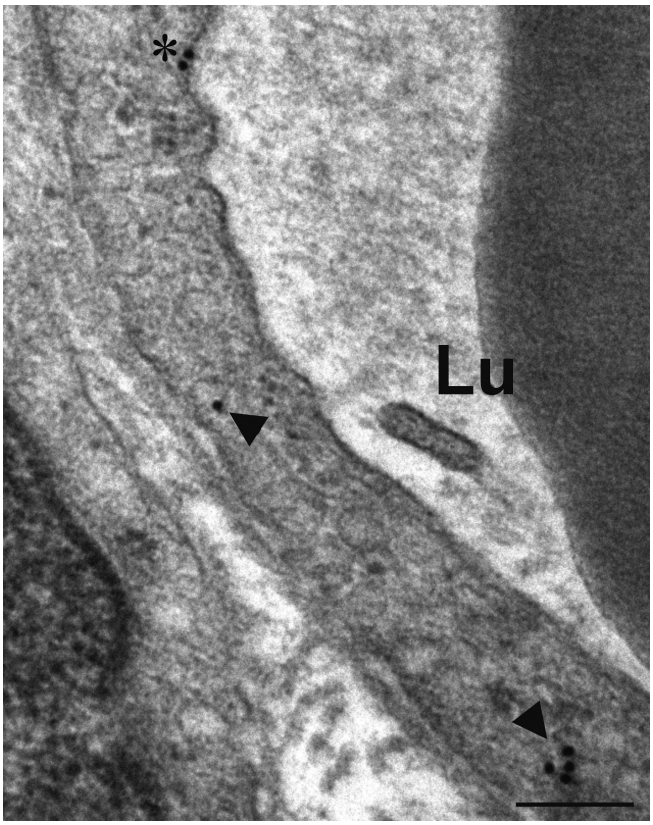


**Fig. 5.** Representative electron micrograph of an intestinal capillary in the duodenum of a control rat after post-embedding immunohistochemistry, using a nNOS-specific antibody. The 18 nm gold particles labelling nNOS were localized to the interior of vesicles (arrowheads) or above the endothelium plasma membrane (asterisk). Lu: capillary lumen Bar: 200 nm.

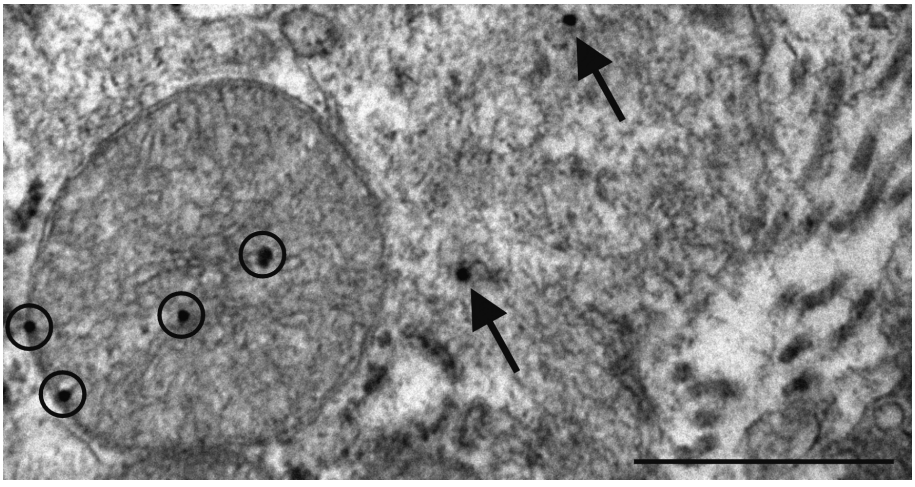

**Fig. 6.** Representative electron micrograph of an enteric smooth muscle cell in the duodenum of an ethanol-treated rat after post-embedding immunohistochemistry, using an eNOS-specific antibody. Most of the eNOS-labelling gold particles accumulated in the mitochondria (circled). Single labels dispersed above the cytoplasm (arrows) were rarely seen in ethanol-treated rats. Bar: 500 nm.

mitochondrial accumulation. iNOS-labelling gold particles were observed in at most 20% of the local mitochondria in the different intestinal regions of both the controls and the alcoholic rats (not shown).

## Discussion

On the basis of post-embedding immunohistochemistry, a previous paper provided evidence of cell type-specific differences in the subcellular distributions of the three NOSs in the rat duodenum (Talapka et al., 2011). The present study was therefore initiated to ascertain whether the pathological condition caused by chronic ethanol consumption influences the normal distribution of the NOSs. Formerly we reported gut region specificity in the susceptibility of myenteric neurons and their microenvironment to different pathological conditions (Krecsmarik et al., 2006; Izbéki et al., 2008; Bódi et al., 2012), and we therefore set out to investigate the possible rearrangements of the cellular and subcellular NOS compartments in three gut segments in response to chronic ethanol treatment.

When the gold particles labelling the three NOSs under normal metabolic conditions were counted, the nNOS labels were in general the most numerous, which is in agreement with the finding of an earlier study that more than 80% of the constitutive NOS (both neuronal and endothelial) activity in the rat small intestine is accounted for by nNOS (Qu et al., 1999). These findings also strengthen the view that nNOS is the main source of NO in the GI tract under physiological conditions. However, the pronounced differences in the number of nNOS labels along the oro-anal axis of the gut (a high number of labels in the duodenum, a low number in the ileum, and then a high number again in the colon) suggest the significance of differences in the microenvironment of NOS in a given intestinal segment. Counting the nNOS labels after chronic ethanol consumption lends support to this suggestion; the numbers of nNOS labels are decreased considerably in the duodenum and the colon, and increased robustly in the ileum. Since the microenvironment of NOS in the subcellular organelles determines the rate and efficiency of its catalysis, which in turn influences NO generation at distinct intracellular locations (Villanueva and Giulivi, 2010), we suggest that these differences are accounted for by the differential changes in the enteric microenvironment in the proximal and distal parts of the small intestine after chronic ethanol consumption. As the changes in the number of gold labels relating to chronic ethanol consumption are in most cases not accompanied by changes in the relative levels of mRNAs, we suggest that the rearrangement of the nNOS compartments between the gut segments in alcoholic rats is the primary event, rather than changes in enzyme synthesis. However, it was also revealed that several molecular mechanisms, both transcriptional and post-transcriptional effects, can modulate the expression of the constitutive isoforms of NOS (Förstermann et al., 1998).

The number and the distribution of the eNOS-labelling gold particles also varied greatly along the GI tract, but the quantitative features of the eNOS labels were changed in the opposite way to those in nNOS after ethanol consumption. Whereas the number of gold particles labelling nNOS in the duodenal ganglia decreased by more than 50%, the number of gold particles labelling eNOS approximately doubled here. The significant increase in the relative level of eNOS mRNA in the duodenum after ethanol administration reveals the up-regulation of eNOS in this particular gut segment. The opposite effects of eNOS and nNOS on the intracellular $Ca^{2+}$ concentration have also been demonstrated. While eNOS inhibited the $Ca^{2+}$ influx, nNOS facilitated the $Ca^{2+}$ outflow from the sarcoplasmic reticulum (Barouch et al., 2002). Additionally, further findings have indicated an increase in expression of one NOS isoform in one cell type, but decreases in the production of other forms in consequence of exposure to ethanol (Deng and Deitrich, 2007). These results suggest the presence of a compensatory mechanism, in which one NOS isoform can replace another, thereby promoting maintenance of the optimum NO production even under pathological conditions.

In agreement with previous studies (McCafferty et al., 1999; Singer et al., 1999; Mancinelli et al., 2001; Roberts et al., 2001), low numbers of iNOS labels were demonstrated in all the cellular compartments and gut segments under normal metabolic conditions. The significant increase in the number of iNOS-labelling gold particles in the myenteric ganglia and smooth muscle cells of the ileum after chronic ethanol exposure was accompanied by an up-regulation of iNOS mRNA. These findings support our former conclusions that the increasing proportion of iNOS-expressing myenteric neurons contributes to the ethanol-induced overproduction of NO and thus results in GI motility disturbances associated with alcohol abuse (Bagyánszki et al., 2011).

The previously reported cell type-specific distribution of the three NOSs in the rat duodenum (Talapka et al., 2011) was confirmed here in the other three intestinal segments. However, depending on the gut segment under investigation, a distinct subcellular realignment of NOS-labelling gold particles was characteristic after chronic ethanol consumption. It was most noteworthy that barely 10% of the mitochondria in the smooth muscle cells of the control duodenum contained eNOS labels, whereas after ethanol administration gold labels indicative of eNOS were demonstrated in more than 60% of the local mitochondria. In contrast, while almost 70% of the mitochondria in the ileal ganglia of the controls contained eNOS labels, after ethanol treatment only 40% of mitochondria were labelled. Increasing evidence suggests that arginase II, which is confined predominantly to the mitochondria, modulates NOS activity by regulating the bioavailability of L-arginine

(Berkowitz et al., 2003; White et al., 2006; Lim et al., 2007). We therefore hypothesize that in the duodenal muscle cells (where the trafficking of eNOS labels into the mitochondria was demonstrated after ethanol exposure) a competition develops between arginase II and eNOS within the mitochondria for their common substrate L-arginine. The balance between arginase II and eNOS might therefore be critical in controlling the NO level under the pathological conditions induced by ethanol consumption.

In conclusion, the present study has demonstrated for the first time that chronic ethanol administration influences the rearrangement of three NOS isoforms in the rat intestine both regionally and cell type specifically. In view of the opposite effects of ethanol administration on the quantitative distribution of the nNOS and eNOS labels, we assume that, in consequence of the presence of different NOSs with similar functions in a given cell type, the gut wall is able to adapt to different pathological conditions through the rearrangement of the subcellular NOS compartments.

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