

## **Introduction**

The mammalian enteric nervous system is comprised of neurons and glial cells in the wall of the esophagus, stomach, intestine, pancreas and gall bladder. The functional and neurochemical complexity of neurons of the enteric nervous system is similar to that of the central nervous system. It consists of approximately 100 million neurons, like the spinal cord of the examined species. The glial cells closely associated with the neurons resemble the astrocytes of the central nervous system, rather than Schwann-cells.

In the anatomical organisation of the enteric nervous system, the neuronal circuits are situated in or between the tissue layers of the gut wall. In two of these neuronal circuits (plexus) the neurons are organised into ganglia and the connection between ganglia is attained through internodal segments. The myenteric plexus is situated between the smooth muscle layers of the gut wall and regulates the motility of the intestine. The submucosa plexus controls the enteroendocrin functions and the secretion and the absorption of the mucosa.

Nitric oxide (NO) is an intracellular and extracellular signaling molecule, which is synthesized by nitric oxide synthases (NOS). In mammals, three NOS isoforms are known: inducible NOS (iNOS) and the constitutively expressed (cNOS) endothelial (eNOS) and neuronal NOS (nNOS).

In the enteric nervous system, NO is the main inhibitory neurotransmitter, it plays a key role in the descending relaxational phase of the peristaltic reflex. Pharmacological and knock-out experiments showed the role of nNOS produced NO in the non-adrenergic non-cholinergic relaxation of intestinal smooth muscle. Specific nNOS inhibitors reduce intestinal smooth muscle relaxations. NO also plays a very important role in the pathological alterations of the gastrointestinal tract. The pathological background in the alterations of intestinal motility often involves the apoptosis and/or dysfunction of myenteric nitrergic neurons. Such as the case in achalasia and diabetes when the motility dysfunction of the intestine is partially due to a downregulation of nNOS protein expression and selective apoptosis of nitrergic myenteric neurons.

It is well-known from the literature that alcohol or ethanol (ethyl-alcohol,  $C_2H_5OH$ ) found in alcoholic beverages interferes with the absorption of certain nutrients and vitamins. Furthermore, alcohol damages the intestinal mucosa and therefore leads to malnutrition which is often observed in alcoholic patients. Alcohol consumption also interferes with the normal immunological functions of the intestine, leading to immunodeficiency and increasing the risk of infections. Generally, the health risk of alcohol consumption is present in men over 40g ethanol/kg body weight/day, in women over 20g ethanol/kg body weight/day. From the total amount of consumed alcohol 20% is absorbed in the stomach and 80% in the proximal part of the intestine, in the duodenum and jejunum by simple diffusion. Ethanol concentration similar to that of the consumed alcohol is found only in the duodenum and jejunum. In the more distal segments of the intestine the luminal alcohol concentration is the same as the alcohol concentration of the blood, indicating that ethanol enters the lumen from the vascular space.

The consumed ethanol has a profound effect on the motility of the intestine, however, the underlying mechanisms remain unknown. Alterations in intestinal peristalsis lead to severe gastrointestinal symptoms such as the accumulation of bacterial toxins and inhibition of absorption. These symptoms largely contribute to the health deterioration not only of alcoholic patients but also of patients suffering from various motility disorders.

## Specific aims

Since NO regulates the descending inhibitory phase of the peristaltic reflex, our hypothesis was that changes in the synthesis of NO or in the expression of NOS proteins contribute to the observed motility alterations following chronic alcohol consumption.

The specific aims followed in the course of this work could be summarized as:

- To determine the changes in intestinal motility *in vivo* following chronic alcohol consumption in the murine small intestine.
- To study the NO-mediated smooth muscle relaxations *in vitro* in myenteric plexus-smooth muscle preparations in the murine jejunum.
- To determine whether the total number of myenteric neurons and nNOS-immunoreactive myenteric neurons change as a consequence of alcohol consumption.
- To verify whether the amount of NO produced by enteric neurons change following chronic alcohol consumption.
- To study the colocalisation of NO producing myenteric neurons and nNOS-immunoreactive neurons in the myenteric plexus.
- Is there a change in the activity of cNOS and iNOS enzymes in different segments of the rat intestine?
- To determine the amount of eNOS and nNOS proteins in different segments of the rat intestine by Western blotting.

## Materials and Methods

### *Animals*

Two-month-old mice were randomly divided into a chronic alcohol-treated group and two control groups, one receiving isocaloric sucrose solution the other receiving tap water. Chronic alcohol-treated mice were given an increasing concentration of ethanol in tap water (v/v%) starting with 10% on the first, 15% on the second and 20% on the third to the fifth week. Ethanol intake and weight was continuously measured during the treatment period. The average daily ethanol intake was 7.8 g ethanol/kg body weight. Wistar rats were randomly divided into two groups. The ethanol-drinking group was given 20% (v/v) ethanol *ad libitum* in drinking water during 8 weeks after two weeks of adaptation period with 10% and 15% ethanol, the control group received water. To determine the time-dependence of ethanol consumption on the number of nNOS-immunoreactive cells, two animals of each group were sacrificed weekly for quantitative immunohistochemistry. Tissue samples from rats sacrificed after 8 weeks of alcohol consumption were used for measurement of NOS activity and for Western blot analysis.

### *In vivo small intestinal transit*

Mice received an intragastric injection of Evans blue into the stomach. Fifteen minutes later the mice were anaesthetized with diethyl ether. The transit front was measured from the pylorus to the most distal point of migration of Evans blue and expressed as percent migration of Evans blue compared to the total length of the small intestine.

### *In vitro smooth muscle relaxation*

The jejunum was opened along the mesenteric border and the mucosal layer was removed. Circular muscle strips were positioned between two electrodes, the lower end of the muscle strip was fixed and the other end was connected to a strain gauge transducer for continuous recording of isometric tension. The non-adrenergic non-cholinergic (NANC) conditions were established by adding guanethidine (3  $\mu$ M) and atropine (1  $\mu$ M). Muscle strips were precontracted with prostaglandin  $F_{2\alpha}$  (0.3  $\mu$ M) and NANC relaxations were induced by electrical field stimulation (EFS 1,2,4 and 8 Hz), nitric oxide (NO added as acidified  $\text{NaNO}_2$ , 10  $\mu$ M, 30  $\mu$ M, 0.1 mM) and glyceryl trinitrate (GTN, 1 $\mu$ M). Nitro-L-arginine (L-NNA, 0.3 mM) was used as NOS

inhibitor. Relaxations are expressed as percentage relaxation of the prostaglandin  $F_{2\alpha}$ -induced contraction.

### ***Immunohistochemistry and cell quantification***

nNOS antibody was used to stain the neuronal isoform of NOS and protein gene product 9.5 (PGP9.5) and HuC/D was used as panneuronal marker. In samples from mice the nNOS and PGP9.5-immunoreactive nerve cell bodies were counted per visual field. Twenty randomly chosen fields in each wholemount were analysed, and the number of immunostained neurons was calculated. In the cases of rat samples twenty digital photographs of identical magnification, size and resolution were taken from each intestinal segment. Stained cells were counted in each digital photograph with the use of the Plexus Pattern Analysis software developed in our laboratory.

### ***Imaging of NO with DAF-FM***

Myenteric plexus-smooth muscle preparations were incubated in DAF-FM and was transferred to an inverted microscope (Zeiss Axiovert 100M) equipped with a x25 water immersion objective (Zeiss Plan-neofluar, numerical aperture 0.8) in a confocal laser scanning microscopy system (Zeiss, LSM510T) compare fluorescent signals between different preparations, settings were fixed for all samples Image data were analyzed using the Zeiss LSM510 analyzing software V2.53.

### ***Measurement of NOS activity***

cNOS and iNOS activities were determined in the duodenum, jejunum, ileum and colon and expressed in fmol/mg protein/min. NO formation in the intestinal tissues was measured by the conversion of [ $^3$ H]L-citrulline from [ $^3$ H]L-arginine.

### ***Western blot analysis***

For Western blotting we used SDS polyacrylamide gel electrophoresis. The primary antibodies were follows: mouse anti-nNOS, anti-eNOS and anti- $\beta$ -actin. Three independent blots stained with either nNOS or eNOS were analyzed by the NIH Image 1.63F. In all cases,  $\beta$ -actin was used as an internal standard.

## Results and discussion

We found that the intestinal transit time of mice was significantly delayed in response to chronic alcohol consumption when compared to the control group. This delay might be due to an inadequate relaxation of small intestinal smooth muscle. In intestinal smooth muscle-myenteric plexus preparations, we observed a significant impairment of NO-mediated smooth muscle relaxations to electrical field stimulus after chronic alcohol-treatment, while relaxations to exogenous NO remained unchanged. This suggests that the effect that ethanol exerts on smooth muscle relaxation is not due to a defective responsiveness of the smooth muscle to NO but appears to be originating from impaired nitrenergic innervation.

The quantification of nNOS-immunoreactive cells and the total number of cells in the myenteric plexus showed that in both mice and rats the number of nNOS-immunoreactive cells significantly decreased while the total number of cells did not change. This indicates an adaptive change in the expression of nNOS in myenteric neurons rather than an apoptotic process.

The fluorescence intensity of the NO-sensitive dye DAF-FM was significantly increased in myenteric neurons after chronic alcohol consumption. This result seemed contradictory to the decrease in nNOS-immunoreactive neurons and to the observed impaired nitrenergic relaxations. In light of this discrepancy and because DAF-FM does not distinguish between NO produced by different NOS isoforms, we performed nNOS immunohistochemistry on DAF-FM loaded preparations. We found that in 22.7% of neurons DAF-FM colocalized with nNOS, 42.3% was stained with nNOS only, and 35% was stained with DAF-FM only. This partial colocalization with nNOS suggests that other NOS isoforms might be present in myenteric neurons, and these isoforms synthesize NO. However this NO produced does not seem to compensate for the impaired nitrenergic transmission.

Measurement of the physiological constitutive NOS (cNOS) activity revealed a 20 times higher activity in the colon than in the small intestine and after chronic ethanol treatment demonstrated a significant decrease in the jejunum, ileum, and colon, while in the duodenum it remained unchanged as compared with the control group. The physiological iNOS activity was higher in the ileum and colon than in the duodenum and jejunum, and these levels were not significantly affected by ethanol. The nNOS protein content measured by Western blotting indicated a significant

decrease in the colon after ethanol consumption, while in other intestinal segments change was not detectable.

## Conclusions

Using two, separate experiments in the mouse and rat intestine, we showed that chronic alcohol consumption profoundly affects the NO system.

- In the murine small intestine we observed a delay in intestinal transit which might indicate that the smooth muscle contraction during peristalsis is not followed by an adequate relaxation.
- This hypothesis was verified in our smooth-muscle relaxation experiments where we detected a decrease in NO-mediated relaxations.
- In both mice and rats we showed a significant decrease in the number of nNOS-immunoreactive neurons while the total number of neurons did not change. The decrease in nNOS might indicate that NO produced by nNOS is decreased also.
- NO synthesis detected by DAF-FM increased after chronic alcohol consumption. The NO-producing neurons showed a limited (20%) colocalisation with nNOS. This indicates that the increased NO synthesis might be due to other NOS isoforms or might be produced in a NOS-independent manner.
- In different segments of the rat intestine we detected a segment-specific decrease in nNOS activity and nNOS protein.

In conclusion, the selective impairment of nNOS in the gastrointestinal tract following chronic alcohol consumption might play an important role in the motility disturbances observed in alcoholic patients and laboratory animals.



### **Full papers:**

1. **Krecsmarik M**, Izbéki F, Bagyánszki M, Linke N, Bódi N, Kaszaki J, Katarova Z, Szabó Á, Fekete É, Wittman T (2006) *Chronic ethanol exposure impairs neuronal nitric oxide synthase in the rat intestine*. Alcohol Clin Exp Res, Vol 30, No 6, 967–973 IF:2.63
2. **Krecsmarik M**, Katarova Z, Bagyánszki M, Szabó G, Fekete É (2004) *Gastrointestinal phenotype of GAD67lacZ transgenic mice with early postnatal lethality*. Histol. Histopathol. 20: 75-82 IF:2.02
3. Román V, Bagyánszki M, **Krecsmarik M**, Horváth A, Resch B. Á, Fekete É (2004) *Spatial pattern analysis of nitrergic neurons in the developing myenteric plexus of the human fetal intestine*. Cytometry 57A(2):108-12. IF:2.11
4. Román V, **Krecsmarik M**, Bagyánszki M, Fekete É (2001) *Evaluation of the total number of myenteric neurons in the developing chicken gut using cuproinic blue histochemical staining and neurofilament immunocytochemistry*. Histochem Cell Biol 116:241-246. IF:2.23

### **Abstracts:**

1. **Krecsmarik M**, Winter BY de, Man JG de, Bagyánszki M, Nassauw L Van, Fekete É, Pelckmans PA, Timmermans J-P *Chronic alcohol consumption impairs nitrergic inhibition in the mouse small intestine*. Digestive Disease Week and the 107th Annual Meeting of the American Gastroenterological Association Institute, Los Angeles, USA. Gastroenterology 2006 Apr; 130 (4 suppl 2) A1-911 IF:12.38
2. Izbéki F, **Krecsmarik M**, Szabó S, Kaszaki J, Kuris A, Bagyánszki M, Rosztóczy A, Fekete É, Wittmann T *Decreased number of nitrergic and serotonergic cells in the small intestine of chronic alcohol-treated rats*. 12th European Neurogastroenterology and Motility Meeting. Neurogastroenterol Motil 2004 16(6) 829-873 IF:2.56