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The role of N-methyl-D-aspartate receptors in colon motility disorders

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Ph.D. Thesis

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LIST OF PAPERS RELATED TO THE SUBJECT OF THE THESIS

List of full papers

I. Kaszaki J, Palásthy Z, **Érces D,** Rácz A, Torday C, Varga G, Vécsei L., Boros M (2008) Kynurenic acid inhibits intestinal hypermotility and xanthine oxidase activity during experimental colon obstruction in dogs. *Neurogastroenterol Motil* **20(1):** 53-62. **IF=3.338**

II. Varga G, **Érces D,** Fazekas B, Fülöp M, Kovács T, Kaszaki J, Fülöp F, Vécsei L, Boros M (2010) N-Methyl-D-aspartate receptor antagonism decreases motility and inflammatory activation in the early phase of acute experimental colitis in the rat. *Neurogastroenterol Motil* **22:** 217-225. **IF=3.349**

III. **Érces D,** Varga G, Fazekas B, Kovács T, Tőkés T, Tiszlavicz L, Fülöp F, Vécsei L, Boros M, Kaszaki J (2012) N-Methyl-D-aspartate receptor antagonist therapy suppresses colon motility and inflammatory activation six days after the onset of experimental colitis in rats. *European Journal of Pharmacology* **691:** 225-234. **IF=2.737**

IV. Kaszaki J, **Érces D,** Varga G, Szabó A, Vécsei L., Boros Mihály (2012) Kynurenines and intestinal neurotransmission: the role of N-methyl-D-aspartate receptors. *J Neural Transm* **119(2):** 211-223. **IF=2.730**

V. **Érces D,** Varga G, Kovács ÁL, Fülöp F, Vécsei L, Boros M, Kaszaki J (2011) N-Methyl-D-aspartate receptor antagonist therapy in experimental colitis. *In: Functional and Motility Disorders of the Gastrointestinal Tract. Proceedings of the Humboldt Kolleg NeurogastRO* pp 41-49.

List of abstracts relating to the subject of the thesis

1. **Érces D**, Varga G, Kovács T, Kaszaki J, Vécsei L, Boros M (2008) Glutamate receptor inhibition improves intestinal function in experimental colitis. *British Journal of Surgery* **95(S6):** 1-104.
2. Varga G, **Érces D**, Fazekas B, Fülöp M, Kovács T, Kaszaki J, Fülöp F, Vécsei L Boros M (2009) N-methyl-D-aspartate receptor inhibition decreases motility and inflammatory activation in experimental colitis. *Shock* **32(S1):** 18.
3. **Érces D**, Rácz A, Kaszaki J, Vécsei L, Boros M (2007) Kinurénsav kezelés hatása a vastagbél keringésre kísérletes ileusban. *Érbetegségek* **S1:** 10.

4. Kaszaki J, **Érces D**, Varga G, Tordai Cs, Vécsei L, Boros M (2007) Szabadgyök képződés gátlása glutamát receptor antagonista kezeléssel akut vastagbél obstrukcióban. *Folia Hepatologica* **11(S3):** 22.

5. Kovács T, Fazekas B, Varga G, **Érces D,** Kaszaki J, Vécsei L, Boros M (2009) Az NMDA-receptorgátlás vizsgálata bélgyulladásos patkánymodellben. *Magyar Sebészet* **62(S3):** 154.

6. Fazekas B, Varga G, **Érces D,** Kovács T, Kaszaki J, Vécsei L, Boros M (2009) Az NMDA-receptor-aktiváció jelentősége kísérletes bélgyulladásban. *Magyar Sebészet* **62(S3):** 153.

7. Palásthy Zs, **Érces D**, Kaszaki J, Vécsei L, Lázár Gy, Boros M (2009) Szabadgyök képződés gátlása glutamát receptor antagonista kezeléssel akut vastagbél obstrukcióban. *Magyar Sebészet* **62(S3):** 153.

8. Varga G, **Érces D,** Fazekas B, Fülöp M, Kovács T, Kaszaki J, Fülöp F, Vécsei L, Boros M (2009) N-methyl-D-Aspartate receptor inhibition decreases motility and inflammatory activation in experimental colitis. *Shock* **32(S1):** 14.

CONTENTS

LIST OF ABBREVIATIONS

Ach	acetylcholine
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BBB	blood-brain barrier
cNOS	constitutive
CNS	central nervous system
CO	cardiac output
CVP	central venous pressure
eNOS	endothelial nitric oxide synthase
ENS	enteric nervous system
GI	gastrointestinal
GMC	giant migrating complex
IBD	inflammatory bowel disease
ICC	interstitial cells of Cajal
iNOS	inducible nitric oxide synthase
IPAN	intrinsic primary afferent neuron
KynA	kynurenic acid
MAP	mean arterial pressure
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	NO synthase
NO_x	NO products (nitrite and nitrate)
nNOS	neuronal NOS
PMN	polymorphonuclear
ROS	reactive oxygen species
SMA	superior mesenteric artery
SOD	superoxide dismutase
TNBS	2,4,6-trinitrobenzenesulfonic acid
TPR	total peripheral resistance
XO	xanthine oxidase
XOR	xanthine oxidoreductase

1. SUMMARY

Gastrointestinal neuroprotection involves the net effects of many mechanisms which protect the enteric nervous system and its cells from death, dysfunction or degeneration. Neuroprotection is also a therapeutic strategy, aimed at slowing or halting the progression of primary neuronal loss following acute or chronic diseases.

This thesis will focus on the roles of glutamate and N-methyl-D-aspartate (NMDA) receptors in the intrinsic neuronal control of gastrointestinal motility; the inflammation linked to gastrointestinal motility changes; and the involvement of tryptophan metabolites, especially kynurenic acid (KynA), in the regulatory function of the enteric nervous system and the modulation of the inflammatory response. We have designed and conducted three experimental studies to investigate whether the blockade of peripheral NMDA-sensitive glutamate receptors alters motility changes after mechanical ileus or chemically induced inflammation in the large intestine, and how this modulation is accomplished.

In *Study* I, we performed experiments on three groups of dogs. Group 1 served as the sham-operated control; in groups 2 and 3, mechanical colon obstruction was maintained for 7 h. Group 3 was treated with the natural NMDA receptor antagonist KynA at the onset of the mechanical ileus. Hemodynamics and motility changes were monitored, and the activities of xanthine oxidoreductase (XOR) and nitric oxide synthase (NOS) were determined from tissue samples. The mechanical ileus induced a hyperdynamic circulatory reaction, significantly elevated the motility index and increased the mucosal leukocyte accumulation and the XOR activity. The KynA treatment augmented the tone of the colon, permanently decreased the motility index of the giant colonic contractions and reduced the increases in XOR and NOS activities.

In *Study* II, the intestinal inflammatory and motility changes were examined in a rat model of colitis. The macrohemodynamics, inflammatory enzyme activities (NOS and XOR) and colonic motility were evaluated 17 h after colitis induction with 2,4,6-trinitrobenzenesulfonic acid (TNBS) and compared with the control conditions. The TNBS enema induced a systemic hyperdynamic circulatory reaction, significantly elevated the mucosal XOR and NOS activities and augmented the colonic motility relative to the controls. The NMDA receptor antagonist KynA or treatment with KynA, a blood-brain barrier-permeable KynA analog, significantly reduced the XOR and NOS activities, decreased the motility and increased the tone of the colon.

In *Study* III, KynA or the synthetic analog SZR-72 was administered 6 days after TNBS induction. The experiments were performed on anesthetized rats that were randomized to control or colitis groups. Large bowel motility parameters and macrohemodynamics were recorded, and the nitrite/nitrate (NO_x) concentration and XOR activity were determined on colon biopsies. TNBS induction elevated the tissue inflammatory enzyme activities and the level of NO_x formation. The NMDA receptor antagonist treatments significantly decreased the signs of inflammatory activation and the levels of NO_x, and normalized the rate of bowel movements in both NMDA receptor antagonist-treated colitis groups in the late phase of experimental colitis.

Overall, the evidence suggests that gastrointestinal neuroprotection against inflammation and glutamate-induced neurotoxicity may be mediated synergistically through the blockade of NMDA receptors, and the inhibition of NOS activity and XOR-dependent superoxide production. These components are likewise significant factors in the pathomechanism of gastrointestinal inflammatory diseases and inflammation-linked motility alterations. Inhibition of the enteric NMDA receptors by KynA or its analogs may provide a novel option via which to influence intestinal hypermotility and inflammatory processes simultaneously.

2. INTRODUCTION

Gastrointestinal (GI) motility changes can be associated with or induced by local inflammation. The different types of mechanical intestinal obstructions are commonly diagnosed during consultations or emergency surgical situations, and these syndromes could be accompanied by severe abdominal inflammation (Bauer et al. 2002, Madl et al. 2003). Moreover, irrespective of the etiology or the type of the intraperitoneal surgical intervention, GI motility disorders are prevailing characteristics in the postoperative period. In general, the essential successful treatment of these clinical entities involves normalization of the GI motility. However, the morbidity rates of these syndromes are still very high, and the therapeutic possibilities of dysmotility are still rather limited, mainly due to the incompletely explored pathophysiology.

Intestinal dysmotility is also present in inflammatory bowel diseases (IBDs), Crohn's disease and ulcerative colitis (Braus and Elliott, 2009). The integrity of the superficial mucosa is breached in these conditions, and the extending inflammatory activation (Stein et al. 1998) may alter the nitrergic (Boughton-Smith 1994) and the adrenergic (Jacobson et al. 1997) neurotransmission, thus causing modulation of the smooth muscle activity. The exact pathways leading to the development of inflammation-induced colon motility changes are unmapped, but recent studies have demonstrated that glutamatergic elements may be involved in the signal transduction during this condition. Glutamate, the main excitatory neurotransmitter in the central nervous system (CNS), is also present in the enteric nervous system (ENS; Giaroni et al. 2003, Liu et al. 1997) and a high proportion of the ENS neurons express the N-methyl-D-aspartate (NMDA)-type glutamate receptors (Giaroni et al. 2003, Kirchgessner et al. 2001, Sinsky et al. 1998, Wiley et al. 1991). In line with these observations, previous data have suggested the involvement of enhanced NMDA receptor activation in nociception (Li et al. 2006, Zhou et al. 2006) as a remote CNS effect of colitis (Coutinho et al. 1996).

2.1. Regulation of bowel motility. The ENS

The reflex circuitries of the ENS have been thoroughly studied in the mammalian gut (Furness et al. 2004, 2006, 2008). Propulsion of the bowel content involves contraction of the circular muscle orally to a bolus in the lumen (the ascending excitatory reflex), and relaxation on the anal side (the descending inhibitory reflex). Three types of stimuli (distension, mechanical distortion of the mucosa, and changes in luminal chemistry) can independently elicit polarized reflex responses in the intestine, excitation in the oral direction and relaxation

in the anal direction (Kunze et al. 1999). Two main patterns of activity are recognized in the small intestine, the fed pattern and the interdigestive state. The latter is characterized by the giant migrating complex (GMC), which passes along the intestine every 80-100 min in humans.

The first neurons in the intrinsic nerve circuits, which are activated by appropriate stimuli, are the intrinsic primary afferent neurons (IPANs). They detect changes in lumenal chemistry, mechanical distortion of the mucosa and mechanical forces in the external musculature. Type II neurones are found in the myenteric and submucosal plexuses of the small and large intestines of all mammals (Furness 2000, Kunze et al. 1999).

Interneurons in the myenteric plexus have been identified by structural studies. One type of orally directed (‘ascending’) and three types of anally directed (‘descending’) interneurons have been found in the small intestine. The ascending neurons are cholinergic and, like the descending neurons, form chains that extend along the gut (Kunze et al. 1999). The descending type of interneurons are involved in local motility reflexes, the conduction of GMCs and secretomotor reflexes, through cholinergic, nitrergic, peptidergic and serotoninergic neurotransmission (Costa et al. 1996, Kunze et al. 1999).

It has subsequently been recognized that motility is automated by the pacemaker cells of the ENS. These are specialized cells known as interstitial cells of Cajal (ICCs), a non-neural cell type with a similar mesenchymal origin to that of the muscle (Sanders et al. 2006, Thomson et al. 1998), which are distributed in specific locations within the tunica muscularis of the GI tract. The ICCs provide pathways for the active propagation of slow waves, are mediators of enteric motor neurotransmission, and play a role in afferent neural signaling. Ultrastructural studies have demonstrated that the neuroeffector junctions within the GI tract involve specialized synapses that exist between enteric nerve terminals and intramuscular ICCs (ICCs-IM). The ICC-IMs are coupled to smooth muscle cells via gap junctions, and postjunctional responses elicited in the ICC-IMs are conducted to neighboring smooth muscle cells (Ward et al. 2006). In the colon, the ICCs located along the submucosal surface of the circular muscle layer (ICC-SMs) also provide a pacemaker function (Smith et al. 1987). A special population of ICCs is distributed over the surface of muscle bundles and within septae that separate muscle bundles, and are termed ICC-SEPs (Lee et al. 2007). Functional neurotransmission cannot occur in the absence of these cells (Ward et al. 2000). Indeed, surgical manipulations of the GI tract, including intestinal resection and anastomosis, lead to dysmotility in association with disruption of the ICC networks (Yanagida et al. 2004).

Extensive physiological studies have revealed that the muscle layers of the stomach and intestines are dually innervated by excitatory and inhibitory motor neurons. The primary transmitter of excitatory motor neurons is acetylcholine (ACh), while antagonists of tachykinin receptors can block residual transmission. Similarly to the excitatory motor neurons, the inhibitory neurons have co-transmitters. The substances that can contribute to transmission include nitric oxide (NO), ATP, vasoactive intestinal peptide, and pituitary adenylyl cyclase-activating peptide. Of these, NO is most frequently implicated as primary transmitter (Furness et al. 1995).

Extrinsic primary afferent neurons are found in the nodose and dorsal root ganglia and transmit sensory information to the CNS. The submucosal primary afferent neurons innervate other submucosal neurons and project to the myenteric plexus. Secretomotor neurons in the submucosal plexus contain choline-acetyltransferase or VIP and are innervated either directly or indirectly, thereby providing the neural circuitry for a secretomotor reflex (Furness et al. 2000, Kirchgessner et al. 2001).

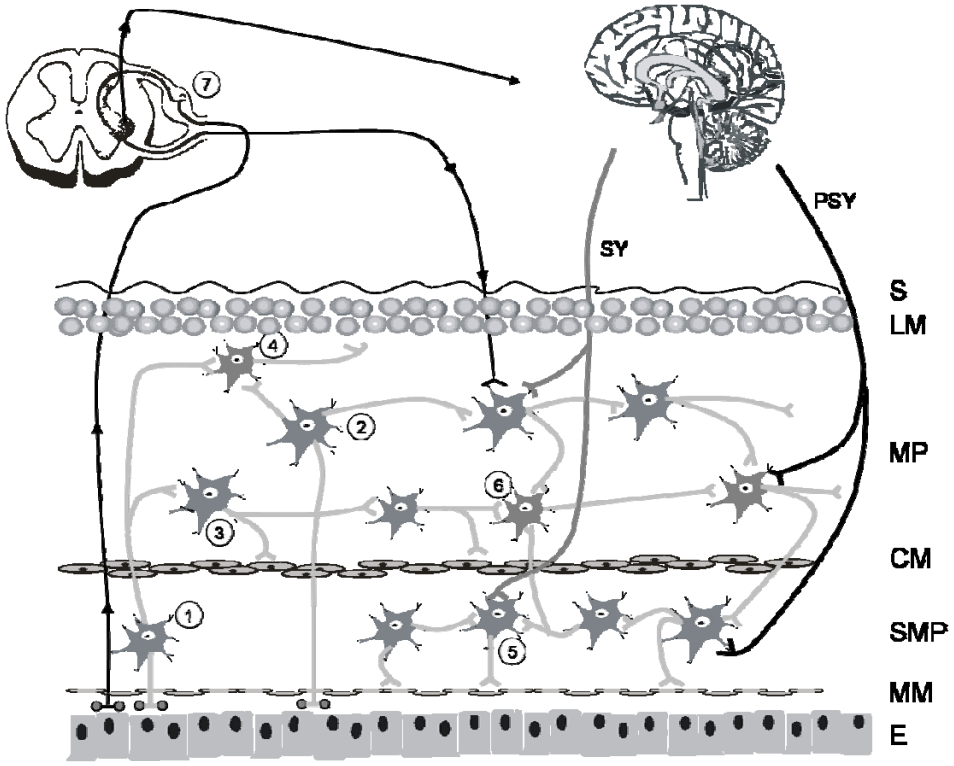

Figure 1. *Simplified representation of the enteric neuronal system and its connections with the CNS. 1. An IPAN with a cell body in the submucosal plexus; 2. an IPAN with a cell body in the myenteric plexus (1 and 2 are AH-type neurons); 3-5. muscle motor neurons; 6. an interneuron (3-6 are S-type neurons); 7. an extrinsic primary afferent neuron; S: serosa; LM: longitudinal muscle; MP: myenteric plexus; CM: circular muscle; SMP: submucosal plexus; MM: muscularis mucosae; E: intestinal epithelium, SY: sympathetic fibers; PSY: parasympathetic fibers. Based on the work of JB Furness (Furness 2000, Kaszaki et al. 2012).*

2.2. Glutamate receptors in the ENS

Glutamate is the major excitatory neurotransmitter in the mammalian CNS, and it has been established that higher glutamate concentrations can lead to overexcitation, which has implications in the development of several disorders (Coyle et al. 1993, Ozawa et al. 1998, Turski et al. 1993, Weinberg et al. 1999). Glutamate not only mediates neurotransmission, but also takes part in the regulation of cell viability (Sattler et al. 2001), differentiation (Maric et al. 2000) and the formation of synapses. The importance of these normal and pathological functions explains the need for appropriately regulated glutamate levels. The findings of Liu strongly supported the hypotheses that there are glutamatergic neurons in the ENS and that glutamate is an enteric neurotransmitter (Liu et al. 1997). It has been shown that glutamate receptors are distributed in the intestinal tract, and glutamate immunoreactivity has been detected in subsets of submucosal and myenteric neurons in the guinea-pig ileum (Moroni et al. 1986). Glutamatergic submucosal neurons are presumed to be intrinsic primary afferent neurons that project axons to the mucosa and can detect mucosal chemical and mechanical stimuli.

The receptors that mediate the glutamate signal are divided into two main classes, which can be subdivided further according to the pharmacological agents that are able to activate the appropriate receptor specifically. The ionotropic receptors, such as kainate, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptors are ligand-gated ion channels, while the metabotropic receptors are coupled to G-proteins.

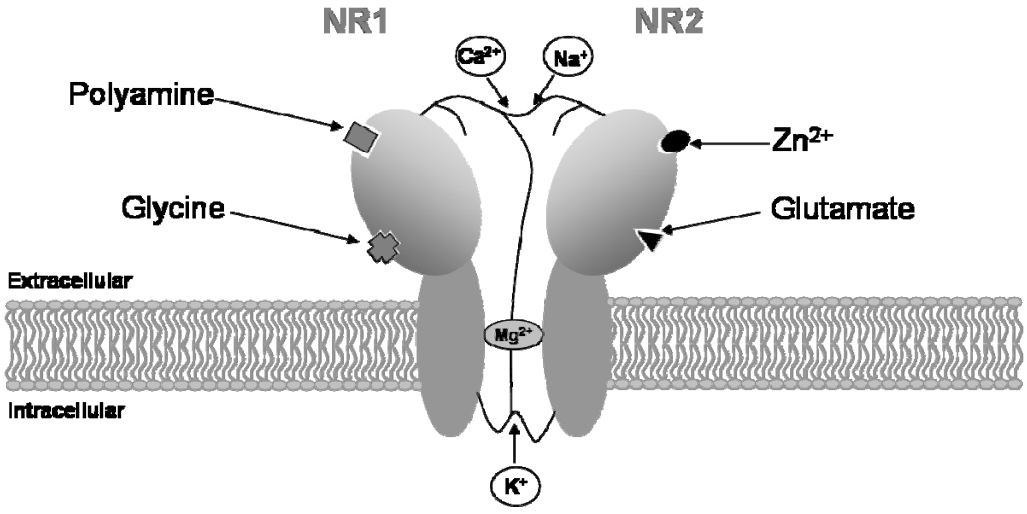

Figure 2. *Schematic illustration of the structure of the NMDA-type glutamate receptor with its main distinct binding sites. The ion channel is blocked by Mg^{2+} in a voltage-dependent manner. NMDA receptor activation (binding of both glycine and glutamate) opens the nonselective cation channel. The activation results in the flow of Na^{+} and Ca^{2+} into the cell and of K^{+} out of the cell (Kaszaki et al. 2012).*

2.3. Roles of NMDA receptors in the regulation of GI motility

GI motility regulation is predominantly cholinergic in nature, but the neurons of the ENS have a wide variety of neurotransmitters (Furness 2006). Ionotropic NMDA-sensitive glutamate receptors are present and abundantly expressed on enteric cholinergic neurons (Liu et al. 1997, Moroni et al. 1986, Shannon et al. 1989, Wiley et al. 1991). Moreover, glutamate is selectively concentrated in terminal axonal vesicles and can be released after the application of an appropriate stimulus (Sinsky et al. 1998, Wiley et al. 1991). These early data were reinforced by studies demonstrating that the activation of glutamate-NMDA receptors enhances ACh release from myenteric neurons in the guinea-pig ileum and colon (Giaroni et al. 2003, Kirchgessner et al. 2001). This latter effect has been proposed as a possible mechanism for glutamate-induced contractions of the guinea-pig ileum (Giaroni et al. 2003, Liu et al. 1997, Sinsky et al. 1998, Wiley et al. 1991) and functional studies have described the significance of glutamate in the modulation of motor and secretory functions in the gut (Cosentino et al. 1995, Sinsky et al. 1998, Wiley et al. 1991).

The GI motility response basically depends on the outcome of myenteric plexus activation, and the net effect combines contractile and relaxant signals. The participation of peripheral NMDA glutamate receptors in this mechanism is of special interest, but the exact mediatory pathways and concomitant neurotransmitter releases have not yet been elucidated clearly. In this respect, it is important to note that non-adrenergic non-cholinergic intrinsic innervations mediate inhibitory or relaxing responses to the peristaltic waves through nitrergic neurotransmission (Kohjitani et al. 2005). There is a clear link between Ca^{2+}-dependent constitutive NO production and the ENS reactions (Ekblad et al. 1994, Furness et al. 2000, Qu et al. 1999). The NMDA receptor is associated with a cation-selective channel that gates Ca^{2+} in the resting state, and this receptor has been shown to be up to 70 times more permeable to Ca^{2+} than AMPA or kainate receptors. For transmitter release, the Ca^{2+}-dependent neuronal NO synthase (nNOS) permits the rapid release of NO, a process associated with the translocation of cytosolic nNOS to the cell membrane. There it binds to postsynaptic density-95 protein in intimate association with NMDA glutamate receptors, permitting a direct route for Ca^{2+} through the NMDA receptor channel to nNOS. The activation of myenteric NMDA receptors, followed by a massive Ca^{2+} influx through the NMDA receptor-ion complex, may stimulate NO synthesis, which in turn would increase an ACh release. An elegant study by Miluseva demonstrated that stimulation of the enteric NMDA receptors by glutamate resulted in increased NADPH-diaphorase staining (reflecting an increase in myenteric nitrergic

neurons) and NOS activity, in line with the enhanced ACh efflux. The NOS inhibitor N^{ω}-nitro-l-arginine effectively opposed both effects (Miluseva et al. 2005). Another study reinforced the functional linkage of peripheral NMDA receptors in the lower esophageal sphincter and the NO-cGMP pathway, leading to smooth muscle relaxation (Kohjitani et al. 2005). Thus, glutamate or its endogenous receptor agonists/antagonists may participate in modulation of the enteric cholinergic function, since activation of the NMDA receptors enhances NO-dependent ACh release from the myenteric neurons in the ileum and colon (Giaroni et al. 2003, Kohjitani et al. 2005, Miluseva et al. 2005).

In contrast with the above, several studies have indicated that NMDA induces contraction of the guinea-pig ileal smooth muscle (Moroni et al. 1986). Electrical field stimulation under various conditions induces contractions instead of relaxation in the lower esophageal sphincter, which proved sensitive to tetrodotoxin (Kohjitani et al. 2005). Moreover, electrically evoked ACh release was demonstrated to be inhibited by NO (Hebeiss et al. 1996), while in other studies NO failed to modulate the release of ACh (Mizhorkova et al. 2001). A feasible explanation of these contradictory observations is that smooth muscle relaxation is modulated in part by the extracellular production of superoxide anions, thereby eliminating the relaxant effect of endogenous NO. It should be added that the reaction of NO with superoxide radicals leads to the formation of peroxynitrite, a known inhibitor of NOS activity and a highly injurious molecule to a variety of cells (Beckman et al. 1990). Superoxide production has been reported to be triggered by NMDA and inhibited by superoxide dismutase (SOD) or MK801 in cultured cerebellar granule cells (Lafon-Cazal et al. 1993). Furthermore, nNOS catalyzes superoxide formation at lower concentrations or in the absence of L-arginine in a Ca^{2+}/calmodulin-dependent manner (Pou et al. 1992). It should be noted that elevated xanthine oxidoreductase (XOR) activity and polymorphonuclear (PMN) cell accumulation are typical components of gastrointestinal inflammation, and the inhibition of PMN leukocyte activation and reduction of the tissue concentrations of PMN- or XOR-derived superoxide radicals can in turn reduce the tissue damage. In this line, the accumulation of glutamate with subsequent activation of the NMDA glutamate receptors can lead to the increased production of nitroso radicals and further formation of reactive oxygen species (ROS) through the stimulation of constitutive NO synthase (cNOS) and XOR activities (Yue et al. 2001).

2.4. Kynurenic acid and neuroprotection in the ENS.

L-Tryptophan is an important essential amino acid used for protein synthesis and it is also a precursor of bioactive molecules. Approximately 1-2% of the intake is metabolized through serotonin synthesis, but most enters the kynurenine pathway, synthesizing L-kynurenine, quinolinic acid, kynurenic acid (KynA) and NAD. The rest of the dietary tryptophan undergoes bacterial degradation or is excreted in the urine (Keszthelyi et al. 2009). Two major products of the tryptophan–L-kynurenine pathway, quinolinic acid and KynA, act on the glutamate receptors. The main route of this pathway generates quinolinic acid, an agonist of the NMDA glutamate receptors and NAD, while only a side-branch is responsible for KynA production.

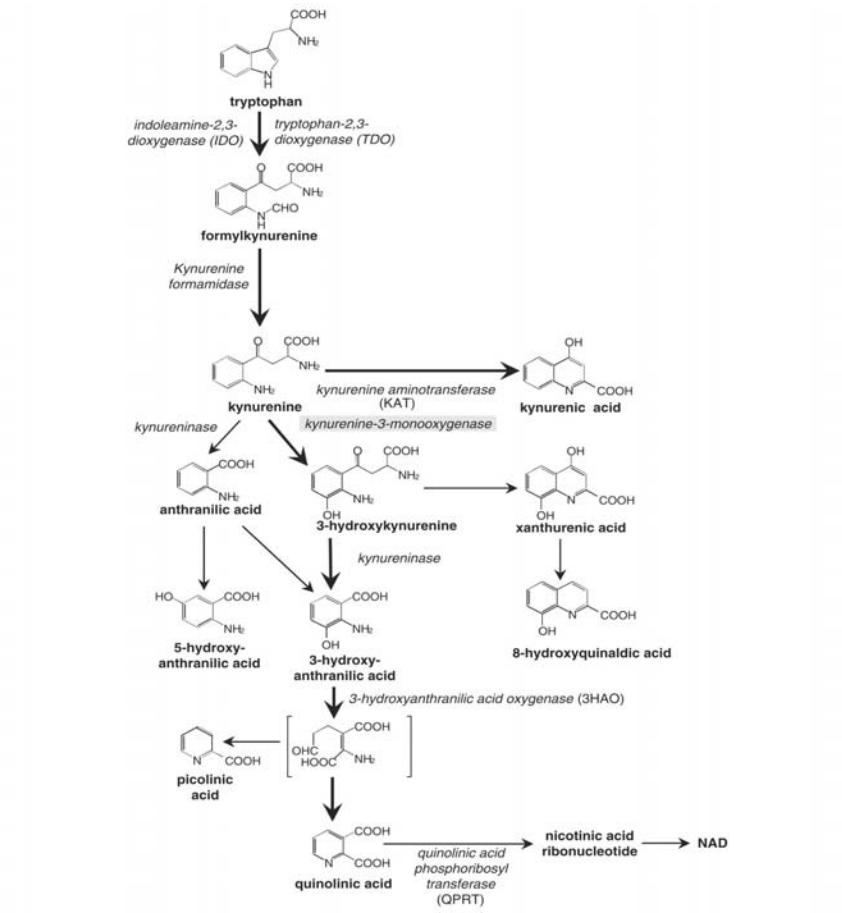

Figure 3. *The L-tryptophan metabolism (Rodgers et al. 2009).*

KynA is an endogenous NMDA receptor antagonist (Klivényi et al. 2004) and is able to reduce excitotoxic damage of the CNS both *in vivo* (Faden et al. 1989, Simon et al. 1986) and *in vitro* (Choi et al. 1988). KynA is considered neuroprotective in neurodegenerative disorders (Klivényi et al. 2004), but it is practically not able to cross the blood–brain barrier (BBB; Fukui et al. 1991). To date, a number of clinical data suggest that the metabolism of

tryptophan along the kynurenine pathway is altered in inflammatory GI disorders, too. The plasma level of L-kynurenine is elevated in IBD patients, and the levels are likewise increased in celiac disease (Forrest et al. 2002, Torres et al. 2007). However, despite these findings, the roles and function of KynA and other l-tryptophan metabolites in the ENS are as yet largely unknown.

3. MAIN GOALS

The main purpose of our studies was to investigate and clarify the roles of NMDA-type glutamate receptors in GI motility disorders associated with inflammation. Since the chronology and time frame of events can be decisive factors during these conditions, we had to design three related series of experiments to explore the most important components of pathophysiological changes.

1. *Study I* was designed to determine whether NMDA receptors play roles in physiological and pathophysiological GI motility changes. Our main aim was to follow the variations in the colonic motility as a function of time during the development of inflammatory reactions. With this aim, we used a large animal model of acute mechanical ileus to monitor inflammatory and motility changes simultaneously. We administered exogenous KynA to outline the *in vivo* consequences of NMDA receptor antagonism on colon motility regulation in this setup.
2. *Study II* was designed to evaluate the level of acute inflammatory activation in the distal colon in a rodent model of experimental colitis. Our aim was to investigate whether alterations in NMDA receptor activation play a role in the regulation of intestinal motility in these settings. However, as KynA is virtually unable to cross the BBB, it can not provide information on possible CNS effects of NMDA receptors in GI regulation. To address this issue, we set out to characterize and compare the *in vivo* effects of the endogenous NMDA glutamate receptor antagonist KynA and its BBB-permeable synthetic analog SZR-72 in the GI tract after the initial inflammatory insult. An additional aim was to characterize the *in vivo* effects of SZR-72 in the GI tract. The compound was originally developed to influence NMDA receptor overexcitation in the CNS and its peripheral ENS effects were still largely unmapped.
3. The goal of *Study III* was to examine whether GI motility alterations could be influenced by NMDA receptor antagonism in the later period of inflammation. It is important to note that medical therapy typically starts after the onset of signs and symptoms, and experimental studies involving delayed treatment are therefore arguably more relevant to the clinical scenario. To address this issue, we set out to characterize the *in vivo* effects of the endogenous NMDA glutamate receptor antagonist KynA and its BBB-permeable synthetic analog, SZR-72, on the inflammatory and motility changes still present 6 days after the onset of colitis. To

acquire further insight into the mechanism of action of KynA, we designed *in vitro* experiments to test the direct effects of KynA on the activity of the ROS-producer enzyme XOR.

4. A further aim was to provide comprehensive comparative data on the role of NMDA receptors in the ENS during baseline and inflammatory conditions. To this end, we have collected data from dogs, Wistar rats and Sprague-Dawley rats, the strains most commonly used for GI inflammation studies.

4. MATERIALS AND METHODS

4.1. Animals

The experiments were performed in adherence to the NIH guidelines for the use of experimental animals. The study was approved by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged.

The experiments in *Study I* were performed on healthy, mongrel dogs of both sexes (average weight 12-18 kg; *n*=16) from the Animal House of the University of Szeged.

The experiments of *Study II* were performed on male Wistar rats (average weight 300 g; *n*=24).

In *Study III,* male Sprague-Dawley rats (average weight 350 g; *n*=21) were used. The species, the numbers of animals in the individual groups, the interventions and the administered agents in the respective *Studies* are shown below.

Studies	Observation time	Groups	Interventions	n
Study I	**7 hr**	1	Sham-operated control	5
(Dogs)		2	Mechanical ileus	6
		3	Mechanical ileus + KynA	5
Study II	**17-23 hrs**	1	Sham-operated	6
(Wistar rats)		2	Colitis	6
		3	Colitis + KynA	6
		4	Colitis + SZR-72	6
Study III	**5 hrs on day 6 (144-149 hrs)**	1	Sham-operated	5
(Sprague-Dawley rats)		2	Colitis	5
		3	Colitis + KynA	6
		4	Colitis + SZR-72	5

Table I. Study groups, treatments and numbers (n) of animals.

4.2. Surgical preparations, experimental protocol in Study I

Surgery was performed under sodium pentobarbital anesthesia (30 mg kg^{-1} iv). The left femoral artery and vein were cannulated for the recording of mean arterial pressure (MAP)

and for fluid and drug administration, respectively. A central venous catheter was introduced into the left jugular vein for central venous pressure (CVP) measurement. A Swan-Ganz thermodilution catheter was positioned into the pulmonary artery via the right femoral vein to measure the cardiac output (CO).

After a midline abdominal incision, the superior mesenteric artery (SMA) was dissected free and an ultrasonic flow-probe was placed around the exposed SMA to measure the mesenteric blood flow. The level of the obstruction was marked by placing a silicone tourniquet catheter around the mid-transverse colon, keeping the neurovascular connections intact. Strain gauge transducers (Experimetria Ltd., Budapest, Hungary) were sutured with an atraumatic technique onto the antimesenteric side of the bowel wall to measure colonic motility at 10 cm proximally from the occlusion point.

The baseline variables were determined during a 30-min control period. Group 1 (n=5) served as the sham-operated control. Dose-response effects (the exact concentrations of KynA required to yield a beneficial effect *in vivo*) were investigated in pilot rat studies. In groups 2 (n=6), and 3 (n=5), complete large bowel obstruction was induced by tightening the tourniquet. The animals in groups 1 and 2 were treated with the vehicle for KynA, while in group 3, KynA (Sigma Chem. USA; 50 mg kg^{-1} iv in a 0.7 ml min^{-1} iv infusion for 30 min in 20 ml 0.1 M NaOH with the pH adjusted to 7.2-7.4) was administered 3 h after the onset of ileus. The animals were observed for 7 h, the beginning of mechanical ileus denoting 0 h. Changes in colonic motility and hemodynamic parameters were registered hourly. At the end of the experiment, tissue samples were taken from the proximal part of the large bowel for the determination of inflammatory enzyme activities (NOS and XOR).

4.3. Surgical preparations and experimental protocols in Studies II and III

The animals were randomly assigned to one or other of the groups, and were deprived of food, but not water, for 12 h prior to the enema. Colitis was induced under transient ether anesthesia by the colonic instillation of 2,4,6-trinitrobenzenesulfonic acid (TNBS; 40 mg kg^{-1} in 0.25 ml of 25% ethanol) through an 8-cm-long soft plastic catheter (Morris et al. 1989). The sham-operated groups were treated with the solvent of TNBS (0.25 ml of 25% ethanol). After the enemas, the rats were returned to their cages and were fed *ad libitum* with standard laboratory chow.

The sham-operated or TNBS-treated animals in *Study II* were anesthetized with sodium pentobarbital (50 mg kg^{-1} bw intraperitoneally) 16 h after the enema. In *Study III*, anesthesia was started 143 h (6 days – 1 h) after the TNBS enema. For instrumentation, the

animals were placed in a supine position on heating pads. Tracheostomy was performed to facilitate spontaneous breathing, and the right jugular vein was cannulated with PE50 tubing for drug administration and Ringer's lactate infusion (10 ml kg^{-1} h^{-1}). A thermistor-tip catheter (PTH-01; Experimetria Ltd., Budapest, Hungary) was positioned into the ascending aorta through the right common carotid artery for CO measurements, using a thermodilution technique with a computer program (SPEL Advanced Cardiosys 1.4, Experimetria Ltd, Budapest, Hungary). The left common carotid artery was isolated and an ultrasonic flow probe (1RS; Transonic Systems Inc., Ithaca, NY, USA) filled with acoustic coupling gel was placed on the carotid artery. The right femoral artery was cannulated with PE40 tubing for MAP and heart rate measurements. After a midline abdominal incision, a strain-gauge transducer (Experimetria Ltd, Budapest, Hungary) was sutured onto the colonic wall by an atraumatic technique with seromuscular stitches 3 cm distal from the cecum for colonic motility detection. After a 30-min recovery period following the end of instrumentation, hemodynamic and colonic motility changes were monitored for 6 h (data were recorded for 15 min hourly) in *Study II* and for 5 h in *Study III*.

The groups in the two rodent studies were basically the same; the only difference between the protocols was the time frame of the experiments (17-23 h or 5 h at the beginning of day 6 after the enema (144-149 h), respectively). In both rat studies, group 1 served as a sham-operated control and group 2 was an untreated colitis group. In group 3, the animals received 25 mg kg^{-1} of KynA (Sigma-Aldrich Inc, St. Louis, MO, USA) dissolved in 0.1 M NaOH in a total volume of 1 ml, with the pH adjusted to 7.2-7.4. Group 4 was treated iv with 10 mg kg^{-1} of SZR-72 in a 1 ml h^{-1} infusion for 60 min. SZR-72 (2-(2-N,N-dimethylaminoethylamine-1-carbonyl)-1H-quinolin-4-one hydrochloride; synthetized by the Institute of Pharmaceutical Chemistry, University of Szeged (Patent No. 104448-1998/Ky/me), was dissolved in 1 ml of saline and the pH was adjusted to 7.2-7.4. The infusion of KynA or SZR-72 started 60 min after the baseline measurements (at 18 h or 1 h on day 6 (145 h) after the enema, respectively) and lasted for 60 min.

4.4. Measurements

4.4.1. Hemodynamic measurements

In *Study I*, the MAP, CVP, portal venous pressure and SMA blood flow were monitored continuously and registered with a computerized data-acquisition system (Haemosys 1.17; Experimetria Ltd., Budapest, Hungary). The CO was determined by thermodilution, using a SPEL Advanced Cardiosys 1.4 computer (Experimetria Ltd., Budapest, Hungary). The total

peripheral vascular resistance (TPR) was calculated via the standard formula (MAP-CVP/CO).

In *Studies II* and *III,* the pressure signals (BPR-02 transducer; Experimetria Ltd, Budapest, Hungary) and carotid artery flow signals (T206 Animal Research Flowmeter; Transonic Systems Inc., Ithaca, NY, USA) were measured continuously and registered with a computerized data-acquisition system (Experimetria Ltd, Budapest, Hungary). Total peripheral vascular resistance was calculated by using the standard formula.

4.4.2. Colonic motility measurements

The strain-gauge transducer sutured onto the colon was connected to an SG-M bridge amplifier, and the signals were recorded continuously with a computerized data-acquisition system (SPEL Advanced Haemosys 1.72; Experimetria Ltd, Budapest, Hungary). At each time point, the duration of sampling was 10 min, with a sampling frequency of 500 Hz; the signal analysis was performed off-line. The qualitative characterization of the motility pattern was based on several components, including the amplitude, the frequency and the tone. The amplitude and frequency contractions were calculated as a function of time, while the tone of the colon was given by the mean value of the minima of the motility curves (Palásthy et al. 2006, Kaszaki et al. 2008).

4.4.3. Preparation of colon biopsies

Colon biopsies kept on ice were homogenized in phosphate buffer (pH 7.4) containing Tris-HCl (50 mM, Reanal, Budapest, Hungary), EDTA (0.1 mM), dithiotreitol (0.5 mM), phenylmethylsulfonyl fluoride (1 mM), soybean trypsin inhibitor (10 μg ml^{-1}) and leupeptin (10 μg ml^{-1}, Sigma-Aldrich GmbH, Steinheim, Germany). The homogenate was centrifuged at 4 °C for 20 min at 24,000 g (Amicon Centricon-100, Millipore Corporation, Bedford, MA, USA). Tissue nitrite/nitrate (NO_x) was determined in the supernatant.

4.4.4. Tissue XOR activity

The XOR activity was determined in the ultrafiltered, concentrated supernatant by a fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine in the presence (total XOR) and absence (xanthine oxidase (XO) activity) of the electron acceptor methylene blue (Beckman et al. 1989).

4.4.5. In vitro XOR activity examination. Inhibition of XOR activity

The activity of 10 mU XOR was measured in the presence of 5 μM xanthine. The chemiluminescence was detected in the presence or in the absence of KynA or allopurinol in the 1 μM - 1 mM concentration range in 50 mM K-phosphate buffer containing 0.1 mM EDTA, pH=7.4 (experimental buffer solution). A previously mixed aliquot of the components (10 mU XOR/5 μM xanthine) was injected into a plastic minivial with a diameter of 10 mm containing luminol (100 μM) dissolved in the experimental buffer solution (pH=7.4) and was inserted into a common Packard potassium glass vial. The total volume of the reaction mixture was 1 ml (Ferdinandy et al. 2000, Onody et al. 2003).

Chemiluminescence was followed for 5 minutes in the tritium channel, in a Packard Tri-Carb 2100 Model liquid scintillation counter set in the out-of-coincidence mode. All manipulations were performed in a dark room with minimal light. Measurements were started after dark adaptation of the sample for 2 min. The luminol blank was determined before adding the sample and the measured count was subtracted from the total output. Chemiluminescence was detected in the presence or in the absence of KynA or allopurinol in the 1 μM - 1 mM concentration range. Results were expressed as inhibition (as a percentage of the control).

4.4.6. Measurement of tissue NO products

The stable end-products of NO, NO_x, were determined in the colonic homogenate by the Griess reaction in *Study III*. This assay depends on the enzymatic reduction of nitrate to nitrite, which is then converted into a colored azo compound detected spectrophotometrically at 540 nm. Total NO_x was calculated and expressed in μmol (mg protein)$^{-1}$ (Moshage et al. 1995).

4.4.7. NOS activity measurements

NO formation in the intestinal tissues was measured via the conversion of [^{3}H]L-citrulline from [^{3}H]L-arginine according to the method of Szabo *et al.* (1993). Briefly, large bowel biopsies kept on ice were homogenized in phosphate buffer (pH 7.4) containing 50 mM Tris-HCl (Reanal, Budapest, Hungary), 0.1 mM EDTA (Serva Feinbiochemica GmbH, Heidelberg, Germany), 0.5 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} soybean trypsin inhibitor and 10 μg ml^{-1} leupeptin. The homogenate was centrifuged at 4 ^{o}C for 20 min at 24,000g and the supernatant was loaded into centrifugal concentrator tubes (Amicon Centricon-100; 100,000 MW cut-off ultrafilter; Millipore Corporation, Bedford,

MA, USA). The tubes were centrifuged at 900g for 150 min and the concentrated supernatant was washed out from the ultrafilter with 250 µl homogenizing buffer. The samples were incubated with a cation-exchange resin (Dowex AG 50W-X8, Na^+ form; The Dow Chemical Company, Midland, MI, USA) for 5 min to deplete endogenous L-arginine. The resin was separated by centrifugation (1500g for 10 min) and the supernatant containing the enzyme was assayed for NOS activity.

For the Ca^{2+}-dependent eNOS activity, 50 µl enzyme extract and 100 µl reaction mixture (pH 7.4, containing 50 mM Tris-HCl buffer, 1 mM NADPH, 10 µM tetrahydrobiopterin, 1.5 mM $CaCl_2$, 100 U ml^{-1} calmodulin and 0.5 µCi [^{3}H]L-arginine (Amersham U.K., specific activity 63 Ci $mmol^{-1}$)) were incubated together for 60 min at 37 ^oC. The reaction was stopped by the addition of 1 ml ice-cold HEPES buffer (pH 5.5) containing 2 mM EGTA and 2 mM EDTA. Measurements were performed with the nonselective NOS inhibitor NNA (Sigma-Aldrich GmbH, Steinheim, Germany, 3.2 mM) to determine the extent of [^{3}H]L-citrulline formation independent of the NOS activity. Inducible NOS (iNOS) was measured without Ca^{2+}-calmodulin and with EGTA (8 mM). 1 ml reaction mixture was applied to Dowex cation-exchange resin (AG 50W-X8, Na^+ form; The Dow Chemical Company, Midland, MI, USA) and eluted with 2 ml distilled water. The eluted [^{3}H]L-citrulline activity was measured with a scintillation counter (Tri-Carb Liquid Scintillation Analyzer 2100TR/2300TR, Packard Instrument Co, Meriden, CT, U.S.A.). Protein contents of samples were determined by the Lowry method.

4.5. Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows; Jandel Scientific, Erkrath, Germany). The distribution of our experimental data was analyzed by the Kolmogorov-Smirnov normality test. Failure of the normality test indicated non-parametric distribution of the data. Accordingly, we employed nonparametric statistical tests. Friedman repeated measures analysis of variance on ranks was applied within groups. Time-dependent differences from the baseline for each group were assessed by Dunn's method, and differences between groups were analyzed with Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison. In the Figures, median values and 75th and 25th percentiles are given; p values <0.05 were considered significant.

5. RESULTS

5.1. Hemodynamics

In *Study I,* the administration of KynA did not significantly influence the MAP in animals with mechanical ileus and, similarly, treatment with KynA did not influence the colitis-induced changes in MAP as compared with the colitis group in *Studies II* and *III* (Table II).

In *Study I*, the obstruction of the large bowel led to a significant CO elevation after 5 h, and KynA treatment did not influence this process (Table IV). However, the treatment with the nonselective NMDA receptor antagonist inhibited the obstruction-induced decrease in TPR. The changes were statistically significant 6 h after obstruction (Table III).

On day 1 of colitis, the CO was significantly higher than in the sham-operated group (Table IV), while the TPR was significantly lower in the TNBS-treated groups in comparison with the sham-operated group. In this case, treatment with KynA did not influence the colitis-induced changes in CO and TPR as compared with the colitis group. At the same time, a significant increase in TPR relative to the colitis group evolved 19 h after colitis induction after SZR-72 treatment (Table III).

There were no significant between-group differences in CO (Table IV) or TPR (Table III) 6 days after the vehicle enema instillation or colitis induction, before the start of NMDA receptor antagonist treatment.

Table II. Changes in MAP [mmHg]. [x] $p<0.05$ between groups *vs* sham-operated group; [#] $p<0.05$ between NMDA antagonist-treated groups

	Parameters	Baseline	1 h after treatment start	2 h after treatment start	At the end of the experiment
		MAP in *Study I*			
Sham-operated	**Median**	**138**	**135**	**135**	**130**
	25p; 75p	*131; 147*	*123; 148*	*123; 135*	*116; 128*
Mechanical ileus	**Median**	**146**	**138**	**134**	**134**
	25p; 75p	*145; 151*	*137; 143*	*129; 139*	*129; 142*
Mechanical ileus + KynA	**Median**	**144**	**136**	**125**	**129**
	25p; 75p	*128; 159*	*115; 153*	*104; 141*	*103; 155*
		MAP in *Study II*			
Sham-operated	**Median**	**134**	**129**	**129**	**126**
	25p; 75p	*131; 137*	*128; 134*	*128; 131*	*125; 129*
Colitis	**Median**	**112** x	**113** x	**108** x	**106** x
	25p; 75p	*98; 117*	*98; 123*	*91; 119*	*98;119*
Colitis + KynA	**Median**	**119** x	**118**	**113** x	**110** x
	25p; 75p	*110; 122*	*106; 123*	*105; 125*	*106; 116*
Colitis + SZR-72	**Median**	**116**	**113**	**115** x	**111** x
	25p; 75p	*103; 125*	*107; 124*	*109; 122*	*105; 114*
		MAP in *Study III*			
Sham-operated	**Median**	**121**	**123**	**118**	**122**
	25p; 75p	*117; 128*	*108; 130*	*113; 129*	*119; 134*
Colitis	**Median**	**123**	**120**	**118**	**116**
	25p; 75p	*107; 123*	*107; 123*	*106; 126*	*110;121*
Colitis + KynA	**Median**	**129**	**127**	**121**	**119**
	25p; 75p	*123; 130*	*120; 130*	*115; 127*	*117; 124*
Colitis + SZR-72	**Median**	**123**	**118**	**124**	**120**
	25p; 75p	*122; 126*	*116; 126*	*121; 129*	*115; 123*

Table III. Changes in TPR [mmHg (ml min)$^{-1}$]. ^{x} $p<0.05$ between groups *vs* sham-operated group; $^{\#}$ $p<0.05$ between NMDA antagonist-treated groups and colitis group.

	Parameters	Baseline	1 h after treatment start	2 h after treatment start	At the end of the experiment
		TPR in *Study I*			
Sham-operated	**Median**	**0.91**	**1.07**	**1.14**	**1.03**
	25p; 75p	*0.90; 1.06*	*1.05; 1.18*	*0.82; 1.18*	*0.94; 1.14*
Mechanical ileus	**Median**	**0.93**	**0.91**	**0.80 x**	**0.69 x**
	25p; 75p	*0.86; 1.01*	*0.89; 1.06*	*0.70; 0.87*	*0.66; 0.78*
Mechanical ileus + KynA	**Median**	**0.93**	**1.05**	**1.03**	**0.98**
	25p; 75p	*0.89; 0.97*	*0.99; 1.07*	*0.99; 1.08*	*0.93; 1.09*
		TPR in *Study II*			
Sham-operated	**Median**	**2.20**	**2.11**	**2.34**	**2.43**
	25p; 75p	*1.73; 2.78*	*1.58; 2.59*	*1.84; 2.66*	*2.07; 2.58*
Colitis	**Median**	**1.47 x**	**1.45 x**	**1.47 x**	**1.45 x**
	25p; 75p	*1.36; 1.57*	*1.42; 1.55*	*1.28; 1.57*	*1.13; 1.60*
Colitis + KynA	**Median**	**1.59 x**	**1.54 x**	**1.55 x**	**1.50 x**
	25p; 75p	*1.36; 1.71*	*1.32; 1.69*	*1.22; 1.75*	*1.18; 1.57*
Colitis + SZR-72	**Median**	**1.55 x**	**1.66 #**	**1.77**	**1.86**
	25p; 75p	*1.47; 1.73*	*1.61; 1.78*	*1.63; 2.00*	*1.45; 2.03*
		TPR in *Study III*			
Sham-operated	**Median**	**1.89**	**1.67**	**1.64**	**2.05**
	25p; 75p	*1.56; 2.30*	*1.46; 1.80*	*1.40; 2.27*	*1.77; 2.22*
Colitis	**Median**	**1.82**	**1.89**	**1.86**	**1.67**
	25p; 75p	*1.43; 2.25*	*1.21; 2.09*	*1.33; 2.39*	*1.08; 2.76*
Colitis + KynA	**Median**	**1.69**	**2.15**	**1.90**	**2.25**
	25p; 75p	*1.29; 2.87*	*1.34; 2.17*	*1.67; 2.62*	*1.50; 2.27*
Colitis + SZR-72	**Median**	**1.85**	**1.93**	**1.61**	**1.76**
	25p; 75p	*1.61; 3.05*	*1.30; 2.79*	*1.09; 2.84*	*1.16; 2.13*

Table IV. Changes in CO [ml kg^{-1}]. ^{x} $p<0.05$ between treated and sham-operated group.

	Parameters	Baseline	1 h after treatment	2 h after treatment	End of the experiment
		CO in *Study I*			
Sham-operated	**Median**	**133**	**122**	**127**	**125**
	25p; 75p	*119; 148*	*118; 136*	*119; 133*	*117; 126*
Mechanical ileus	**Median**	**141**	**154**	**153 x**	**168 x**
	25p; 75p	*131; 150*	*139; 173*	*142; 160*	*137; 181*
Mechanical ileus + KynA	**Median**	**136**	**144**	**145**	**144 x**
	25p; 75p	*125; 147*	*136; 149*	*138; 152*	*142; 160*
		CO in *Study II*			
Sham-operated	**Median**	**154**	**147**	**151**	**165**
	25p; 75p	*120; 188*	*125; 185*	*106; 190*	*133; 188*
Colitis	**Median**	**215 x**	**224 x**	**228 x**	**235 x**
	25p; 75p	*197; 244*	*211; 275*	*206; 260*	*208; 255*
Colitis + KynA	**Median**	**205**	**196**	**217**	**239 x**
	25p; 75p	*184; 220*	*185; 241*	*193; 242*	*226; 245*
Colitis + SZR-72	**Median**	**215**	**185**	**195**	**193**
	25p; 75p	*183; 227*	*173; 203*	*188; 202*	*181; 223*
		CO in *Study III*			
Sham-operated	**Median**	**190**	**194**	**178**	**167**
	25p; 75p	*164; 213*	*167; 223*	*162; 208*	*158; 183*
Colitis	**Median**	**222**	**227**	**214**	**201**
	25p; 75p	*213; 230*	*198; 246*	*197; 223*	*141; 262*
Colitis + KynA	**Median**	**205**	**212**	**223**	**157**
	25p; 75p	*187; 232*	*183; 237*	*210; 233*	*149; 177*
Colitis + SZR-72	**Median**	**211**	**219**	**192**	**221**
	25p; 75p	*143; 269*	*161; 288*	*152; 238*	*191; 256*

5.2. Colonic motility changes

The colon motility index and the amplitude of the GMCs did not change in the sham-operated group during the experiments in *Study I*. The motility of the colon segment proximal to the obstruction was only slightly elevated until 5 h after the obstruction; subsequently, an approximately 1.65-fold increase gradually evolved. This change was significant by the end of the observation period.

The KynA treatment significantly inhibited the ileus-induced increase in the motility index and decreased the amplitude of the GMCs as compared with the nontreated mechanical ileus group (Figure 4A). The tone of the proximal colon was significantly decreased after obstruction, and this change was significantly inhibited by KynA treatment after 6 h (Figure 4B). The frequency of the contractions did not differ in the sham-operated and mechanical ileus groups during the observation period (Figure 5B). However, the administration of KynA caused a significant elevation in the frequency of the GMCs, which were characterized by a decreased amplitude (Figure 5A).

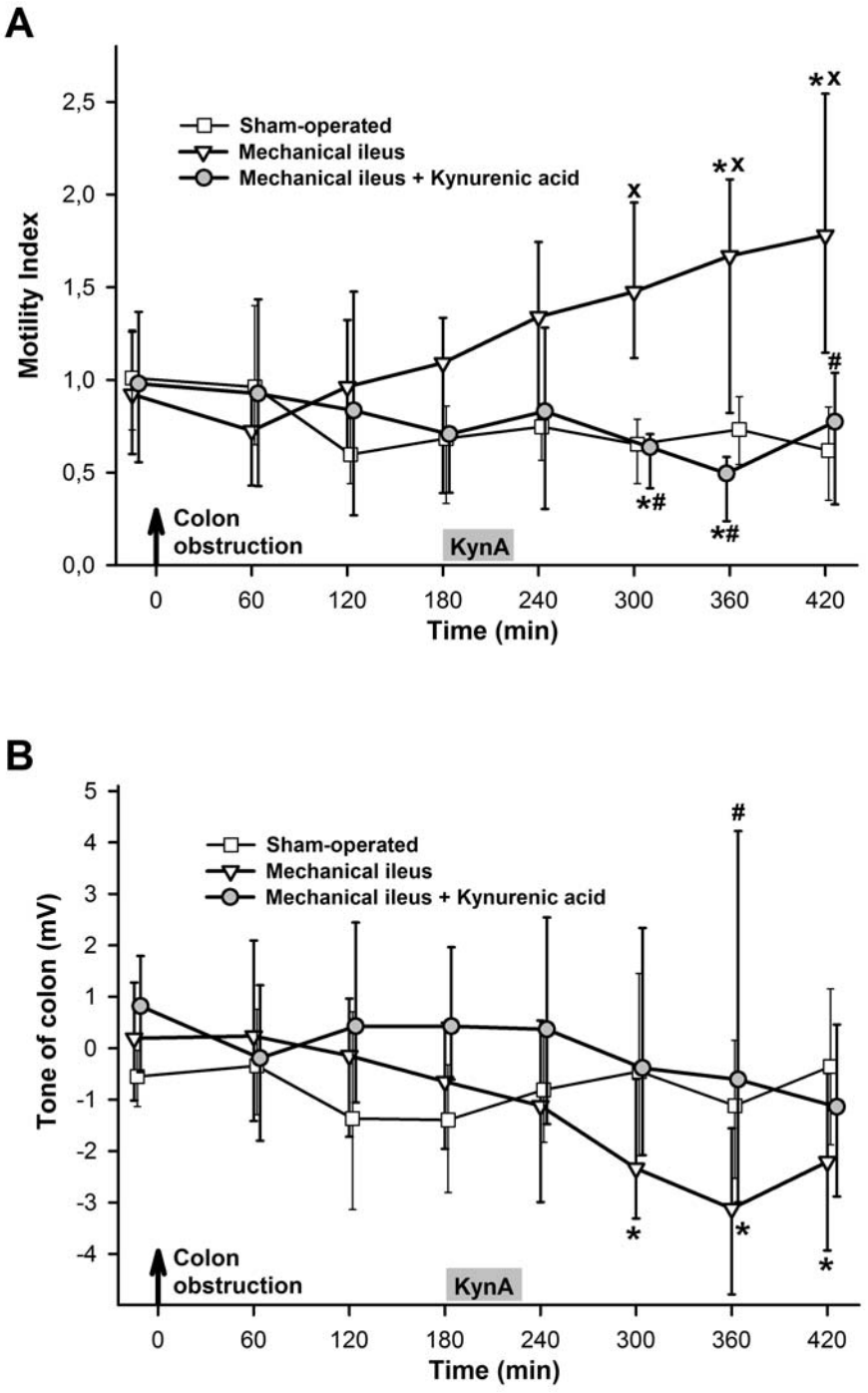

Figure 4. *Changes in motility index in Study I* ***(A)****, tone* ***(B)*** *of GMCs of the proximal colon in the sham-operated (empty squares with thin continuous line), mechanical ileus (empty triangles with continuous line), and KynA-treated mechanical ileus (shaded circles with continuous line) groups. An arrow indicates the beginning of obstruction and a gray box the infusion of KynA. The plots demonstrate the median values and the 25th (lower whisker) and 75th (upper whisker) percentiles, * p<0.05 within groups vs baseline values, ^{x} p<0.05 between groups vs sham-operated group values, $^{\#}$ p<0.05 between KynA-treated group vs obstructed group values.*

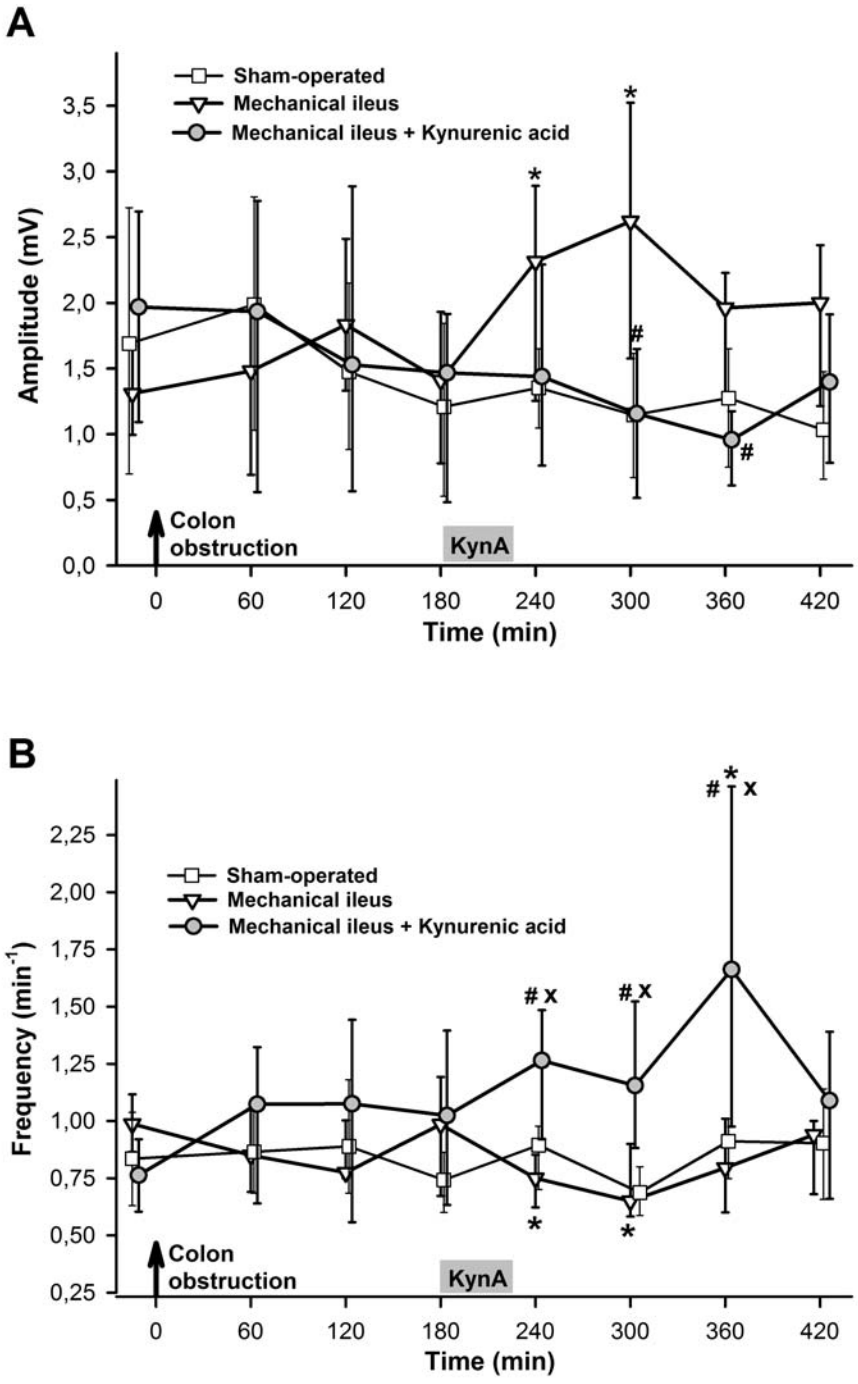

Figure 5. *Changes during Study I in amplitude **(A)** and frequency **(B)** of GMCs of the proximal colon in the sham-operated (empty squares with thin continuous line), mechanical ileus (empty triangles with continuous line), and KynA-treated mechanical ileus (shaded circles with continuous line) groups. An arrow indicates the beginning of obstruction and a gray box the infusion of KynA. The plots demonstrate the median values and the 25th (lower whisker) and 75th (upper whisker) percentiles, * p<0.05 within groups vs baseline values, ^{x} p<0.05 between groups vs sham-operated group values, $^{\#}$ p<0.05 between KynA-treated group vs obstructed group values.*

In *Study II*, the motility index and the tone of the colon were used as markers to estimate the excitatory status of the ENS (Figure 6AB). During the observation period, these parameters did not differ significantly from the baseline in the sham-operated group. At the beginning of the observation period, the motility index was higher in the TNBS-treated groups than in the sham-operated animals and it remained significantly higher during the later phase of the experiments.

After their administration, both NMDA antagonists decreased the motility index, but the characteristics of the changes were different. KynA reduced the motility promptly and had a shorter effect, while SZR-72 exerted a more gradual and prolonged inhibitory effect. The motility effects of the NMDA receptor antagonist compounds were similar 1 h after the end of the treatments (i.e. at 20 h of colitis). The tone of the colon gradually decreased in the colitis group in response to the inflammation. Administration of the NMDA antagonists in both cases significantly reversed the colitis-induced decreased colonic tone, and this parameter in these groups did not differ from the level in the sham-operated group. However, the effect of KynA was again shorter than that following SZR-72 administration.

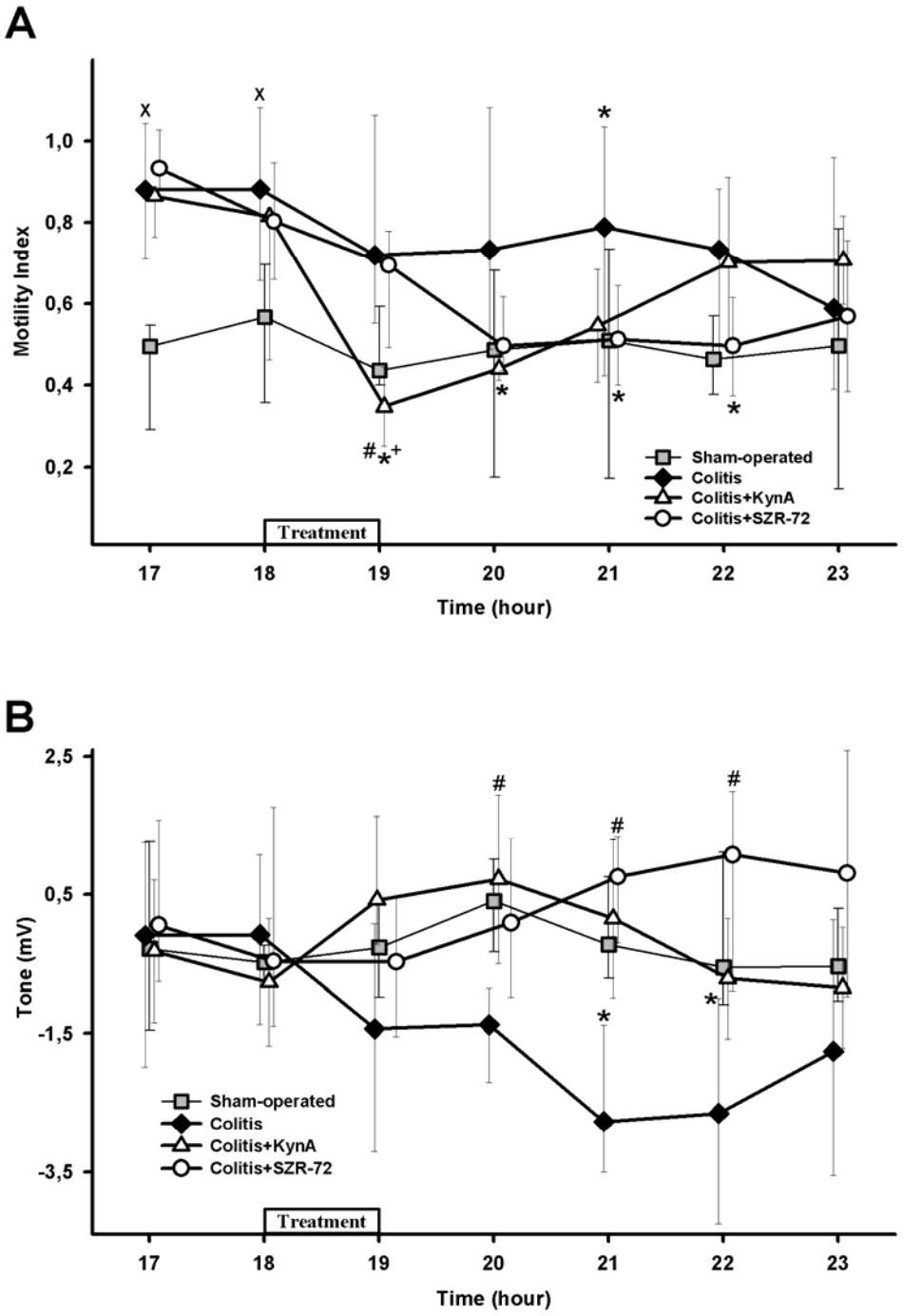

Figure 6. *Changes in motility index in Study II* ***(A)*** *and tone* ***(B)*** *of the colon in the sham-operated (shaded squares with a thin continuous line), colitis (black diamonds with a thick line), SZR-72 (empty circles with a thick line) and KynA-treated colitis (empty triangles with a thick line) groups. The box indicates the treatment with NMDA antagonists. The plots demonstrate the median values and the 25th (lower whisker) and 75th (upper whisker) percentiles, * $p<0.05$ within groups vs baseline values, ^{x} $p<0.05$ between groups and sham-operated group values, $^{\#}$ $p<0.05$ between NMDA antagonist-treated groups and colitis group, $^{+}$ $p<0.05$ between KynA-treated group and SZR-72-treated group values.*

In *Study III*, the KynA and SZR-72 *per se* did not influence the colonic motility (Figure 7A). The frequency and amplitude of intestinal movements (Figure 8AB) and the colonic tone were used as accurate markers to characterize the motility status of the large intestine 6 days after colitis induction; by this stage, the tone did not differ from that in the sham-operated controls (Figure 7B). No significant change was observed in the amplitude of the contractions in the TNBS-treated groups as compared with the vehicle-treated animals, but the frequency of intestinal movements was increased significantly.

The administration of the NMDA receptor antagonists effectively decreased the rate of bowel movements in both treated groups with colitis. KynA treatment significantly increased the smooth muscle tone of the intestine 1 h after administration and this elevation was maintained until the end of the observation period. SZR-72 caused a similar elevation in the colonic tone, which started somewhat later, ~ 2 h after the treatment, and the tone elevation likewise persisted until the end of the experiments.

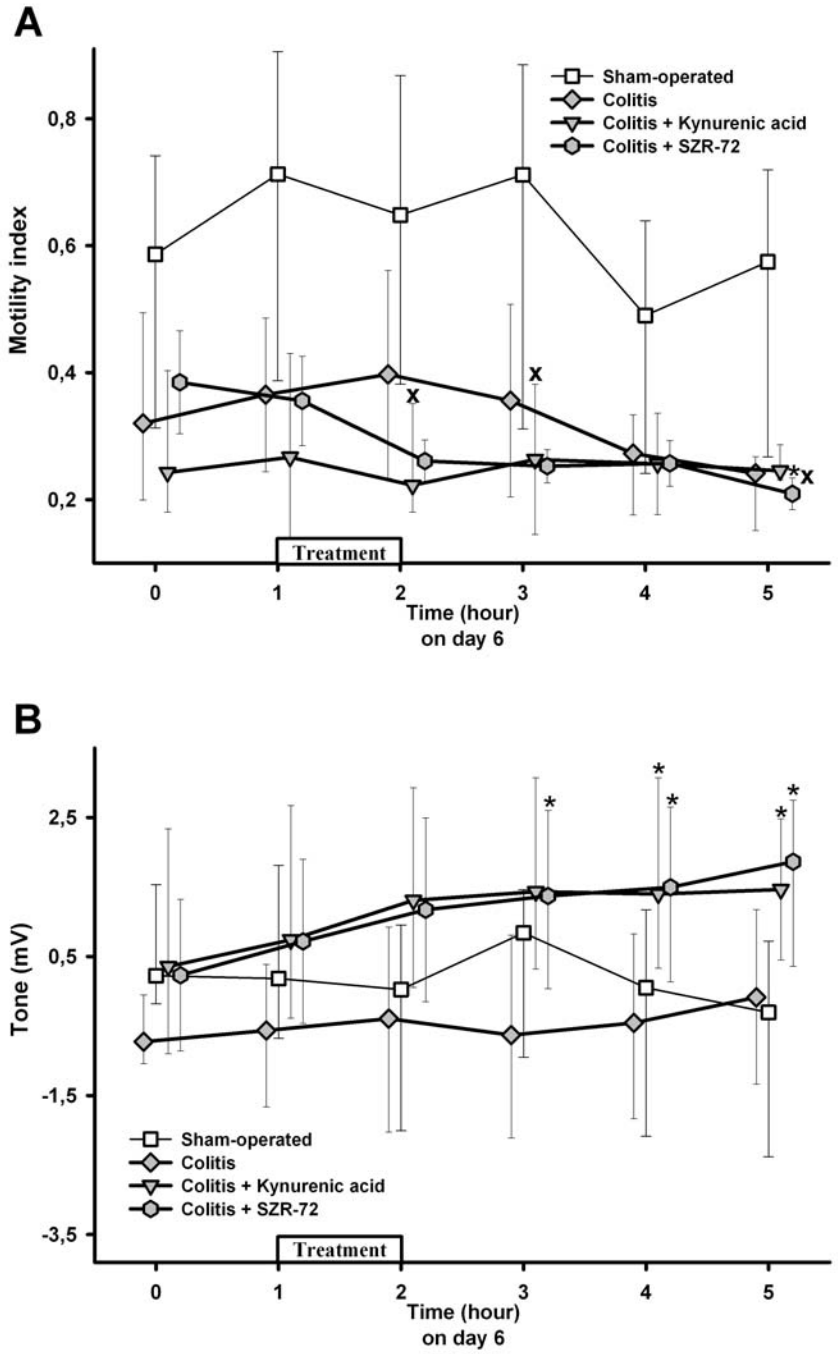

Figure 7. *Changes on day 6 after enema in motility index **(A)** and tone **(B)** of the contractions of the proximal colon in the sham-operated (empty squares with thin continuous line), colitis (shaded diamonds with solid line), SZR-72 (shaded hexagons with solid line) and KynA-treated colitis (shaded triangles with solid line) groups. The box indicates the treatment with NMDA antagonists. The plots demonstrate the median values and the 25th (lower whisker) and 75th (upper whisker) percentiles, * $p<0.05$ within groups and baseline values, ^{x} $p<0.05$ between groups and sham-operated group values, $^{\#}$ $p<0.05$ between NMDA antagonist-treated group and colitis group.*

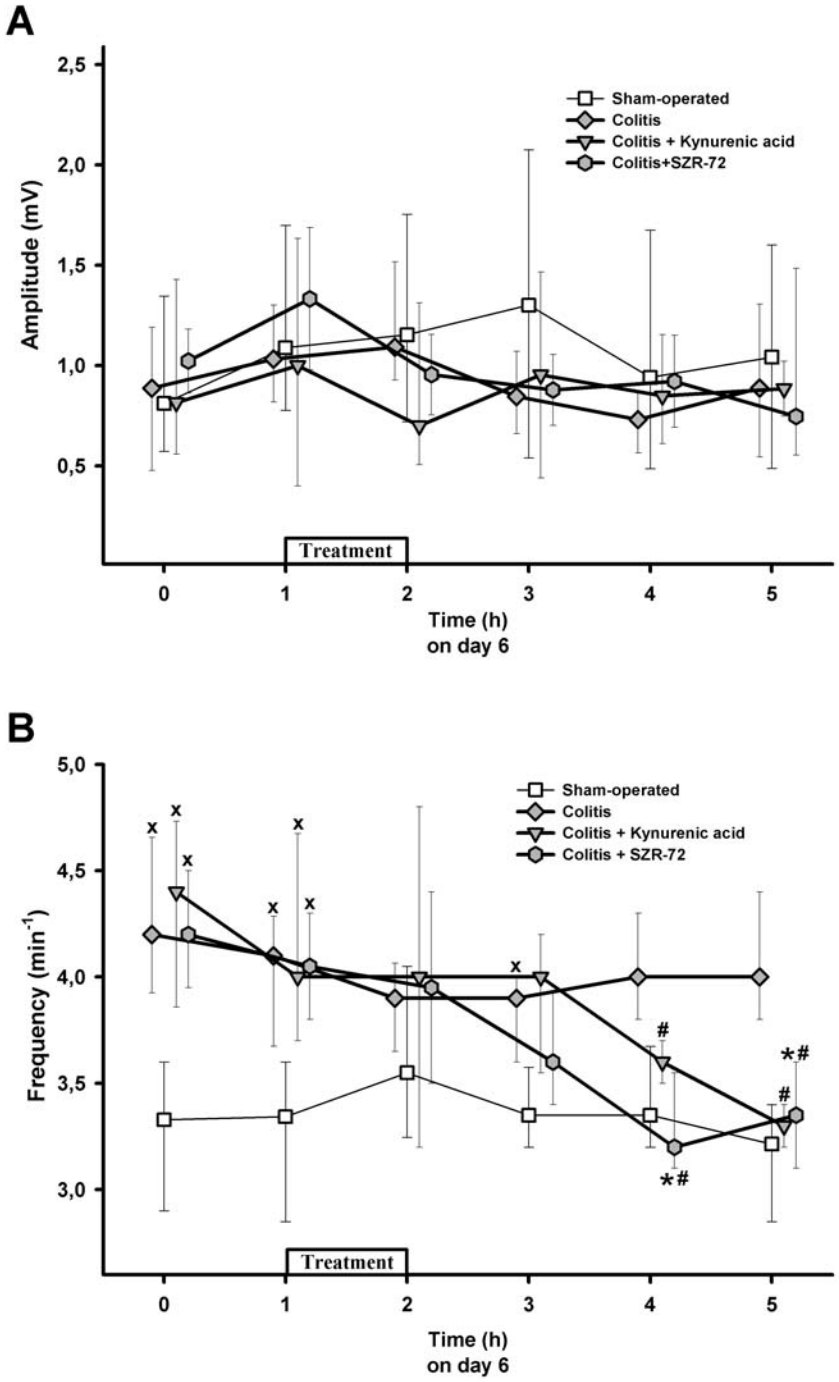

Figure 8. *Changes on day 6 after enema in amplitude* ***(A)*** *and frequency* ***(B)*** *of the contractions of the proximal colon in the sham-operated (empty squares with thin continuous line), colitis (shaded diamonds with solid line), SZR-72 (shaded hexagons with solid line) and KynA-treated colitis (shaded triangles with solid line) groups. The box indicates the treatment with NMDA antagonists. The plots demonstrate the median values and the 25th (lower whisker) and 75th (upper whisker) percentiles, * $p<0.05$ within groups and baseline values, ^{x} $p<0.05$ between groups and sham-operated group values, $^{\#}$ $p<0.05$ between NMDA antagonist-treated group and colitis group.*

5.3. NO production

NOS activity is responsible for the production of NO, an important regulator of the colonic motility. In *Study I*, after the obstruction we found significantly elevated NOS activity. NMDA antagonist KynA treatment was capable of reducing the NOS activity in the colonic tissue (Figure 9).

Colitis resulted in a significant increase in the total NOS activity over the value for the sham-operated group. In *Study II*, both of the NMDA antagonist treatments significantly decreased the colonic NOS activity as compared with the nontreated colitis group (Figure 10A).

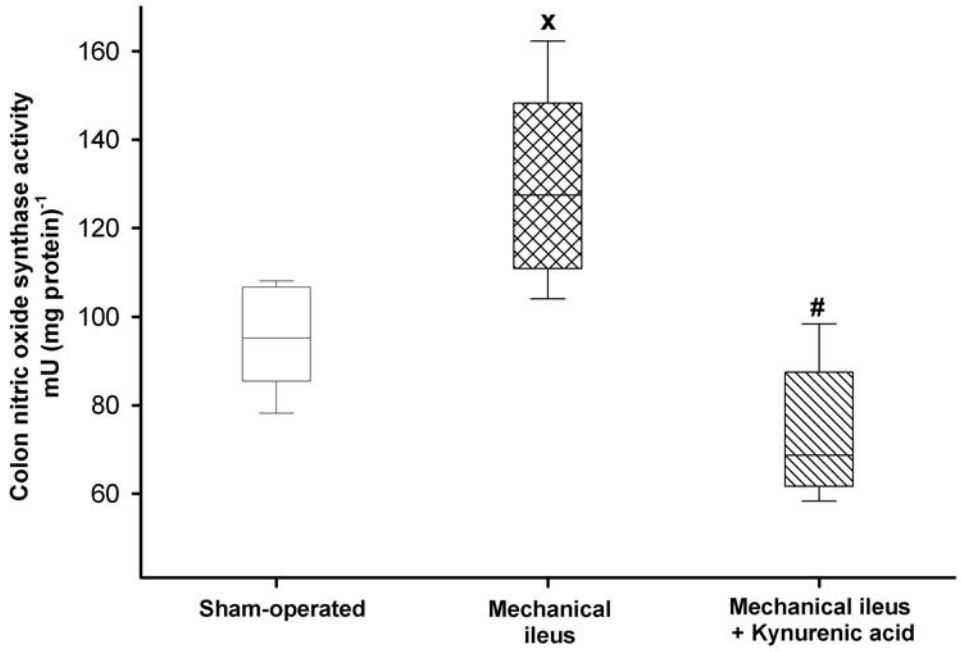

Figure 9. *Changes in activity of NOS in Study I, in colonic tissue from sham-operated (empty box), mechanical ileus (checked box), and mechanical ileus + KynA-treated (striped box) groups. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker), and 75th (upper whisker) percentiles.* ^{x} *$p<0.05$ between groups vs sham-operated group values,* $^{\#}$ *$p<0.05$ between KynA-treated groups vs obstructed group values.*

In the untreated colitis group in *Study III*, a significant elevation in NO_x level was observed in the colonic tissue relative to the control. Both NMDA antagonists decreased the NO_x levels to the control level (Figure 10B).

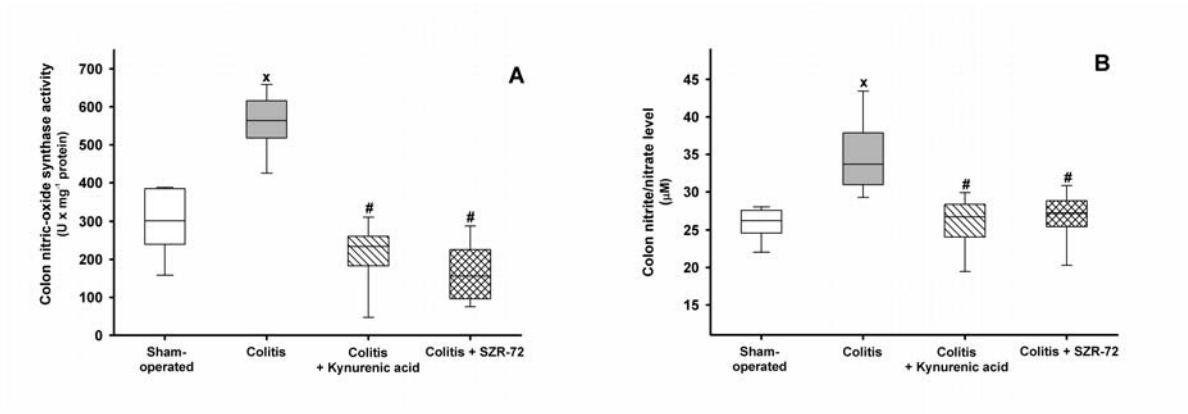

Figure 10. A: *Changes in activity of nitric oxide synthase on day 1 after colitis induction in colonic tissue from sham-operated (empty box), colitis (shaded box), KynA-treated colitis (striped box) and SZR-72-treated (checked box) groups. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker), and 75th (upper whisker) percentiles.* ^{x} *$p<0.05$ between groups and sham-operated group values,* $^{\#}$ *$p<0.05$ between NMDA antagonist-treated groups and colitis group values.* **B:** *Changes in tissue NO_x concentration on day 6 after colitis induction in the sham-operated (empty box), colitis (shaded box), KynA-treated colitis (striped box) and SZR-72-treated (checked box) groups. The plots demonstrate the median (horizontal line in the box) and the 25th and 75th percentiles.* ^{x} *$p<0.05$ between groups and sham-operated group values,* $^{\#}$ *$p<0.05$ between NMDA antagonist-treated groups and colitis group values.*

5.4. Changes in intestinal XOR activity

XOR is activated during the inflammation process and produces a considerable amount of superoxide radicals. During the experiments of *Study I*, in the sham-operated group, the XOR activities did not differ significantly. The activity of the superoxide anion-producing XOR was significantly increased after the colon obstruction. The treatment with the nonselective NMDA receptor antagonist significantly inhibited the ileus-induced increases in XOR activity (Figure 11A).

In *Study II*, 23 h following TNBS treatment, a significant elevation in colonic XOR activity was measured relative to the sham-operated group. The KynA and SZR-72 treatments each significantly decreased the XOR activity to the level for the sham-operated animals (Figure 11B). In *Study III*, the NMDA antagonist treatment on day 6 was still effective in reducing the XOR activity, but SZR-72 was less effective in this case (Figure 11C).

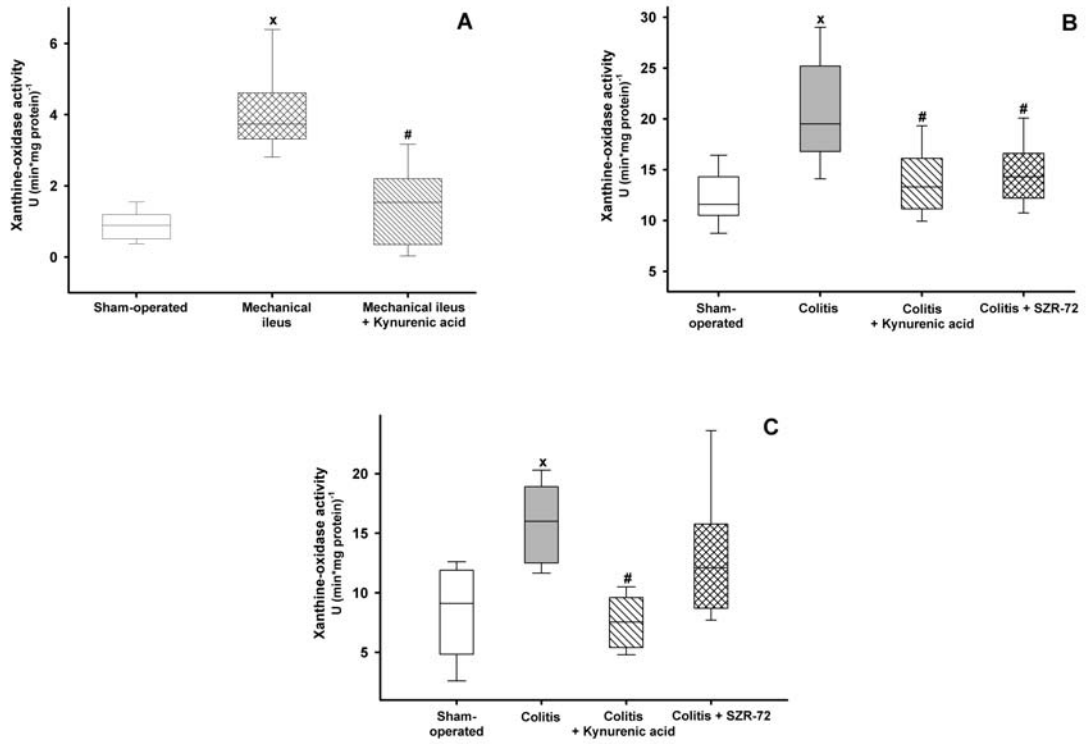

Figure 11. A: *Changes in activity of XOR in colonic tissue from sham-operated (empty box), mechanical ileus (checked box), and mechanical ileus + KynA-treated (striped box) groups. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker), and 75th (upper whisker) percentiles.* ^{x} *p<0.05 between groups vs sham-operated group values,* $^{\#}$ *p<0.05 between KynA-treated groups vs obstructed group values.* **B:** *Changes in XOR activity on day 1 after colitis induction in colonic tissue from sham-operated (empty box), colitis (shaded box), KynA-treated colitis (striped box) and SZR-72-treated (checked box) groups. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker), and 75th (upper whisker) percentiles.* ^{x} *p<0.05 between groups vs sham-operated group values,* $^{\#}$ *p<0.05 between NMDA antagonist-treated groups vs colitis group values.* **C:** *Changes in colon XOR activity in the sham-operated (empty box), colitis (shaded box), KynA-treated colitis (striped box) and SZR-72-treated (checked box) groups. The plots demonstrate the median (horizontal line in the box) and the 25th and 75th percentiles.* ^{x} *p<0.05 between groups and sham-operated group values,* $^{\#}$ *p<0.05 between NMDA antagonist-treated groups and colitis group values.*

5.5. In vitro effects of KynA on the luminol-enhanced chemiluminescence generated by xanthine/XOR

The inhibition of chemiluminescence was expressed as a percentage of the control. The effects of KynA were examined in the 1 μM - 5 mM concentration range. KynA proved to be an effective chemical tool in blocking chemiluminescence generated by the xanthine/XO system (Figure 12A). The effects of allopurinol, a specific inhibitor of XOR, were examined in the same concentration range and the inhibitory effect of 1 μM allopurinol proved to be

nearly the same as that for KynA. Allopurinol at 10 μM and 100 μM was more effective than KynA in the same concentration in blocking the chemiluminescence generated by the xanthine/XOR system, while a higher (1 mM) allopurinol concentration disturbed the chemiluminescence measurements.

Dose-dependent effects of KynA on XOR activity were further investigated by *in vitro* fluorometric assay. Between 0,1 mM and 1 mM, KynA effectively inhibited the XOR activity by 60-98% of the control. The vechicle did not influence the basic XOR activity (Figure 12B).

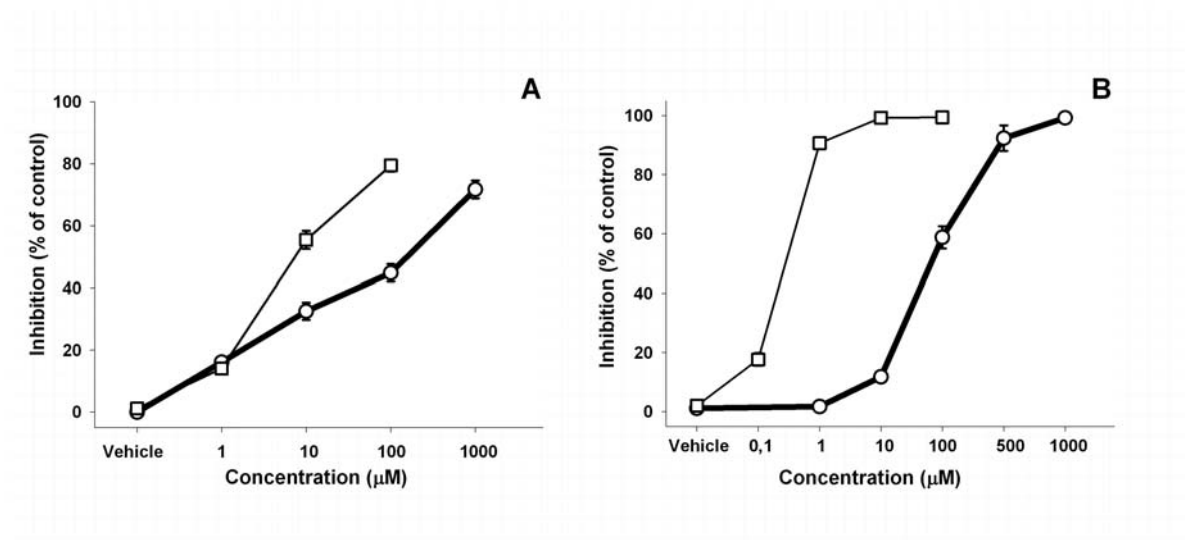

Figure 12. *Dose-dependent in vitro inhibitory effects of KynA.* **A:** *Changes in chemiluminescence in the presence of test compounds after induction of the X/XO reaction. The degree of inhibition of chemiluminescence was measured with increasing KynA (thick line with circles) and allopurinol (thin line with squares) concentrations.* **B:** *Inhibitory effects of KynA on XO activity in the fluorometric assay. The bars indicate means ± SEM.*

5.6. The motility effects of NMDA antagonist treatments in acute, inflammation-associated colon disorders in different species

In all acute GI inflammation models, regardless of the species, an increased bowel motility index was observed, accompanied by a decreased tone of the colonic smooth muscles. These motility changes were associated with increased levels of inflammatory enzyme activities. Regardless of species, the NMDA receptor antagonist treatment was able to decrease the motility index, increase the tone and reduce the XOR activity and the level of NO production (Table V). It is important to note that TNBS-induced inflammation can be identified only 14-17 h following enemas (data not shown) in rodents. This phase was considered the acute period of TNBS-induced colitis that is comparable with the early (1-7-h)

period of mechanical ileus-induced inflammation in larger animals. The comparative data collected from *Studies I-III* are shown below.

Species	Observation time	Intervention	Motility index	Bowel tone	NO production	XOR activity
Dog	7 hrs	Mechanical ileus	↑	↓	↑	↑
		Ileus + Kyna	↓	↑	↓	↓
Wistar rat	17-23 hr	Colitis	↑	↓	↑	↑
		Colitis + KynA	↓↓	↑	↓	↓
		Colitis + SZR-72	↓	↑↑	↓	↓
Sprague-Dawley rat	17-23 hr	Colitis	↑	↓	↑	↑
		Colitis + KynA	↓	↑	↓	↓
		Colitis + SZR-72	↓	↑	↓	↓

Table V. *Data on mechanical ileus and colitis in dogs and rats (for the sake of clarity, other time frames of colitis (between 17 and 23 h) of Sprague-Dawley rats are not presented in other parts of the thesis).*

6. DISCUSSION

Local inflammation is either the inducer of GI motility changes or is associated with altered motility. The entire integrative circuitry for enteric reflexes is located in the wall of the small intestine and colon, and the production of inflammatory mediators and metabolites therefore has significant consequences in the pathogenesis of all motility disorders (Lakhan et al. 2010, Törnblom et al. 2005). Indeed, the activation of resident macrophages in the tunica muscularis and the upregulated cytokine production may affect the smooth muscle contractility (Won et al. 2006). There is now good evidence that a postoperative ileus initiates the activation of transcription factors, upregulates proinflammatory cytokines, and increases the release of kinetically active mediators (inducible NO and prostaglandins), important factors in the recruitment of leukocytes and the suppression of motility (Kalff et al. 2003). On the other hand, Hellström *et al.* have demonstrated that low doses of endotoxin change the myoelectric activity in the small intestine, with repetitive bursts of spike potentials and a simultaneous increase in the transit of the intestinal contents (Hellström et al. 1997). On the basis of these observations, it should be emphasized that motility alterations are time-dependent, characteristically changing in parallel with the modified neurotransmitter release, or the impairment of the function of the enteric neurons during the development of inflammation (Kaszaki et al. 1987).

We used a study design to follow the steps of the pathways leading to inflammation-associated colon motility changes. First we investigated the time course of inflammatory and motility changes in the large intestine in acute mechanical ileus. Experimental blockade of the passage increased the large bowel motility 5 h after obstruction, and triggered significant NO production and XOR activation in the proximal colon (Palásthy et al. 2006). The nonselective, natural NMDA receptor antagonist KynA decreased the motility index, together with the NOS activity and ROS produced by XOR, which indicated that glutamate-NMDA receptors contribute to the excitatory profile of the motility pattern in the early phase of the inflammatory process (Kaszaki et al. 2008). The relative weight of KynA treatment in the modification of the obstruction-induced motility dysfunction was significant.

As next step, the role of glutamate receptors was further investigated in the early and later phases of TNBS-induced colitis. In this model, enhanced colon motility was present at 17-23 h, but 5-7 days later the motility had decreased significantly. Our results are consistent with the findings of Gurung *et al*. (Gurung et al. 2007), and the different patterns of the early and the later motility changes may be ascribed to different responses to neurotransmitters

(Hosseini et al. 1999). Hypermotility in the early phase is a sign of increased excitation in the ENS, while the later decrease in motility reflects the ensuing overexcitatory damage of the myenteric plexus and the loss of the regulatory function of the ENS. It should be noted here that glutamate neurotoxicity (necrosis and apoptosis) has been observed in a subset of enteric neurons in both intact bowel preparations and cultured myenteric ganglia (Kirchgessner et al. 1997).

The inflammatory and motility changes in TNBS-induced colitis were effectively modified by KynA and also by treatment with SZR-72, a BBB-permeable synthetic KynA analog (Knyihár-Csillik et al. 2008, Varga et al. 2010). Since KynA is unable to penetrate the BBB (Kiss et al. 2005), the iv administered compound acted at the periphery only and targeted the NMDA receptors of the ENS. In these studies, both treatments decreased the motility index significantly, normalized the smooth muscle tone of the colon to the control level and exerted an inhibitory effect on the colitis-induced high NOS and XOR activities in the early phase of colitis. This effect of NMDA antagonists suggests that the glutamate receptors mainly contribute to the excitatory profile of the motility pattern and inflammation in the examined time frame (Varga et al. 2010).

6.1. Role of NMDA receptors in primary motility disorders: Study I

The results of *Study I* have revealed a significant potential for KynA to decrease the facilitatory pathways of the colonic motility. Our study design allowed us to follow the variations in time of the inflammatory and motility changes in the large intestine in the acute phase of mechanical ileus, and to investigate the role of the glutamate receptors in this scenario. In this canine model, experimental blockade of the intestinal passage increased the large bowel motility, and triggered a hyperdynamic circulatory reaction 5 h after obstruction, accompanied by significant NO production and XOR activation in the proximal colon. This hyperdynamic cardiovascular response may be regarded as a compensatory reaction through which the organism strives to accommodate to the evolving septic metabolic changes (Bone et al. 1991, Palásthy et al. 2006).

It is clear that, besides being one of the main excitatory transmitters in the CNS, glutamate can act either as a neurotransmitter in the peripheral nervous system or at least as a modulator of classical transmitter systems (Liu et al. 1997, Kirchgessner et al. 2001, Wiley et al. 1991, Sinsky et al. 1998). In particular, there is now evidence for glutamate release from neurons and the presence of glutamate receptors in the intestine in nonhuman species (Ren et al. 1999), and receptors of the NMDA subtype in the myenteric plexus (Moroni et al. 1986).

This subtype is preferentially activated by quinolinic acid and blocked by KynA (Stone et al. 2003, Perkins et al. 1982, Stone et al. 2001). These data therefore indicated that NMDA subtype receptors play a role in the gut motility, and activation by glutamate could increase the contractile activity. Our results have revealed that glutamatergic facilitation does indeed take part in an obstruction-induced increase in colon motility.

The enzymes of the kynurenine pathway are activated by inflammation and immune stimulation, leading to large increases in the generation of the NMDA agonist quinolinic acid and its antagonist, KynA (Stone et al. 2001, Mackay et al. 2006). The balance between the relative concentrations of these substances during an inflammatory response could therefore have a profound influence on the excitability of the enteric neurons and hence on the motility of the gut (Forrest et al. 2002, 2003). Moreover, quinolinic acid can increase the formation of ROS, both through a direct Fenton-like interaction with iron, and through the NMDA receptor-activated increase in intracellular Ca^{2+} level, which results in a higher XOR activity (Rios et al. 1991).

The relative weight of KynA treatment in the modification of the ileus-induced motility dysfunction was significant. Our results indicate that mainly glutamate receptors contribute to the excitatory profile of the motility pattern in the examined time frame, since nonselective NMDA receptor antagonism treatment significantly decreased the motility index and amplitude of the GMCs.

The link between constitutive NO production and the gastrointestinal nervous system is of special interest (Ekblad et al. 1994, Furness et al. 2000, Qu et al. 1999). For transmitter release, nNOS permits the rapid release of NO, and this process is associated with the translocation of cytosolic nNOS to the cell membrane. There, it is bound to postsynaptic density 95 protein in intimate association with NMDA glutamate receptors, permitting a direct route for Ca^{2+} through the NMDA receptor channel to nNOS. The excessive accumulation of glutamate with subsequent activation of the ligand-gated, ion channel NMDA glutamate receptors appears to result in excitotoxicity due to the breakdown of ionic homeostasis, stimulation of nNOS activity with the increased production of NO leading to the formation of ROS and nitroso species (Liu et al. 1997, Furness et al. 2000). These results suggest that neuroprotection against glutamate by KynA might be mediated synergistically through the blockade of NMDA receptors and inhibition of nNOS.

6.2. Neuroprotection in inflammation-induced motility disorders: Studies II and III

The applied TNBS enema causes extensive transmural colitis that usually lasts longer than 8 weeks in rodents. This experimental IBD model is characterized by a weight loss with visceral hyperalgesia (Zhou et al. 2008), significant elevations of NOS activities (Kiss et al. 1997, Yue et al. 2001) and tissue granulocyte accumulation (Kiss et al. 1997). The development of TNBS-caused colonic inflammation shares many similarities with fulminant episodes of human IBDs, but the acute motility consequences differ from the later changes. In this regard, we designed two studies to monitor the motility and inflammatory changes in the large intestine during the early onset and in a later, more established phase of the inflammation after an acute TNBS challenge.

On day 1, the intracolonic TNBS administration enhanced the large bowel motility significantly and induced serosal hyperemia with a hyperdynamic macrocirculatory reaction. These changes were accompanied by tissue NOS and XOR activation in the proximal colon. The study design additionally allowed us to investigate the functional role of the NMDA glutamate receptors; the results revealed a significant potential for NMDA receptor antagonists to decrease both inflammatory activation and the parallel facilitation of colonic motility in this scenario.

However, the later phase of the established inflammation on day 6 was characterized by normal systemic hemodynamics, accompanied by biochemical signs of nitrosative stress. According to our observations in *Studies II* and *III,* differences between the two phases were truly present. The colon was hypermotilitic on the first day after TNBS enema administration, and the analysis of the motility parameters on day 6 furnished evidence of a tendency to a return toward normal homeostasis (Kaszaki et al. 2012). These differences may be ascribed to time-dependent responses to neurotransmitters; early hypermotility is a sign of increased excitation in the ENS (Hosseini et al. 1999). The ensuing loss of the regulatory neural function is clearly multifactorial, but several lines of indirect evidence have suggested a role for NMDA receptors in this scenario. Glutamate neurotoxicity (necrosis and apoptosis) has been observed in a subset of enteric neurons in both intact bowel preparations and cultured myenteric ganglia (Kirchgessner et al. 1997). In *Study I*, we demonstrated that treatment with the NMDA receptor antagonist KynA decreased the obstruction-caused intestinal hypermotility in dogs, and the use of specific antagonists to block NMDA receptors had similar effects in an acute TNBS-induced colitis model in rats (Kaszaki et al. 2008, Varga et al. 2010).

However, there were no significant changes in hemodynamics on day 6 after the TNBS enema; during the early phase of colitis evolution, the barrier function of the mucosal epithelium was rapidly lost (Appleyard et al. 2002, Krimsky et al. 2003), leading to bacterial translocation and a characteristic cardiovascular response. The hyperdynamic process is regarded as a compensatory change through which the organism strives to accommodate to the emerging septic metabolic changes (Bone et al. 1991). In TNBS-treated animals, this reaction was evidenced by decreased MAP and TPR and elevated CO levels. Treatment with the NMDA receptor antagonist KynA did not significantly influence the overall hemodynamics, but the analog SZR-72 caused a moderate TPR elevation. The difference between the systemic circulatory effects of the examined NMDA antagonists may be linked to the divergent permeabilities of SZR-72 and KynA through the BBB. This assumption is supported by the observation that L-glutamate and NMDA microinjection into the nucleus tractus solitarius produced dose-dependent decreases in blood pressure, mesenteric blood flow and iliac vascular resistance. However, previous administration of the NMDA receptor antagonist MK-801 into the same CNS area significantly attenuated this NMDA-induced depressor effect (Tian et al. 1994). Unlike KynA (Bari et al. 2006, Vécsei et al. 1992), SZR-72 readily crosses the BBB and in this way peripheral hemodynamic reactions may be modulated by CNS effects.

A further important feature of NMDA receptor antagonist treatment modalities was the parallel influence on distinct elements of the inflammatory response, including the inhibition of local NO synthesis. The matching effects on the microcirculatory, motility and biochemical responses may be indicative of a connection between the action on local NMDA receptors and the NOS systems. Indeed, the *in vitro* study by Lomax *et al*. provided evidence that the altered neural regulation of the gastrointestinal microvasculature contributes significantly to the pathogenesis of TNBS colitis (Lomax et al. 2007).

It has recently been demonstrated that NMDA receptors are expressed not only on neurons, but also on a number of non-neuronal cell types, including endothelial cells and immune-competent cells, and this points to the action of a common regulatory mechanism (Boldyrev et al. 2004, Hinoi et al. 2004, Miglio et al. 2005, Reijerkerk et al. 2010). Indeed, Mashkina *et al.* (2010) have established that the population of inactive lymphocytes contains only a few cells with NMDA receptors, and expression is induced after appropriate stimuli. Moreover, it has been reported that NMDA receptor expression and a normal function are required for activation of the respiratory burst of PMN granulocytes (Kim-Park et al. 1997). On the other hand, NMDA activation by glutamate can alter the ion balance between the

intra- and the extracellular space by elevation of the intracellular Ca^{2+} level, and the increased Ca^{2+} influx may result in excitotoxicity. It is therefore suggested that a regulatory interplay between the neuronal and cellular immune systems is highly likely in the gastrointestinal tract through NMDA receptor activation.

The pathogenic role of NO-derived species such as peroxynitrite in IBDs is supported by the fact that the intracolonic administration of exogenous peroxynitrite induces severe inflammation, which mimics the features of human ulcerative colitis. Peroxynitrite produced by the reaction of NO with superoxide is highly cytotoxic and able to induce structural damage (Ko et al. 2005, Linden et al. 2005). ROS produced in the inflamed mucosa are mainly generated by activated phagocytic leukocytes via NADPH oxidase and the XOR system. The colonic XOR activity was elevated significantly 6 days after TNBS induction, although the level was somewhat lower than in the early phase of colitis (Varga et al. 2010).

In *Study III,* we have demonstrated that the amplitude of the contractions and the tone of the colonic smooth muscle have normalized by day 6, but the frequency of bowel movements is still increased over the baseline level. This implies that the regulation of frequency involves the distinct action of a mediator or a regulatory system; indeed, the relevant literature data suggest that this response may be linked to NO and altered NO synthesis. Bossone *et al.* (2001) reported that the contraction frequency changes observed after repeated applications of TNBS are critically connected to the activation of iNOS. In *Study II,* we also presented evidence of the role of activation of the NMDA receptors in NO synthesis in the colonic tissue. It was found that on day 6 the TNBS-induced increased peristaltic frequency is associated with significantly increased plasma NO_x levels. It is important to note that an augmented Ca^{2+} influx through the NMDA receptor-ion complex may stimulate Ca^{2+}-dependent NO synthesis (Petersson et al. 2007, Yue et al. 2001). Thus, the data might suggest that activation of the ligand-gated, ion channel NMDA glutamate receptors can lead to the stimulation of cNOS isoforms (endothelial NOS and nNOS), with increased NO production and an increased frequency of intestinal contractions. The activation of NMDA receptors seems to be critically involved in this phenomenon since the inhibition of the NMDA receptors by the NMDA antagonists markedly decreased the rate of contractions.

Our results demonstrated that enhanced colon motility is present after 17–23 h of colitis, but in the later phase (on day 6) the motility usually decreases (Gurung et al. 2008). The different patterns of the early and the later motility changes may be ascribed to different responses to neurotransmitters (Hosseini et al. 1999). Hypermotility in the early phase is a sign of the increased excitation in the ENS, while the ensuing decrease in motility reflects

overexcitatory damage of the neurons of the myenteric plexus and loss of the regulatory function of the ENS. Both KynA and SZR-72 treatments decreased the motility index significantly and normalized the smooth muscle tone of the colon to the control level in *Study II*. This effect of NMDA antagonists in the early phase of colitis suggests that the glutamate receptors mainly contribute to the excitatory profile of the motility pattern in the examined time frame.

6.3. KynA directly inhibits XOR activity in vitro

Besides the motility-influencing effects, KynA is inhibitory on the activity of XOR *in vivo*. A possible explanation for the decreased XOR activity could be a substrate analog, nonspecific inhibitory effect of KynA, since there is structural similarity to hypoxanthine/xanthine, the substrate for XOR. Therefore, a series of investigations in a cell-free, *in vitro* environment were designed to gain insight into the direct effects of KynA on the XOR activity. Indeed, the effect of KynA on the activity of XOR was assessed for the first time, and a significant inhibitory potential was observed for the compound. These findings reveal that KynA not only significantly influences the motility pattern in the colon, but also exerts a protective, anti-inflammatory effect due to direct and indirect inhibition of XOR-derived oxygen radical production. Our results further indicate that KynA may offer a pharmacologic approach to inhibit XOR activity in the GI tract.

It remains to be established whether the findings in these experimental models are applicable to humans. However, together with previous observations, these data strongly suggest that suppression of the hypermotility function of the NMDA receptors might be beneficial in serving as a supplementary tool with which to influence excitotoxicity complications in the intestine. In conclusion, since the etiopathogenesis of inflammation-associated motility disorders is multifactorial, multifunctional therapy is clearly required to address the various pathological aspects of these diseases. In this regard, we suggest that the blockade of enteric NMDA receptors might provide greater therapeutic efficacy by targeting motility and local inflammatory changes.

7. SUMMARY OF FINDINGS

- NMDA receptor antagonist treatment effectively influences the colonic motility changes (increased motility index and decreased smooth muscle tone) during acute inflammation. This suggests that the activation of enteric NMDA-sensitive glutamate receptors participates in the mediation of inflammation-related motility changes in the GI tract.
- Parallel to the motility reactions, nonselective NMDA receptor antagonist treatment decreases pro-inflammatory activation in the colon. We demonstrated for the first time the direct inhibitory effect of KynA on the XOR activity, and thus the anti-inflammatory effect of KynA could be related to the elimination of XOR-dependent oxygen radical production in the colon.
- The significant anti-inflammatory effects of NMDA antagonist treatment can be detected even at later phases of inflammation (beyond 6 days) in experimental colitis.
- The peripheral administration of KynA and its BBB-permeable analog SZR-72, proved to be equally effective in this condition.
- NMDA antagonist treatment was capable of influencing the motility and inflammatory changes in different models of colonic inflammation, which strongly indicates that glutamate-induced neurotoxicity has a basic role in the pathogenesis of inflammation-associated GI motility disorders in a species-independent manner.

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10. ANNEX