Protective compounds in animal models of trigeminal activation and neurodegeneration

Ph.D. thesis

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Szeged

2009

Original publications directly related to the PhD thesis

I. **Vámos E**, Párdutz A, Varga H, Bohár Z, Tajti J, Fülöp F, Toldi J, Vécsei L. (2009) L-kynurenine combined with probenecid and the novel synthetic kynurenic acid derivative attenuate nitroglycerin-induced nNOS in the rat caudal trigeminal nucleus. *Neuropharmacology* **57:**425-9.
IF: 3,383

II. **Vámos E**, Párdutz A, Fejes A, Tajti J, Toldi J, Vécsei L. (2009) Modulatory effects of probenecid on the nitroglycerin-induced changes in the rat caudal trigeminal nucleus. *European Journal of Pharmacology* (in press)
IF: 2,787

III. **Vámos E**, Vörös K, Zádori, Vécsei L, Klivényi P. (2009) Neuroprotective effects of probenecid in a transgenic animal model of Huntington's disease. *Journal of Neural Transmission* **116:**1079-86.
IF: 2,514

Total impact factor: 8,684

Publications not directly related to the thesis

I. **Vámos E**, Fejes A, Koch J, Tajti J, Fülöp F, Toldi J, Párdutz Á and Vécsei L. (2009) Kynurenate derivative attenuates the nitroglycerin-induced CamKIIα and CGRP expression changes. *Headache* (in press)
IF: 3,081

II. **Vámos E**, Vörös K, Vécsei L, Klivényi P. (2009) Neuroprotective effects of L-carnitine in a transgenic animal model of Huntington's disease. *Biomedicine and Pharmacotherapy* (in press)
IF: 2,198

III. **Vámos E,** Párdutz Á, Klivényi P, Toldi J, Vécsei L. (2009) The role of kynurenines in disorders of the central nervous system: possibilities for neuroprotection. *Journal of the Neurological Sciences* **283:** 21-7.
IF: 2,359

IV. Zádori D, Klivényi P, **Vámos E,** Fülöp F, Toldi J and Vécsei L. (2009) Kynurenines in chronic neurodegenerative disorders: future therapeutic strategies. *Journal of Neural Transmission* (in press)
IF: 2,514

V. Varga H, Párdutz Á, **Vámos E**, Plangár I, Egyud E, Tajti J, Bari F, Vécsei L. (2007) Cox-2 inhibitor attenuates NO-induced nNOS in rat caudal trigeminal nucleus. *Headache* **47:**1319-25.
IF: 3,081

VI. Varga H, Párdutz Á, **Vámos E**, Bohár Z, Bagó F, Tajti J, Bari F, Vécsei L. (2009) Selective inhibition of cyclooxygenase-2 attenuates nitroglycerin-induced calmodulin-dependent protein kinase II alpha in rat trigeminal nucleus caudalis. *Neuroscience Letters* **451**:170-3.
IF: 2,2

VII. Fülöp F, Szatmári I, **Vámos E**, Zádori D, Toldi J, Vécsei L. (2009) Syntheses, transformations and pharmaceutical applications of kynurenic acid derivatives. Current Medicinal Chemistry (in press)
IF: 4,94

Total impact factor: 20,373

Other papers

I. **Vámos E,** Csáti A, Vécsei L, Klivényi P. (2009) Effects of valproate on the dopaminergic system in mice. *Neurological Research* **31:**217-9.
IF: 1,63

II. Zádori D, Geisz A, **Vámos E,** Vécsei L, Klivényi P. (2009) Valproate ameliorates the survival and the motor performance in a transgenic mouse model of Huntington's disease. Pharmacology Biochemistry and Behavior (in press)
IF: 2,751

Total impact factor: 4,381

Patent

Use of kynurenic acid and its analogues in the treatment of headaches. (It is covered by Patent reference number #P0900281)

Cumulative impact factor: 33,438

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List of abbreviations

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BBB – blood-brain barrier

bodyweight - bw

CamKIIα – calmodulin-dependent protein kinase II alpha

CGRP – calcitonin gene-related peptide

CNS – central nervous system

COX - cyclooxigenase

HD – Huntington's disease

HPLC - High performance liquid chromatograpy

i.p. - intraperitoneal

IR - immunoreactive

KYNA – kynurenic acid

L-KYN – L-kynurenine

MRP – multidrug resistance-associated protein

NMDA – N-methyl-D-aspartate

nNOS – neuronal nitric oxide synthase

NO – nitric oxide

NSAID – non steroid anti-inflammatory drug

NTG - nitroglycerin

PBS – phosphate buffer saline

PFA – paraformaldehide

s.c. - subcutaneous

TNC – caudal trigeminal nucleus

Summary

In some neurological disorders, there are only functional changes in the nervous system, but there are diseases where there is a progressive cell death in the pathomechanism. Sometimes the functional and morphological disorders coexist and distinguishing them can be difficult. In the present works we chose to examine migraine headache, a disorder with functional abnormalities and Huntington's disease, which involves neurodegenerative process.

Migraine is one of the most common neurological disorders. Despite recent and continuous advance in its research, the precise pathomechanism remains unclear. Systemic administration of the nitric oxide donor nitroglycerin can trigger an attack in migraineurs. In the rat, nitroglycerin activates second-order neurons in the caudal trigeminal nucleus, and increases expression of neuronal nitric oxide synthase and calmodulin-dependent protein kinase II alpha and decreases that of the calcitonin gene-related peptide there.

Huntington's disease is an autosomal dominant inherited disorder, caused by an expanded polyglutamine region of a protein called huntingtin. The excitotoxicity, oxidative damage, mitochondrial dysfunction, the altered membrane transport and the inflammatory process may have important roles in the pathogenesis of Huntington's disease.

In the present works our findings demonstrate that L-kynurenine (metabolized to kynurenic acid, which is an antagonist of the excitatory amino acid receptors) and 2-(2-*N*,*N*-dimethylaminoethylamine-1-carbonyl)-1*H*-quinolin-4-one hydrochloride (a novel synthesized derivative of kynurenic acid) exert a modulating effect on the trigeminal activation in the nitroglycerin model of migraine possibly via the glutamate receptors. These drugs may have a modulating effect on trigeminal activation and sensitization in the nitroglycerin model of migraine, and it may therefore open up novel therapeutic opportunities in headache management.

We have also demonstrated that probenecid pretreatment successfully attenuated the nitroglycerin-induced decrease of the area covered by calcitonin gene-related peptide-immunoreactive fibres and the increase of the neuronal nitric oxide synthase- and calmodulin-dependent protein kinase II alpha-positive neurons in the rat caudal trigeminal nucleus. Overall, our results suggest that the systemic administration of probenecid exerts an indirect effect on the caudal trigeminal nucleus by blocking the inflammatory and sensitization processes, also crucial in the pathogenesis of migraine, mitigating the activation properties of nitric oxide in the trigeminal system. These data may be important

to in pointing to new therapeutic strategies against migraine attacks and other headache syndromes.

Our findings demonstrate that probenecid administration to N171-82Q transgenic mice extends the survival time, improves the motor performance and decreases the number of intranuclear inclusions, these parameters being important in the pathomechanism of Huntington's disease. These results have shown that probenecid treatment results in the delay of symptom development and lessening of symptom severity.

These data may contribute to a better understanding of the pathomechanisms of migraine headache and HD and the relevance of the excitotoxical and neuroinflammatory processes in these conditions.

I. Introduction

Neurological diseases are disorders, which primarily involve the nervous system and the treatment pose a great challenge. In some of these disorders, there are only functional changes in the nervous system, but there are diseases where there is a progressive cell death in the pathomechanism. Sometimes the functional and morphological disorders coexist and distinguishing them can be difficult. In the present works we chose to examine migraine, a disorder with functional abnormalities and Huntington's disease (HD) which involves neurodegeneration.

I.1. *Migraine*

Migraine headache is one of most common neurological disorders. The prevalence of migraine is up to 12 % among the population [1-4]. Despite of the intensive research the exact pathomechanism of migraine remains unknown [3]. Although the long term outcome of migraine is benign, the social and economic impact of this disorder due to its high prevalence is enormous. Despite recent advances of the therapy the appropriate treatment is yet to be achieved.

The central concepts of the pathogenesis of migraine which occurs during an attack are based on the activation of the trigeminovascular system. The trigeminal nerves, which innervate intracranial and extracranial tissues, account for head pain and other symptoms in migraine. Human and animal experiments have shown that the pain during migraine is likely to result both from activation and sensitization of the first- and second-order trigeminal nerve fibres which innervate pain-producing intracranial structures [3]. These results may suggest that treatment affecting the initiation of central sensitization should be administered immediately after the onset of migraine pain to prevent intracranial hypersensitivity.

Recently, a decisive progress in the pharmacology of migraine has been achieved with the discovery of the involvement of nitric oxide (NO). The importance of endogenous NO production during the headache phase of migraine seems to be evidence in an experimental study where the inhibition of nitric oxide synthase (NOS) relieved the symptoms of spontaneous migraine with high efficacy [5].

The systemic administration of the NO donor nitroglycerin (NTG) triggers a delayed attack without aura in many migraineurs, but not in healthy subjects [6]. The delay between the administration and the headache does not favour a purely vascular action. In view of the similarity to normal migraine, the role of the trigeminovascular system is implicated in the NTG model [7]. Surprisingly in the view of short half-lives of NTG and NO [8] in vivo, the migraine occurs several hours after NTG infusion [9]. Thus it appears that NO is a cause of migraine through mechanisms that develop over a long period of time. This is consistent with the possibility of a delayed and sustained production of NO by NOS in a large number of tissues [10,11].

In the rat, NTG administration increased the number of *c-fos*- [12] and neuronal NOS (nNOS) - [13] immunoreactivity in neurons of the lower caudal trigeminal nucleus (TNC) (including the upper cervical spinal cord), where most of the trigeminal nociceptors project. The NTG-induced nNOS increase may be related to the activation of primary trigeminal afferents by NO, leading to a self-amplifying process [13]. Systemic administration of NTG also selectively increases the expression of Ca^{2+}/calmodulin-dependent protein kinase II alpha (CamKIIα) in the spinal TNC [14]. The most likely explanation for this increased CamKIIα immunoreactivity is the secondary activation of second order nociceptive neurons and/or interneurons because of excitation of their peripheral afferents. In the lower TNC of the rat, NTG causes a decrease in the area covered by calcitonine gene-related peptide (CGRP)-immunoreactive (IR) fibres, probably due to an increased release [15,16].

Overall, after a delay of several hours, the area covered by CGRP-IR fibres in the TNC is decreased, suggesting activation of the peripheral afferents there [16], while the numbers of nNOS- [13] and CamKIIα- [14] IR neurons in the same area are increased, which may be regarded as a self-amplifying process, causing central sensitization at the level of second-order nociceptors. Similar trigeminal sensitization has been demonstrated in migraineurs [17].

I.2. *Huntington's disease*

Neurodegenerative disorders are morphologically featured by progressive, often long-lasting cell death in the central nervous system (CNS). Basic processes inducing neurodegeneration caused by genetic, environmental and endogenous factors such as abnormal protein functions, mitochondrial dysfunctions and neuroinflammatory processes.

This degenerative process usually associated with cytoskeletal protein aggregates forming intracytoplasmic and/or intranuclear inclusions in neurons. These mechanisms often interrelated and induce programmed cell death cascades. In the present study we focused on HD, which is a "polyglutamine disease" with a clear genotype-phenotype correlation.

Huntington's disease is an autosomal dominantly inherited neurodegenerative disorder. Although the prevalence of HD is rather low (~ 5/100 000; [18,19]), the disease displays a progressive nature. The disease manifested by psychiatric, cognitive and motor symptoms starting in mid-life and progressing to death. The motor symptoms involve the loss of coordination of voluntary- and involuntary movements, including chorea and dystonia. Despite recent and continuous research advances, the precise mechanism of the neurodegeneration in HD remains unknown.

Huntington's disease is caused by expansion of the cytosine–adenine–guanine (CAG) repeat in the protein coding region of the IT15 gene. The gene was identified in 1993 [20], located on the short arm of chromosome 4 (4p16.3). The physiological HD alleles comprise 6 to 35 CAGs, while alleles with 36 to 39 CAGs are characterized by reduced penetrance. Above 39 CAGs, there is obligatory disease development. The greater the expansion, the earlier the disease is manifested (reviewed by [21]). Expansion of the polyglutamine repeat in huntingtin alters its interactions with cellular proteins disrupting their functions and contributing to pathology [22,23].

The pathological abnormalities seem to be restricted to the CNS, with preferential vulnerability in the caudate, putamen and deep layers of the cerebral cortex. The pathological alterations mainly affect the CNS and especially the striatum in which the loss of γ-aminobutyric acidergic medium-sized spiny neurons is the most pronounced feature (reviewed by [24,25]). The N-terminal fragments of mutant huntingtin accumulate in the nuclei of the affected neurons and form intranuclear aggregates [26,27]. Long polyglutamine tracts are known to form hydrogen bonded, β-sheets that are prone to aggregation [28,29]. Indeed, aggregates called inclusion bodies have been identified in human disease tissue for all polyglutamine disorders [30,31]. Aggregates were found to affect vital cellular functions and accelerate cell death [32]. Suppression of aggregate formation has been shown to be beneficial in cell models for HD and is accepted as one of the markers of successful therapeutic approaches [33].

An advance facilitating study of the pathogenesis of disease was the introductions of transgenic mouse models of HD. Transgenic mice (line N171-82Q) expressing exon 1 of the human HD gene with an expanded CAG repeat develop a progressive neurological

disorder [34]. These transgenic mice have CAG repeat lengths of 82, under the control of the mouse prion protein promoter [35]. The disease starts at the age of 8 weeks, while in the following 4–6 weeks the mice not only fail to gain weight but they also lose weight. At 12–16 weeks of age, the transgenic mice begin to exhibit an irregular, uncoordinated gait, hypokinesis, stereotypic movements and tremor. The brain of the transgenic mice is slightly smaller and exhibits striatal atrophy and neuronal intranuclear inclusions that are immunopositive for huntingtin and ubiquitin [35]. Consequently, these transgenic mice develop neurological symptoms that resemble many of those seen in HD.

Despite the basic difference of the two mentioned neurological disorders several factors are well known to be important in their pathogenesis, including glutamate-induced excitotoxicity [36-38] and inflammatory processes (reviewed by [39,40]).

I.3. Glutamate-induced excitotoxicity

The enhanced release of glutamate, which is the main excitatory amino acid in the brain, leads to the prolonged stimulation of its receptors and due to a complex pathomechanism induce the devastation of postsynaptic neurons. The process is called excitotoxicity, and it was first described nearly four decades ago [41]. It is important to mention that there could be a link between glutamate-induced excitotoxicity and neurodegenerative processes, because the energy impairment can lead to a partial membrane depolarization resulting in the relief of magnesium block of the N-methyl-D-aspartate (NMDA) channel. Glutamate-induced excitotoxicity, caused by the overactivation of NMDA receptor or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, is well known to induce neuronal damage. It has been implicated in various neurological disorders (reviewed by [42,43]).

Both human and animal studies have demonstrated the important role of glutamate in migraine pathogenesis. In migraine patients, higher glutamate levels have been measured in the plasma [44]. The glutamate-induced excitability is mediated via the ionotropic NMDA, AMPA / kainate and metabotropic glutamate receptors [45]. These receptors are present in the superficial lamina of the TNC, in the trigeminal ganglion and in the thalamus, regions which are involved in the pathogenesis of migraine [46,47]. Synaptic transmission between first- and second-order trigeminal neurons is partially mediated by glutamatergic mechanisms [48]. The experimental data suggest that NMDA receptors are involved in central sensitization of the sensory system [49,50]. Furthermore, research

findings demonstrate that excitatory amino acids such as glutamate participate in the migraine attack-triggering process [51].

Glutamate excitotoxicity may play an important role in the development of HD, too [52-54]. This overactivation is due to the impairments in energy metabolism caused by the altered huntingtin gene of HD patients. The selective impairment of medium-sized spiny neurons could be explained in one hand by that they receive a massive glutamaterg input from the cortex and the thalamus [55,56] . In the other hand, the NMDA receptors can be found in an especially excessive amount on the spines of medium-sized spiny neurons and the expression pattern of the receptor subunits is also different compared to the other striatal neurons [57].

I.4. Inflammatory process

Some prostaglandins act as important inflammatory mediators that contribute to the progression of inflammation. Prostaglandins are thought to play a role in many neurological functions, including nociceptive processing. Several isoforms of their synthesizing enzyme are known: cyclooxygenase-1 (COX-1) and -2 and the most recently discovered and described COX-3 [58,59]. Each type of COX facilitates the production of different types of prostaglandins. In particular, COX-1 is involved in the production of prostaglandins that are needed for various regulatory functions in the body. COX-2, on the other hand, is involved in the production of prostaglandins that mediate inflammation.

Inducible COX-2 is expressed in the superficial dorsal horn of the rat spinal cord [60] and plays a role in central sensitization [61]. It has been proposed that prostaglandins synthesized by COX-2 assist in synaptic transmission [62] and enhance postsynaptic activity of both excitatory and inhibitory neurotransmitters [63]. It also mediates most of the analgesic effects of non-steroidal anti-inflammatory drugs (NSAIDs) [64]. After hindpaw inflammation in mice some of the COX-2 expressing cells in laminae I-II are also positive for nNOS [65] suggesting an interaction between the two enzymes. NSAIDs, such as acetylsalicylic acid (Aspirin®), are effective in the treatment of acute migraine headache [66]. This effect could be due to their inhibitory action on COX-2 and prostaglandins in the spinal trigeminal complex [67]. Since these substances can play an important role in the process of trigeminal activation during migraine [68,69].

Casper et al. [70] examined the effects of three types of NSAIDs on damage caused by toxicity due to glutamate. One way by which glutamate mediates cell death is by

activating COX-2, thereby increasing the production of inflammatory prostaglandins. Though less well characterized than in Huntington's, inflammatory cytokines are present and increase with disease stage in these disorders as well [71]. Recent study suggests that elevated levels of cytokines are present in blood and spinal fluid of HD patients as early as 16 years before expected symptom onset, and that these levels increase with the stage of disease, both before and after symptom development [72].

Taken together, the mechanisms mentioned above, play an important role in both migraine where functional alteration of the trigeminal system is prominent and HD, where neurodegeneration is present. Thus the study of the modulation of the glutamate-induced excitotoxicity and neuroinflammatory process can be relevant in this context.

II. Aims

The aims of our studies were to

(i) study the effects of the glutamate-induced excitotoxicity in the animal model of migraine. We examined effects of kynurenic acid (KYNA) which is one of the few known endogenous broad spectrum antagonists of excitatory amino acid receptors. KYNA penetrates the blood-brain barrier (BBB) poorly [73], which hampers its therapeutic use. Peripheral treatment with L-kynurenine (L-KYN) combined with probenecid, a known inhibitor of the transport of organic acid from the cerebrospinal fluid [74], dose-dependently increases the concentration of the neuroprotective KYNA in the brain [75]. In our experiments we tested the effects of the co-administration of L-KYN and probenecid and a novel synthesized derivative of KYNA (2-(2-N,N-dimethylaminoethylamine-1-carbonyl)-1H-quinolin-4-one hydrochloride) (Figure 1.) on the NTG-induced nNOS expression in the rat TNC.

(ii) to examine the effects of probenecid in the animal models of trigeminal activation and neurodegeneration. It is well known that probenecid is a known non-selective inhibitor of multidrug resistance-associated proteins (MRPs) and organic anion transporters (surveyed by [76]). It has been demonstrated that inhibition by probenecid of the ability of the organic acid transporters to cross the BBB can raise the level of KYNA in the brain dose-dependently [77]. The modulation of the probenecid-sensitive transporters can alter the concentration of inflammatory products such as prostaglandins in the CNS [78].

To acquire further data on the effectivity of probenecid

- in trigeminal pain processing, we studied the effects of probenecid on the NTG-induced expressions of CGRP, nNOS and CamKIIα in the rat TNC.
- in the N171-82Q transgenic mouse model of HD, we studied the effects of probenecid on the survival, behaviour and immunohistochemical changes.

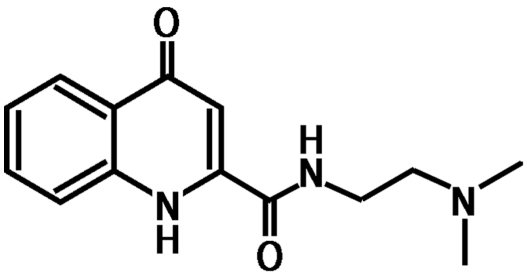

Figure 1. Chemical structure of the novel synthesized derivative of KYNA

III. Materials and methods

III.1. Animal model of migraine:

Animals:

The procedures utilized in this study followed the guidelines of the International Association for the Study of Pain and the European Communities Council (86/609/ECC). They were approved by the Ethics Committee of the Faculty of Medicine, University of Szeged. Adult male Sprague-Dawley rats (weighting between 200 and 250 g) were used. The animals were raised and maintained under standard laboratory conditions, with tap water and regular rat chow available *ad libitium* on a 12-h dark 12-h light cycle.

Drugs:

L-KYN sulphate and probenecid were obtained from Sigma (Steinheim, Germany), while the new compound – is covered by Patent reference number #P0900281 - was synthetized in the Department of Pharmaceutical Chemistry, University of Szeged.

III.1.1. Pretreatment of L-KYN combined with probenecid or a novel derivative of KYNA

a., Drug administration:

The doses of L-KYN and probenecid that were chosen were based on earlier work [79,80]. We used three experimental set-ups, for immunohistochemistry, Western blotting and chromatography, respectively. In the immunohistochemistry (IHC)- and Western blot (Wb) analyses, the animals were divided into three groups. In the first group the animals did not receive drug pretreatment; instead, they were injected with vehicle solution (n_{IHC}=12, n_{Wb}=8). The rats in the second group received an intraperitoneal (i.p.) injection of L-KYN (300 mg/kg bw diluted to 2 ml, pH 7.4) and probenecid (200 mg/kg bw diluted to 1.5 ml, pH 7.4) (n_{IHC}=12, n_{Wb}=8). In the third group, the rats were injected i.p. with KYNA derivative (300 mg/kg bw diluted to 2 ml, pH 7.4) (n_{IHC}=12, n_{Wb}=8). Thirty minutes later, half of the animals in each group (n_{IHC}=6, n_{Wb}=4) were injected subcutaneously (s.c.) with NTG (Nitrolingual°; Pohl-Boskamp GmbH, Hohenlockstedt, Germany) at a dose of 10

mg/kg bw, and the other half of the rats received a s.c. injection of the vehicle (Pohl-Boskamp GmbH, Hohenlockstedt, Germany). For the high-performance liquid chromatography (HPLC) measurements, the animals were divided into two groups. The rats in the first group, were injected with vehicle solution (n_{HPLC}=6). In the second group the rats received an i.p. injection of L-KYN (300 mg/kg bw diluted to 2 ml, pH 7.4) and probenecid (200 mg/kg bw diluted to 1.5 ml, pH 7.4) (n_{HPLC}=6).

b., Immunohistochemistry:

Four hours after the NTG or placebo injections, the rats were deeply anaesthetized with chloral hydrate (0.4 g/kg bodyweight, Fluka Analytical, Buchs, Switzerland, 23100) and perfused transcardially with 100 ml of phosphate-buffered saline (PBS, 0.1 M, pH 7.4) followed by 500 ml of 4% PFA (Merck, Darmstadt, Germany) in PBS. The cervical (C1-C2) spinal cord (between -5 and -11 mm caudal from the obex), representing the lowest part of the TNC, was removed. The tissue blocks were postfixed overnight for immunohistochemistry in the same fixative. After cryoprotection of the tissue blocks (10% sucrose for two hours, 20% sucrose until the blocks sank, and 30% sucrose overnight), 30 µm thick cryostat sections were cut and serially collected in 18 wells containing cold PBS. Each well received sections at 0.5 mm intervals throughout the rostro-caudal extent of the C1-C2 spinal cord. After pretreatment with 0.3% H_2O_2 for 30 min to suppress endogenous peroxidase activity, the free-floating sections were rinsed several times in 0.1 M PBS containing 1% Triton X-100 (Spektrum-3D, Debrecen, Hungary, 3.20150). The samples were then kept for 2 nights at 4 ^{o}C in anti-nNOS antibody (Euro-Diagnostica, Arnhem, The Netherlands, B220-1) at a dilution of 1:5000. The immunocytochemical reaction was visualized with the avidin-biotin kit of Vectastain (Vector Laboratories Inc., Burlingame CA, USA, PK6101 for nNOS) and stained with nickel ammonium-sulphate-intensified 3´,3´-diaminobenzidine. The specificity of the immune reactions was checked by omission of the primary antiserum. The sections were dried overnight on glass slides, and coverslipped with DPX mounting medium (Scharlau Chemie S.A., Barcelona, Spain, 5.720050). The sections were analysed under a Nikon Phase Contrast (085 59762, Tokyo, Japan) light microscope and photomicrographs were taken with Olympus DP70 (Tokyo, Japan) camera equipment. The digital photos were analysed through the use of Image-Pro® Plus 6.2 software (MediaCybernetics, Bethesda, MD, USA).

c., Western blotting:

In both groups four hours after the injections, the rats were deeply anaesthetized with chloral hydrate and perfused transcardially with 100 ml PBS. The dorsal part of the spinal cord segments (C1 and C2) was homogenized in cold lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% igepal, 0.1% cholic acid, 2 µg/ml leupeptin, 2 mM phenylmethylsulphonyl fluoride (PMSF), 1 µg/ml pepstatin, 2 mM EDTA and 0.1% sodium dodecyl sulphate (SDS) (all chemicals were from Sigma, Steinheim, Germany). Lysates were precleared by centrifugation, and supernatants were stored at -20 °C.

Protein concentration was measured according to BCA protein assay method (BCA Protein Assay Kit, Novagen®, Darmstadt, Germany. 71285-3), with bovine serum albumin (BSA) as a standard, and equal amounts of protein samples (30 µg/lane) were separated by standard SDS polyacrylamide gel electrophoresis (SDS-PAGE) procedures at 180 V for 45 min and transferred to membrane (Amersham, Buckingamshire, England). Following the transfer and blocking in 5% non-fat dry milk, membranes were incubated overnight at 4 °C with the nNOS antibody, diluted to 1:2500 (BD Biosciences, San Jose, CA, USA, 610308). Alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, Steinheim, Germany, A3562) at 1:2000 dilution in the blocking buffer was used as secondary antibody for 1 h at room temperature. Protein bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT, Sigma, Steinheim, Germany B5655) as substrate. After the detection of nNOS protein, the membranes were stripped and reprobed for ß-actin antibody (Calbiochem, Gibbstown NJ, USA, CP01) diluted to 1:2000, which was used as an internal control.

d., KYNA detection in the TNC:

Thirty minutes after the drug or vehicle treatment, the rats were deeply anaesthetized with chloral hydrate and rapidly perfused transcardially with 100 ml ice-cold PBS. The dorsal parts of the spinal cord segments (C1 and C2) were homogenized in 250 µl distilled water. The samples were deproteinated with 62.5 µl 8% perchloric acid and centrifuged (12,000 rpm, 10 min, 24 °C). The supernatant were filtered with a syringe-driven filter unit (Millipore, Bedford, USA, SLHV 013 NL). The KYNA concentrations of the samples were quantitated with the Agilent 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany) with fluorescence detection (excitation: 344 nm, emission: 398 nm). Briefly, 50 µl samples were applied onto a Hypersil 5 ODS HPLC column (150 mm × 4 mm, Thermo Fisher Scientific, Waltham, USA, 30105-154030), and chromatographed

isocratically at a flow rate of 1 ml/min with a mobile phase consisting of 0.2 M zinc acetate (pH 6.2) containing 5% acetonitrile. The retention time of KYNA was about 6 min. The results were expressed as pmol/g wet weight.

III.1.2. Pretreatment of probenecid

a., Drug administration

For immunohistochemistry, the animals were divided into two groups (n=12 per group). In the first group, the animals were injected i.p. with probenecid at a dose of 200 mg/kg bw (diluted in 1.5 ml 0.1 N NaOH, pH 7.4). The animals in the second group were treated with an i.p. injection of the solvent of probenecid. One hour later, half of the animals in both groups received a s.c. injection of NTG (10 mg/kg bw; Pohl-Boskamp GmbH, Hohenlockstedt, Germany), and the other half of the rats received a s.c. injection of the vehicle of NTG (Pohl-Boskamp GmbH, Hohenlockstedt, Germany).

For HPLC measurements, the animals were divided into two groups (n=6 per group). The rats in the first group were injected with a solution of the vehicle of probenecid. In the second group the rats received an i.p. injection of probenecid (200 mg/kg bw diluted in 1.5 ml 0.1 N NaOH, pH 7.4).

b., Immunohistochemistry

The protocol was the same as described above for nNOS staining.

The samples were kept for 2 nights at 4 ^{o}C in anti-nNOS antibody (Euro-Diagnostica, Arnhem, The Netherlands, B220-1) at a dilution of 1:5000, for 4 nights at 4 °C in anti-CamKIIα antibody (Sigma, Steinheim, Germany, C-265) at a dilution of 1:2000 and for 2 nights at 4 °C in anti-CGRP antibody (Sigma, Steinheim, Germany, C-8198) at a dilution of 1:20000.

c., KYNA detection in the TNC

One hour after the probenecid or vehicle treatment the protocol was the same as described above.

Data evaluation

nNOS- and CamKIIα-IR cells were counted in laminae I-II of the cervical spinal cord, in three different series of sections in each animal. The individual sections in these series were taken at 0.5 mm intervals along the rostrocaudal axis.

CGRP-IR fibres in laminae I-II of the cervical dorsal horns were determined by video imaging by means of Image Pro Plus® 6.2 image analysis software (Media Cybernetics, Silver Spring, Md, USA). Stained sections were examined under a bright field with a Nikon microscope and a 4x objective. Images were recorded with an Olympus DP70 CCD camera (Tokyo, Japan) and transmitted to the frame grabber, which converted the image into a digital matrix of 1600 x 1200 pixels. After image acquisition, a threshold gray level was established in order to detect IR fibres in the digitized microscopic image, the discrimination step. To avoid the subjective bias of manual thresholding, the threshold was determined on the basis of the density histogram displayed by the program. It was set at the point where the flat part of the histogram (pixels with high densities) started to rise steeply. The program expressed the area innervated by the IR fibres as the number of pixels with densities above the threshold. For the calibration, known areas of different shapes were measured. Measurements were made in a blinded fashion from at least 16 sections for each staining in each animal group and averaged.

Higher magnification images of nNOS-, CamkIIα- and CGRP immunohistochemistry were taken using a Zeiss AxioImager microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) supplied with a PixeLINK CCD camera (PixeLINK, Ottawa, ON, Canada).

Group values are given as means ± SEM. Statistical comparisons between the control and NTG-treated groups, Western blot optical densities and HPLC measurements were carried out by using analysis of variance (ANOVA) followed by the Scheffe test. Both analyses were implemented in SPSS (Version 11.0 for Windows, SPSS Inc.), with $p < 0.05$ taken as statistically significant.

III.2. Transgenic mouse model of HD

Animals

All animal experiments were carried out in accordance with the European Union Guide for the Care and Use of Laboratory Animals and were approved by the local animal care

committee. Male transgenic mice of the N171-82Q strain were originally obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and were maintained on the B6C3F1 background and bred locally. The offsprings were genotyped by using a PCR assay on the tail DNA at the age of 4 weeks. The mice of mixed genotype were housed together, with same-sex animals per cage, under standard laboratory conditions with ad libitum access to tap-water and regular mouse food. They were kept under natural light in 12-h cycles.

Survival test

Eleven animals received i.p. injections of probenecid (Sigma, Steinheim, Germany) at a dose of 150 mg/bw kg 3 times a week starting at 6 weeks of age until death; 19 animals received i.p. injections of the vehicle of probenecid in the same volume at the same times.

Behaviour testing

Drug treatment

A separate set of 6-week-old transgenic- and wild-type mice were used for behavioural, immunohistochemical studies. The transgenic mice were divided into two groups (n = 6 per group). The animals in the first group received probenecid (150 mg/kg bodyweight, i.p., 5 ml/kg). In the second group, the transgenic mice received the vehicle of probenecid (5 ml/kg, i.p.). The wild-type mice in the control group received the vehicle of probenecid (5 ml/kg, i.p.). The same experimental protocol and drug administration were used as above for immunohistochemistry (n = 6 per group). In the HPLC measurements, the animals were divided into two groups (n = 6 per group). The group of the wild-type control mice was injected with vehicle solution (5 ml/kg, i.p.). The second group of the wild-type mice received an i.p. injection of probenecid (150 mg/kg bw, i.p., 5 ml/kg) daily for 1 week.

Open-field test

The Conducta system and programme (Experimetria Ltd., Hungary) were used to detect and evaluate the changes in spontaneous motor activity in the open-field test. The Conducta apparatus, situated on the floor under dispersed light conditions, registered inter alia the ambulation distance, the mean velocity, the immobility time, the local time, and the duration of rearings and jumpings which are markers of the spontaneous locomotor activity and explorative behaviour. Each mouse was placed in the centre of the arena and its behaviour was recorded for 5 min. Between sessions, the open field was cleaned with

alcohol and dried. Tests were performed once a week for 10 weeks at the same day and the same time of day to avoid alterations due to the diurnal rhythm. The tests for behaviour were carried out the following day of the probenecid or vehicle injection.

Immunohistochemistry

At the age of 16 weeks, the mice were deeply anaesthetized with isofluranum (Abbott Laboratories Ltd., Queenborough, UK) and perfused transcardially with 15 ml phosphate-buffered saline, followed by 15 ml 4% paraformaldehyde (PFA) in PBS. The brains were rapidly removed *in toto* and postfixed overnight in the same fixative. After cryoprotection (10% glycerol), 30-µm thick cryostat sections were cut to obtain sections from the entire striatum with the overlying cortex. Serial sections were immunostained with a polyclonal antibody recognizing the first 256 amino acids of human huntingtin (EM48, Chemicon International Inc., Temecula, CA, USA) at dilutions of 1:500. The specificity of the immune reactions was controlled by omitting the primary antiserum. An additional series of sections from each case were Nissl-stained with cresyl violet. The sections were dried overnight on glass slides, and coverslipped using DPX as mounting medium. The sections were analysed in a Nikon Phase Contrast (085 59762, Tokyo, Japan) light microscope and photomicrographs were taken using Olympus DP70 (Tokyo, Japan) camera equipment. The digital photos were analysed using Image-Pro_ Plus 6.2 software (MediaCybernetics Inc., Bethesda, MD, USA).

HPLC measurements

Animals were dissected and their brains were immediately removed and placed on ice. The cortices and the striata were rapidly dissected out and homogenized in 250 µl distilled water. The samples were deproteinated with 62.5 µl 8% perchloric acid and centrifuged. The supernatant were filtered with a syringe-driven filter unit (Millipore, Bedford, MA, USA). The KYNA concentrations of the samples were quantitated with the Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) with fluorescence detection (excitation: 344 nm, emission: 398 nm). In brief, 50-µl amounts of sample were applied onto a Hypersil 5 ODS HPLC column (Thermo Fisher Scientific, Waltham, MA, USA), and chromatographed isocratically at a flow rate of 1 ml/min with a mobile phase consisting of 0.2 M zinc acetate containing 5% acetonitrile. The retention time of KYNA was about 6 min. The results were expressed as pmol/g wet weight.

Statistical analysis

Kaplan–Meier analysis and the Mantel-Cox log rank test were used to determine the survival differences between groups. One-way ANOVA followed by Fisher's LPSD test was used to determine significant differences between groups in the behaviour tests and to compare the level of KYNA between the probenecid-treated wild-type mice and the vehicle-treated wild-type mice. Huntingtin-IR cells in the striatum and the cortex and the cresyl violet-stained neurons in the striatum were counted by an observer blinded to the procedures in five different series of sections in each animal. The independent Student t-test was used to detect the difference between the probenecid-treated transgenic mice and the vehicle-treated transgenic mice in case of huntingtin aggregates. All data were expressed as means ± standard error of the mean (x ± SEM). Statistical significance was taken as $p<0.05$.

IV. Results

IV.1. Animal model of migraine

IV.1.1. Pretreatment of L-KYN combined with probenecid and a novel derivative of KYNA

The HPLC measurements clearly indicated a significantly increased KYNA level in the TNC thirty minutes after L-KYN and probenecid administration (Figure 2).

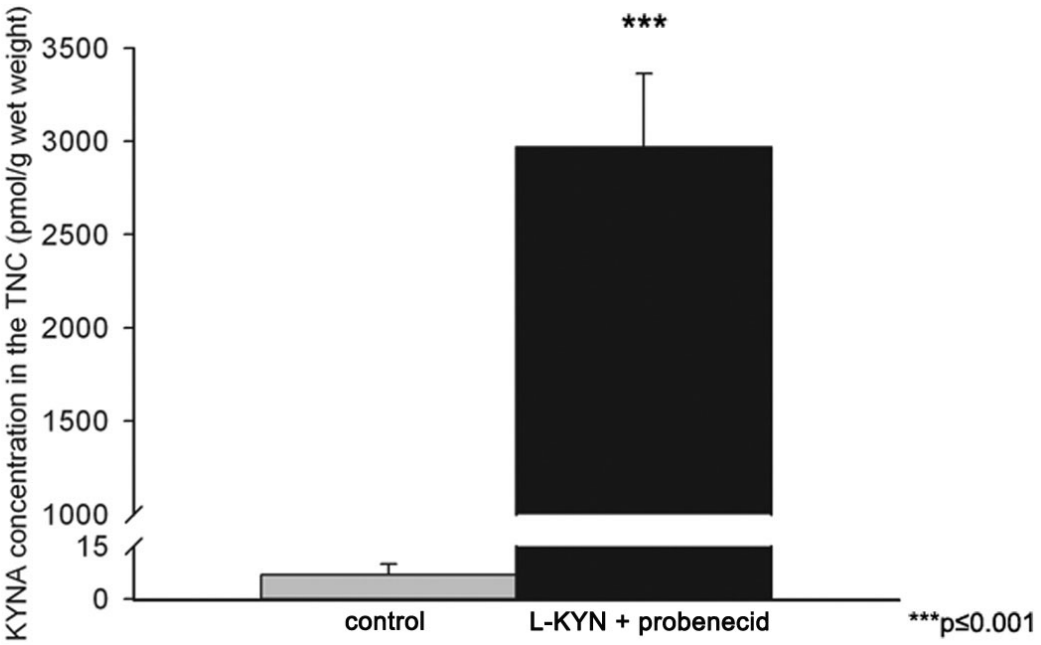

Figure 2. Histogram showing the KYNA level in the TNC after vehicle or L-KYN-probenecid treatment (means+S.E.M., n=6 per group).

The transverse sections of the cervical spinal cord demonstrated numerous nNOS-IR neurons in the superficial layers of the dorsal horns. There was no significant difference in the numbers of NOS-positive cells at different levels of the C1–C2 region. In non-pretreated rats, NTG produced a significant increase in the number of nNOS-IR neurons in the superficial layers of the TNC (Figure 3a and b). The administration of L-KYN-probenecid (Figure 3c and d) or of the new KYNA analogue (Figure 3e and f) significantly attenuated the effect of NTG on the number of nNOS-IR cells in the TNC. The results of the statistical analysis are presented in the histogram in Figure 4.

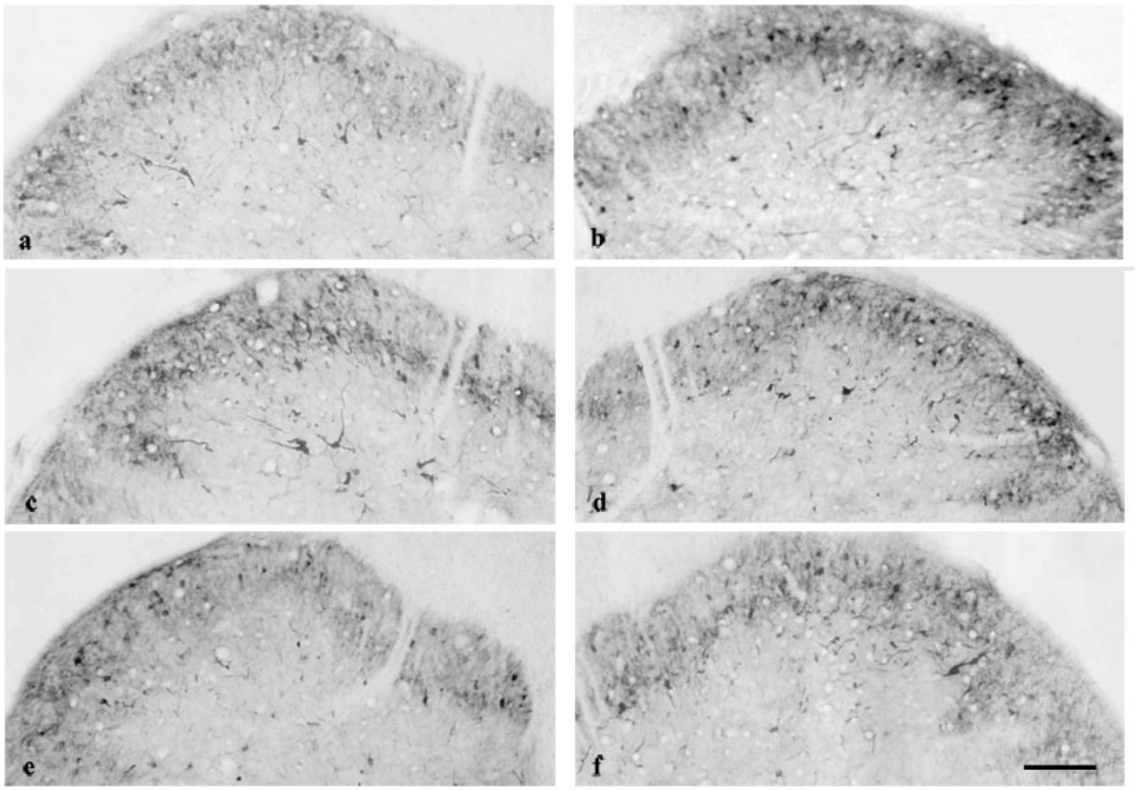

Figure 3. nNOS-immunoreactivity in the upper cervical spinal cord in control (a,b), the L-KYN-probenecid-pretreated (c,d) and the KYNA derivative-pretreated (e,f) rats after placebo (a,c,e) or NTG (b,d,f) injections. Both L-KYN combined with probenecid and the new KYNA derivative successfully attenuated the NTG-induced nNOS activation. Scale bar = 50 µm.

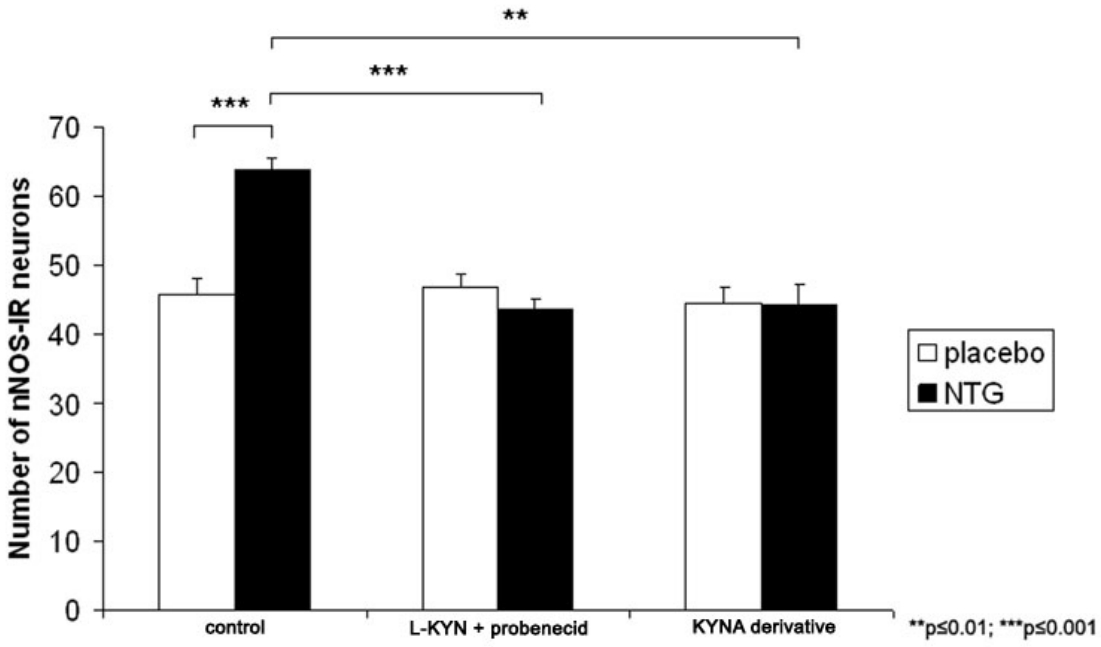

Figure 4. Histogram showing the mean number of nNOS-IR cells in the superficial layers of the dorsal horns of the L-KYN-probenecid-pretreated and the KYNA derivative-pretreated animals after vehicle (light bars) or NTG (dark bars) injections (means+S.E.M., n=6 per group).

Western blot analysis of the C1 and C2 region confirmed the results obtained by immunohistochemistry. A band characteristic of the nNOS protein was identified at ~155 kDa (Figure 5.). Densitometric analyses confirmed that the nNOS band in the Western blots was significantly enhanced in segments C1 and C2 after NTG administration (Figure 6.)

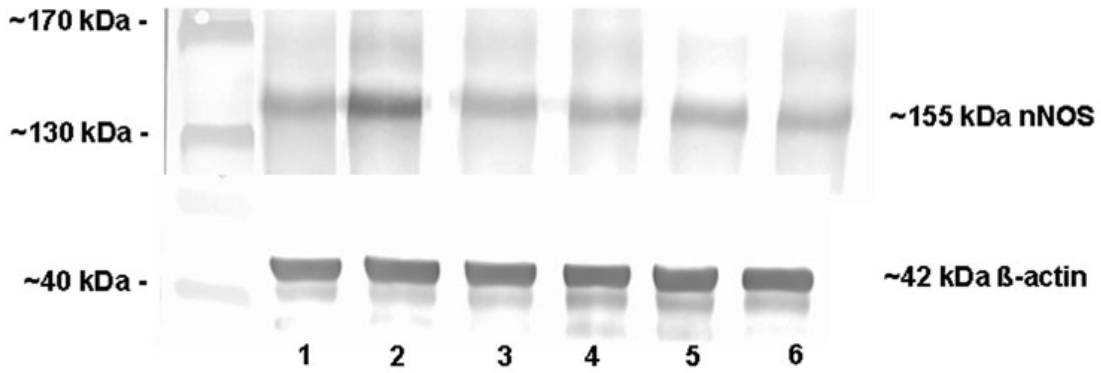

Figure 5. Western blotting of nNOS in the C1-C2 segments of the spinal cord in the control (lanes 1,2), the L-KYN-probenecid (lanes 3,4) and the KYNA derivative (lanes 5,6) animals. The corresponding ß-actin bands are shown below for each animal group.

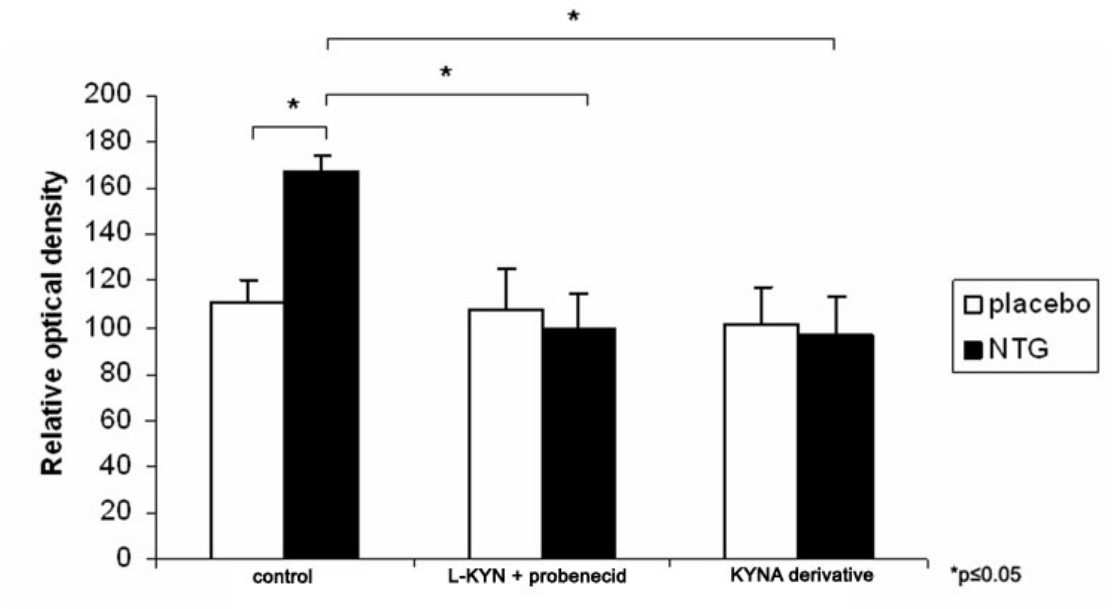

Figure 6. Histogram showing the optical densities of nNOS Western blots in the C1-C2 segments of the three animal groups four hours after s.c. injection of vehicle (light bars) or NTG (dark bars) (means+S.E.M., n=4 per group). Data are expressed as proportions of ß-actin.

IV.1.2. Pretreatment of probenecid

Transverse sections of the cervical spinal segments revealed abundant CGRP-positive fibres in the superficial layers of the caudal trigeminal nucleus. The areas covered by these fibres were not significantly different at the various rostro-caudal levels, nor on the different sides of the C1-C2 segments. The CGRP-innervated area for the NTG-treated group was significantly smaller than that for the placebo-treated group ($P<0.01$; Figure 5.A,B and Figure 6.). This decrease was successfully attenuated by probenecid pretreatment (Figure 5.C,D and Figure 6.).

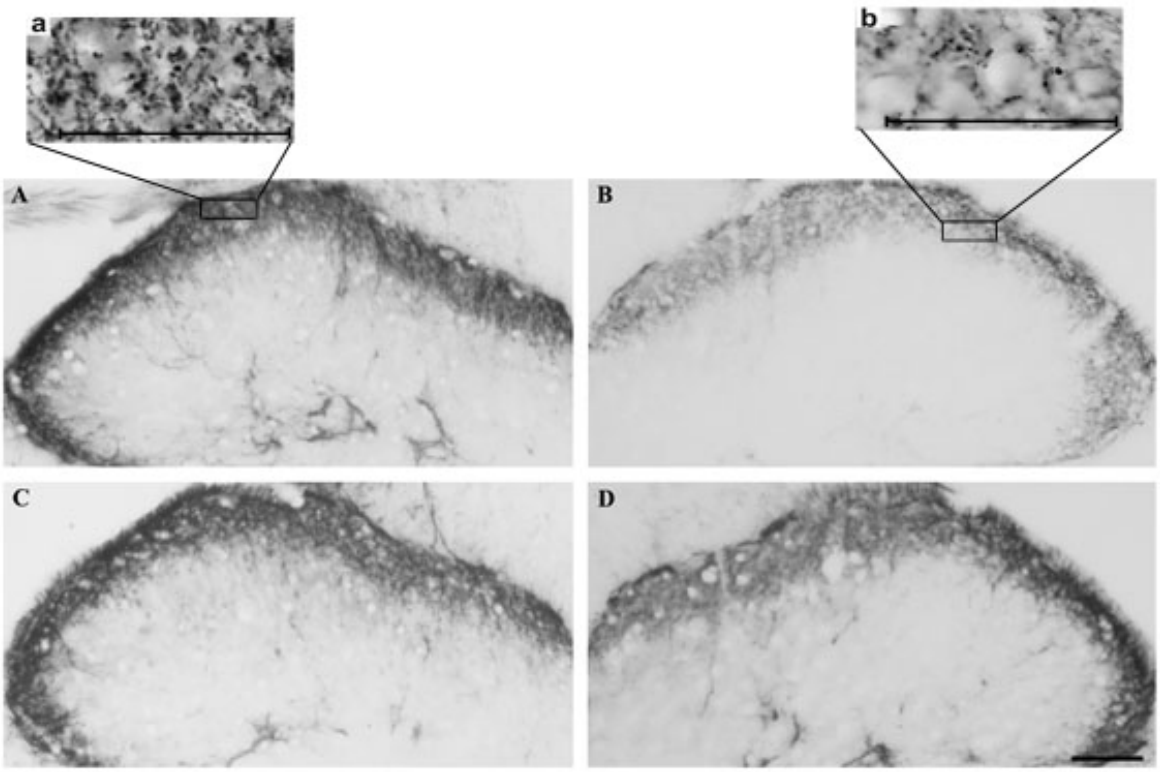

Figure 7. CGRP immunoreactivity in the upper cervical spinal cord in control (A,B) and probenecid-pretreated (C,D) rats after placebo (A,C) or NTG (B,D) injections. CGRP-IR fibres and buttons under 63x objective (a,b). Scale bar = 50 µm.

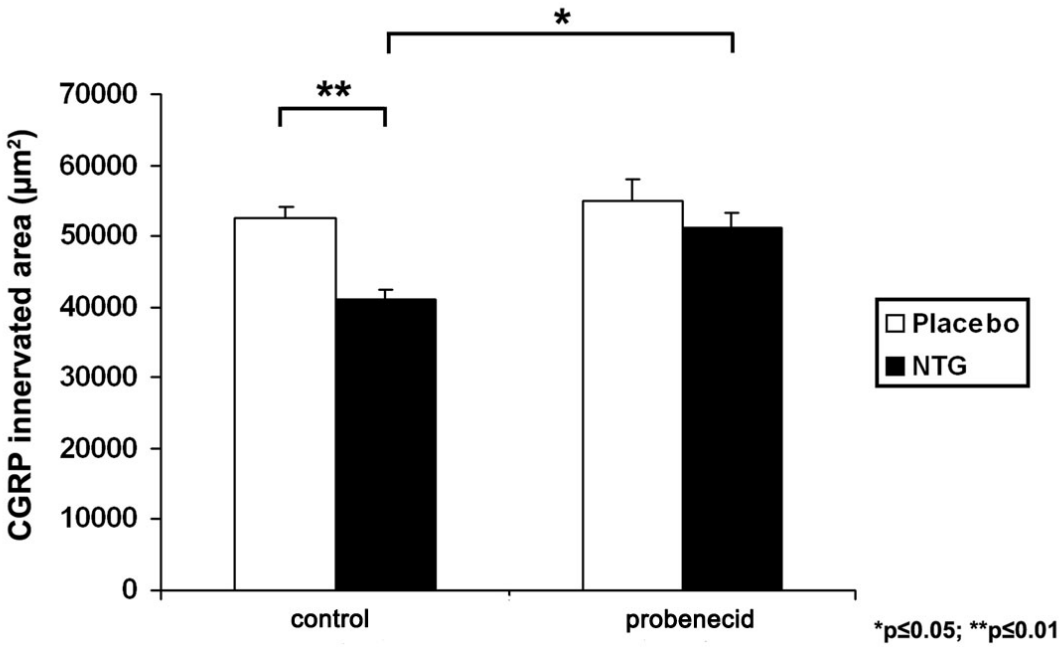

Figure 8. Histogram showing the area in μm^2 covered by CGRP-IR fibres in superficial laminae I-II of the TNC at C1-C2 in both groups after vehicle (light bars) or NTG (dark bars) (means±S.E.M, n=6 per group).

Transverse sections of the cervical spinal cord demonstrated numerous nNOS-IR neurons in the superficial laminae of the dorsal horns. There was no significant difference in the number of nNOS-positive cells at the different levels of the C1-C2 region. In the non-pretreated rats, NTG produced a significant increase in the number of nNOS-IR neurons in the superficial layers of the TNC (Figure 9.A,B). The administration of probenecid (Figure 9.C,D) significantly attenuated the effect of NTG on the number of nNOS-IR cells in the TNC. The results of the statistical analysis are presented in the histogram in Figure 10.

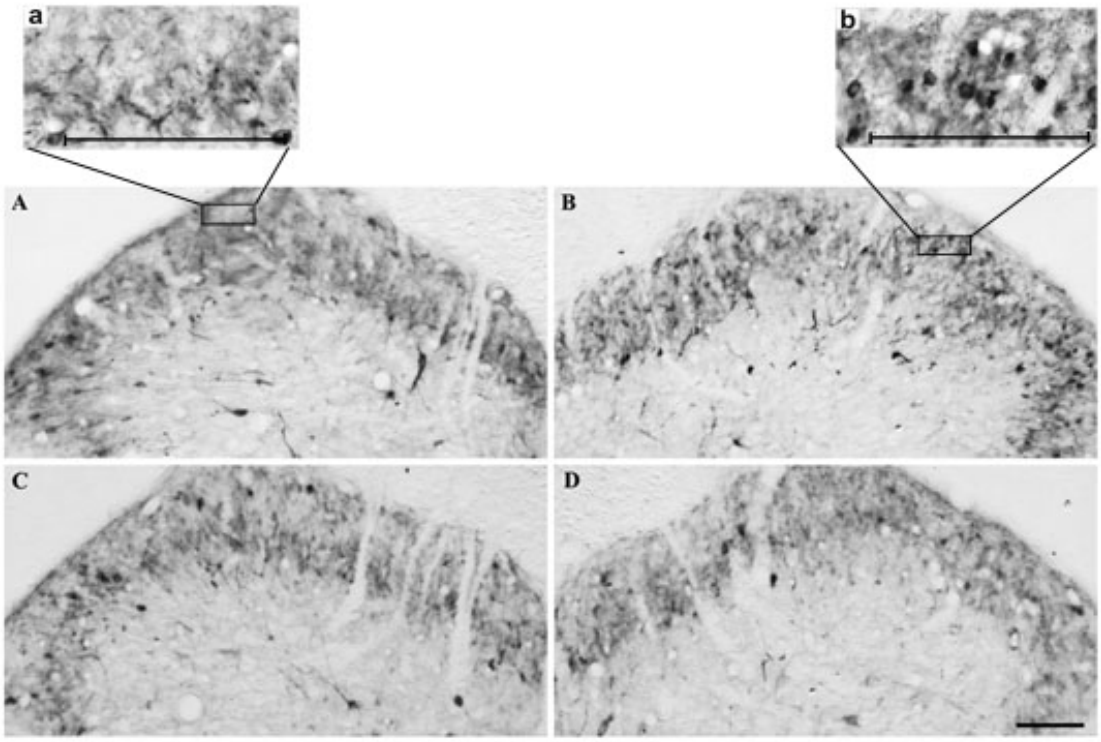

Figure 9. nNOS immunoreactivity in the upper cervical spinal cord in control (A,B) or probenecid-pretreated (C,D) rats after placebo (A,C) or NTG (B,D) injections. nNOS-IR neurons under 63x objective (a,b). Scale bar = 50 µm.

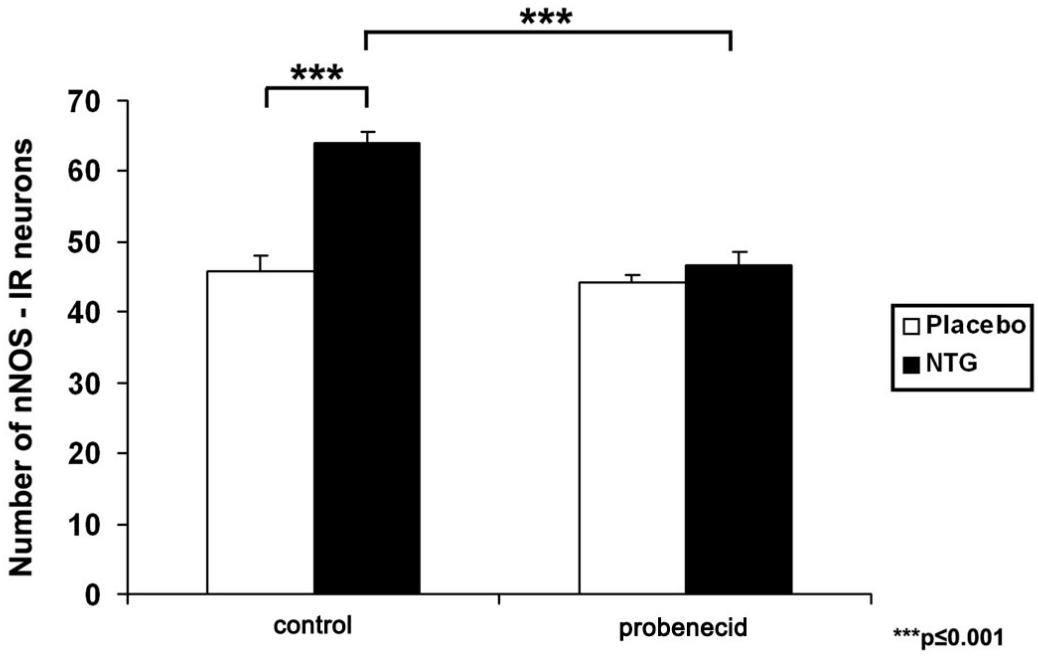

Figure 10. Histogram showing the mean number of nNOS-IR cells in the superficial layers of the dorsal horns of both groups after vehicle (light bars) or NTG (dark bars) injections (means±S.E.M., n=6 per group).

On microscopic examination of immunostained transverse sections, CamKIIα immunoreactivity was found in the neurons of the TNC and in the neuropil of lamina II. CamKIIα-IR cells were abundant in the superficial layers of the TNC. The numbers of cells were not significantly different at the various rostro-caudal levels, nor on the different sides of the TNC. NTG induced a significant increase in the number of CamKIIα-positive cells in the superficial layers of the TNC in the non-pretreated rats (Figure 11.A,B and Figure 12.). Probenecid attenuated the CamKIIα increase (Figure 11.C,D and Figure 12.).

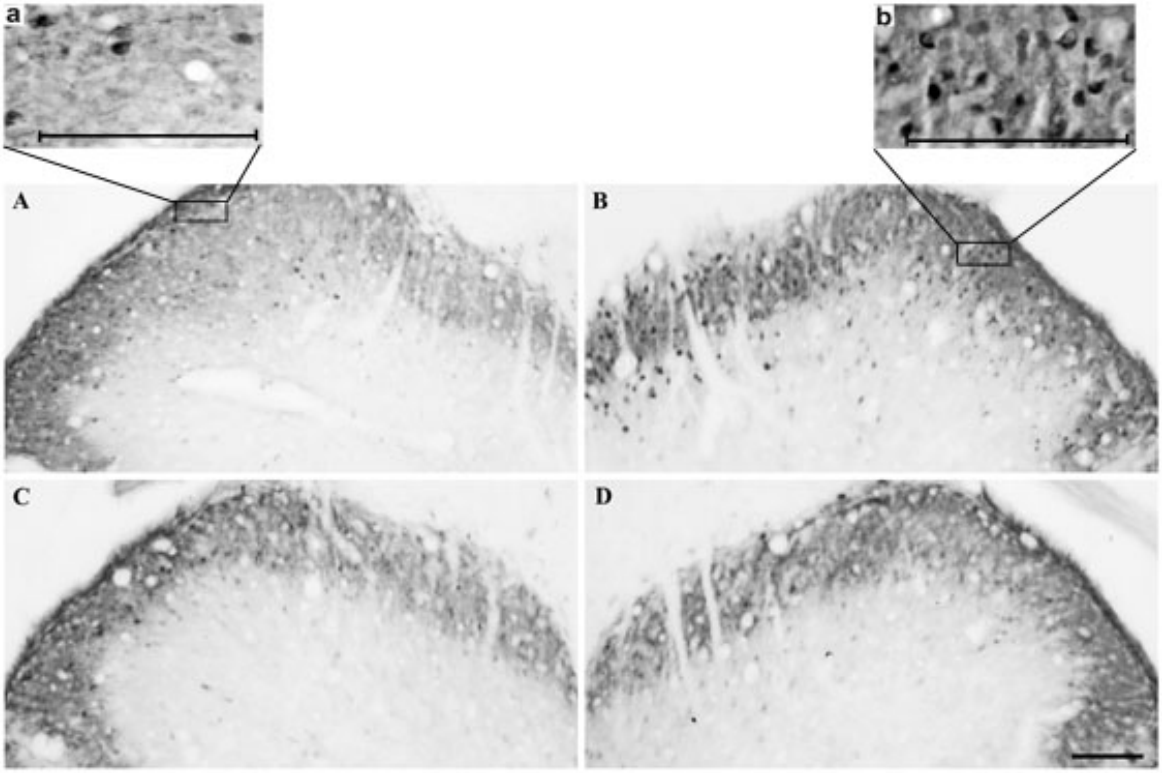

Figure 11. CamKIIα immunoreactivity in the upper cervical spinal cord in control (A,B) or probenecid-pretreated (C,D) rats after placebo (A,C) or NTG (B,D) injections. CamKIIα-IR neurons under 63x objective (a,b). Scale bar = 50 μm.

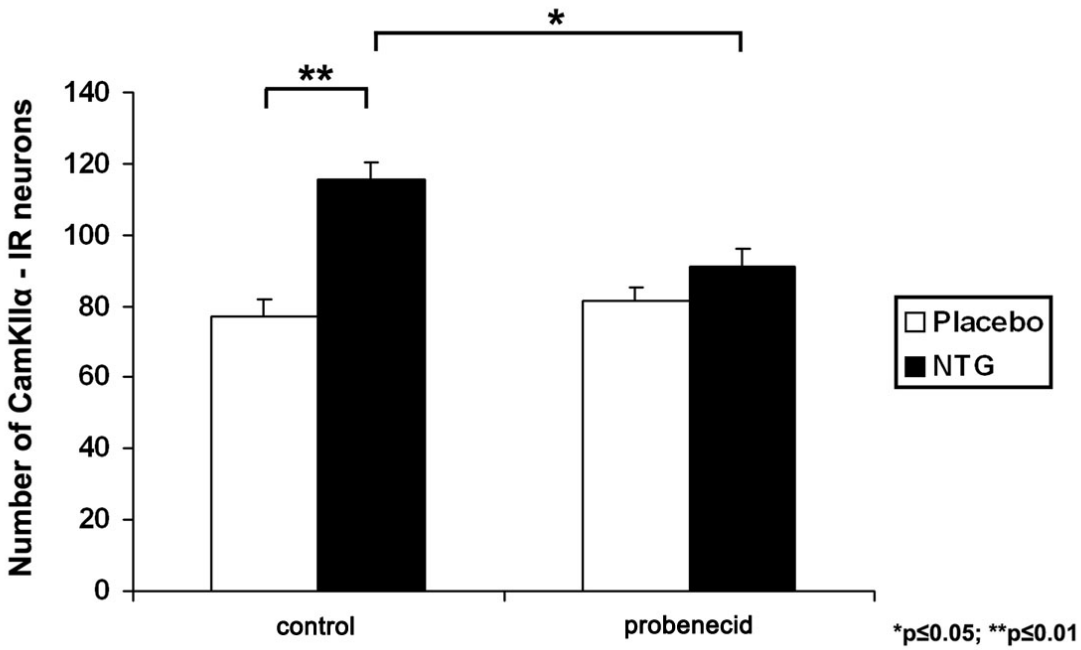

Figure 12. Histogram showing the mean number of CamKIIα-IR cells in the superficial layers of the dorsal horns of both groups after vehicle (light bars) or NTG (dark bars) injections (means±S.E.M., n=6 per group).

IV.2. Transgenic mouse model of HD

Even 1 week of administration of probenecid resulted in an increased level of KYNA in the wild-type mouse cortex over that in the control group but not in the striatum (Figure 13.).

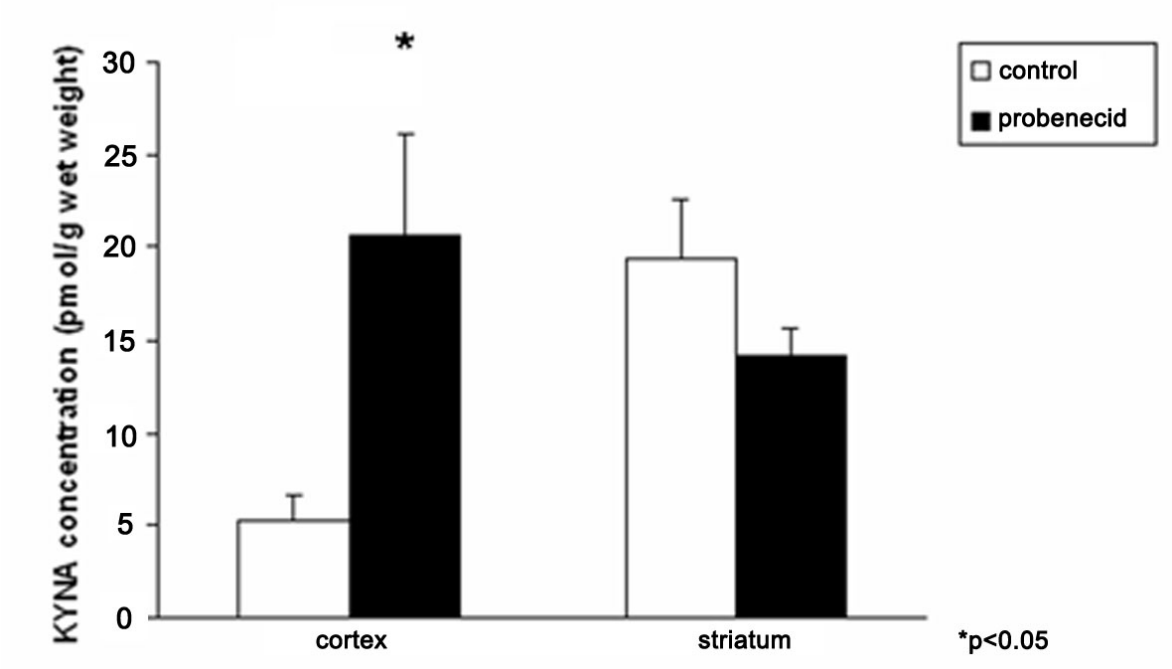

Figure 13. Histogram showing the KYNA level in the cortex after vehicle and probenecid treatment (mean±S.E.M).

The mean duration of survival of the vehicle-treated N171- 82Q mice was 118.1 days. Probenecid administration produced a highly significant increase in the survival of 35% (159.6 days) (Figure 14.).

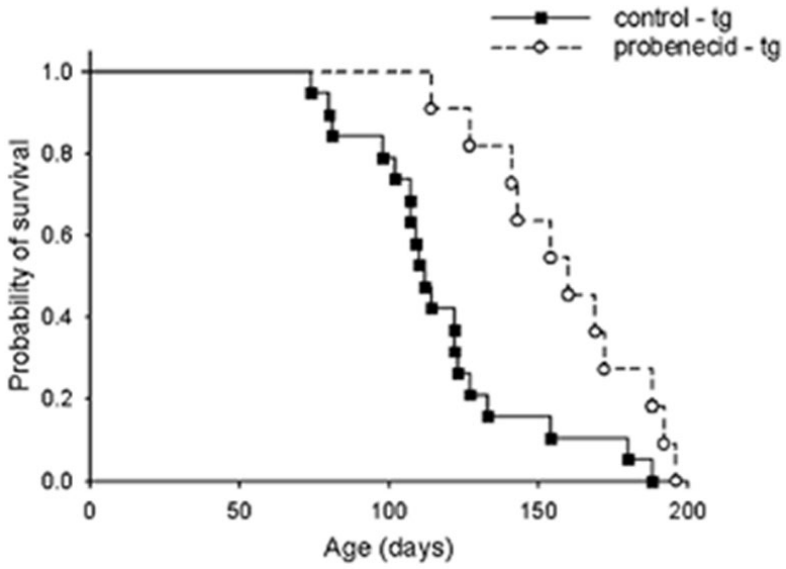

Figure 14. Probenecid significantly increased the survival time of N171-82Q mice. In fact, according to the Kaplan–Meyer survival curve, probenecid-treated animals showed an increased survival compared to the control group (*p<0.05).

Our earlier experiments showed that chronic probenecid treatment did not cause significant motor activity changes ($p_{ambulation\ distance} = 0.76$; $p_{mean\ velocity} = 0.73$; $p_{immobility\ time} = 0.2$; $p_{rearing\ count} = 0.9$) between the probenecid-treated wild-type mice (n = 9) and the vehicle-treated control wild-type mice (n = 9). The ambulation distance moved is shown in Figure 15.a. The transgenic mice treated with probenecid moved significantly more than the controls by the age of 14 weeks. The probenecid-treated group spent significantly less time in the same place compared with the vehicle-treated transgenic group (Figure 15.c). The probenecid treatment induced a slight, but not significant increase in the mean velocity (Figure 15.b). The frequency of rearing was significantly lower in the control transgenic mice than in the probenecid-treated group (Figure 15.d).

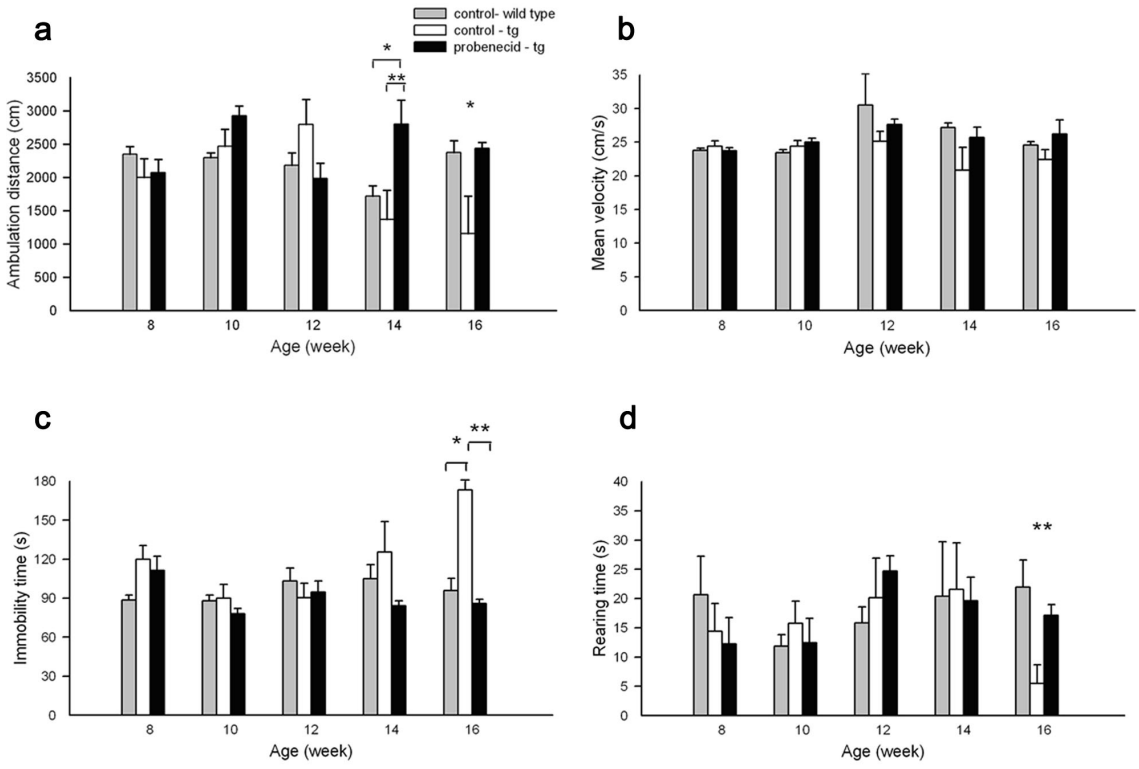

Figure 15. Behavioral assessment of N171-82Q transgenic mice in open-field tests. Control transgenic mice showed significantly reduced total locomotor counts in open-field test compared with probenecid treated mice by 16 weeks of age

Probenecid treatment also ameliorated the striatal neuronal loss in these animals. Our quantitative analysis demonstrated that the probenecid-treated transgenic animals had a

significantly higher number of surviving striatal neurons relative to the vehicle-treated group (Figure 16.).

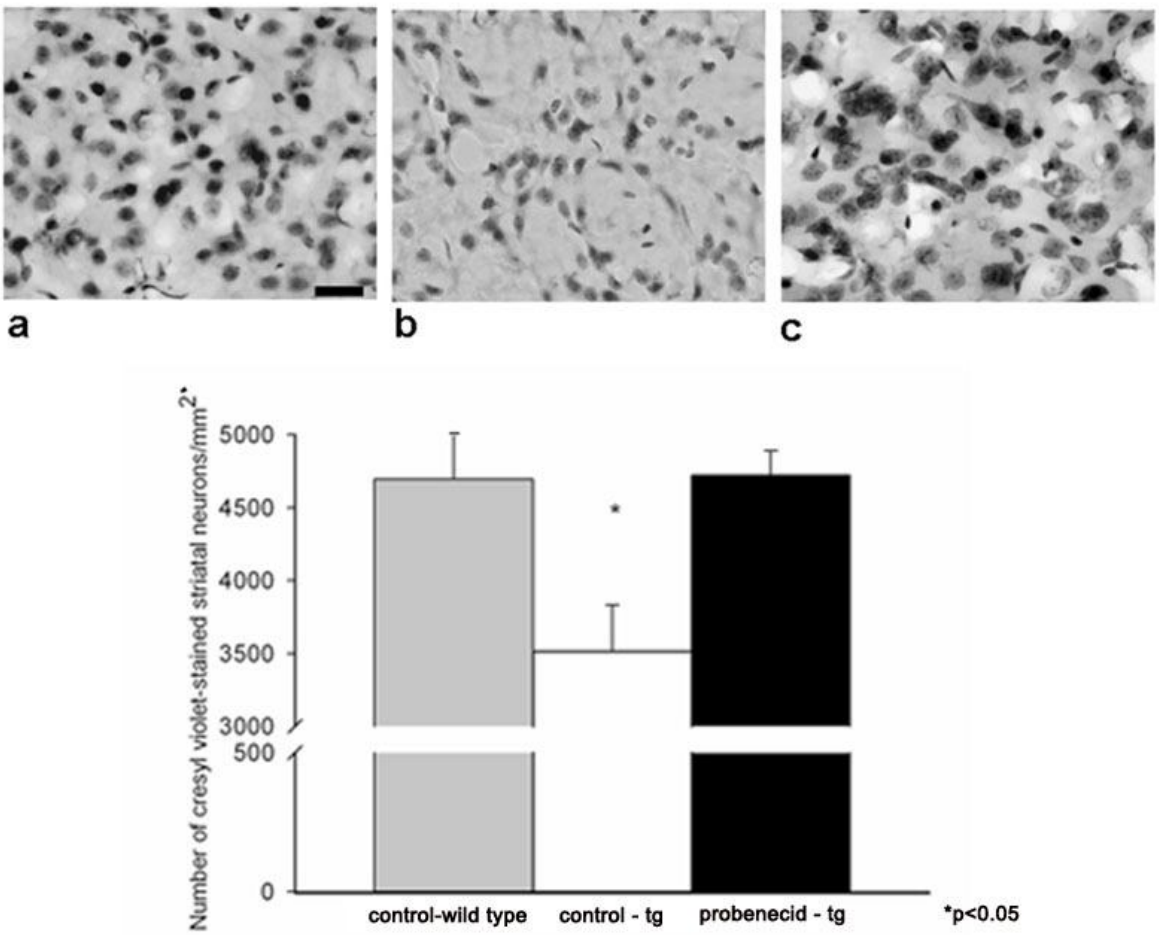

Figure 16. Cresyl violet-stained neurons in the striatum in the wild type (a), control transgenic (b) anf probenecid-treated (c) transgenic groups. Scale bar = 50 µm.
Diagram showing the mean number of cresyl violet-stained neurons in the striatal area of wild-type mouse (gray bar), control transgenic mouse (light bar) and probenecid-treated N171-82Q mouse (dark bar) at 16 weeks of age (mean+S.E.M., n=6 per group).

Huntingtin-IR aggregates were significantly more numerous in the outer lamina of the pyriform cortex (layer II), which is an important area of the N171-82Q transgenic mice as concern the EM48 positivity, and within the lateral striatum. The aggregates were much more prominent within the cortex as compared with the neostriatum. In the probenecid-treated group, fewer EM48 positive neurons were detected in both areas than the vehicle-treated transgenic group. Probenecid treatment significantly reduced the numbers of striatal and cortical positive neurons (Figure 17.).

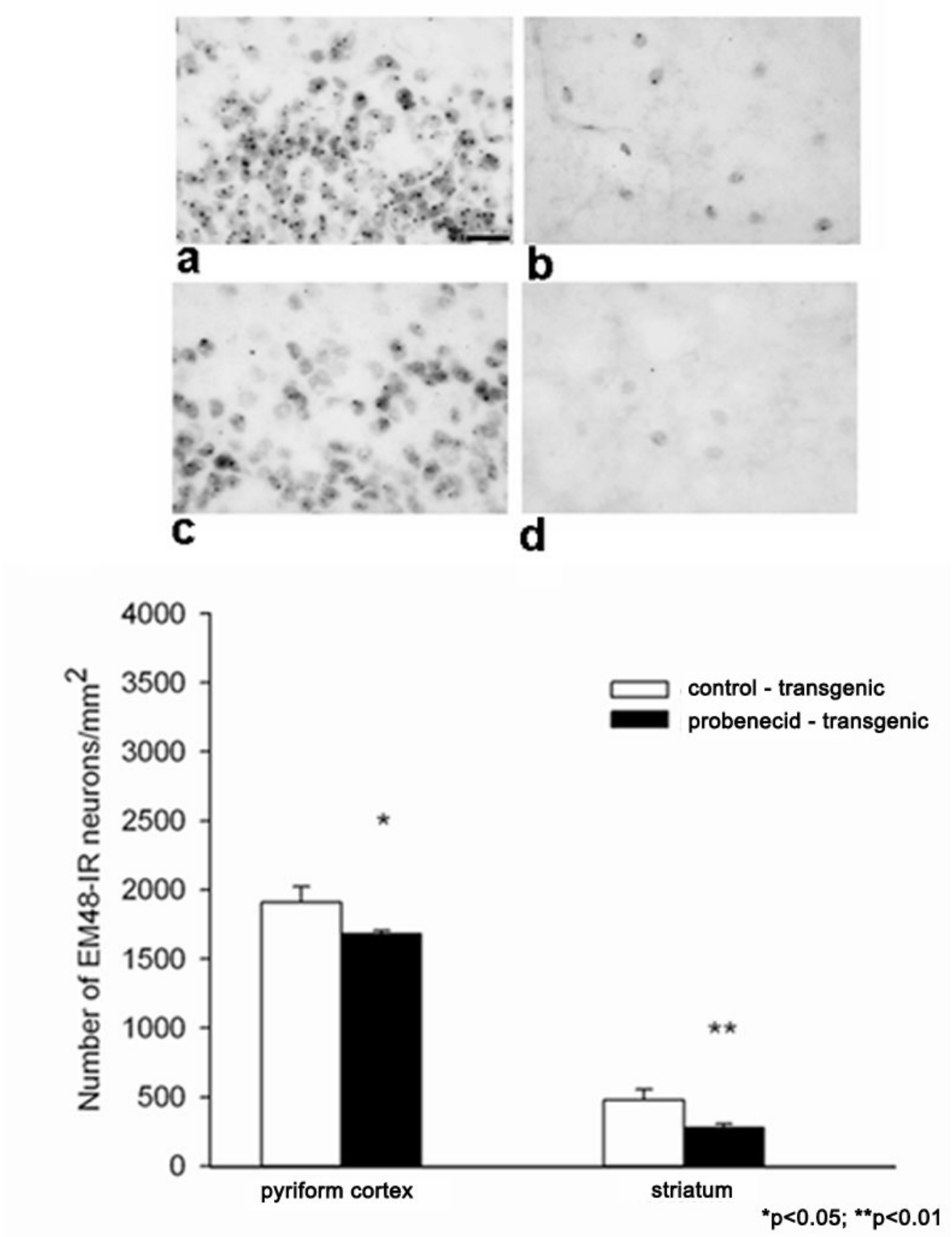

Figure 17. Polyclonal EM48 antibody staining of cortical sections (A,C) and striatal sections (B,D) at 16-week-old control and probenecid-treated transgenic mouse brains. Scale bar = 50 µm.
Diagram showing the mean number of EM48-IR cells in the pyriform cortex and in the striatum of probenecid-treated (dark bar) and control (light bar). (mean+S.E.M., n=6 per group).

V. Discussion

Our HPLC data confirm earlier findings and observations [81], we found that the administration of L-KYN in combination with probenecid caused a robust increase in the level of KYNA in the rat TNC. In the present study, this combination or a novel KYNA derivative (2 - (2 - *N*,*N* – dimethylaminoethylamine – 1 - carbonyl) - 1*H* – quinolin – 4 - one hydrochloride) attenuated the NTG-induced enhancement of nNOS in the most caudal portion of the rat TNC.

The most probable way for NTG to enhance nNOS expression is via the peripheral afferents, causing a self-amplifying process in the TNC, where glutamate may also play a role. Indeed, all the major glutamate receptor classes have been identified in the superficial lamina of the rat TNC [46]. NMDA receptors seem to be relevantly involved in the process of NO synthesis [82]. Moreover, it has been shown that the activation of NMDA receptors is pivotal for the development of central sensitization in the dorsal horn [83,84]. Moreover, glutamate is involved in neuronal sensitization at the level of the trigeminal nucleus, a process involved in allodynia [85]. Earlier data have revealed that NTG can also enhance the level of CamKIIα in the TNC of the rat [14], which is in line with other data, since phosphorylation of the NMDA receptors initiated prolonged increases in the excitability of the spinal cord neurons [84]. NMDA-receptor phosphorylation is increased in the spinal dorsal horn neurons in animals displaying neuropathic pain or capsaicin-induced hyperalgesia [86,87]**.** The central nervous system uptake of KYN and its metabolism to KYNA may act to reduce NMDA receptor-mediated central sensitization [88].

Besides the NMDA antagonistic effect [89], KYNA is able to act on the AMPA / kainate receptors [90]. A synthetic NMDA receptor antagonist, MK-801, and a synthetic AMPA receptor antagonist, GYKI-52466, effectively block trigeminovascular nociception [91]. Indeed, the mixed AMPA / kainate receptor antagonist LY293558 has been shown to be effective in the acute treatment of migraine [45]. Thus, glutamate plays a key role in trigeminal activation and sensitization [92] and modulation of the glutamate receptors appears crucial in the pathogenesis of migraine.

In our experiments, in the N171-82Q transgenic mouse model of HD the chronic administration of a novel analogue of KYNA improved the survival time, motor activity changes as compared with that in the control transgenic mice (data not shown). Thus, our results have shown that the KYNA derivative treatment in N171-82Q mouse model of HD results the delay of symptom development and lessening of symptom severity. Previous

data suggest abnormalities in different metabolites of the kynurenine pathway in the neostriatum of HD patients [93-96]. Earlier studies have shown that the KYNA levels are depleted in the striatum and cortex of patients with HD [97,93]. The populations of neurons that degenerate in HD, the medium-sized spiny neurons of the striatum, are NMDA receptor-rich and therefore more vulnerable to excitotoxic cell death than other cell types (reviewed by [21]). Further experiments have proved that some synthetic kynurenines affect the excitatory response of the striatal neurons in a structure-related and dose-dependent manner [98].

Despite recent advances in the therapy, appropriate treatment of migraine and HD is yet to be achieved. Since KYN metabolites may modulate different neuronal targets in head pain conditions and HD, the side-effect profile and hence the possible indications and contraindications would be different relative to the drugs already in use.

Taken together, these observations suggest the involvement of metabolites of the kynurenine pathway (see Appendix) on various sites of nociception and neurodegeneration. One strategy with the aim of increasing the therapeutic potential of KYNA is to develop synthetic analogues which can readily penetrate the BBB, and act as glutamate receptor antagonists. Our results show that L-KYN (metabolized to KYNA) and 2-(2-*N*,*N*-dimethylaminoethylamine-1-carbonyl)-1*H*-quinolin-4-one hydrochloride (a novel KYNA derivative) exert a modulating effect on the trigeminal activation in the NTG model of migraine and the neurodegenerative processes in the transgenic mouse model of HD, possibly via the glutamate receptors. Thus, KYNA derivatives may afford novel therapeutic opportunities in the management of these neurological disorders.

As a non-selective inhibitor of MRPs and organic acid transporters in the BBB, probenecid can inhibit the elimination of the excitatory amino acid receptor antagonist KYNA produced in the brain (reviewed by [99,76]). Our HPLC data confirmed the earlier finding that the administration of probenecid causes a significant increase of KYNA in the TNC [100-103]. Although probenecid treatment increased the KYNA level, it did not attain the concentrations needed to block NMDA receptors. KYNA has been reported to antagonize not only the NMDA receptor [104], but also AMPA/kainate receptor [105-107] and the G protein-coupled GPR35 receptor [108], but these effects also require a few hundred nanomolar or even higher KYNA concentrations. Overall, these data suggest that the modulatory effects of probenecid are only marginally linked to the above mechanisms. The endogenous KYNA increase observed after probenecid administration may be sufficient to modulate the alpha7-nicotinic acetylcholine receptor function [109]; activation

of this receptor at the presynaptic site is involved in the regulation of glutamate release [110] and thus the indirect modulation of trigeminal activation and neurodegenerative process by glutamatergic mechanisms might be possible.

It has been suggested that probenecid is able to inhibit MRP4 [111], which can transport prostaglandins E1 and E2 with higher affinity than for other MRPs. MRP4 catalyses the uptake of the key inflammatory mediators prostaglandins E1 and E2 in a time- and ATP-dependent manner [112]. Several lines of evidence indicate that prostaglandins induce the release of neurotransmitters such as excitatory amino acids, CGRP and NO. Conversely, glutamate, CGRP, cytokines and NO enhance prostaglandin release [113].

CGRP is a key transmitter in primary nociceptive afferents, and both basic research and clinical studies have provided evidence of its role in the pathomechanism of migraine [114-116]. The concentration of CGRP in the jugular venous blood plasma is elevated during the headache phase of migraine [114]. Peripheral trigeminal activation is provided by the release of CGRP in migraine [15,114], and CGRP can be released from the peripheral afferents in animals by NO-mediated mechanisms [117]. Peripheral sensitization of meningeal trigeminal nociceptors and the increased release of CGRP are thought to activate second-order neurons that mediate central sensitization, which is important in the pathology of migraine. The decrease in CGRP immunoreactivity after systemic NTG administration is probably due to the activation of primary trigeminal Aδ and C fibres and their interactions with second-order trigeminal neurons [16]. The release of neuropeptides such as CGRP from peripheral nerve endings induces a painful state of local neurogenic inflammation in the cerebral dura. In our experiments, probenecid attenuated the NTG-induced depletion of CGRP in the most caudal portion of the rat TNC, inhibiting CGRP release from the sensory nerve endings by blocking the inflammation process, which plays a key role in trigeminal activation.

The most likely explanation for the NTG-induced nNOS increase is the secondary activation of the peripheral afferents interacting with second-order trigeminal neurons, leading to a self-amplifying process in the TNC. In migraine patients, early activation of the L-arginine/NO pathway and a late rise in the synthesis of prostanoids has been demonstrated after the onset of headache [68] and increases in the release of prostaglandin E2 and in the NO production of monocytes have been found in migraineurs without aura [69], suggesting an interaction. Moreover, acetylsalicylic acid and a selective COX-2 inhibitor, NS398, inhibited the NTG-induced nNOS expression changes in the rat, which

indicates that prostanoids are involved in this process [118,119]. Probenecid, which inhibits MRP4 [111], may block the sensitization process via the prostanoids at the level of the TNC.

NTG can also enhance CamKIIα in the TNC of the rat by similar mechanisms [14]. The level of CamKIIα immunoreactivity in the superficial laminae of the spinal cord is increased after s.c. injections of formalin [120] or capsaicin [121], or after intrathecal injections of substance P [122], and these increases can be blocked by CamKII inhibitors [121]. Such findings suggest that CamKIIα has an important role in nociceptive processing and contributes to central sensitization [121]. A CamKII inhibitor dose-dependently mitigates inflammation-induced thermal hyperalgesia and mechanical allodynia [123], while pretreatment with acetylsalicylic acid and the selective COX-2 inhibitor NS398 attenuates the NTG-induced changes in CamKIIα immunoreactivity [118,124]; accordingly, the inhibition of MRP4 by probenecid [111], which affects prostanoid transport, may have a key role in the modulatory process.

It has been well known that excitotoxicity, energy deficit, oxidative stress, protein aggregation and inflammatory process also play an important role in the pathogenesis of HD (reviewed by [39]). In this study, we used the N171-82Q transgenic mouse model of HD to determine the chronic effects of probenecid on the survival time, motor activity changes neuronal loss and on the formation of the mutant huntingtin aggregates. The spontaneous locomotor activity (involving the total distance moved, the immobility time and the velocity) and the duration of rearings were significantly different in the transgenic control group by 14 weeks of age. The administration of probenecid significantly improved the motor activity in the N171-82Q mice as compared with that in the control transgenic mice. Thus, our results have shown that probenecid treatment in N171-82Q mouse model of HD results in the delay of symptom development and lessening of symptom severity. These improvements may be accounted for by the protection of striatal cells observed in probenecid-treated transgenic group. The N-terminal fragments of mutant huntingtin accumulate in the nucleus of affected neurons and form intranuclear aggregates [26,27]. The role of the intranuclear aggregates is still controversial, but the effect on the neuronal loss may be hypothesized. In our result of this study, there were significant decreases in EM48 immunoreactivity in the striatum and in the pyriform cortex of the probenecid-treated N171-82Q mice relative to the PBS-treated transgenic group. We demonstrated that probenecid treatment delays the aggregate formation. Our findings suggest that a decrease in nuclear aggregates may decelerate the progress of the disease which is in accordance

with other experiments [125,126]. Inhibition of MRP4 by probenecid may contribute to the protection of neurons in this transgenic animal model of HD by blocking inflammatory signals.

On the other hand, recent studies have demonstrated that probenecid inhibits the pannexin-1 channel [127], which is largely expressed in the spinal cord [128] and may play an important role in neuronal inflammatory processes [129] and hence in pain processing. P2X7 receptors, which are closely related to pannexin-1, are present in the sensory ganglia [130] and associated with neuron-glia communication [131] and involved in nociception [132]. Moreover, the pannexin-1-related P2X7 activation that occurs in the spinal dorsal horns is implicated in the development of chronic neuropathic pain, allodynia and the sensitization process [133]. Its inhibition may be responsible for the attenuation of NTG-induced CGRP, nNOS and CamKIIα expression-changes and for the abolition of the peripheral trigeminal activation and the central sensitization process in the TNC.

Pannexin 1 can be activated not only by inflammatory process but apoptotic signals. Pannexin 1 is a potential candidate protein of ATP release during high extracellular potassium-induced cell death [129]. The pathomechanism of HD involves neuronal atrophy and cell death and thus probenecid may also exert protective effect on this process. Interestingly, extracellular ATP has been recently reported to elicit neuronal death through stimulation of P2X7 receptors. These are ATP-gated cation channels known to modulate neurotransmitter release from neuronal presynaptic terminals and to regulate cytokine production and release from microglia. Recent findings demonstrate that alteration in P2X7-mediated calcium permeability may contribute to HD synaptic dysfunction and increased neuronal apoptosis. In cultured neurons expressing mutant huntingtin showed increased susceptibility to apoptosis triggered by P2X7-receptor stimulation. Furthermore, *in vivo* data strongly suggest that altered P2X7-receptor level and function contribute to HD pathogenesis and highlight the therapeutic potential of P2X7 receptor antagonists [134].

Overall, inhibition by probenecid of the ability of the organic acid transporters to cross the BBB can raise the excitatory amino acid antagonist KYNA level of the brain dose-dependently [135]. In this model, the relatively slight KYNA concentration increase lead us to suggest that the glutamatergic modulatory effects of probenecid in the trigeminal complex and the striatum may be only marginal, and the effects of probenecid on prostanoids and pannexin-1 could play a more significant role. The fact that probenecid-influenced mechanisms are involved in this self-amplifying process in the trigeminal area

and in the neurodegenerative process in HD as well may furnish further details about the pathophysiology of migraine and HD. Despite these encouraging results, further studies are clearly needed to elucidate the exact mechanism of the modulatory effect of probenecid in these neurological disorders.

It is noteworthy that the pre-treatment with the KYNA analogue or L-KYN and probenecid or probenecid alone produced the same effect on the NTG induced nNOS activation in the trigeminal system. This suggests that this modulatory action involves at least two mechanisms one related to glutamate the other to probenecid. The conclusion is similar to the observations in the HD model. Chronic administration of KYNA derivative or probenecid improved the survival time and the motor activity changes in the transgenic mouse model of HD possibly in two different ways.

VI. Conclusions

Our results show that L-KYN (metabolized to KYNA) and 2-(2-*N*,*N*-dimethylaminoethylamine-1-carbonyl)-1*H*-quinolin-4-one hydrochloride (a novel KYNA derivative) have a modulating effect on trigeminal activation and sensitization in the NTG model of migraine, and it may therefore open up novel therapeutic opportunities in headache management.

Overall, our results suggest that the systemic administration of probenecid exerts an indirect effect on the TNC by blocking the inflammatory and sensitization processes, also crucial in the pathogenesis of migraine, mitigating the activation properties of NO in the trigeminal system. These data may be important to in pointing to new therapeutic strategies against migraine attacks and other headache syndromes.

We have demonstrated that probenecid administration to N171-82Q transgenic mice extends the survival time, improves the motor performance and decreases the number of intranuclear inclusions, these parameters being important in the pathomechanism of HD.

These data may contribute to a better understanding of the pathomechanisms of migraine headache and HD and the relevance of the excitotoxical and neuroinflammatory processes in these conditions.

VII. Acknowledgements

I would like to express my gratitude to my supervisors, Árpád Párdutz M.D., Ph.D. and Péter Klivényi M.D., Ph.D. for their scientific guidance and continuous support of my research activities.

I would also like to thank to Professor László Vécsei, Member of the Hungarian Academy of Sciences, Head of the Department of Neurology, University of Szeged for the opportunity to work in his laboratory and his scientific advices.

I wish to thank to all co-workers with whom I performed the experiments, especially Hedvig Varga, Dénes Zádori M.D., Annamária Fejes.

I would like to acknowledge the technical assistance of Valéria Vékony Széll and Andrea Varga.

I wish to express my special gratitude to my family and friends for their endless support during my work

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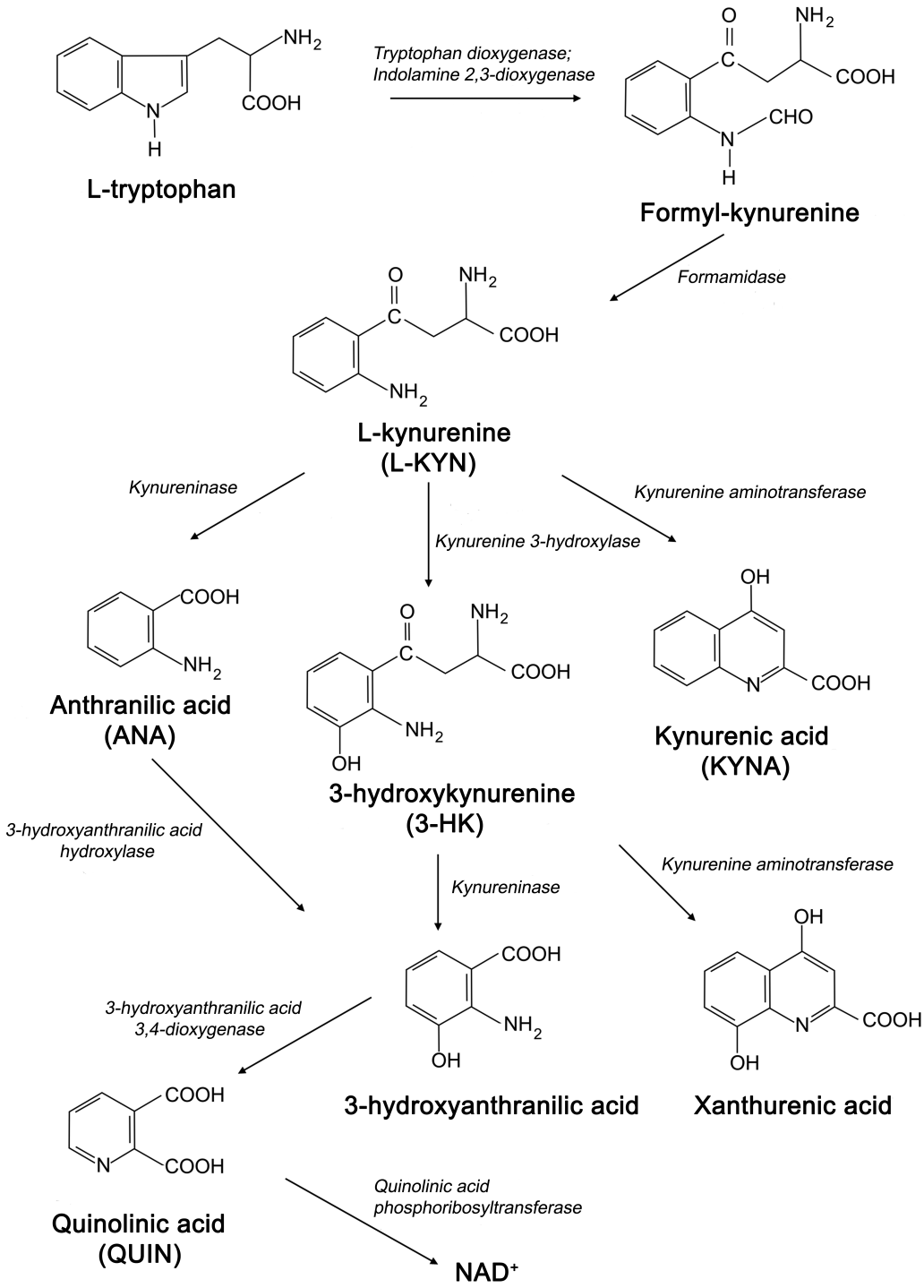
Appendix

1. Kynurenine pathway:



Az idegrendszer károsodásával járó neurológiai kórképek terápiája, kialakulásuk és a kórlefolyások sokszínűsége miatt a mai napig nagy kihívás jelent. Egyes betegségek hátterében az idegrendszer funkcionális zavara áll, és léteznek olyan morfológiai változásokkal járó neurológiai betegségek, amelyek patomechanizmusában progresszív sejtpusztulást írtak le. Ezek alapján kísérleteinkhez választottuk a migrént, amely elsősorban funkcionális változásokkal jár, valamint a neurodegeneratív csoportba tartozó Huntington-kórt.

A migrénes fejfájás a népesség közel 12%-át érinti és ezzel a leggyakoribb neurológiai kórképnek tekinthető. Az intenzív kutatások ellenére, a betegség pontos patomechanizmusa nem ismert. Annak ellenére, hogy az utóbbi időben több, igen hatásos gyógyszer jelent meg a migrénes roham enyhítésére, a biztos kezelés nem minden esetben megoldott. A migrén egyik humán modellje a nitrogén-monoxid donor nitroglicerin (NTG) szisztémás adása, amely a migréneseknél több órás latenciával aura nélküli rohamot provokálhat, feltehetőleg a trigeminovascularis rendszer aktiválása révén. Kísérleti állatokban a NTG többek között képes aktiválni a trigeminalis rendszer neuronjait és megemeli a neuronális nitrogén-monoxid szintáz (nNOS)- és kalmodulin-függő protein kináz II alpha (CamKIIα) immunreaktív sejtek számát, míg hatására lecsökken a calcitonin gén-relációs peptid (CGRP)-immunreaktív rostok által lefedett terület nagysága a caudalis trigeminalis magban (TNC). Ez a jelenség egy önerősítő folyamatot sejtet a trigeminalis rendszerben, amely centrális szenzitizációt hozhat létre, amely folyamat migréneseknél is ismert

A Huntington-kór egy autoszomális dominánsan öröklődő neurodegeneratív kórkép. A Huntington-kór prevalenciája ugyan nem túl magas (~5/10000), de mivel a kórkép progresszív jellegű, és biztosan halálhoz vezet, fontos a betegség patomechanizmusának megismerésére és terápiás hatású vegyületek kifejlesztésére komoly hangsúlyt fektetni. Az intenzív kutatások ellenére azonban a mai napig nem lehet tudni pontosan, hogy milyen folyamatok állnak a betegség hátterében és a biztos kezelés sem megoldott tekintve a kór lefolyása alatt jelentkező tünetek komplexitását. Vizsgálatainkhoz az N171-82Q egértörzset használtuk. A transzgén jelenléte által előidézett tünetek a születést követően körülbelül két hónapon belül kezdenek kialakulni. Az egér súlya tovább nem gyarapodik, tremor, hypokinesis és koordinációs zavar fejlődik ki, a járás

abnormálissá válik. A „betegség” előrehaladtával szövettanilag intranukleáris zárványok jelennek meg főként a cortexben és a striatumban.

A fő agyi excitátoros aminosavként számon tartott glutamát fokozott mértékű felszabadulása receptorainak elhúzódó stimulációjához vezet, mely különböző mechanizmusok révén az afferens sejtek pusztulását okozza. A glutamát előidézte neuronális sejtpusztulást (excitotoxicitás) közel négy évtizede írták le először. Humán- és állatkísérletes eredmények alapján ismert, hogy a glutamát fontos szerepet játszik a migrén és a Huntington-kór patomechanizmusában egyaránt.

A prosztaglandinok, mint gyulladásos mediátorok, hozzájárulnak a gyulladásos folyamatok kialakulásához. A prosztaglandinokról tudott, hogy részt vesznek a nociceptív információ kialakulásában, de szerepüket leírták a Huntington-kór patomechanizmusában is.

Mindezek alapján kísérleteink célja az volt, hogy megvizsgáljuk az excitátoros aminosav receptor gátló kinurénsav előanyagának-, az L-kinureninnek, valamint egy új, szintetikus kinurénsav származéknak a hatását a NTG-indukált nNOS immunreaktivitás változásra patkány TNC-ben. Továbbá, hogy a probenecid önálló hatását vizsgáljuk a NTG-indukált expresszió-változásra patkány TNC-ben és megnézzük, hogy miként befolyásolja a N171-82Q transzgenikus egerek túlélését, motoros aktivitását és miként hat a szövettani változásokra.

Eredményeink alapján a probeneciddel kombinált L-kinurenin, valamint a szintetikus kinurénsav származék egyaránt kivédik a NTG-indukált nNOS emelkedést és a patkány TNC felszínes rétegeiben. Eredményeink alapján az L-kinurenin modulálja a trigeminalis fájdalomérző működést a migrén nitroglicerines modelljében, feltehetőleg NMDA receptorokon keresztül. A kinurénsav analóg újabb támadáspontú anyagként szóba jöhet a fejfájások kezelésében.

A probenecid, mint nem-szelektív transzporter gátló képes volt kivédeni patkányban a NTG-indukált CGRP-immunreaktivitás csökkenést, valamint a nNOS- és a CamKIIα- immunpozitív sejtek számának emelkedését a TNC területén. Valamint a probenecid az N171-82Q transzgenikus modellben szignifikáns mértékben növelte az állatok túlélését, és javította azok spontán motoros teljesítményét. A túlélés- és magatartásvizsgálatokkal kapott eredményeket a szövettani vizsgálat is megerősítette, azaz a probenecid kivédte a striatalis sejtpusztulást és csökkentette az aggregátumokat tartalmazó immunreaktív sejtek számát. A probenecid feltehetőleg a prosztaglandinok

gátlásán keresztül a gyulladásos folyamatokra hatva fejtette ki hatását a vizsgált paraméterek tekintetében.