Modulatory effect of synthetic kynurenic acid derivatives on the nitroglycerin-induced trigeminal activation and sensitization in rats

ANNAMÁRIA FEJES-SZABÓ

Ph.D. Thesis

Department of Neurology
Faculty of Medicine
Albert Szent-Györgyi Clinical Centre
University of Szeged

Szeged
2014

Original publications directly related to the Ph.D. thesis:

I. **Fejes-Szabó A**, Bohár Z, Vámos E, Nagy-Grócz G, Tar L, Veres G, Zádori D, Szentirmai M, Tajti J, Szatmári I, Fülöp F, Toldi J, Párdutz A, Vécsei L.
Pre-treatment with new kynurenic acid amide dose-dependently prevents the nitroglycerine-induced neuronal activation and sensitization in cervical part of trigemino-cervical complex.
J. Neural. Transm. 2014 Jul;121(7):725-38.
IF: 2.871

II. Vámos E, **Fejes A**, Koch J, Tajti J, Fülöp F, Toldi J, Párdutz Á, Vécsei L.
Kynurenate derivative attenuates the nitroglycerin-induced CamKIIalpha and CGRP expression changes.
Headache 2010 May;50(5):834-43.
IF: 2.642

Total impact factor: 5.513

Publications not directly related to the Ph.D. thesis:

I. Vámos E, Párdutz Á, **Fejes A**, Tajti J, Toldi J, Vécsei L.
Modulatory effects of probenecid on the nitroglycerin-induced changes in the rat caudal trigeminal nucleus.
Eur. J. Pharmacol. 2009 Oct 25;621(1-3):33-7.
IF: 2.585

II. Tajti J, Párdutz A, Vámos E, Tuka B, Kuris A, Bohár Z, **Fejes A**, Toldi J, Vécsei L.
Migraine is a neuronal disease.
J. Neural. Transm. 2011 Apr;118(4):511-24.
IF: 2.730

III. **Fejes A**, Párdutz Á, Toldi J, Vécsei L.
Kynurenine metabolites and migraine: experimental studies and therapeutic perspectives.
Curr. Neuropharmacol. 2011 Jun;9(2):376-87.
IF: 2.847

IV. Párdutz Á, **Fejes A**, Bohár Z, Tar L, Vécsei L.
Kynurenines and headache.
J. Neural. Transm. 2012 Feb;119(2):285-96.
IF: 3.052

V. Bohár Z, **Fejes-Szabó A**, Tar L, Varga H, Tajti J, Párdutz A, Vécsei L.
Evaluation of c-Fos immunoreactivity in the rat brainstem nuclei relevant in migraine pathogenesis after electrical stimulation of the trigeminal ganglion.
Neurol. Sci. 2013 Sep;34(9):1597-604.
IF: 1.495

VI. **Fejes-Szabó A**, Bohár Z, Nagy-Grócz G, Vámos E, Tar L, Pődör B, Tajti J, Toldi J, Vécsei L, Párdutz A.
Effect of probenecid on the pain-related behaviour and morphological markers in orofacial formalin test of the rat.
CNS Neurol. Disord. Drug Targets – in press
IF: 2.702

Total impact factor: 15.411

Cumulative impact factor: 20.924

Table of contents

List of abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
α7-nACh	α7-nicotinic acetylcholine
bw	body weight
C1-C2	cervical part of spinal trigeminal nucleus pars caudalis
CaMKIIα	calcium/calmodulin-dependent protein kinase II α
CGRP	calcitonin gene-related peptide
GPR-35	G-protein-coupled receptor-35
HPLC	high performance liquid chromatography
i.p.	intraperitoneal
IR	immunoreactive
KYNAa1	*N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride
KYNAa2	*N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride
L-KYN	L-kynurenine
LLOQ	lower limit of quantitation
LOD	limit of detection
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NTG	nitroglycerin
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline containing 1% Triton X-100
PROB	probenecid
S.E.M.	standard error of the mean
TBST	Tris-buffered saline containing Tween 20
TNC	spinal trigeminal nucleus pars caudalis

Summary

Migraine is one of the most common neurological disorders. Its pathomechanism is only partially known and therapy is not always effective. In migraine, activation of trigeminal system plays an important role involving peptides/enzymes like calcitonin gene-related peptide (CGRP), neuronal nitric oxide synthase (nNOS) and calcium/calmodulin-dependent protein kinase II α (CaMKIIα) and glutamate and α7-nicotinic acetylcholine (α7-nACh) receptors.

Kynurenic acid is an endogenous antagonist of the above mentioned receptors, but its poor ability to cross the blood-brain barrier limits its therapeutic potential, therefore in our experiments we investigated effect of its pro-drug, L-kynurenine (L-KYN) combined with probenecid (PROB) and two newly synthesized kynurenic acid amides (KYNAa1, KYNAa2) on kynurenic acid concentration of the central nervous system in rats and on morphological and behavioural changes induced by systemic administration of nitroglycerin (NTG), an animal model of migraine.

Our results showed that kynurenic acid concentration in cervical part of spinal trigeminal nucleus pars caudalis (C1-C2) - where most of the trigeminal nociceptors convey - was significantly increased one hour after L-KYN-PROB. Moreover, KYNAa2 also enhanced kynurenic acid levels suggesting that this new derivative, at least partially, transforms to kynurenic acid resulting in a possible direct and indirect action in the central nervous system. The significant decrease of CGRP and increase of c-Fos, nNOS and CaMKIIα four hours after NTG treatment in C1-C2 measured by immunohistochemistry and Western blotting reflect the activation and sensitization of the trigeminal system. These changes were attenuated by the pre-treatment with L-KYN-PROB, KYNAa1 and in a dose-dependent manner with KYNAa2 probably due to their effects on the target receptors of kynurenic acid located in the peripheral and central part of the trigeminal system. The NTG-induced lower ambulation distance demonstrated by Open Field Test may indicate pain condition in rats, which can not be observed after pre-treatment with KYNAa2. Furthermore, the KYNAa2 administration lowers the basic ambulation distance which can reflect its central effect.

Our results suggest that by modulating the activation and sensitization of the trigeminal system in animals, kynurenic acid or its derivatives can be potential drug candidates in the treatment of headaches.

Introduction

Migraine

Migraine, an idiopathic neurological disorder belonging to the primary headache group in the classification of the International Headache Society, is characterised by spontaneous, recurrent headache attacks followed by symptom free intervals (Headache Classification Committee of the International Headache Society, 2013). The headache is moderate or severe, unilateral, throbbing and is associated with nausea or vomiting, photophobia, phonophobia, or sensitivity to other sensory stimuli (Headache Classification Committee of the International Headache Society, 2013). One attack lasts 4-72 hours and blocks the normal daily routine, as the normal physical activity exacerbates the pain (Headache Classification Committee of the International Headache Society, 2013). Migraine is often accompanied with allodynia, when non-nociceptive stimuli cause pain sensation, which suggests a sensitization process at the level of second- and third-order trigeminal neurones (Burstein et al., 2000).

Migraine is very common, its prevalence is about 12% (Stovner and Andree, 2010). It peaks in the period which is most productive in life, and the attacks are relatively abundant, worsening the quality of life and making the patients disabled (Lipton et al., 2005). It is the seventh-highest specific cause of disability in the world (Vos et al., 2012) resulting in an immense direct and indirect economic deficit reaching up to €1,222 per-person a year in eight countries representing 55% of the European Union's adult population (Linde et al., 2012).

Several theories have been suggested regarding the pathophysiology of migraine, but none of them can explain the full spectrum of the disorder. According to the first, so-called vascular theory, migraine attacks are induced by the initial intracranial vasoconstriction which is followed by sudden, rebound vasodilatation (Graham and Wolff, 1938). This theory explains the throbbing quality of headache, the efficacy of vasoconstrictive agents such as ergotamine or triptans and the migraine inducing effects of vasodilator compounds such as nitroglycerin (NTG) or calcitonin gene-related peptide (CGRP). The neurogenic theory was introduced by Mike Moskowitz and coworkers, who presumed that onset of a migraine attack is related to the activation of the trigeminovascular system, which results in sterile, neurogenic inflammation in dura mater and in the consequential sensitization of primary trigeminal neurones and the activation of the central trigeminal nociceptive pathways (Buzzi

et al., 1995). Since vascular and neurogenic theories failed to explain all aspects of migraine, e.g. central symptoms like nausea, vomiting and sensitivity to light and sound, other theories were also suggested.

Functional imaging studies have revealed activation of the dorsolateral pons and the dorsal midbrain including nucleus raphe dorsalis, nucleus raphe magnus, locus coeruleus and periaqueductal grey matter during a migraine attack (Weiller et al., 1995), which are components of the pain-modulatory pathways and are in functional relationship with the trigeminal system and can be associated with migraine pathogenesis (Fejes et al., 2011).

Another phenomenon partially explaining numerous symptoms of migraine is the theory linked to the cortical spreading depression (Bhaskar et al., 2013), which is an intense but slow continuous spread of excitation across the cerebral cortex followed by depression (Leao, 1944) and accompanied by slowly spreading cortical hypoperfusion as well (Olesen et al., 1981). This phenomenon is considered to be the physiological analogue of migraine aura, which is confirmed by studies using functional brain imaging (Hadjikhani et al., 2001). Cortical spreading depression was also able to activate the trigeminovascular afferents (Moskowitz et al., 1993) and could increase the persistent blood flow and cause plasma protein extravasation in the dura mater (Bolay et al., 2002).

Nevertheless, migraine is considered as a primarily genetically determined disease with a polygenic background (Pietrobon, 2007) and a large amount of candidate genes were implicated in migraine by genetic studies, but the mechanism, by which they actually participate in its pathophysiology, is still poorly understood (Bhaskar et al., 2013). A rare monogenic subtype of migraine is the familial hemiplegic migraine which causes attacks indistinguishable from the classical form and is associated with prominent aura symptoms (Pietrobon, 2007). The three most common mutations include the missense mutation of the P/Q-type calcium channel alpha l subunit gene (CACNAlA) on chromosome 19, the mutation in the ATP1A2 gene, which encodes a Na^+–K^+-ATPase and the mutation of a Na^+ channel gene (SCN1A), all of which lead to an increase of glutamate level in synaptic cleft producing a hyperexcitable state in migraineurs (Pietrobon, 2007).

One of the common points in these different pathomechanism theories is the activation of the trigeminal system during the attacks. The trigeminal system is responsible for most of the pain processing originating from the area of head (Carpenter and Sutin, 1983) and provides an

important pain-transmitting link from the cranial vasculature to the central nervous system (Edvinsson et al., 2012). The cell bodies of the pseudounipolar first-order trigeminal neurones are located in the trigeminal ganglion (Carpenter and Sutin, 1983) and contain numerous neurotransmitters such as glutamate, CGRP, substance-P, pituitary adenylate cyclase-activating polypeptide and neurokinin A (Edvinsson, 2011), which are released both at the periphery and in the central nervous system during the activation of trigeminal system. Consequently, enhanced CGRP level was measured in venous blood sampled from the external jugular vein ipsilateral to the side of headache during the migraine attack (Goadsby et al., 1990), which induces cranial vasodilatation (Ho et al., 2010) and greatly contributes to the peripheral plasma extravasation and neurogenic inflammation (Edvinsson et al., 2012). The released inflammatory substances rapidly sensitize the trigeminal first-order neurones (Strassman et al., 1996) resulting in a throbbing head pain, which is aggravated by activities increasing intracranial pressure, for example pulsations of the arteries, physical exercise, bending down, coughing and sneezing (Burstein et al., 2000).

The neurotransmitters released from the central terminals of the primary nociceptors activate the second-order neurones in the spinal trigeminal nucleus pars caudalis (TNC) located in the medullary part of the brain stem blending into the substantia gelatinosa of the first two cervical spinal segments (Carpenter and Sutin, 1983). Animal studies show that in response to activation induced by thermal, mechanical and chemical noxious stimulation as a marker, the c-Fos transcription factor increases in the neurones located in the area of spinal cord and TNC corresponding to the terminal fields of primary nociceptive afferent fibres and to the distribution of nociresponsive neurones (Harris, 1998). The main transmitter released from first-order trigeminal neurones is glutamate, which acts on its own ionotropic and metabotropic receptors located in the trigeminal system (Tallaksen-Greene et al., 1992). Continuous activation originating from the sensitized primary nociceptors induces plastic and long-term changes in the second-order neurones mainly through the glutamate receptors resulting in the central sensitization, which manifests clinically in cutaneous allodynia of scalp and face (Burstein et al., 2000), when the non-nociceptive stimuli including combing the hair, scalp tenderness, and wearing glasses and contact lenses cause pain and discomfort. Central sensitization has a clinical significance as well, since the efficacy of acute attack treatment with triptans is decreased after its development (Burstein et al., 2005). In these

plastic changes, calcium/calmodulin-dependent protein kinase II alpha (CaMKIIα) plays important role (Willis, 2001) interacting with N-methyl-D-aspartate (NMDA) glutamate receptors (Garry et al., 2003) similarly to the long-term potentiation process related to learning and memory (Sanhueza and Lisman, 2013).

Despite the extensive experimental data regarding the pathomechanism of migraine, it is not clearly known how migraine attacks are triggered. Furthermore, the current preventive and attack therapies are sometimes only moderately effective and often poorly tolerated. Animal experimental models can help us to elucidate emerging questions and to develop new drugs.

Nitroglycerin model of migraine headache

NTG is a highly lipophilic organic nitrate, which has been used for decades in the therapy of angina pectoris and myocardial infarction (Chiariello et al., 1976). It readily crosses biological membranes, including the blood-brain barrier (Torfgard et al., 1989) and is converted to nitric oxide (NO) in the body mainly enzymatically (Harrison and Bates, 1993). Endogenously, NO is synthesized by nitric oxide synthases (NOSs) and is chemically identical to endothelium derived relaxing factor (Moncada et al., 1988). It activates cytoplasmic guanylate cyclases, causing an increase of intracellular guanosine 3,5'-monophosphate and cytosolic calcium and resulting in vasodilatation (Axelsson et al., 1979). Furthermore, it is involved in inflammatory responses (Coleman, 2002), cell communication (Morris et al., 1992), non-adrenergic and non-cholinergic nerve activity (Toda and Okamura, 1990), regulation of glutamate release in the spinal cord (Okuda et al., 2001), pain transmission (Meller and Gebhart, 1993) and development of hyperalgesia (Dolan et al., 2000).

One of the most common side-effects of NTG is headache (Ashina et al., 2000) due to vasodilatation induced by NO, which occurs immediately after its administration. However in most of the migraineurs, the immediate headache is followed by a delayed, typical migraine headache without aura 4–6 hours after infusing NTG, which can not be attributed to NO’s prompt vasodilator effect (Sicuteri et al., 1987; Di Clemente et al., 2009). This observation and the results of a pilot study showing that treatment with a NOS inhibitor attenuates spontaneous migraine headaches in 67% of subjects (Lassen et al., 1997), contributed to the implication of NO in migraine pathogenesis.

In addition to the migraine-like headache induced by systemic administration of NTG, which was critically evaluated by several independent groups, NTG evolves numerous changes, which can be observed during a regular migraine attack too. These properties make systemic administration of NTG to a reliable, reproducible, generally accepted and easy to use model of human migraine. NTG can trigger migraine with aura as well, during which activation in the primary visual area of the occipital cortex was demonstrated (Afridi et al., 2005). NTG induced premonitory symptoms (yawning, tiredness, irritability, neck stiffness, frequency of urination, hunger and low mood) in migraineurs, who have usually these symptoms before spontaneous migraine attack (Afridi et al., 2004). Both during genuine migraine attack (Weiller et al., 1995) and after taking of NTG by migraine patients, significant activation in the dorsal rostral brain stem and dorsal lateral pons can be observed during the pain, which was ipsilateral to the site of headache (Afridi et al., 2005). In healthy subjects, sublingual NTG induced changes in evoked cortical responses (Di Clemente et al., 2009), which are comparable to those found immediately before and during a regular migraine attack (Afra et al., 2000).

Furthermore, NTG can sensitize the trigeminal system as well. In healthy subjects, sublingual NTG induced changes in trigeminal nociceptive blink reflex and pain thresholds, which are consequences of NO's action on structures of the central nervous system (Di Clemente et al., 2009) and similar changes have been reported during migraine attacks and thought to reflect sensitization of neurones in TNC (Kaube et al., 2002). After oral administration of NTG, migraineurs without aura exhibited changes reflecting sensitization of second-order nociceptive neurones within the TNC, including a significant heat pain threshold reduction and an increment in the amplitude of laser evoked potentials during headache when the supraorbital zones were stimulated (de Tommaso et al., 2004), which were similar to those observed during spontaneous migraine attack (de Tommaso et al., 2002). A typical feature of sensitization of the pain pathways - a significant facilitation in temporal summation of pain at spinal level - can be observed in migraineurs after NTG administration (Woolf, 2007), which was significantly facilitated in migraineurs, who developed migraine after NTG (Perrotta et al., 2011).

The migraine-provoking effect of NTG is further supported by the fact that numerous symptoms induced by NTG respond to acute and prophylactic medications known to have an

effect in genuine migraine. Sumatriptan reduced the NTG-induced headache and decreased the middle cerebral artery blood flow velocity in healthy volunteers (Fullerton et al., 1999). NTG-induced a more pronounced decrease of intracranial blood flow velocity in the middle cerebral artery in migraine patients, which disappeared one hour after zolmitriptan or sumatriptan administration (Thomaides et al., 2003). Similarly to spontaneous migraine attack (Goadsby et al., 1990), plasma CGRP concentration increased significantly during the migraine-like headache induced by sublingual administration of NTG in migraineurs without aura, while this change cannot be observed during immediate headache and in the subjects who do not have migraine-like headache (Juhasz et al., 2003). Interestingly, CGRP level showed significant positive correlations with migraine headache intensity (Juhasz et al., 2003) and sumatriptan was able to reduce the intensity of headache and the plasma concentration of CGRP (Juhasz et al., 2005). Prophylactically or ictally administered valproate – a well documented preventive drug in migraine treatment (Hering and Kuritzky, 1992) - was effective against the NTG-induced migraine-like headache in migraineurs without aura (Thomaides et al., 2008).

Data from humans concerning NTG administration led to the hypothesis that NTG administration can function as an animal experimental model of migraine too, which was verified by experiments, since NTG can induce changes in animals similar to that of what happens in humans during the NTG-induced attack.

Injection of NTG was able to trigger scratching head reactions, climbing cage, red ear and photophobia (Zhu et al., 2011; Markovics et al., 2012). Furthermore, NTG facilitated the face-grooming behaviour - considered to reflect a specific nocifensive response - induced by CGRP, which was blocked by sumatriptan (Yao and Sessle, 2008).

NTG administration induces dural and pial artery dilation (Gozalov et al., 2007) and leakage of plasma proteins from dural blood vessels (Reuter et al., 2001). It can evolve increased meningeal and cortical regional blood flow (Read et al., 1999; Greco et al., 2011; Markovics et al., 2012), which were attenuated by sumatriptan (Read et al., 1999) and which has been described in migraine and associated with migraine with aura (Olesen et al., 1981).

NTG treatment can trigger release of transmitters present in the trigeminal system: in rats a significant decrease of the area innervated by CGRP-immunoreactive (IR) afferents in the cervical part of TNC (C1-C2) (Pardutz et al., 2002) and a marked increase of CGRP and

substance-P in plasma was detected, which latter was suppressed by local botulinum toxin type A injection, which is also effective in chronic migraineurs (Shao et al., 2013). Pituitary adenylate cyclase-activating polypeptide-38 and -27 immunoreactivity was increased significantly in the TNC of rats after NTG injection (Tuka et al., 2012), and similar tendency was observed in the plasma of migraineurs during the attack compared to the interictal period (Tuka et al., 2013).

NTG treatment activates the trigeminal system reflected by changes of c-fos mRNA and c-Fos protein expression - an anatomical marker of neuronal activity (Harris, 1998) - in the trigeminal ganglion and in the TNC (Tassorelli and Joseph, 1995; Markovics et al., 2012; Ramachandran et al., 2012), which were attenuated by pre-treatment with sumatriptan (Ramachandran et al., 2012).

Behavioural, electrophysiological and morphological studies prove that NTG can cause the sensitization of the trigeminal system, which phenomenon is also present in migraineurs. A dose-dependent and prolonged NTG-induced thermal and mechanical allodynia was detected in mice, which were alleviated by sumatriptan (Bates et al., 2010). NTG caused a hyperalgesic response in the tail-flick test, which was attenuated by pre- or co-treatment with non-peptide CGRP receptor antagonist MK-8825 (Greco et al., 2013), inhibition of which receptor is effective in migraine too (Olesen et al., 2004). In rats, epidural NTG induces hyperalgesia at the level of the spinal cord (Masue et al., 1999), which was dose-dependently suppressed by epidural non-steroidal anti-inflammatory drugs e.g. indomethacin, diclofenac and ibuprofen (Masue et al., 1999) used generally against migraine headache (Silberstein, 2000). Chronic intermittent administration of NTG to mice resulted in acute and chronic hyperalgesia, where the acute but not the chronic one was significantly reduced by sumatriptan, whereas both the acute and chronic hyperalgesia was significantly attenuated by topiramate which is effective in migraine prophylaxis (Pradhan et al., 2014).

Electrophysiological results show, that infusions of NTG to cats increased the mean basal discharge rate of all second-order neurones in the TNC receiving sensory input from the superior sagittal sinus, a clear indication of a sensitization process, which can be decreased by iontophoretic application of the serotonin (1B/1D) receptor agonist eletriptan (Lambert et al., 2002).

Increase in neuronal NOS- (nNOS) and CaMKIIα-IR neurones in the C1-C2 were detected four hours after systemic administration of NTG in male rats, suggesting the presence of self-amplifying mechanisms in this area, possibly linked to a central sensitization process (Pardutz et al., 2000; 2007). The changes observed in expression of sensitization markers were attenuated by lysine acetylsalicylate and by NS398 (selective cyclooxygenase-2 inhibitor) (Pardutz et al., 2004; Varga et al., 2007; 2009). Interestingly, these alterations of nNOS and CaMKIIα immunoreactivity can not be observed in the thoracic dorsal horn (Pardutz et al., 2000; 2007) showing a selective effect of NTG administration on the trigeminal system.

To summarize, numerous experimental data in animals confirm that NTG is able to activate and sensitize the trigeminal system - phenomena also observed during migraine attacks - making NTG administration an appropriate animal experimental model of migraine headache.

Kynurenines

The kynurenine pathway is an endogenous metabolic route, which is responsible for the 95% of tryptophan metabolism (Wolf, 1974) and takes place in the macrophages and microglial cells and in part in neurones (Guillemin et al., 2003; 2007). Its main end products are nicotinamid adenine dinucleotide and nicotinamid adenine dinucleotide phosphate (Beadle et al., 1947).

Several metabolites of this pathway have neuroactive properties with toxic or protective effects. 3-hydroxykynurenine and 3-hydroxyanthranilic acid are neurotoxic through producing free radicals and elevating the oxidative stress level, causing neuronal damage and under pathological conditions, an increase in their concentration have been demonstrated (Fejes et al., 2011). Quinolinic acid has excitotoxic effect causing significant destruction of neurones after intrastriatal administration (Guidetti and Schwarcz, 1999) presumably through agonism on the NMDA receptor (Stone and Perkins, 1981) or by the stimulation of the release and inhibition of uptake of glutamate (Tavares et al., 2002). It also induces lipid peroxidation (Rios and Santamaria, 1991) and the production of reactive oxygen species (Behan et al., 1999). Changes of its concentration in the cerebrospinal fluid have been demonstrated in various neurodegenerative disorders (Klivenyi et al., 2004). Xanthurenic acid is considered as a part of a detoxification process that reduces the concentration of 3-hydroxykynurenine (Gobaille et al., 2008). Its administration in high dosages to rats seems to induce a degree of

sedation and analgesia (Heyliger et al., 1998). Experimental data strongly indicate a physiological role for xanthurenic acid in synaptic signalling (Gobaille et al., 2008).

L-kynurenine (L-KYN) itself is not a neuroactive compound, but it has a central role in the kynurenine pathway, since it is the precursor of kynurenic acid, which is the main neuroprotective end product of this metabolic route. Its protective effect is demonstrated by several experimental results, where kynurenic acid reduced brain damage induced by hypoxic-ischemia (Andine et al., 1988), reversed the contralateral turning caused by quinolinic acid-induced lesions of the nigrostriatal dopaminergic pathway (Miranda et al., 1999) and induced neuroprotection in mouse cortical neuron cultures (Klein et al., 2013). This feature is probably related to various receptorial actions, like NMDA antagonism at 7.9 μM by attaching to its glycine-binding site (Kessler et al., 1989), inhibition of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors at high micromolar concentration (Prescott et al., 2006), the blockade of α7-nicotinic acetylcholine (α7-nACh) receptors in a non-competitive manner at low concentrations (30–100 nM) by binding to sites located in the N-terminal domain (Pereira et al., 2002) and the ability to agonise the G-protein-coupled receptor-35 (GPR-35) at 7.4 μM concentration (Wang et al., 2006). Changes in its concentration in pathological circumstances were also described, namely it decreases in epilepsy, infantile spasm, Parkinson's disease, multiple sclerosis, Huntington's disease and Alzheimer's disease and increases in viral infections (Klivenyi et al., 2004; Nemeth et al., 2006) suggesting its role in pathomechanism of various neurological disorders. Furthermore, emerging evidence underlines that kynurenic acid can be crucial in nociception including migraine too. Its peripheral and central administration proved to be anti-nociceptive in animal experimental models of pain (Kristensen et al., 1993; Mecs et al., 2009) and enhancement of its concentration in the central nervous system attenuated the increase of NTG-induced nNOS immunoreactivity (Vamos et al., 2009). Based on these observations, manipulation of kynurenic acid levels in the nervous system could provide a therapeutic option in many neurological diseases especially in migraine. Unfortunately, this is rather restricted because it has only a very limited ability to cross the blood–brain barrier (Fukui et al., 1991). To resolve this issue, L-KYN and probenecid (PROB) are given together, since L-KYN crosses the blood-brain barrier easily (Fukui et al., 1991) and PROB inhibits the excretion of kynurenic acid from the brain resulting a dose-dependent and robust increase in the level of kynurenic

acid in the central nervous system (Vecsei et al., 1992). Furthermore, recent experimental results underline the importance of the application of various derivatives of kynurenic acid with a presumed better central nervous system penetration and similarity regarding their pharmacological effect (Knyihar-Csillik et al., 2008; Vamos et al., 2009; Marosi et al., 2010; Zadori et al., 2011b; Demeter et al., 2012; Gellert et al., 2012).

Aims

The aims of our studies were to

I. Determine the kynurenic acid concentration in the C1–C2 spinal cord segments of the rat, receiving most of the trigeminal nociceptive input, using high performance liquid chromatography (HPLC) either 60 minutes after intraperitoneal treatment with L-KYN combined with PROB, or 60 and 300 minutes after the administration of a newly synthesized kynurenic acid derivative.
II. Examine the modulatory effect of the pre-treatment with L-KYN combined with PROB or the administration of two new kynurenic acid amides on the NTG-induced activation of primary trigeminal neurones by measuring the transmitter release from the central terminals indicated by CGRP expression in the C1-C2 using immunohistochemistry and Western blotting.
III. Investigate the suspected dose-dependent influence of one of the two new kynurenic acid derivatives on the activation of second-order trigeminal neurones located in the C1-C2 reflected by c-Fos immunohistochemistry.
IV. Test the supposed effect of pre-treatment with L-KYN combined with PROB and with two new kynurenic acid amides on the NTG-induced sensitization of second-order trigeminal neurones reflected by the nNOS and CaMKIIα expression measured by immunohistochemistry and Western blotting in the same area.
V. Study the potential modulatory effect of NTG administration on the behaviour of rats in Open Field Test and detect the modulatory effect of pre-treatment of one of the two new kynurenic acid amides on the NTG-induced changes.

Materials and Methods

Animals

The procedures utilized in this study followed the guidelines for the Use of Animals in Research of the International Association for the Study of Pain and the directives of the European Economic Community (86/609/ECC). They were approved by the Committee of Animal Research at the University of Szeged (I-74-12/2012) and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XI./352/2012). One hundred and forty-four adult male Sprague-Dawley rats weighing 200-250 g were used. The animals were maintained on a 12-h dark – 12-h light cycle under standard laboratory conditions, with tap water and regular rat chow available *ad libitum*.

Drugs

L-KYN and PROB were obtained from Sigma-Aldrich.

The new kynurenic acid amides (Patent number #P0900281/PCT/HU2010/00050), *N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride (KYNAa1) and *N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride (KYNAa2) (Figure 1), were synthesized in the Department of Pharmaceutical Chemistry, University of Szeged with regard to the following structural properties: (i) the presence of a water-soluble side-chain, (ii) the inclusion of a new cationic centre, and (iii) side-chain substitution to facilitate brain penetration (Fulop et al., 2012). Coupling between kynurenic acid and 2-dimethylaminoethylamine was achieved by using *N*,*N*'-diisopropylcarbodiimide in the presence of 1-hydroxybenzotriazole hydrate, yielding KYNAa1 (Fulop et al., 2012). KYNAa2, containing the tertiary nitrogen in pyrrole ring system, were prepared by reacting kynurenic acid with 2-pyrrolidinoethylamine (Fulop et al., 2012).

NTG and its Placebo were obtained from Pohl-Boskamp.

The dosages of drugs were chosen on the basis of earlier works (Vecsei et al., 1992; Pardutz et al., 2000; Vamos et al., 2009).

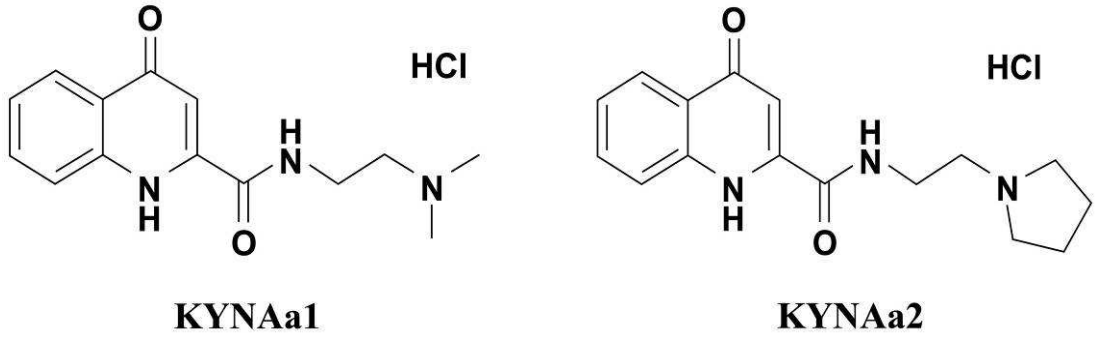

Figure 1. The structure of the new kynurenic acid amides, *N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride (KYNAa1) and *N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride (KYNAa2).

Drug administration

For the HPLC measurements: The animals were divided into three groups. In the control group, the animals received only the intraperitoneal (i.p.) vehicle solution (physiological saline) as treatment (n=4), without drug. In the second group (n=4), the rats received an i.p. injection of L-KYN (300 mg/kg body weight (bw), diluted to 2 ml, pH 7.4) and PROB (200 mg/kg bw, diluted to 1.5 ml, pH 7.4). In the third group (n=10), the rats were treated with an i.p. injection of 1 mmol/kg bw KYNAa2 (diluted to 2 ml, pH 7.4).

For immunohistochemistry: The animals were divided into six groups. The animals of the control group (n=12) received only the i.p. vehicle solution (physiological saline) as pre-treatment. The rats in the second group (n=12) received an i.p. injection of L-KYN (300 mg/kg bw, diluted to 2 ml, pH 7.4) combined with PROB (200 mg/kg bw, diluted to 1.5 ml, pH 7.4). In the third group (n=12), the rats were injected intaperitoneally with the KYNAa1 (1 mmol/kg bw, diluted to 2 ml, pH 7.4). In the remaining 3 groups (n=10 per group), the rats were pre-treated with an i.p. injection of KYNAa2 at dosages of 0.1, 0.5 and 1 mmol/kg bw, respectively (diluted to 2 ml, pH 7.4). One hour after the pre-treatment in each group, half of the animals (n=6 or 5) received an i.p. injection of NTG (10 mg/kg bw), while the other half of the rats (n=6 or 5) received an i.p. injection of the Placebo of NTG.

For Western blotting: The animals were divided into two groups (n=10 per group). The animals in the control group received i.p. vehicle solution (physiological saline) only as pre-treatment. In the second group, the rats were pre-treated with an i.p. injection of 1 mmol/kg

bw KYNAa2. The administration of Placebo and NTG was performed as described above, i.e. one hour after the pre-treatments.

For the Open Field Test: The animals were separated into two groups (n=20 per group). In the control group, the animals received i.p. vehicle solution (physiological saline) as pre-treatment, without drug. In the second group, the rats were pre-treated with an i.p. injection of KYNAa2 at the dose of 1 mmol/kg bw. The administration of Placebo and NTG was performed as described above.

Kynurenic acid detection with HPLC

Sampling and sample preparation: At determined time points following the i.p. injection (60 minutes in the control and L-KYN-PROB groups and 60 and 300 minutes in the KYNAa2 group), the rats were deeply anesthetized with chloral hydrate (0.4 g/kg bw, Sigma-Aldrich) and transcardially perfused with 100 ml phosphate-buffered saline (PBS, 0.1 M, pH 7.4) for 5 minutes. The C1-C2 segments were removed and stored at −70 °C until analysis. Then the samples were weighed and sonicated in an ice-cooled solution (250 µl) comprising perchloric acid (2.5% w/w, Scharlau), internal standard (3-nitro-L-tyrosine, 2 µM, Sigma-Aldrich,) and distilled water for 1.5 minutes in an Eppendorf tube. The content of the Eppendorf tube was centrifuged at 12,000 rpm for 10 minutes at 4 °C. From the supernatant 100 µl was transferred to a test vial.

Chromatographic conditions: The kynurenic acid concentrations of the samples were quantified based on the slightly modified method of Herve and colleagues (Herve et al., 1996), with an Agilent 1100 HPLC system (Agilent Technologies). The system was equipped with a fluorescent and an UV detector. The former was applied for the determination of kynurenic acid and the latter for the determination of the internal standard. Chromatographic separations were performed on an Onyx Monolithic C18 column, 100 mm x 4.6 mm I.D. (Phenomenex Inc.) after passage through a Hypersil ODS pre-column, 20 x 2.1 mm I.D., 5 µm particle size (Agilent Technologies) with a mobile phase composition of 0.2 M zinc acetate (Sigma-Aldrich)/acetonitrile (Scharlau) = 95/5 (v/v), the pH of which was adjusted to 6.2 with acetic acid (VWR International), applying isocratic elution. The flow rate and the injection volume were 1.5 ml/minute and 50 µl, respectively. The fluorescent detector was set

at excitation and emission wavelengths of 344 and 398 nm. The UV detector was set at 365 nm wavelength. The retention time of kynurenic acid was about 6 minutes.

HPLC method validation: *Calibration curve and linearity* - Calibrants were prepared at 6 different concentration levels, from 1 to 100 nM and 0.5 to 5 µM for kynurenic acid and the internal standard, respectively. Three parallel injections of each solution were made under the chromatographic conditions described above. The peak area responses were plotted against the corresponding concentration, and the linear regression computations were carried out by the least square method with the freely available R program (R Foundation for Statistical Computing, R Development Core Team). Very good linearity was observed throughout the investigated concentration ranges for kynurenic acid and the internal standard when either fluorescence or UV detection was applied. *Selectivity* - The selectivity of the method was checked by comparing the chromatograms of kynurenic acid and the internal standard for a blank central nervous system sample and those for a spiked sample. All compounds could be detected in their own selected chromatograms without any significant interference. *LOD and LLOQ* - Limit of detection (LOD) and lower limit of quantitation (LLOQ) was determined via signal-to-noise ratio with threshold 3, according to the ICH guidelines (Validation of Analytical Procedures: Text and Methodology Q2 (R1), International Conference on Harmonization). The LOD was 0.4 nM, while the LLOQ was 1 nM for kynurenic acid, respectively. *Precision* - Replicate HPLC analysis showed that the relative standard deviation was $\leq$ 3.1% for the concentration and $\leq$ 0.1% for the retention time. *Recovery* - The relative recoveries were estimated by measuring spiked samples of kynurenic acid at 2 different concentrations with 3 replicates of each. No significant difference was observed for the lower and higher concentrations. The recoveries ranged from 82 to 92% for kynurenic acid.

Immunohistochemistry

Histological procedure: Four hours after treatment with Placebo or NTG, the rats, anaesthetized with chloral hydrate as described above, were perfused transcardially with 100 ml PBS, followed by 500 ml 4% paraformaldehyde in PBS and the C1-C2 segment of the cervical spinal cord was removed. After overnight postfixation in the same fixative and cryoprotection (10% sucrose for 2 h, 20% sucrose until the blocks sank, and 30% sucrose overnight), 30 µm cryostat sections were cut, serially collected in 18 wells containing cold

PBS, where each well received sections at a 0.54-mm distance throughout the rostrocaudal extent of the C1-C2 segments and treated as free-floating sections. After suppression of the endogenous peroxidase activity with 0.3% H_2O_2 in PBS for 30 minutes and several rinses in PBS containing 1% Triton X-100 (PBST, VWR International), sections were incubated in PBST containing (i) rabbit anti-rat CGRP polyclonal antibody (Sigma-Aldrich, C-8198, dilution: 1:20,000, incubation: overnight at room temperature) or (ii) rabbit anti-rat c-Fos (H-125) polyclonal antibody (Santa Cruz Biotechnology, sc-7202, dilution: 1:1,000, incubation: overnight at room temperature) or (iii) rabbit anti-rat nNOS polyclonal antibody (EuroProxima, 2263B220-1, dilution: 1:5,000, incubation: two nights at 4 °C) or (iv) mouse anti-rat CaMKIIα monoclonal antibody (Sigma-Aldrich, C-265, dilution: 1:2,000, incubation: four nights at 4 °C). The immunohistochemical reaction was visualized by using Vectastain Elite avidin-biotin kits (Vector Laboratories; PK6101 for c-Fos, nNOS and CGRP; PK6102 for CaMKIIα). The sections were incubated at room temperature for 2 hours in PBST containing goat anti-rabbit or horse anti-mouse biotinylated secondary antibody, respectively. After several rinses in PBST, and incubation at room temperature for 2 hours in PBST containing avidin and biotinylated horseradish peroxidase, the sections were stained with 3,3'-diaminobenzidine (Sigma-Aldrich) intensified with nickel ammonium sulphate (VWR International). The specificity of the immune reactions was checked by omitting the primary antiserum.

Data evaluation: All evaluations were performed by an observer blind to the experimental groups. The area covered by CGRP-IR fibres in laminae I and II of the cervical dorsal horns was determined by video imaging, using Image Pro Plus 6.2® image analysis software (Media Cybernetics). Photomicrographs of sections were taken under the 4x objective of a Nikon Optiphot-2 light microscope fitted with an Olympus DP70 CCD camera (Olympus Corporation) and transmitted to the frame grabber, which converted the image into a digital matrix of 1600 x 1200 pixels. After image acquisition, the laminae I-II in dorsal horn were determined manually as area of interest by means of The Rat Brain in Stereotaxic Coordinates Atlas (Paxinos and Watson, 2007) and a threshold grey level was established with the Image Pro Plus 6.2® image analysis software in order to detect IR fibres in the digitalized microscopic image. 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 on the

point where the flat part of the histogram 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, we measured formations with known area. The area covered by IR fibres per dorsal horn was calculated as the multiplication of the average number of pixels for the individual dorsal horn and the area of one pixel.

The c-Fos-, nNOS- and CaMKIIα-IR cells were counted by an observer blinded to the procedure under the 10x objective of a Nikon Optiphot-2 light microscope (Nikon Instruments) in laminae I-II of the C1-C2. The c-Fos-positive neurones were scored as those with obvious specific nuclear staining; the nNOS-IR neurones as those exhibiting cytoplasmic and dendritic staining; and the CaMKIIα-labelled cells with a clearly increased immunoreactivity relative to the background. In three different series of sections in each animal, the number of IR neurones per section was calculated, i.e. the numbers of immunopositive neurones present on the two dorsal horns were summed. Thereafter, the results on the individual sections were averaged for each animal.

Photomicrographs were taken under 4x objective of Nikon Optiphot-2 light microscope fitted with an Olympus DP70 CCD camera or under the 40x and 63x objective of a Zeiss Axio Imager M2 Upright Microscope (Carl Zeiss MicroImaging) fitted with an AxioCam MRc camera (Carl Zeiss MicroImaging) and Adobe Photoshop CS2 9.0 graphics program was used to create the artworks.

Western blotting

Sampling and sample preparation: In both groups four hours after the NTG or Placebo injections, the rats were deeply anaesthetized with chloral hydrate as described above, perfused transcardially with 100 ml ice cold PBS and the dorsal horn of C1-C2 segment was removed. Until measurements, the samples were stored at −70 °C. The C1-C2 segments were sonicated in ice 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, 1 μg/ml pepstatin, 2 mM EDTA and 0.1% sodium dodecyl sulphate (all chemicals were from Sigma-Aldrich). The lysates were cleared from cellular debris by centrifugation at 12,000 rpm for 10 minutes at 4 °C and supernatants were aliquoted and stored at -20 °C. Protein concentration was measured according to BCA protein assay method with BCA Protein Assay Kit

(Novagen) using bovine serum albumin as a standard. Samples were cooled on ice during the whole procedure. Prior to loading, each sample was mixed with sample buffer, and denaturated by boiling for 3 minutes. Equal amounts of protein samples (20 μg/lane) were separated by standard SDS polyacrylamide gel electrophoresis on 10% (for nNOS and β-actin) and 15% (for CGRP) Tris–Glycine gel and electrotransferred onto Amersham Hybond-ECL nitrocellulose membrane (0.45 and 0.2 μm pore size selectively, GE Healthcare). The Page Ruler Prestained Protein Ladder (Fermentas, 10-170 kDa) was used to determine approximate molecular weights. Following the transfer, membranes were blocked for one hour at room temperature in Tris-buffered saline containing Tween 20 (TBST, MP Biomedicals) and 5% non fat dry milk powder and incubated in TBST containing 1% non fat dry milk and (i) rabbit anti-rat CGRP polyclonal antibody (Sigma-Aldrich, C-8198, dilution: 1:2,000, incubation: overnight at room temperature) or (ii) mouse anti-rat nNOS monoclonal antibody (BD Biosciences, 610308, dilution: 1:2,500, incubation: overnight at room temperature) or (iii) mouse anti-rat β-actin monoclonal antibody (Calbiochem, CP01, dilution: 1:10,000, incubation: overnight at room temperature). Next day after several rinses, membranes were incubated in TBST containing 1% non fat dry milk and horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology; sc-2030 and sc-2031) for two hours at room temperature. Protein bands were visualized after incubation of membranes with the SuperSignal West Pico Chemiluminescent Substrate (Pierce) using Carestream Kodak BioMax Light film (Kodak).

Data evaluation: For densitometric analyses, films were scanned and quantified using Java ImageJ 1.47v analysis software (National Institutes of Health). The results were normalized to the absolute control animals. β-actin served as a control to ensure loading of equivalent amounts of sample proteins.

Open Field Test

Before the Open Field Test, animals were habituated in the dimly lit, quiet test room for at least 30 minutes. The experiments were performed between 11 a.m. and 4 p.m. during the light cycle of rats. To avoid misinterpretation related to the acute cardiovascular effects of NTG and in parallel with earlier studies showing delayed behavioural alterations after systemic NTG in mice (Markovics et al., 2012), we tested the rats three hours and forty

minutes after the NTG or Placebo injections. The animals were placed in the Open Field box (48x48x40 cm, Experimetria Ltd.) and ambulation time, ambulation distance, local time and the number of rearings were registered for 15 minutes, and were evaluated using the Conducta 1.0 behaviour analysis program (Experimetria Ltd.). The rats did not receive any food or water during the observation period. The test box was cleaned and decontaminated after each animal.

Statistical analysis

Statistical analysis of the measurement data was carried out with IBM SPSS Statistics software (Version 20, IBM), using one-way analysis of variance followed by the Tukey, Schaffe or Tamhane *post hoc* test (except for the data of HPLC analyses), depending on the variances of the data, with $p<0.05$ taken as statistically significant. Group values are reported as means ± standard error of the mean (S.E.M.). The independent Student t-test was used to analyse the results of HPLC measurements to compare the vehicle-treated and the L-KYN-PROB-treated groups. While to compare the vehicle-treated and the KYNAa2-treated groups, the non-parametric Kruskal-Wallis test was applied for group comparisons followed by Mann-Whitney U test for *post hoc* analysis, with $p<0.05$ taken as statistically significant, where the group values are reported as median and interquartile range.

Results

Kynurenic acid detection with HPLC

The HPLC measurements of kynurenic acid concentration clearly indicated a robust, statistically significant increase in kynurenic acid level in the C1-C2 one hour after administration of L-KYN combined with PROB (2967,42±395.76 pmol/g wet weight) as compared with control group (11.76±1.67 pmol/g wet weight) (***p<0.001) (Figure 2).

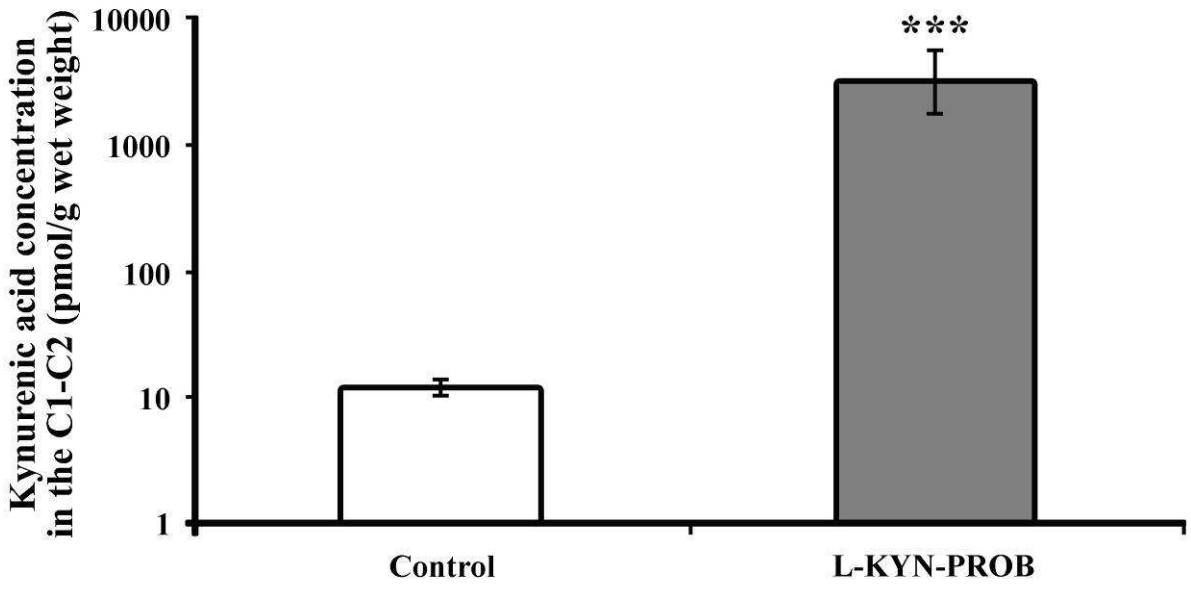

Figure 2. Histogram showing the kynurenic acid level in the C1-C2 spinal cord segments after vehicle or L-KYN combined with PROB treatment (means ± S.E.M., n = 4 per group). L-KYN-PROB significantly increased the kynurenic acid level in the C1–C2 segments as compared with the control group (***p<0.001).

The HPLC measurements showed a significant (*p<0.05), more than two-fold increase in kynurenic acid level in the C1-C2 60 minutes after 1 mmol/kg bw KYNAa2 administration (25.38 pmol/g wet weight, interquartile range: 23.26-40.80 pmol/g wet weight) compared to vehicle treated samples (11.48 pmol/g wet weight, interquartile range: 8.85-15.00 pmol/g wet weight) (Table 1), while the concentration of kynurenic acid decreased to baseline at 300 minutes (9.02 pmol/g wet weight, interquartile range: 6.43-30.92 pmol/g wet weight) (Table 1). Although, it requires a different validation method, we screened the kynurenic acid concentration on the blood samples too. Preliminary results suggest a robust increase (approximately 50x) in the peripheral kynurenic acid levels reaching µmolar concentrations (data not shown).

Table 1 - Results of HPLC measurements from the C1-C2 at different time points after KYNAa2 treatment

Groups		**Kynurenic acid concentrations (pmol/g wet weight; median and interquartile range)**
Control (saline) (n=4)	60 min	11.48 (8.85-15.00)
1 mmol/kg bodyweight KYNAa2 (n=10)	60 min (n=5)	25.38 (23.26-40.80)*
	300 min (n=5)	9.02 (6.43-30.92)

*p<0.05

Immunohistochemistry

The transverse sections of the C1-C2 demonstrated abundant CGRP-positive fibres, and c-Fos-, nNOS- and CaMKIIα-IR neurones in the superficial layers (laminae I-II) of the dorsal horns. The area covered by IR fibres and the number of IR cells did not differ significantly between sections located at the various levels along the rostrocaudal axis or between the right and left dorsal horns of the cervical segments.

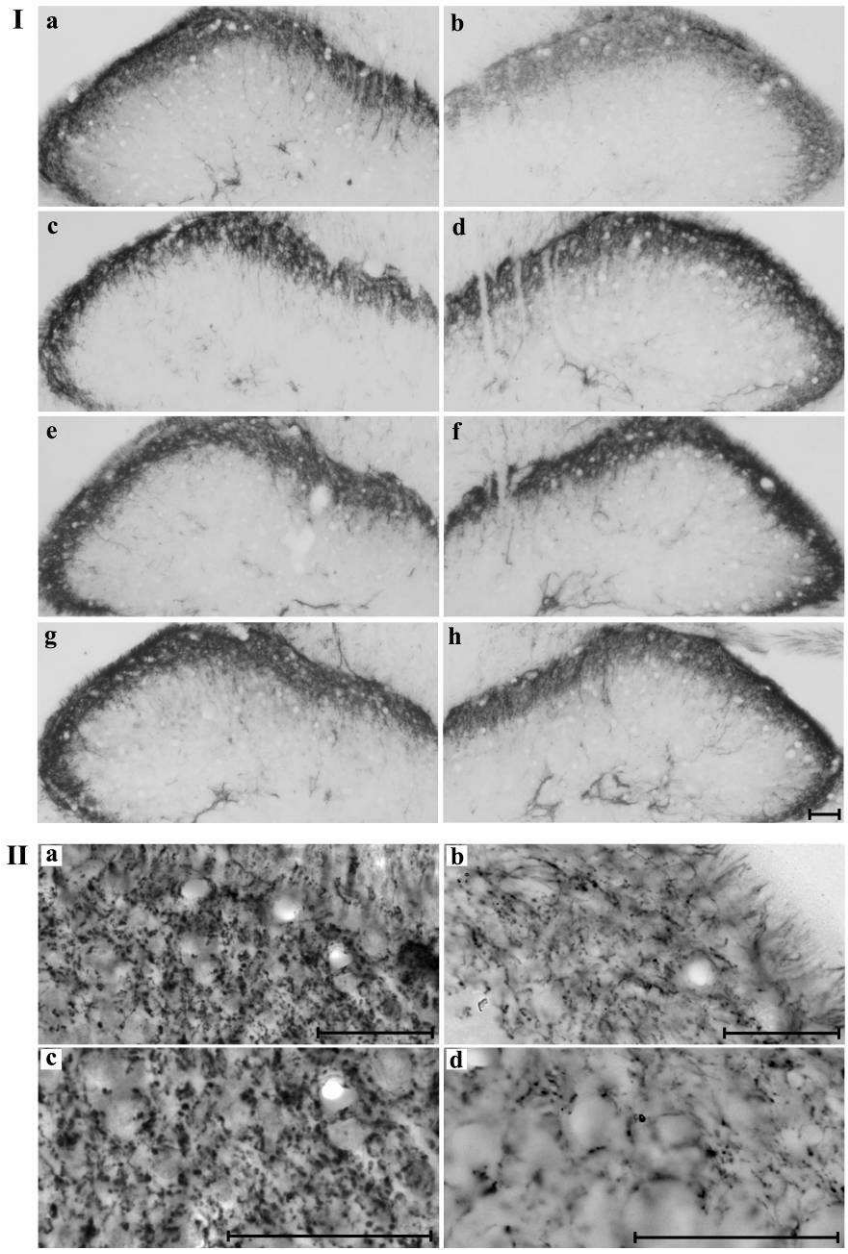

Figure 3. Representative photos of sections from the C1-C2 segments of the rat after CGRP immunohistochemistry. (I) CGRP immunoreactivity in the C1-C2 in control (a, b), L-KYN-PROB-pre-treated (c, d), KYNAa1-pre-treated (e, f), and KYNAa2-pre-treated (g, h) rats after Placebo (a, c, e, g) or NTG (b, d, f, h) injections. (II) CGRP-IR fibres and buttons under 40x objective (a, b) and under 63x objective (c, d) in Placebo (a, c) and NTG-treated (b, d) rats of the controls. In the control group (I/a, b and II), the IR staining of the CGRP fibres is decreased after the NTG injection (I/b, II/b, d) as compared with the Placebo-treated animals (I/a, II/a, b). After L-KYN-PROB (I/c, d) or 1 mmol/kg bw KYNAa1 (I/e, f) or 1 mmol/kg bw KYNAa2 (I/g, h) pre-treatment, the changes otherwise induced by NTG cannot be observed (I/d, f, h) compared to the animals treated with Placebo (I/c, e, g). Scale bar = 50 µm.

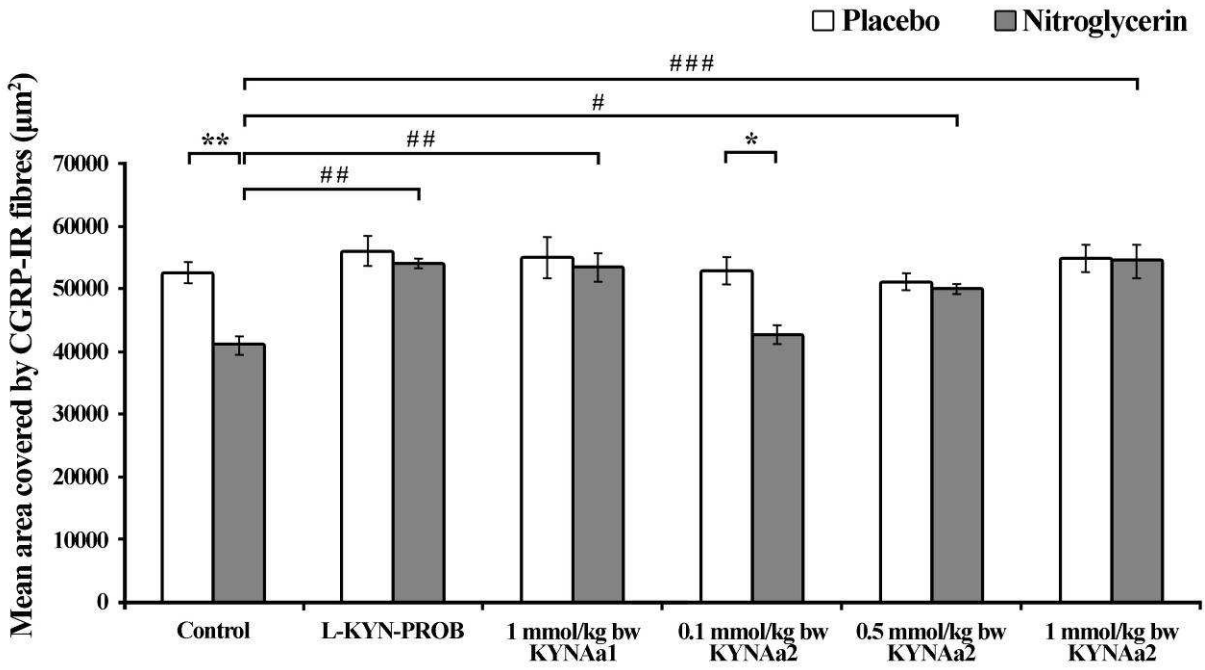

Figure 4. Histogram showing the mean area in μm^2 covered by CGRP-IR fibres in superficial laminae I and II of the C1-C2 in the six animal groups after Placebo (white bars) or NTG (grey bars) (means ± S.E.M., n = 6 or 5 per subgroup). In the control group, NTG injection significantly decreased the mean area covered by CGRP-IR fibres in the C1-C2 as compared with the Placebo-treated animals (**p<0.01). The combined pre-treatment with L-KYN-PROB and KYNAa1 (1mmol/kg bw) attenuated the NTG-induced changes. The significant difference between Placebo and NTG treated animals also appeared in the group pre-treated with 0.1 mmol/kg bw KYNAa2 (*p<0.05), but this phenomenon disappeared in the groups pre-treated with 0.5 and 1 mmol/kg bw KYNAa2. The effects of various pre-treatments are also reflected by the significant differences between the values of the NTG groups (#p<0.05; ##p<0.01; ###p<0.001).

In the control group, reduced CGRP staining can be observed in laminae I-II of the C1-C2 segments after NTG treatment compared to Placebo treated rats (Figure 3). This decrease is reflected in area covered by CGRP as well, i.e. significant difference can be measured in NTG-treated rats as compared with Placebo-treated animals (**p<0.01; Figure 4).

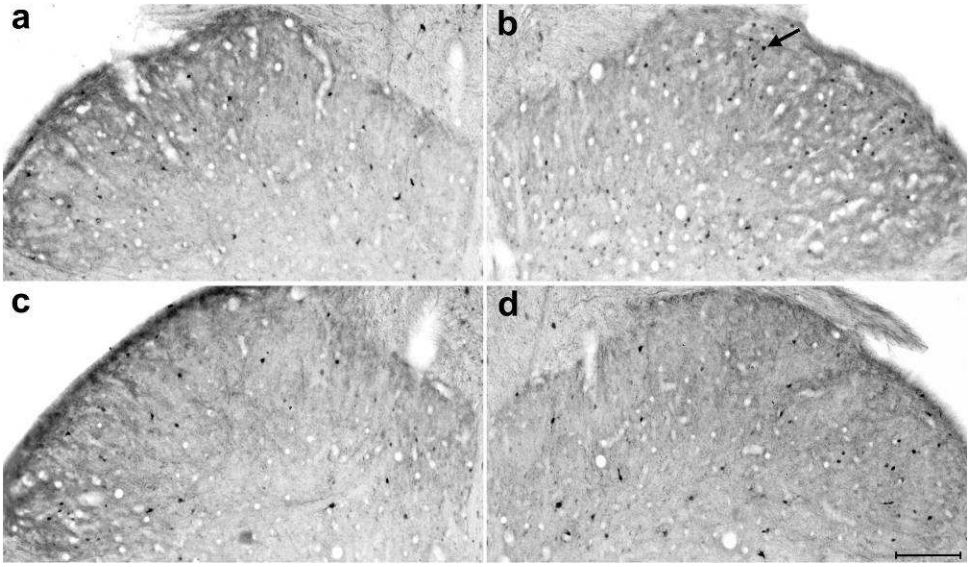

Figure 5. Representative photos of c-Fos immunoreactivity in the C1-C2 segments of the rat. In the control group (a, b), more c-Fos-IR cells can be observed in the superficial laminae of the C1-C2 after NTG injection (b) as compared with the Placebo-treated animals (a). This NTG-induced change is not detected after 1 mmol/kg bw KYNAa2 pre-treatment (c, d). The black arrow in "b" indicates a c-Fos-positive cell. Scale bar: 100 μm

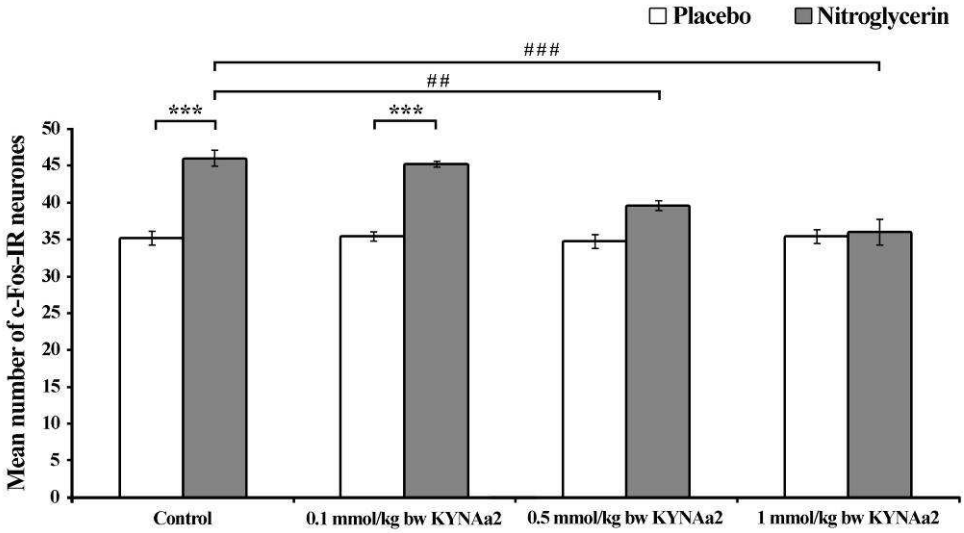

Figure 6. The mean number of c-Fos-IR neurones (means ± S.E.M., n = 6 or 5 per subgroup). NTG injection significantly increased the mean number of c-Fos-IR cells in the C1-C2 in the control group as compared with the Placebo-treated rats (***$p<0.001$), which persisted after a pre-treatment with low dosage of KYNAa2 (0.1 mmol/kg bw) (***$p<0.001$) and disappeared when the dosage of KYNAa2 was higher (0.5 mmol/kg and 1 mmol/kg bw). We could not detect significant difference between NTG-treated rats of control group and of group receiving 0.1 mmol/kg bw KYNAa2, while there was significant difference between NTG-treated animals of control group and of groups pre-treated with 0.5 and 1 mmol/kg bw dose of KYNAa2 (##$p<0.01$; ###$p<0.001$).

In the control group, increased c-Fos, nNOS and CaMKIIα immunoreactivity (Figures 5, 7, 9) can be observed in the superficial layers of the C1-C2 after NTG treatment compared to Placebo. The differences in the numbers of IR neurones proved to be statistically significant for all three immunostainings (***$p<0.001$; Figures 6, 8, 10).

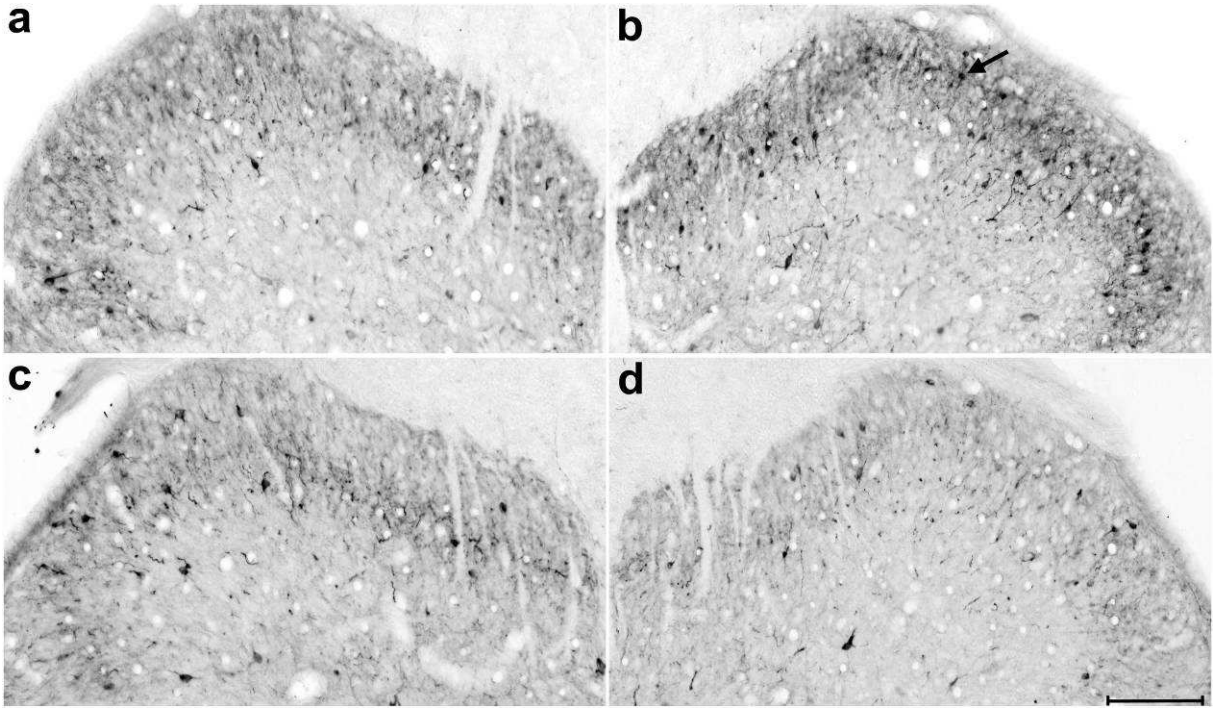

Figure 7. Representative photos of nNOS-stained neurones in the C1-C2 of the rat. In the control group (a, b), an increased nNOS immunoreactivity can be observed in the superficial laminae of the C1-C2 after NTG injection (b) as compared with the Placebo-treated animals (a). In the group pre-treated with 1 mmol/kg bw KYNAa2 (c, d), the NTG-induced change can not be seen. The black arrow on photo (b) points to a nNOS-IR neurone. Scale bar: 100 μm

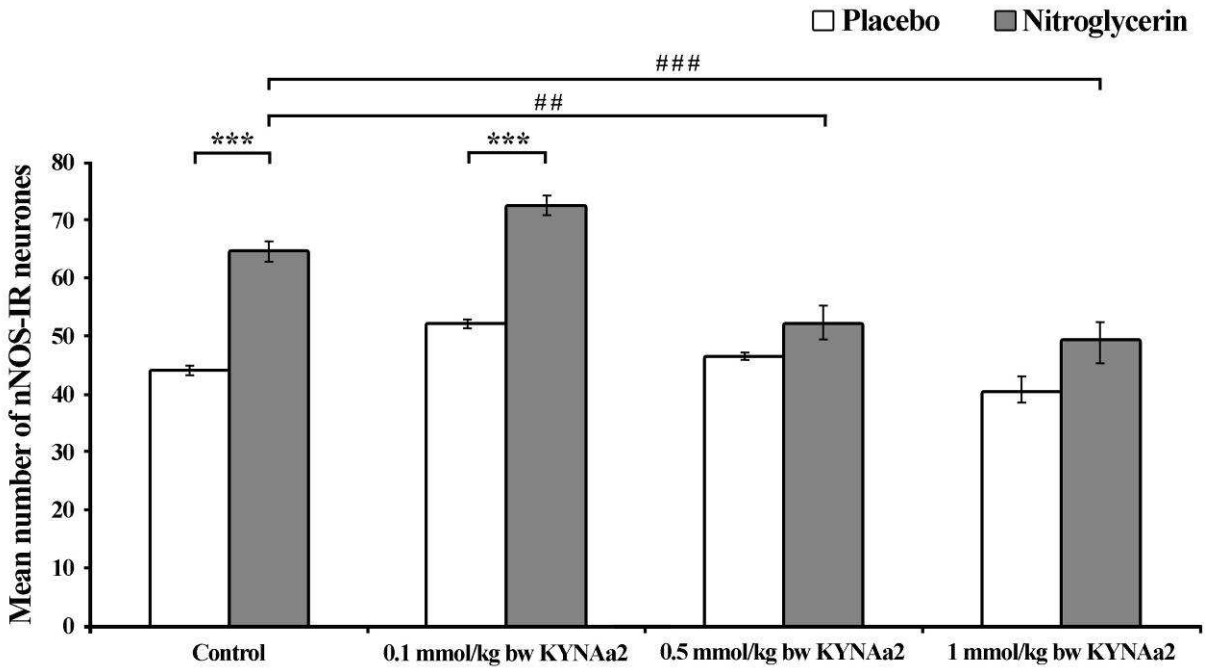

Figure 8. The average number of nNOS-IR cells (means ± S.E.M., n = 6 or 5 per subgroup). NTG injection significantly enhanced the mean number of nNOS-IR neurones in the C1-C2 in the controls as compared with the rodents treated with Placebo (***$p<0.001$). This significant increase was still present after pre-treatment with a low dosage of KYNAa2 (0.1 mmol/kg bw) (***$p<0.001$), while it disappeared in groups pre-treated with higher dosages of KYNAa2 (0.5 and 1 mmol/kg bw). The effects of different pre-treatments are also reflected by the significant differences between the values of the NTG groups (##$p<0.01$; ###$p<0.001$).

Both L-KYN-PROB and KYNAa1 pre-treatment successfully attenuated the decrease in CGRP and increase in CaMKIIα immunopositivity (Figures 3, 4, 9, 10). The pre-treatment with KYNAa2 affected dose-dependently the NTG-induced changes in the immunoreactivity of all studied markers (Figures 3-10).

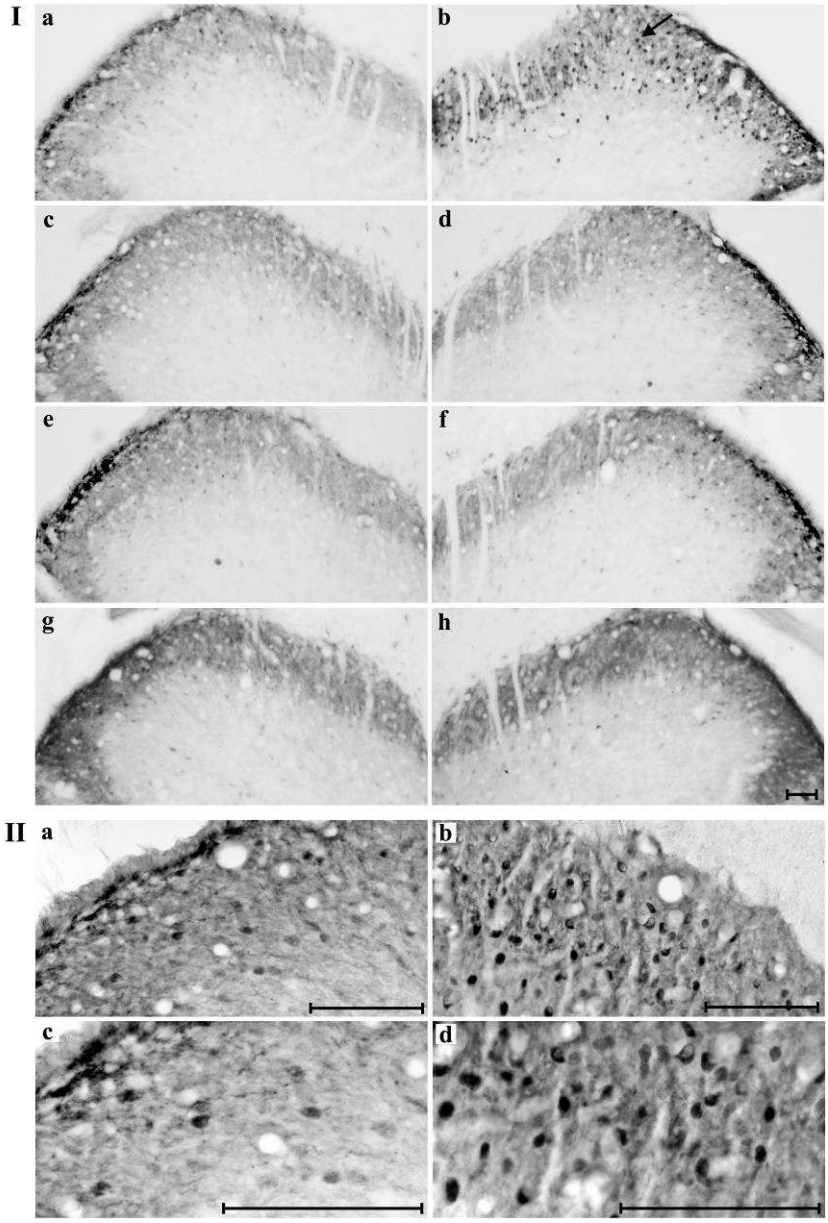

Figure 9. Typical photos showing CaMKIIα-IR cells in the C1-C2 segments of the rat. (I) CaMKIIα immunopositivity in the C1-C2 in control (a, b), L-KYN-PROB-pre-treated (c, d), KYNAa1-pre-treated (e, f) and KYNAa2-pre-treated (g, h) rats after Placebo (a, c, e, g) or NTG (b, d, f, h) injections. (II) CaMKIIα-IR neurones under 40x objective (a, b) and under 63x objective (c, d) in Placebo (a, c) and NTG-treated (b, d) animals of the controls. Increased CaMKIIα staining can be observed in the superficial laminae of the C1-C2 in the control group (I/a, b and II) after NTG injection (I/b, II/b, d) as compared with the Placebo-treated animals (I/a, II/a, c). In the groups pre-treated with L-KYN-PROB (I/c, d), 1 mmol/kg bw KYNAa1 (I/e, f) and 1 mmol/kg bw KYNAa2 (I/g, h), this enhancement is not visible after NTG injection (I/d, f, h) relative to the Placebo treatment (I/c, e, g). The black arrow in "I/b" points to a CaMKIIα-immunopositive neurone. Scale bar: 50 μm

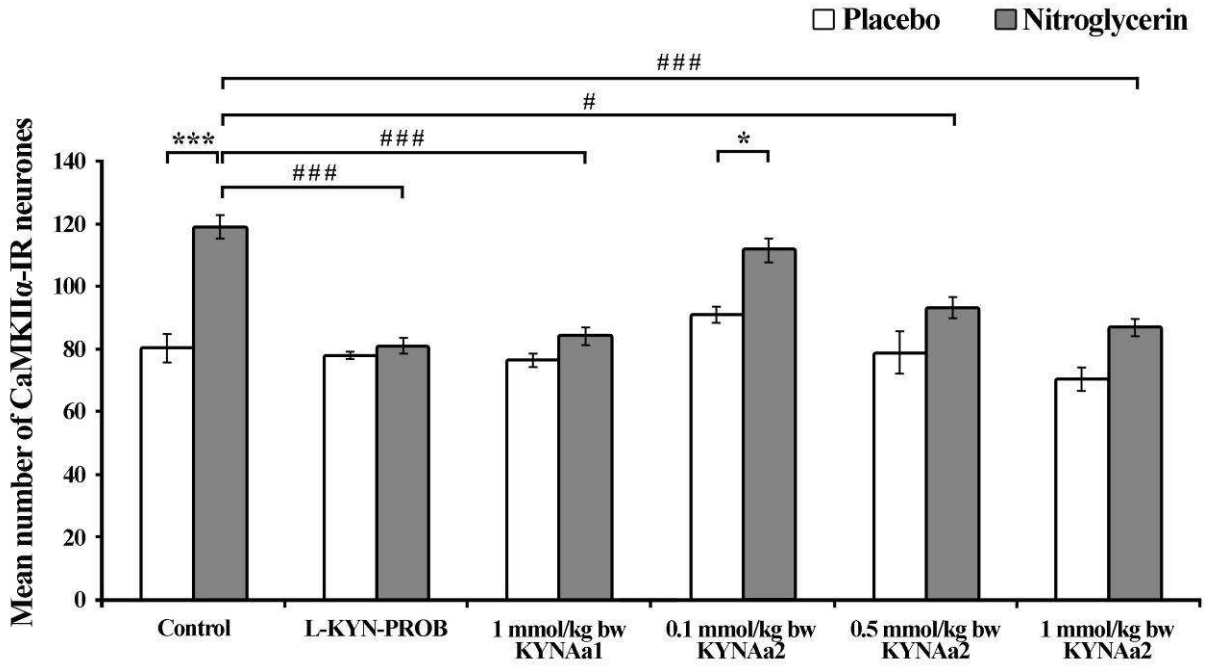

Figure 10. Histogram showing the mean number of CaMKIIα-IR cells in the C1-C2 in the six groups after Placebo (white bars) or NTG (grey bars) injections (means ± S.E.M., n = 6 or 5 per subgroup). There was a significant increase in the CaMKIIα-IR cells after the NTG injection in the control group (***$p<0.001$) and in the group pre-treated with the lowest dosage of KYNAa2 (0.1 mmol/kg bw) (*$p<0.05$). However, this enhancement did not appear in the L-KYN-PROB-pre-treated and the 1 mmol/kg bw KYNAa1-pre-treated animals and in the rats pre-treated with the higher dosages of KYNAa2 (0.5 and 1 mmol/kg bw). The effects of different pre-treatments are also reflected by the significant differences between the values of the NTG groups (#$p<0.05$; ###$p<0.001$).

Western blotting

Western blotting analysis of the C1-C2 region confirmed the results obtained by CGRP and nNOS immunohistochemistry. A band characteristic of the CGRP peptide was identified at 15 kDa and nNOS protein was identified at 155 kDa (Figure 11). Densitometric analyses confirmed the immunohistochemical results, since the CGRP bands were significantly decreased (*$p<0.05$) and nNOS bands were significantly enhanced (***$p<0.001$) in dorsal horn of C1-C2 segments after NTG administration as compared with the Placebo-treated animals (Figure 11). This effect of NTG on CGRP and nNOS was attenuated by pre-treatment with 1 mmol/kg bw KYNAa2 (Figure 11).

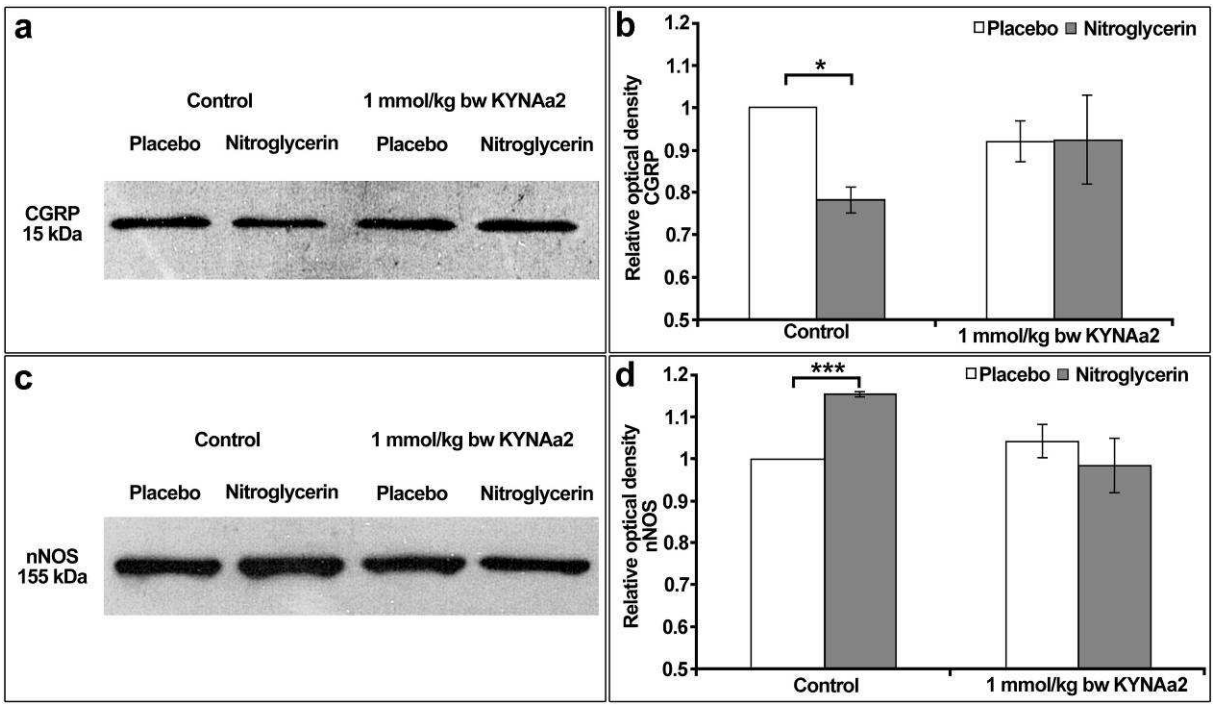

Figure 11. Western blotting of CGRP and nNOS in the C1–C2 segments of the spinal cord (means ± S.E.M., n = 5 per subgroup). Density of CGRP band decreased (a, b) and density of nNOS band increased (c, d) significantly after NTG administration compared to the Placebo in the control group (*p<0.05; ***p<0.001), which was attenuated by pre-treatment with 1 mmol/kg bw KYNAa2

Open Field Test

Treatment with NTG significantly decreased the ambulation distance of the animals compared to Placebo-treated rats (*p<0.05; Figure 12). Pre-treatment with 1 mmol/kg bw KYNAa2 attenuated this difference, but a tendency for a lower basic ambulation distance was observed after KYNAa2 pre-treatment (Figure 12). There were no significant changes in ambulation time, local time or in the number of rearing between the subgroups (data not shown).

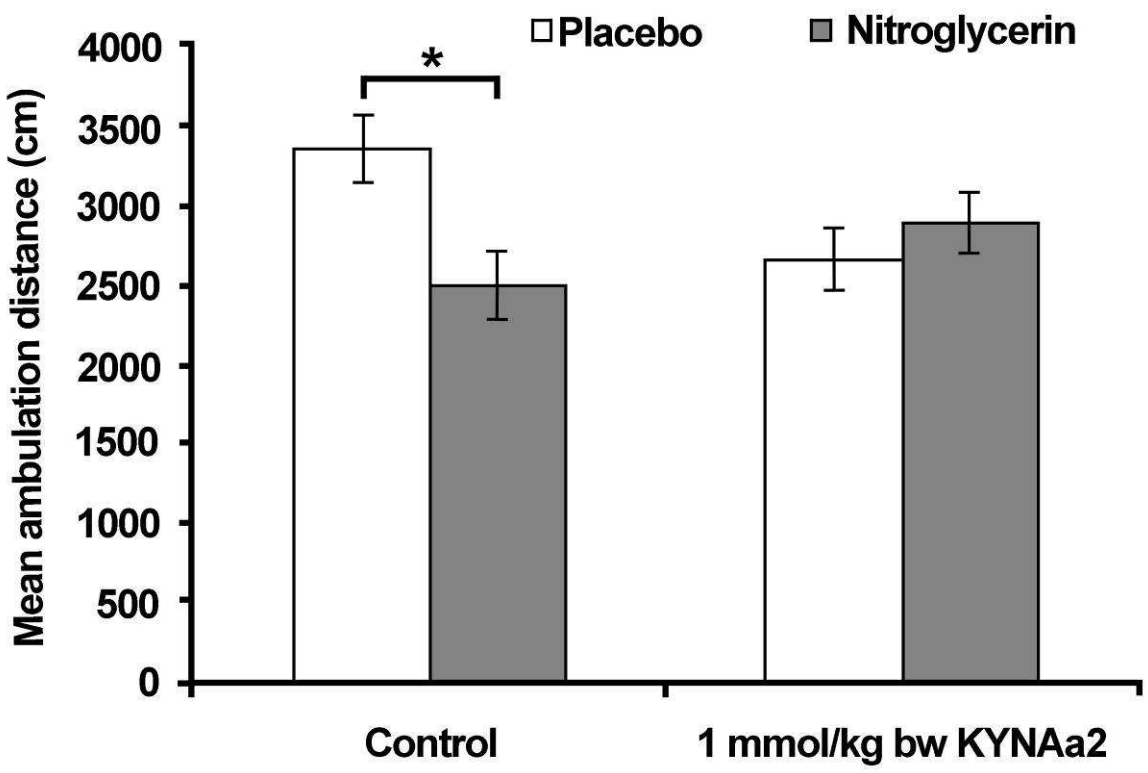

Figure 12. Diagram showing the results of Open Field Test (means ± S.E.M., n = 10 per subgroup). In the control group, treatment with NTG decreased the ambulation distance significantly compared to Placebo-treated rats (*$p<0.05$). Pre-treatment with 1 mmol/kg bw KYNAa2 attenuated this effect of NTG, however, a tendency for a lower basic ambulation distance can be observed in KYNAa2 group as compared with Placebo treated rats of control group as well.

Discussion

Kynurenic acid concentrations in the C1-C2

The HPLC data obtained after L-KYN-PROB show a robust increase of kynurenic acid concentration in the C1-C2, which corresponds to previous findings (Vecsei et al., 1992) and supported by further studies (Chauvel et al., 2013). Kynurenic acid has a poor blood-brain barrier penetrance (Fukui et al., 1991), but administration of its precursor, L-KYN increases kynurenic acid concentration in the central nervous system dose-dependently. In experimental conditions, this effect of L-KYN can be enhanced with the co-administration of PROB by blocking the excretion of kynurenic acid, by inhibiting the organic acid transporters (Cunningham et al., 1981), which are involved in the transport of kynurenic acid from brain through the blood-brain barrier (Colin-Gonzalez and Santamaria, 2013).

Using of kynurenic acid derivatives with a presumed better blood-brain barrier penetrance is an alternative option to increase the kynurenic acid concentration or mimic its effect. After the systemic administration KYNAa2, we witnessed a significant increase in the level of kynurenic acid in the C1-C2, suggesting that KYNAa2 is transformed at least partially to kynurenic acid. Similarly, partial transformation of KYNAa1 to kynurenic acid has been described after i.p. administration in serum of mice, parallel to which robust enhancement of KYNAa1 can be observed as well (Zadori et al., 2011a). Thus, central nervous effects of kynurenic acid derivatives might be related both to their direct action and to indirect influence *via* forming kynurenic acid.

Activation of primary trigeminal neurones

CGRP is an essential transmitter in the primary nociceptors and several lines of evidence prove its importance in migraine pathogenesis (Durham, 2006).

Systemic NTG was able to reduce the CGRP content of the C1-C2, which is in the line with earlier experiments (Pardutz et al., 2002). NO released from NTG stimulates the first-order trigeminal nociceptors resulting in transmitter release from the central terminals of Aδ and C fibres targeting the second-order trigeminal nerve cells. This phenomenon was already described after the activation of primary trigeminal neurones (Zhang et al., 1994) and increased release or turnover of CGRP was reported at the level of the spinal cord in various

pain models (Sluka et al., 1992; Garry et al., 2000). Moreover in another model of trigeminal activation, morphological alterations were observed at the level of the distal terminals located in the dura mater suggesting transmitter release after the electrical stimulation of the trigeminal ganglion (Knyihar-Csillik et al., 2000). In earlier studies, it was also proven that the size of the CGRP-positive buttons is smaller in the C1-C2 after NTG, which also suggestive for transmitter release (Pardutz et al., 2002). These animal experimental results can be paralleled by the observations showing increased CGRP concentration in the jugular vein of migraineurs during the attack (Goadsby et al., 1990). Interestingly, the effect of NTG seems to be selective to the trigeminal system, since we did not observe any changes at the level of the thoracic spinal cord (Pardutz et al., 2002).

Our results indicate that L-KYN-PROB, KYNAa1 and in a dose-dependent manner KYNAa2 can mitigate the CGRP decrease in the trigeminal system caused by systemic NTG. Attenuation of CGRP release from the first-order trigeminal nociceptors suggest that kynurenic acid and its derivatives have a marked peripheral effect probably by inhibition of receptors involved in nociception located in the trigeminal system. This is also supported by the observation that kynurenic acid is able to reduce allodynia when administered topically in the joints (Mecs et al., 2009) and to decrease the pain sensitivity in both the tail-flick and the hot-plate tests after its intraperitoneal injection in rats (Heyliger et al., 1998). Moreover, the systemic administration of 5,7-dichlorokynurenic acid, a kynurenic acid derivative with limited central access, dose-dependently inhibited the nocifensive behaviour evoked by formalin-induced tissue injury and inflammation in Phase II. 5,7-Dichlorokynurenic acid was also effective in two models of neuropathic pain, where it reversed cold allodynia in the chronic constriction injury model, and tactile allodynia in animals subjected to spinal nerve ligation (Christoph et al., 2005).

Glutamate receptors can be found on the peripheral arm of the trigeminal system (Watanabe et al., 1994; Quartu et al., 2002) and their inhibition mitigates CGRP release (Garry et al., 2000). α7-nACh receptors are also present on the soma of primary trigeminal neurones (Liu et al., 1998) and glutamate release (Carpenedo et al., 2001) and CGRP induced facial vasodilation (Just et al., 2005) are reduced after their inhibition. Expression of GPR-35 can be observed within the sensory ganglia and spinal cord, activation of which can modulate nociceptive signalling (Ohshiro et al., 2008).

The blockade of the CGRP release at the level of first-order trigeminal nociceptors indicates that kynurenic acid and its derivatives are able to influence the pain processing on the periphery.

Activation of second-order trigeminal neurones

The NO-induced c-Fos expression observed at the level of the C1-C2 reflecting an activation of the second-order trigeminal nerve cells was already observed in earlier studies (Tassorelli and Joseph, 1995) and appears to be selective to the trigeminal system (Pardutz et al., 2000). NO might activate primary sensory fibres in the trigeminal system, since the depletion of neurotransmitter by capsaicin from these neurones abolishes this effect of NTG (Tassorelli et al., 1997).

The alteration of c-Fos expression reflects the dose-dependent modulatory effect of KYNAa2 on the activation of second-order trigeminal neurones located in the C1-C2, which might be related to the inhibition of the peripheral nociceptors, but it may also arise central effects namely action on receptors located in the central part of the trigeminal system. Glutamatergic receptors can be detected on the second-order trigeminal neurones (Tallaksen-Greene et al., 1992) and they are involved in nociceptive processing. Inhibition of these receptors can mitigate c-Fos increase in pain conditions (Mitsikostas et al., 1998; 1999). α7-nACh receptors can be found on primary trigeminal nociceptors (Liu et al., 1998) and their inhibition by kynurenic acid may block glutamate release from the nerve endings (Carpenedo et al., 2001) and thus reduce the activation of secondary trigeminal neurones (Carstens et al., 2000).

Central action of KYNAa2 can be suggested in these experimental settings as well, since kynurenic acid and its derivatives administered intrathecally directly into the central nervous system can produce similar effects. Kynurenic acid significantly reduced the carrageenan-induced c-Fos expression in laminae I-II and IV-V of ipsilateral spinal cord (Zhang et al., 2003). 7-Chlorokynurenic acid significantly inhibited electrophysiological responses of convergent dorsal horn neurones in the second phase of formalin test (Chapman and Dickenson, 1995). Kynurenic acid and 7-chlorokynurenic acid demonstrated a dose-dependent anti-nociceptive action in various animal models of nociception in mice and rats (Nasstrom et al., 1992; Kristensen et al., 1993) and anti-hyperalgesic effect in rats injected with carrageenan (Ren et al., 1992; Zhang et al., 2003). Similarly, 5,7-dichlorokynurenic acid

showed anti-hyperalgesic properties in Mg-deficient rats (Begon et al., 2001). Moreover, i.p. KYNAa1 was more effective mitigating c-Fos activation in the C1-C2 after NTG administration when compared to kynurenic acid alone probably due to its better blood-brain barrier penetrance (Knyihar-Csillik et al., 2008).

KYNAa2, a derivative of kynurenic acid can attenuate the NTG-induced activation of second-order trigeminal neurones by direct or indirect (forming kynurenic acid) manner probably by acting both in the periphery and in the central nervous system.

Sensitization of second-order trigeminal neurones

nNOS is present in the dorsal horn neurones of the spinal cord suggesting a role in sensory and pain processing (Saito et al., 1994). NOS inhibitors block the activation of dorsal horn neurones after nociceptive stimuli (Wang et al., 1999) and can inhibit the trigeminovascular system (Akerman et al., 2002) and preliminary studies show that they might be effective treating migraine (Lassen et al., 1997) and tension type headache (Ashina, 2002). Previously, it was demonstrated that systemic NTG was able to enhance nNOS expression at the level of the C1-C2 (Pardutz et al., 2000) and similar changes were seen in the spinal cord after intradermal capsaicin administration (Wu et al., 2001) and in the TNC after facial formalin injections (Leong et al., 2000). In our experiments, NO derived from NTG might act *via* the stimulation of Aδ and C fibres of the primary trigeminal afferents (Pardutz et al., 2000). These observations suggest that NO donors may trigger a self-amplifying process at the level of central projection site of the trigeminal system by increasing endogenous NO synthesis, which might be relevant in migraine pathogenesis, where the central sensitization process is essential (Burstein et al., 2000).

CaMKIIα is present in the superficial layers of the spinal dorsal horns (Fang et al., 2002). NTG increased CaMKIIα in the second-order neurones of the trigeminal system, which was also shown in previous studies (Pardutz et al., 2007). This phenomenon can be paralleled with the results showing increased expression of CaMKIIα in the spinal cord after subcutaneous formalin (Liang et al., 2004), capsaicin (Fang et al., 2002) and intrathecal substance-P (Choi et al., 2005) administration. Moreover CaMKIIα is able of autophosphorilation, which enhances its activity (Thiel et al., 1988). It can attach to NMDA receptors in rats and its alpha subunit enhances ion currents through AMPA and NMDA receptors (Kolaj et al., 1994) and activates adenylate cyclase related to spinal cord sensitization in animals (Wei et al., 2006).

L-KYN-PROB, KYNAa1 and KYNAa2 was able to inhibit the NTG-induced increase of nNOS and CaMKIIα expression at the level of second-order trigeminal neurones in the C1-C2, which points to the attenuation of the central sensitization phenomena. Both nNOS and CaMKIIα seem to be key players in this process (Fang et al., 2002; Chacur et al., 2010), where neurones undergo plastic anatomical and functional changes involving the activation of AMPA and NMDA receptors (Latremoliere and Woolf, 2009) after strong noxious stimulation. Both AMPA and NMDA receptor blockers inhibit long term potentiation of the C fibre-mediated response in wide dynamic range neurones at the level of lumbar dorsal horn, which is thought to be related to central sensitization (Svendsen et al., 1998). LY235959 - a competitive NMDA antagonist - attenuated the behavioural responses of the second phase after formalin administration, which corresponds to the sensitization process (Davis and Inturrisi, 2001). Taken together, these results suggest that besides peripheral action, kynurenic acid derivatives may inhibit the increases of nNOS and CaMKIIα expression i.e. the NTG induced central sensitization through a possible blockade of AMPA and/or NMDA receptors. It can be also hypothesised that the inhibition of α7-nACh can play a role in the inhibition of the central sensitization process, since glutamate release can be lowered by antagonizing these receptors with kynurenic acid at the presynaptic level (Carpenedo et al., 2001).

Behavioural effect of NTG

NTG administration, which causes the activation and sensitization of the trigeminal system (Tassorelli and Joseph, 1995; Pardutz et al., 2000), significantly decreased the ambulation distance of the rats in the control group, which can be paralleled with other experiments, when pain conditions such as experimental spinal canal stenosis also reduced the ambulation distance (Li et al., 2013). On the other hand, NTG- and Placebo-treated subgroup did not show any significant difference of ambulation distance when pre-treated with KYNAa2 suggesting an anti-nociceptive effect of the latter. Nonetheless, we found a tendency of lower basic ambulation distance in KYNAa2 pre-treated groups suggesting a direct central action of KYNAa2. This theory is also supported by findings that a glycine-site NMDA antagonist passing the blood-brain barrier can modulate the ambulation distance in the Open Field Test, while the antagonist with limited central access did not produce such effect (Christoph et al., 2005).

Conclusions

In these experiments, we managed to manipulate the kynurenine system in rats either by increasing brain kynurenic acid levels by administrating L-KYN combined with PROB or injecting a newly synthesized kynurenic acid amide, which might act directly with a presumed similar receptorial action or can be metabolized to kynurenic acid and thus have an indirect mechanism of action.

Kynurenic acid and its derivatives mitigated the NTG-induced trigeminal activation at the level of first- and second-order neurones. Moreover, they abolished changes related to the central sensitization process at the level of the C1-C2, where most of the trigeminal nociceptors are located. Since these events have a particular importance in the pathomechanism of headaches, kynurenic acid and its derivatives might have a potential role in the treatment of cephalalgias.

Acknowledgements

I would like to express my gratitude to László Vécsei, M.D., Ph.D., D.Sc. for scientific advice and opportunity to work at Department of Neurology.

I would like to thank my supervisor, Árpád Párdutz, M.D., Ph.D. for his scientific guidance and continuous support of my research activities.

I wish to thank all co-workers with whom I performed the experiments, especially, Enikő Vámos, M.Sc., Ph.D., Zsuzsanna Bohár, M.Sc., Lilla Tar, M.D., Gábor Nagy-Grócz, M.Sc. and Dénes Zádori, M.D., Ph.D..

I am grateful to Mrs. Valéria Vékony for the technical assistance, constructive suggestions and her patience.

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

Refecences

Afra J, Sandor PS, Schoenen J. Habituation of visual and intensity dependence of auditory evoked cortical potentials tends to normalize just before and during the migraine attack. Cephalalgia. 2000; 20(8): 714-719.

Afridi SK, Kaube H, Goadsby PJ. Glyceryl trinitrate triggers premonitory symptoms in migraineurs. Pain. 2004; 110(3): 675-680.

Afridi SK, Matharu MS, Lee L, Kaube H, Friston KJ, Frackowiak RS, Goadsby PJ. A PET study exploring the laterality of brainstem activation in migraine using glyceryl trinitrate. Brain. 2005; 128(Pt 4): 932-939.

Akerman S, Williamson DJ, Kaube H, Goadsby PJ. Nitric oxide synthase inhibitors can antagonize neurogenic and calcitonin gene-related peptide induced dilation of dural meningeal vessels. Br J Pharmacol. 2002; 137(1): 62-68.

Andine P, Lehmann A, Ellren K, Wennberg E, Kjellmer I, Nielsen T, Hagberg H. The excitatory amino acid antagonist kynurenic acid administered after hypoxic-ischemia in neonatal rats offers neuroprotection. Neurosci Lett. 1988; 90(1-2): 208-212.

Ashina M. Nitric oxide synthase inhibitors for the treatment of chronic tension-type headache. Expert Opin Pharmacother. 2002; 3(4): 395-399.

Ashina M, Bendtsen L, Jensen R, Sakai F, Olesen J. Possible mechanisms of glyceryl-trinitrate-induced immediate headache in patients with chronic tension-type headache. Cephalalgia. 2000; 20(10): 919-924.

Axelsson KL, Wikberg JE, Andersson RG. Relationship between nitroglycerin, cyclic GMP and relaxation of vascular smooth muscle. Life Sci. 1979; 24(19): 1779-1786.

Bates EA, Nikai T, Brennan KC, Fu YH, Charles AC, Basbaum AI, Ptacek LJ, Ahn AH. Sumatriptan alleviates nitroglycerin-induced mechanical and thermal allodynia in mice. Cephalalgia. 2010; 30(2): 170-178.

Beadle GW, Mitchell HK, Nyc JF. Kynurenine as an Intermediate in the Formation of Nicotinic Acid from Tryptophane by Neurospora. Proc Natl Acad Sci U S A. 1947; 33(6): 155-158.

Begon S, Pickering G, Eschalier A, Mazur A, Rayssiguier Y, Dubray C. Role of spinal NMDA receptors, protein kinase C and nitric oxide synthase in the hyperalgesia induced by magnesium deficiency in rats. Br J Pharmacol. 2001; 134(6): 1227-1236.

Behan WM, McDonald M, Darlington LG, Stone TW. Oxidative stress as a mechanism for quinolinic acid-induced hippocampal damage: protection by melatonin and deprenyl. Br J Pharmacol. 1999; 128(8): 1754-1760.

Bhaskar S, Saeidi K, Borhani P, Amiri H. Recent progress in migraine pathophysiology: role of cortical spreading depression and magnetic resonance imaging. Eur J Neurosci. 2013; 38(11): 3540-3551.

Bolay H, Reuter U, Dunn AK, Huang Z, Boas DA, Moskowitz MA. Intrinsic brain activity triggers trigeminal meningeal afferents in a migraine model. Nat Med. 2002; 8(2): 136-142.

Burstein R, Levy D, Jakubowski M. Effects of sensitization of trigeminovascular neurons to triptan therapy during migraine. Rev Neurol (Paris). 2005; 161(6-7): 658-660.

Burstein R, Yarnitsky D, Goor-Aryeh I, Ransil BJ, Bajwa ZH. An association between migraine and cutaneous allodynia. Ann Neurol. 2000; 47(5): 614-624.

Buzzi MG, Bonamini M, Moskowitz MA. Neurogenic model of migraine. Cephalalgia. 1995; 15(4): 277-280.

Carpenedo R, Pittaluga A, Cozzi A, Attucci S, Galli A, Raiteri M, Moroni F. Presynaptic kynurenate-sensitive receptors inhibit glutamate release. Eur J Neurosci. 2001; 13(11): 2141-2147.

Carpenter MB and Sutin J. Human Neuroanatomy. Eighth edition. 1983; Williams & Wilkins: Baltimore; ISBN: 0-683-01461- AACR2.

Carstens E, Simons CT, Dessirier JM, Carstens MI, Jinks SL. Role of neuronal nicotinic-acetylcholine receptors in the activation of neurons in trigeminal subnucleus caudalis by nicotine delivered to the oral mucosa. Exp Brain Res. 2000; 132(3): 375-383.

Chacur M, Matos RJ, Alves AS, Rodrigues AC, Gutierrez V, Cury Y, Britto LR. Participation of neuronal nitric oxide synthase in experimental neuropathic pain induced by sciatic nerve transection. Braz J Med Biol Res. 2010; 43(4): 367-376.

Chapman V and Dickenson AH. Time-related roles of excitatory amino acid receptors during persistent noxiously evoked responses of rat dorsal horn neurones. Brain Res. 1995; 703(1-2): 45-50.

Chauvel V, Schoenen J, Multon S. Influence of ovarian hormones on cortical spreading depression and its suppression by L-kynurenine in rat. PLoS One. 2013; 8(12): e82279.

Chiariello M, Gold HK, Leinbach RC, Davis MA, Maroko PR. Comparison between the effects of nitroprusside and nitroglycerin on ischemic injury during acute myocardial infarction. Circulation. 1976; 54(5): 766-773.

Choi SS, Seo YJ, Kwon MS, Shim EJ, Lee JY, Ham YO, Lee HK, Suh HW. Increase of phosphorylation of calcium/calmodulin-dependent protein kinase-II in several brain regions by substance P administered intrathecally in mice. Brain Res Bull. 2005; 65(5): 375-381.

Christoph T, Reissmuller E, Schiene K, Englberger W, Chizh BA. Antiallodynic effects of NMDA glycine(B) antagonists in neuropathic pain: possible peripheral mechanisms. Brain Res. 2005; 1048(1-2): 218-227.

Coleman JW. Nitric oxide: a regulator of mast cell activation and mast cell-mediated inflammation. Clin Exp Immunol. 2002; 129(1): 4-10.

Colin-Gonzalez AL and Santamaria A. Probenecid: an emerging tool for neuroprotection. CNS Neurol Disord Drug Targets. 2013; 12(7): 1050-1065.

Cunningham RF, Israili ZH, Dayton PG. Clinical pharmacokinetics of probenecid. Clin Pharmacokinet. 1981; 6(2): 135-151.

Davis AM and Inturrisi CE. Attenuation of hyperalgesia by LY235959, a competitive N-methyl-D-aspartate receptor antagonist. Brain Res. 2001; 894(1): 150-153.

de Tommaso M, Guido M, Libro G, Losito L, Sciruicchio V, Monetti C, Puca F. Abnormal brain processing of cutaneous pain in migraine patients during the attack. Neurosci Lett. 2002; 333(1): 29-32.

de Tommaso M, Libro G, Guido M, Difruscolo O, Losito L, Sardaro M, Cerbo R. Nitroglycerin induces migraine headache and central sensitization phenomena in patients with migraine without aura: a study of laser evoked potentials. Neurosci Lett. 2004; 363(3): 272-275.

Demeter I, Nagy K, Gellert L, Vecsei L, Fulop F, Toldi J. A novel kynurenic acid analog (SZR104) inhibits pentylenetetrazole-induced epileptiform seizures. An electrophysiological study : special issue related to kynurenine. J Neural Transm. 2012; 119(2): 151-154.

Di Clemente L, Coppola G, Magis D, Gerardy PY, Fumal A, De Pasqua V, Di Piero V, Schoenen J. Nitroglycerin sensitises in healthy subjects CNS structures involved in migraine

pathophysiology: evidence from a study of nociceptive blink reflexes and visual evoked potentials. Pain. 2009; 144(1-2): 156-161.

Dolan S, Field LC, Nolan AM. The role of nitric oxide and prostaglandin signaling pathways in spinal nociceptive processing in chronic inflammation. Pain. 2000; 86(3): 311-320.

Durham PL. Calcitonin gene-related peptide (CGRP) and migraine. Headache. 2006; 46 Suppl 1: S3-8.

Edvinsson L. Tracing neural connections to pain pathways with relevance to primary headaches. Cephalalgia. 2011; 31(6): 737-747.

Edvinsson L, Villalon CM, MaassenVanDenBrink A. Basic mechanisms of migraine and its acute treatment. Pharmacol Ther. 2012; 136(3): 319-333.

Fang L, Wu J, Lin Q, Willis WD. Calcium-calmodulin-dependent protein kinase II contributes to spinal cord central sensitization. J Neurosci. 2002; 22(10): 4196-4204.

Fejes A, Pardutz A, Toldi J, Vecsei L. Kynurenine metabolites and migraine: experimental studies and therapeutic perspectives. Curr Neuropharmacol. 2011; 9(2): 376-387.

Fukui S, Schwarcz R, Rapoport SI, Takada Y, Smith QR. Blood-brain barrier transport of kynurenines: implications for brain synthesis and metabolism. J Neurochem. 1991; 56(6): 2007-2017.

Fullerton T, Komorowski-Swiatek D, Forrest A, Gengo FM. The pharmacodynamics of sumatriptan in nitroglycerin-induced headache. J Clin Pharmacol. 1999; 39(1): 17-29.

Fulop F, Szatmari I, Toldi J, Vecsei L. Modifications on the carboxylic function of kynurenic acid. J Neural Transm. 2012; 119(2): 109-114.

Garry EM, Moss A, Delaney A, O'Neill F, Blakemore J, Bowen J, Husi H, Mitchell R, Grant SG, Fleetwood-Walker SM. Neuropathic sensitization of behavioral reflexes and spinal NMDA receptor/CaM kinase II interactions are disrupted in PSD-95 mutant mice. Curr Biol. 2003; 13(4): 321-328.

Garry MG, Walton LP, Davis MA. Capsaicin-evoked release of immunoreactive calcitonin gene-related peptide from the spinal cord is mediated by nitric oxide but not by cyclic GMP. Brain Res. 2000; 861(2): 208-219.

Gellert L, Varga D, Ruszka M, Toldi J, Farkas T, Szatmari I, Fulop F, Vecsei L, Kis Z. Behavioural studies with a newly developed neuroprotective KYNA-amide. J Neural Transm. 2012; 119(2): 165-172.

Goadsby PJ, Edvinsson L, Ekman R. Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. Ann Neurol. 1990; 28(2): 183-187.

Gobaille S, Kemmel V, Brumaru D, Dugave C, Aunis D, Maitre M. Xanthurenic acid distribution, transport, accumulation and release in the rat brain. J Neurochem. 2008; 105(3): 982-993.

Gozalov A, Jansen-Olesen I, Klaerke D, Olesen J. Role of BK(Ca) channels in cephalic vasodilation induced by CGRP, NO and transcranial electrical stimulation in the rat. Cephalalgia. 2007; 27(10): 1120-1127.

Graham JR and Wolff HG. Mechanism of migraine headache and action of ergotamine tartrate. Arch NeurPsych. 1938; 39(4): 737-763.

Greco R, Mangione A, Siani F, Blandini F, Vairetti M, Nappi G, Sandrini G, Buzzi M, Tassorelli C. Effects of CGRP receptor antagonism in nitroglycerin-induced hyperalgesia. Cephalalgia. 2013; 34(8): 594-604.

Greco R, Meazza C, Mangione AS, Allena M, Bolla M, Amantea D, Mizoguchi H, Sandrini G, Nappi G, Tassorelli C. Temporal profile of vascular changes induced by systemic nitroglycerin in the meningeal and cortical districts. Cephalalgia. 2011; 31(2): 190-198.

Guidetti P and Schwarcz R. 3-Hydroxykynurenine potentiates quinolinate but not NMDA toxicity in the rat striatum. Eur J Neurosci. 1999; 11(11): 3857-3863.

Guillemin GJ, Cullen KM, Lim CK, Smythe GA, Garner B, Kapoor V, Takikawa O, Brew BJ. Characterization of the kynurenine pathway in human neurons. J Neurosci. 2007; 27(47): 12884-12892.

Guillemin GJ, Smith DG, Smythe GA, Armati PJ, Brew BJ. Expression of the kynurenine pathway enzymes in human microglia and macrophages. Adv Exp Med Biol. 2003; 527: 105-112.

Hadjikhani N, Sanchez Del Rio M, Wu O, Schwartz D, Bakker D, Fischl B, Kwong KK, Cutrer FM, Rosen BR, Tootell RB, Sorensen AG, Moskowitz MA. Mechanisms of migraine aura revealed by functional MRI in human visual cortex. Proc Natl Acad Sci U S A. 2001; 98(8): 4687-4692.

Harris JA. Using c-fos as a neural marker of pain. Brain Res Bull. 1998; 45(1): 1-8.

Harrison DG and Bates JN. The nitrovasodilators. New ideas about old drugs. Circulation. 1993; 87(5): 1461-1467.

Headache Classification Committee of the International Headache Society. The International Classification of Headache Disorders, 3rd edition (beta version). Cephalalgia. 2013; 33(9): 629-808.

Hering R and Kuritzky A. Sodium valproate in the prophylactic treatment of migraine: a double-blind study versus placebo. Cephalalgia. 1992; 12(2): 81-84.

Herve C, Beyne P, Jamault H, Delacoux E. Determination of tryptophan and its kynurenine pathway metabolites in human serum by high-performance liquid chromatography with simultaneous ultraviolet and fluorimetric detection. J Chromatogr B Biomed Appl. 1996; 675(1): 157-161.

Heyliger SO, Goodman CB, Ngong JM, Soliman KF. The analgesic effects of tryptophan and its metabolites in the rat. Pharmacol Res. 1998; 38(4): 243-250.

Ho TW, Edvinsson L, Goadsby PJ. CGRP and its receptors provide new insights into migraine pathophysiology. Nat Rev Neurol. 2010; 6(10): 573-582.

Juhasz G, Zsombok T, Jakab B, Nemeth J, Szolcsanyi J, Bagdy G. Sumatriptan causes parallel decrease in plasma calcitonin gene-related peptide (CGRP) concentration and migraine headache during nitroglycerin induced migraine attack. Cephalalgia. 2005; 25(3): 179-183.

Juhasz G, Zsombok T, Modos EA, Olajos S, Jakab B, Nemeth J, Szolcsanyi J, Vitrai J, Bagdy G. NO-induced migraine attack: strong increase in plasma calcitonin gene-related peptide (CGRP) concentration and negative correlation with platelet serotonin release. Pain. 2003; 106(3): 461-470.

Just S, Arndt K, Doods H. The role of CGRP and nicotinic receptors in centrally evoked facial blood flow changes. Neurosci Lett. 2005; 381(1-2): 120-124.

Kaube H, Katsarava Z, Przywara S, Drepper J, Ellrich J, Diener HC. Acute migraine headache: possible sensitization of neurons in the spinal trigeminal nucleus? Neurology. 2002; 58(8): 1234-1238.

Kessler M, Terramani T, Lynch G, Baudry M. A glycine site associated with N-methyl-D-aspartic acid receptors: characterization and identification of a new class of antagonists. J Neurochem. 1989; 52(4): 1319-1328.

Klein C, Patte-Mensah C, Taleb O, Bourguignon JJ, Schmitt M, Bihel F, Maitre M, Mensah-Nyagan AG. The neuroprotector kynurenic acid increases neuronal cell survival through neprilysin induction. Neuropharmacology. 2013; 70: 254-260.

Klivenyi P, Toldi J, Vecsei L. Kynurenines in neurodegenerative disorders: therapeutic consideration. Adv Exp Med Biol. 2004; 541: 169-183.

Knyihar-Csillik E, Mihaly A, Krisztin-Peva B, Robotka H, Szatmari I, Fulop F, Toldi J, Csillik B, Vecsei L. The kynurenate analog SZR-72 prevents the nitroglycerol-induced increase of c-fos immunoreactivity in the rat caudal trigeminal nucleus: comparative studies of the effects of SZR-72 and kynurenic acid. Neurosci Res. 2008; 61(4): 429-432.

Knyihar-Csillik E, Tajti J, Csillik AE, Chadaide Z, Mihaly A, Vecsei L. Effects of eletriptan on the peptidergic innervation of the cerebral dura mater and trigeminal ganglion, and on the expression of c-fos and c-jun in the trigeminal complex of the rat in an experimental migraine model. Eur J Neurosci. 2000; 12(11): 3991-4002.

Kolaj M, Cerne R, Cheng G, Brickey DA, Randic M. Alpha subunit of calcium/calmodulin-dependent protein kinase enhances excitatory amino acid and synaptic responses of rat spinal dorsal horn neurons. J Neurophysiol. 1994; 72(5): 2525-2531.

Kristensen JD, Post C, Gordh T, Jr., Svensson BA. Spinal cord morphology and antinociception after chronic intrathecal administration of excitatory amino acid antagonists in the rat. Pain. 1993; 54(3): 309-316.

Lambert GA, Boers PM, Hoskin KL, Donaldson C, Zagami AS. Suppression by eletriptan of the activation of trigeminovascular sensory neurons by glyceryl trinitrate. Brain Res. 2002; 953(1-2): 181-188.

Lassen LH, Ashina M, Christiansen I, Ulrich V, Olesen J. Nitric oxide synthase inhibition in migraine. Lancet. 1997; 349(9049): 401-402.

Latremoliere A and Woolf CJ. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. J Pain. 2009; 10(9): 895-926.

Leao AAP. Spreading depression of activity in the cerebral cortex. J Neurophysiol. 1944; 7: 359-390.

Leong S, Liu H, Yeo J. Nitric oxide synthase and glutamate receptor immunoreactivity in the rat spinal trigeminal neurons expressing Fos protein after formalin injection. Brain Res. 2000; 855(1): 107-115.

Li Q, Liu Y, Chu Z, Chen J, Dai F, Zhu X, Hu A, Yun C. Brain-derived neurotrophic factor expression in dorsal root ganglia of a lumbar spinal stenosis model in rats. Mol Med Rep. 2013; 8(6): 1836-1844.

Liang DY, Li X, Clark JD. Formalin-induced spinal cord calcium/calmodulin-dependent protein kinase II alpha expression is modulated by heme oxygenase in mice. Neurosci Lett. 2004; 360(1-2): 61-64.

Linde M, Gustavsson A, Stovner LJ, Steiner TJ, Barre J, Katsarava Z, Lainez JM, Lampl C, Lanteri-Minet M, Rastenyte D, Ruiz de la Torre E, Tassorelli C, Andree C. The cost of headache disorders in Europe: the Eurolight project. Eur J Neurol. 2012; 19(5): 703-711.

Lipton RB, Bigal ME, Stewart WF. Clinical trials of acute treatments for migraine including multiple attack studies of pain, disability, and health-related quality of life. Neurology. 2005; 65(12 Suppl 4): S50-58.

Liu L, Chang GQ, Jiao YQ, Simon SA. Neuronal nicotinic acetylcholine receptors in rat trigeminal ganglia. Brain Res. 1998; 809(2): 238-245.

Markovics A, Kormos V, Gaszner B, Lashgarara A, Szoke E, Sandor K, Szabadfi K, Tuka B, Tajti J, Szolcsanyi J, Pinter E, Hashimoto H, Kun J, Reglodi D, Helyes Z. Pituitary adenylate cyclase-activating polypeptide plays a key role in nitroglycerol-induced trigeminovascular activation in mice. Neurobiol Dis. 2012; 45(1): 633-644.

Marosi M, Nagy D, Farkas T, Kis Z, Rozsa E, Robotka H, Fulop F, Vecsei L, Toldi J. A novel kynurenic acid analogue: a comparison with kynurenic acid. An in vitro electrophysiological study. J Neural Transm. 2010; 117(2): 183-188.

Masue T, Dohi S, Asano T, Shimonaka H. Spinal antinociceptive effect of epidural nonsteroidal antiinflammatory drugs on nitric oxide-induced hyperalgesia in rats. Anesthesiology. 1999; 91(1): 198-206.

Mecs L, Tuboly G, Nagy E, Benedek G, Horvath G. The peripheral antinociceptive effects of endomorphin-1 and kynurenic acid in the rat inflamed joint model. Anesth Analg. 2009; 109(4): 1297-1304.

Meller ST and Gebhart GF. Nitric oxide (NO) and nociceptive processing in the spinal cord. Pain. 1993; 52(2): 127-136.

Miranda AF, Sutton MA, Beninger RJ, Jhamandas K, Boegman RJ. Quinolinic acid lesion of the nigrostriatal pathway: effect on turning behaviour and protection by elevation of endogenous kynurenic acid in Rattus norvegicus. Neurosci Lett. 1999; 262(2): 81-84.

Mitsikostas DD, Sanchez del Rio M, Waeber C, Huang Z, Cutrer FM, Moskowitz MA. Non-NMDA glutamate receptors modulate capsaicin induced c-fos expression within trigeminal nucleus caudalis. Br J Pharmacol. 1999; 127(3): 623-630.

Mitsikostas DD, Sanchez del Rio M, Waeber C, Moskowitz MA, Cutrer FM. The NMDA receptor antagonist MK-801 reduces capsaicin-induced c-fos expression within rat trigeminal nucleus caudalis. Pain. 1998; 76(1-2): 239-248.

Moncada S, Radomski MW, Palmer RM. Endothelium-derived relaxing factor. Identification as nitric oxide and role in the control of vascular tone and platelet function. Biochem Pharmacol. 1988; 37(13): 2495-2501.

Morris R, Southam E, Braid DJ, Garthwaite J. Nitric oxide may act as a messenger between dorsal root ganglion neurones and their satellite cells. Neurosci Lett. 1992; 137(1): 29-32.

Moskowitz MA, Nozaki K, Kraig RP. Neocortical spreading depression provokes the expression of c-fos protein-like immunoreactivity within trigeminal nucleus caudalis via trigeminovascular mechanisms. J Neurosci. 1993; 13(3): 1167-1177.

Nasstrom J, Karlsson U, Post C. Antinociceptive actions of different classes of excitatory amino acid receptor antagonists in mice. Eur J Pharmacol. 1992; 212(1): 21-29.

Nemeth H, Toldi J, Vecsei L. Kynurenines, Parkinson's disease and other neurodegenerative disorders: preclinical and clinical studies. J Neural Transm Suppl. 2006; (70): 285-304.

Ohshiro H, Tonai-Kachi H, Ichikawa K. GPR35 is a functional receptor in rat dorsal root ganglion neurons. Biochem Biophys Res Commun. 2008; 365(2): 344-348.

Okuda K, Sakurada C, Takahashi M, Yamada T, Sakurada T. Characterization of nociceptive responses and spinal releases of nitric oxide metabolites and glutamate evoked by different concentrations of formalin in rats. Pain. 2001; 92(1-2): 107-115.

Olesen J, Diener HC, Husstedt IW, Goadsby PJ, Hall D, Meier U, Pollentier S, Lesko LM. Calcitonin gene-related peptide receptor antagonist BIBN 4096 BS for the acute treatment of migraine. N Engl J Med. 2004; 350(11): 1104-1110.

Olesen J, Larsen B, Lauritzen M. Focal hyperemia followed by spreading oligemia and impaired activation of rCBF in classic migraine. Ann Neurol. 1981; 9(4): 344-352.

Pardutz A, Hoyk Z, Varga H, Vecsei L, Schoenen J. Oestrogen-modulated increase of calmodulin-dependent protein kinase II (CamKII) in rat spinal trigeminal nucleus after systemic nitroglycerin. Cephalalgia. 2007; 27(1): 46-53.

Pardutz A, Krizbai I, Multon S, Vecsei L, Schoenen J. Systemic nitroglycerin increases nNOS levels in rat trigeminal nucleus caudalis. Neuroreport. 2000; 11(14): 3071-3075.

Pardutz A, Multon S, Malgrange B, Parducz A, Vecsei L, Schoenen J. Effect of systemic nitroglycerin on CGRP and 5-HT afferents to rat caudal spinal trigeminal nucleus and its modulation by estrogen. Eur J Neurosci. 2002; 15(11): 1803-1809.

Pardutz A, Szatmari E, Vecsei L, Schoenen J. Nitroglycerin-induced nNOS increase in rat trigeminal nucleus caudalis is inhibited by systemic administration of lysine acetylsalicylate but not of sumatriptan. Cephalalgia. 2004; 24(6): 439-445.

Paxinos G and Watson C. The rat brain in stereotaxic coordinates. Sixth edition. 2007; Elsevier: Amsterdam; ISBN: 978-0125476126.

Pereira EF, Hilmas C, Santos MD, Alkondon M, Maelicke A, Albuquerque EX. Unconventional ligands and modulators of nicotinic receptors. J Neurobiol. 2002; 53(4): 479-500.

Perrotta A, Serrao M, Tassorelli C, Arce-Leal N, Guaschino E, Sances G, Rossi P, Bartolo M, Pierelli F, Sandrini G, Nappi G. Oral nitric-oxide donor glyceryl-trinitrate induces sensitization in spinal cord pain processing in migraineurs: a double-blind, placebo-controlled, cross-over study. Eur J Pain. 2011; 15(5): 482-490.

Pietrobon D. Familial hemiplegic migraine. Neurotherapeutics. 2007; 4(2): 274-284.

Pradhan AA, Smith ML, McGuire B, Tarash I, Evans CJ, Charles A. Characterization of a novel model of chronic migraine. Pain. 2014; 155(2): 269-274.

Prescott C, Weeks AM, Staley KJ, Partin KM. Kynurenic acid has a dual action on AMPA receptor responses. Neurosci Lett. 2006; 402(1-2): 108-112.

Quartu M, Serra MP, Ambu R, Lai ML, Del Fiacco M. AMPA-type glutamate receptor subunits 2/3 in the human trigeminal sensory ganglion and subnucleus caudalis from prenatal ages to adulthood. Mech Ageing Dev. 2002; 123(5): 463-471.

Ramachandran R, Bhatt DK, Ploug KB, Olesen J, Jansen-Olesen I, Hay-Schmidt A, Gupta S. A naturalistic glyceryl trinitrate infusion migraine model in the rat. Cephalalgia. 2012; 32(1): 73-84.

Read SJ, Manning P, McNeil CJ, Hunter AJ, Parsons AA. Effects of sumatriptan on nitric oxide and superoxide balance during glyceryl trinitrate infusion in the rat. Implications for antimigraine mechanisms. Brain Res. 1999; 847(1): 1-8.

Ren K, Williams GM, Hylden JL, Ruda MA, Dubner R. The intrathecal administration of excitatory amino acid receptor antagonists selectively attenuated carrageenan-induced behavioral hyperalgesia in rats. Eur J Pharmacol. 1992; 219(2): 235-243.

Reuter U, Bolay H, Jansen-Olesen I, Chiarugi A, Sanchez del Rio M, Letourneau R, Theoharides TC, Waeber C, Moskowitz MA. Delayed inflammation in rat meninges: implications for migraine pathophysiology. Brain. 2001; 124(Pt 12): 2490-2502.

Rios C and Santamaria A. Quinolinic acid is a potent lipid peroxidant in rat brain homogenates. Neurochem Res. 1991; 16(10): 1139-1143.

Saito S, Kidd GJ, Trapp BD, Dawson TM, Bredt DS, Wilson DA, Traystman RJ, Snyder SH, Hanley DF. Rat spinal cord neurons contain nitric oxide synthase. Neuroscience. 1994; 59(2): 447-456.

Sanhueza M and Lisman J. The CaMKII/NMDAR complex as a molecular memory. Mol Brain. 2013; 6: 10.

Shao YF, Zhang Y, Zhao P, Yan WJ, Kong XP, Fan LL, Hou YP. Botulinum toxin type a therapy in migraine: preclinical and clinical trials. Iran Red Crescent Med J. 2013; 15(10): e7704.

Sicuteri F, Del Bene E, Poggioni M, Bonazzi A. Unmasking latent dysnociception in healthy subjects. Headache. 1987; 27(4): 180-185.

Silberstein SD. Practice parameter: evidence-based guidelines for migraine headache (an evidence-based review): report of the Quality Standards Subcommittee of the American Academy of Neurology. Neurology. 2000; 55(6): 754-762.

Sluka KA, Dougherty PM, Sorkin LS, Willis WD, Westlund KN. Neural changes in acute arthritis in monkeys. III. Changes in substance P, calcitonin gene-related peptide and glutamate in the dorsal horn of the spinal cord. Brain Res Brain Res Rev. 1992; 17(1): 29-38.

Stone TW and Perkins MN. Quinolinic acid: a potent endogenous excitant at amino acid receptors in CNS. Eur J Pharmacol. 1981; 72(4): 411-412.

Stovner LJ and Andree C. Prevalence of headache in Europe: a review for the Eurolight project. J Headache Pain. 2010; 11(4): 289-299.

Strassman AM, Raymond SA, Burstein R. Sensitization of meningeal sensory neurons and the origin of headaches. Nature. 1996; 384(6609): 560-564.

Svendsen F, Tjolsen A, Hole K. AMPA and NMDA receptor-dependent spinal LTP after nociceptive tetanic stimulation. Neuroreport. 1998; 9(6): 1185-1190.

Tallaksen-Greene SJ, Young AB, Penney JB, Beitz AJ. Excitatory amino acid binding sites in the trigeminal principal sensory and spinal trigeminal nuclei of the rat. Neurosci Lett. 1992; 141(1): 79-83.

Tassorelli C and Joseph SA. Systemic nitroglycerin induces Fos immunoreactivity in brainstem and forebrain structures of the rat. Brain Res. 1995; 682(1-2): 167-181.

Tassorelli C, Joseph SA, Nappi G. Neurochemical mechanisms of nitroglycerin-induced neuronal activation in rat brain: a pharmacological investigation. Neuropharmacology. 1997; 36(10): 1417-1424.

Tavares RG, Tasca CI, Santos CE, Alves LB, Porciuncula LO, Emanuelli T, Souza DO. Quinolinic acid stimulates synaptosomal glutamate release and inhibits glutamate uptake into astrocytes. Neurochem Int. 2002; 40(7): 621-627.

Thiel G, Czernik AJ, Gorelick F, Nairn AC, Greengard P. Ca2+/calmodulin-dependent protein kinase II: identification of threonine-286 as the autophosphorylation site in the alpha subunit associated with the generation of Ca2+-independent activity. Proc Natl Acad Sci U S A. 1988; 85(17): 6337-6341.

Thomaides T, Karagounakis D, Spantideas A, Katelanis S. Transcranial Doppler in migraine attacks before and after treatment with oral zolmitriptan or sumatriptan. Headache. 2003; 43(1): 54-58.

Thomaides T, Karapanayiotides T, Kerezoudi E, Avramidis T, Haeropoulos C, Zoukos Y, Spantideas A. Intravenous valproate aborts glyceryl trinitrate-induced migraine attacks: a clinical and quantitative EEG study. Cephalalgia. 2008; 28(3): 250-256.

Toda N and Okamura T. Mechanism underlying the response to vasodilator nerve stimulation in isolated dog and monkey cerebral arteries. Am J Physiol. 1990; 259(5 Pt 2): H1511-1517.

Torfgard K, Ahlner J, Axelsson KL, Norlander B, Bertler A. Tissue distribution of glyceryl trinitrate and the effect on cGMP levels in rat. Pharmacol Toxicol. 1989; 64(4): 369-372.

Tuka B, Helyes Z, Markovics A, Bagoly T, Nemeth J, Mark L, Brubel R, Reglodi D, Pardutz A, Szolcsanyi J, Vecsei L, Tajti J. Peripheral and central alterations of pituitary adenylate cyclase activating polypeptide-like immunoreactivity in the rat in response to activation of the trigeminovascular system. Peptides. 2012; 33(2): 307-316.

Tuka B, Helyes Z, Markovics A, Bagoly T, Szolcsanyi J, Szabo N, Toth E, Kincses ZT, Vecsei L, Tajti J. Alterations in PACAP-38-like immunoreactivity in the plasma during ictal and interictal periods of migraine patients. Cephalalgia. 2013; 33(13): 1085-1095.

Vamos E, Pardutz A, Varga H, Bohar Z, Tajti J, Fulop F, Toldi J, Vecsei L. l-kynurenine combined with probenecid and the novel synthetic kynurenic acid derivative attenuate nitroglycerin-induced nNOS in the rat caudal trigeminal nucleus. Neuropharmacology. 2009; 57(4): 425-429.

Varga H, Pardutz A, Vamos E, Bohar Z, Bago F, Tajti J, Bari F, Vecsei L. Selective inhibition of cyclooxygenase-2 attenuates nitroglycerin-induced calmodulin-dependent protein kinase II alpha in rat trigeminal nucleus caudalis. Neurosci Lett. 2009; 451(2): 170-173.

Varga H, Pardutz A, Vamos E, Plangar I, Egyud E, Tajti J, Bari F, Vecsei L. Cox-2 inhibitor attenuates NO-induced nNOS in rat caudal trigeminal nucleus. Headache. 2007; 47(9): 1319-1325.

Vecsei L, Miller J, MacGarvey U, Beal MF. Kynurenine and probenecid inhibit pentylenetetrazol- and NMDLA-induced seizures and increase kynurenic acid concentrations in the brain. Brain Res Bull. 1992; 28(2): 233-238.

Vos T, Flaxman AD, Naghavi M, Lozano R, Michaud C, Ezzati M, Shibuya K, Salomon JA, Abdalla S, Aboyans V, Abraham J, Ackerman I, Aggarwal R, Ahn SY, Ali MK, et al. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet. 2012; 380(9859): 2163-2196.

Wang H, Nie H, Zhang RX, Qiao JT. Peripheral nitric oxide contributes to both formalin- and NMDA-induced activation of nociceptors: An immunocytochemical study in rats. J Neurosci Res. 1999; 57(6): 824-829.

Wang J, Simonavicius N, Wu X, Swaminath G, Reagan J, Tian H, Ling L. Kynurenic acid as a ligand for orphan G protein-coupled receptor GPR35. J Biol Chem. 2006; 281(31): 22021-22028.

Watanabe M, Mishina M, Inoue Y. Distinct gene expression of the N-methyl-D-aspartate receptor channel subunit in peripheral neurons of the mouse sensory ganglia and adrenal gland. Neurosci Lett. 1994; 165(1-2): 183-186.

Wei F, Vadakkan KI, Toyoda H, Wu LJ, Zhao MG, Xu H, Shum FW, Jia YH, Zhuo M. Calcium calmodulin-stimulated adenylyl cyclases contribute to activation of extracellular signal-regulated kinase in spinal dorsal horn neurons in adult rats and mice. J Neurosci. 2006; 26(3): 851-861.

Weiller C, May A, Limmroth V, Juptner M, Kaube H, Schayck RV, Coenen HH, Diener HC. Brain stem activation in spontaneous human migraine attacks. Nat Med. 1995; 1(7): 658-660.

Willis WD. Role of neurotransmitters in sensitization of pain responses. Ann N Y Acad Sci. 2001; 933: 142-156.

Wolf H. The effect of hormones and vitamin B6 on urinary excretion of metabolites of the kynurenine pathway. Scand J Clin Lab Invest Suppl. 1974; 136: 1-186.

Woolf CJ. Central sensitization: uncovering the relation between pain and plasticity. Anesthesiology. 2007; 106(4): 864-867.

Wu J, Fang L, Lin Q, Willis WD. Nitric oxide synthase in spinal cord central sensitization following intradermal injection of capsaicin. Pain. 2001; 94(1): 47-58.

Yao D and Sessle BJ. Nitroglycerin facilitates calcitonin gene-related peptide-induced behavior. Neuroreport. 2008; 19(13): 1307-1311.

Zadori D, Ilisz I, Klivenyi P, Szatmari I, Fulop F, Toldi J, Vecsei L, Peter A. Time-course of kynurenic acid concentration in mouse serum following the administration of a novel kynurenic acid analog. J Pharm Biomed Anal. 2011a; 55(3): 540-543.

Zadori D, Nyiri G, Szonyi A, Szatmari I, Fulop F, Toldi J, Freund TF, Vecsei L, Klivenyi P. Neuroprotective effects of a novel kynurenic acid analogue in a transgenic mouse model of Huntington's disease. J Neural Transm. 2011b; 118(6): 865-875.

Zhang RX, Mi ZP, Qiao JT. Changes of spinal substance P, calcitonin gene-related peptide, somatostatin, Met-enkephalin and neurotensin in rats in response to formalin-induced pain. Regul Pept. 1994; 51(1): 25-32.

Zhang YQ, Ji GC, Wu GC, Zhao ZQ. Kynurenic acid enhances electroacupuncture analgesia in normal and carrageenan-injected rats. Brain Res. 2003; 966(2): 300-307.

Zhu X, Han Y, Xiong W, Wang H, Li J, Liu W, Fan Z. [Heat coagulation of middle meningeal artery affects plasma CGRP and substance P levels in migraine rat triggered by nitroglycerin]. Lin Chung Er Bi Yan Hou Tou Jing Wai Ke Za Zhi. 2011; 25(10): 460-462, 468.

Appendix

Értekezés tézisének magyar nyelvű összefoglalója

Az elsődleges fejfájások közé tartozó migrén az egyik leggyakoribb neurológiai betegség, amelynek pontos patomechanizmusa nem ismert. A migrénes rohamok kialakulásában azonban alapvető a trigeminális rendszer aktiválódása és szenzitizációja, amelynek során az elsődleges trigeminális neuronok centrális és perifériás idegvégződéseiből neurotranszmitterek szabadulnak fel, mint például a glutamát vagy a kalcitonin gén-relációs peptid (CGRP). Következésképpen migrénesekben a roham alatt a vena jugularis externában emelkedett CGRP szint mérhető. A centrálisan felszabadult neuroaktív anyagok a másodlagos trigeminális neuronokat aktiválják, amelyek a nyúltvelőben lévő, de az első két cervikális gerincvelői szegmentumig lehúzódó *nucleus tractus spinalis nervi trigemini pars caudalis* (TNC) magban helyezkednek el. Az aktiválódás általánosan használt markere a c-Fos transzkripciós faktor. A perifériáról érkező folyamatos, hosszan tartó ingerlés hatására a másodlagos idegsejtekben létrejön a centrális szenzitizáció, amelyben a glutamát receptorok, a neuronális nitrogén-monoxid szintáz (nNOS) és a kalcium/kalmodulin-függő protein kináz II alfa (CaMKIIα) fontos szerepet játszanak. A centrális szenzitizáció a migrénes roham során az allodynia jelenségében nyilvánul meg, amely terápiás szempontból kritikus, hiszen kialakulása után jelentősen romlik a roham kezelhetősége.

A nitrogén-monoxid (NO) donor nitroglicerin (NTG) szisztémás adása a migrén egyik humán és állatkísérletes modellje. A NTG általánosan ismert mellékhatása egy, a beadást követően azonnal kialakuló fejfájás, amelyet migrénesekben néhány óra elteltével egy típusos, aura nélküli migrénes roham követ, amelyet a migrén preventív és rohamterápiájában használt gyógyszerek képesek kivédeni vagy megszüntetni. Humán vizsgálatokban az NTG képes aktiválni és szenzitizálni a trigeminális rendszert és ez a jelenség állatkísérletes körülmények között is kimutatható: a TNC cervikális szakaszán (C1-C2) a NTG (i) csökkenti a CGRP-t tartalmazó rostok által lefedett területet, amely a transzmitter felszabadulást és az elsődleges trigeminális nociceptorok aktiválódását jelzi, (ii) növeli a c-Fos expressziót tükrözve a másodlagos trigeminális idegsejtek aktiválódását, valamint (iii) fokozza a nNOS és CaMKIIα immunreaktivitást, amelyek a centrális szenzitizációs folyamatok jelenlétére utalnak.

A kinurénsav L-kinureninből (L-KYN) keletkezik az endogén triptofán metabolizmus 95%-áért felelős kinurenin útvonal során. Kísérletes eredmények alapján a kinurénsav neuroprotektív és hatékony a fájdalomcsillapításban is, amely feltehetően annak köszönhető,

hogy képes gátolni a fájdalomérzés patomechanizmusában is fontos szerepet játszó ionotróp glutamát N-metil-D-aszpartát (NMDA) és α-amino-3-hidroxil-5-metil-4-izoxazol-propionát (AMPA), valamint az α7-nikotinos acetilkolin (α7-nACh) receptorokat, továbbá képes serkenteni a G-protein-kapcsolt-35 receptort (GPR-35). Terápiás alkalmazhatóságának viszont határt szab az, hogy csak kis mennyiségben képes átjutni a vér-agy gáton, ezért kísérletes körülmények között sokszor az L-KYN-t használják, amely önmagában nem neuroaktív vegyület, viszont könnyen átjut a vér-agy gáton és dózisfüggő módon képes megemelni a kinurénsav koncentrációját a központi idegrendszerben. Ez utóbbi jelentősen fokozható probeneciddel (PROB), amely a vér-agy gáton is jelenlévő és a kinurénsav központi idegrendszerből történő kiürüléséért is felelős szerves sav transzportereket gátolva éri el hatását. További lehetőséget jelent az olyan, újonnan szintetizált kinurénsav származékok használata, amelyek feltételezhetően jobban átjuthatnak a vér-agy gáton és megtartják a kiindulási vegyület biológiai hatásait, és/vagy a központi idegrendszerben visszaalakulnak kinurénsavvá.

A fentiek alapján kísérleteink célja az volt, hogy PROB-el kombinált L-KYN-el és két újonnan szintetizált kinurénsav származékkal történt kezelést követően (i) megmérjük a kinurénsav koncentrációját a C1-C2-es régióban; (ii) megvizsgáljuk az esetleges moduláló hatásukat a NTG-indukálta trigeminális aktivációra és szenzitizációra; továbbá (iii) kimutassuk és moduláljuk a NTG kezelés állatok viselkedésére gyakorolt hatását.

Az állatkísérleti irányelveket követő és a helyi hatóságok által engedélyezett kísérleteinkben felnőtt hím Sprague-Dawley patkányokat használtunk. A két új, oldallánc szubsztituált kinurénsav amidot a Szegedi Tudományegyetem Gyógyszerkémiai Intézetében szintetizálták (*N*-(2-*N*,*N*-dimetilaminoetil)-4-oxo-1*H*-quinolin-2-karboxamid hidroklorid - KYNAa1 és *N*-(2-*N*-pirrolidiniletil)-4-oxo-1*H*-quinolin-2-karboxamid hidroklorid - KYNAa2). A C1-C2 régió kinurénsav tartalmának meghatározásához intraperitoneális (i.p.) fiziológiás sóoldattal (n=4) vagy i.p. L-KYN (300 mg/testsúly kg (tskg)) és PROB (200 mg/tskg) kombinációjával (n=4) vagy i.p. KYNAa2-vel (1 mmol/tskg, n=2x5) kezeltük az állatokat. Meghatározott időpontokban (60 perc a kontroll és L-KYN-PROB csoportban; 60 és 300 perc a KYNAa2 csoportban) az eltávolított C1-C2-es szegmentumok kinurénsav tartalmát nagy hatékonyságú folyadékkromatográfia (HPLC) segítségével mértük. A morfológiai vizsgálatokhoz a patkányokat i.p. fiziológiás sóoldattal (n=12) vagy i.p. L-KYN (300 mg/tskg) és PROB (200

mg/tskg) kombinációjával (n=12) vagy i.p. KYNAa1-el (1 mmol/tskg, n=12) vagy különböző dózisú i.p. KYNAa2-vel kezeltük (0,1, 0,5 és 1 mmol/tskg, n=10/csoport), amelyek után egy órával az állatok i.p. NTG-t (10 mg/tskg) vagy i.p. Placebo injekciót kaptak (n=5/6 csoportonként). Négy óra elteltével a C1-C2-es régiót eltávolítottuk és CGRP, c-Fos, nNOS és CaMKIIα immunhisztokémiai festéseket végeztünk rajtuk. A C1-C2-es régió I-II laminájában a CGRP rostok által lefedett területet mértük és a c-Fos-, nNOS- és CaMKIIα-immunreaktív (IR) neuronokat számoltuk. A Western blot analízishez a patkányokat i.p. fiziológiás sóoldattal (n=10) vagy i.p. KYNAa2-vel (1 mmol/tskg, n=10) kezeltük, amit egy óra elteltével i.p. NTG (10 mg/tskg) vagy i.p. Placebo injekció követett (n=5/csoport), majd négy órával ez után a C1-C2-es szegmentumokat eltávolítottuk és Western blot technika segítségével meghatároztuk a CGRP, nNOS és β-actin tartalmukat. Az Open Field Teszthez az állatok i.p. fiziológiás sóoldatot (n=20) vagy i.p. KYNAa2-t (1 mmol/tskg, n=20) kaptak, amit egy óra után i.p. NTG (10 mg/tskg) vagy i.p. Placebo injekció követett (n=10/csoport), majd három óra negyven perc elteltével 15 percig mértük a mozgással töltött időt, a megtett távolságot, a nyugalomban töltött időt és az ágaskodások számát.

HPLC eredményeink alapján a kinurénsav koncentrációja egy órával az L-KYN-PROB kombinált kezelés után és a KYNAa2 kezelést követően is szignifikánsan megemelkedett a C1-C2-es régióban a kontroll csoporthoz viszonyítva, majd öt órával az utóbbi kezelés után visszacsökkent alap szintre. A C1-C2-es szakasz transzverzális metszetein a NTG szignifikánsan lecsökkentette a CGRP rostok által lefedett területet, és szignifikánsan megemelte a c-Fos-, nNOS- és CaMKIIα-IR sejtek számát a Placeboval kezelt állatokhoz képest, amelyet az L-KYN-PROB, KYNAa1 és dózisfüggő módon a KYNAa2 képes volt kivédeni. A Western blot mérésekből származó eredmények megerősítették az immunhisztokémiai adatokat, miszerint a CGRP mennyisége szignifikánsan csökkent és az nNOS mennyisége szignifikánsan nőtt a C1-C2-es szegmentumban NTG hatására a Placebohoz képest, amelyet a KYNAa2-vel történt előkezelés kivédett. Az Open Field Tesztben a NTG szignifikánsan lecsökkentette a patkányok által megtett távolságot a Placebo csoporttal összehasonlítva, míg ez a különbség a KYNAa2 előkezelést kapott állatoknál nem volt megfigyelhető.

L-KYN-PROB kezelések hatására megfigyelhető jelentős kinurénsav emelkedés a C1-C2-es szegmentumban megerősíti a korábbi kísérleti eredményeket. A kinurénsav koncentrációjának

növekedése KYNAa2 kezelést követően a C1-C2-ben arra enged következtetni, hogy ez a származék legalább részben képes visszaalakulni a kiindulási vegyületté hasonlóan a KYNAa1-hez, amelyről kimutatták, hogy egér szérumban a kinurénsav koncentrációt is jelentősen megemeli. Mindezek alapján feltételezhetjük, hogy ezen származékok központi idegrendszeri hatásai részben direkt módon hatva és részben indirekt módon, visszaalakulva kinurénsavvá valósulnak meg.

A migrén patomechanizmusának egyik főszereplője a CGRP, amelynek NTG-indukálta csökkenése a C1-C2-es régióban az elsődleges trigeminális neuronokból történő transzmitter felszabadulást és egyben ezen idegsejtek aktiválódását jelzi, amely korábbi kísérletek alapján specifikus a trigeminális rendszerre. Ezek az adatok összhangban vannak más eredményekkel, amelyek szerint a trigeminális rendszer aktiválódása CGRP felszabadulással jár a perifériás és a centrális nyúlványokból és ennek következtében a migrénes roham során emelkedett CGRP szint mérhető. Mivel az L-KYN-PROB, KYNAa1 és dózisfüggő módon a KYNAa2 képes volt kivédeni a NTG-indukálta CGRP csökkenést, feltételezhetjük, hogy ez a hatás a fájdalomérzésben fontos szerepet játszó, és a perifériás trigeminális rendszerben jelen lévő glutamát és α7-nACh receptorok gátlása vagy a GPR-35 receptorok serkentése révén valósulhat meg, amely hatások bizonyítottan képesek csökkenteni a CGRP felszabadulást vagy annak következményeit vagy modulálni a nociceptív jelátvitelt.

A másodlagos trigeminális neuronok NTG-indukált aktiválódását jelzi a C1-C2 felszíni rétegeiben megfigyelt c-Fos immunreaktivitás növekedés, ami korábbi vizsgálatok alapján szintén specifikus a trigeminális rendszerre és feltételezhetően az elsődleges trigeminális neuronok aktiválásán keresztül valósul meg. A c-Fos expresszió növekedését a KYNAa2-vel történt előkezelés dózisfüggő módon védte ki, amely egyrészt megvalósulhat a perifériás nociceptorok gátlása révén, de ugyanakkor központi idegrendszeri hatás is valószínűsíthető. Ez utóbbit alátámasztja az, hogy a glutamát és α7-nACh receptorok jelen vannak az agyban elhelyezkedő trigeminális területeken és gátlásukkal csökkenthető a c-Fos expresszió vagy a glutamát felszabadulás. Továbbá, a közvetlenül a központi idegrendszerbe juttatott kinurénsav és analógjai fájdalomcsillapító hatásúak, és a perifériásan adagolt kinurénsavval ellentétben a KYNAa1 képes volt csökkenteni a NTG-indukált c-Fos expressziót a C1-C2-ben.

Irodalmi adatok alapján az önerősítő folyamatokban fontos szerepet játszó nNOS és CaMKIIα expressziójának emelkedése a C1-C2-ben a NTG hatására kialakuló és a migrén

patogenezisében is nagyon fontos szerepet játszó centrális szenzitizációs folyamatokat mutatja a trigeminális rendszerben, amely korábbi eredmények alapján szintén specifikus. Ezek a megfigyelések összhangban vannak azokkal az adatokkal, amelyek szerint fájdalmas stimulus hatására expressziójuk megemelkedik a másodlagos fájdalomérző neuronokban, és amelyek egyben azt is bizonyítják, hogy szerepük van a fájdalom idegrendszeri feldolgozásában a trigeminális rendszer területén is. A KYNAa2 dózisfüggő módon kivédte a NTG-indukált nNOS expresszió emelkedését és az L-KYN-PROB, KYNAa1 és szintén dózisfüggően a KYNAa2 csökkentette a CaMKIIα-IR sejtek számát, amely eredmények arra utalnak, hogy ezek az anyagok képesek kivédeni a centrális szenzitizációs folyamatokat, feltételezhetően főként az ionotróp glutamát receptorok gátlása révén. Az NMDA és AMPA receptorok alapvetőek a fájdalmas inger hatására kialakuló plasztikus anatómiai és funkcionális változások létrejöttében, amelyet az is bizonyít, hogy ezen receptorok gátlásával csökkenthetőek a centrális szenzitizációra utaló morfológiai és magatartásbeli változások. Ugyanakkor valószínűsíthető, hogy az előkezelések gátló hatásában a preszinaptikus α7-nACh receptorok blokkolása is szerepet játszhat csökkentve a glutamát felszabadulását az idegvégződésekből.

Az Open Field Teszt során a megtett távolságban NTG hatására megfigyelt csökkenés párhuzamba állítható más kísérletekkel, ahol a fájdalmas stimulus szintén csökkentette a megtett távolságot. Ez a csökkenés a KYNAa2 előkezelést követően nem jelent meg jelezve annak esetleges fájdalomcsillapító hatását. Érdekes módon, a megtett távolság alapszintje a KYNAa2-vel és Placeboval kezelt állatokban kismértékben mérséklődött, amely utalhat a kinurénsav származék közvetlen központi idegrendszeri hatására, amelyet alátámaszt az a megfigyelés is, hogy egy, a vér-agy gáton jól penetráló NMDA antagonista szintén csökkentette ezt a paramétert ellentétben egy nem jól penetráló antagonistával.

Összefoglalva, az L-KYN-PROB és a két kinurénsav származékkal történt előkezelések hatékonyak a NTG-indukált perifériás és centrális trigeminális aktiváció és szenzitizáció csökkentésében, amely egyrészt annak köszönhető, hogy megemelik a kinurénsav koncentrációját a központi idegrendszerben vagy az utóbbiak direkt módon is hathatnak feltehetően a kiindulási vegyület támadáspontjain. Adataink újabb és szélesebb körű eredményekkel támasztják alá azt a korábbi felvetést, miszerint a kinurénsav vagy származékai potenciális gyógyszerjelöltként jöhetnek szóba a migrén kezelésében.

Original publications directly related to the Ph.D. thesis:

I.

Pre-treatment with new kynurenic acid amide dose-dependently prevents the nitroglycerine-induced neuronal activation and sensitization in cervical part of trigemino-cervical complex.

Fejes-Szabó A, Bohár Z, Vámos E, Nagy-Grócz G, Tar L, Veres G, Zádori D, Szentirmai M, Tajti J, Szatmári I, Fülöp F, Toldi J, Párdutz A, Vécsei L.

J. Neural. Transm. 2014 Jul;121(7):725-38.

IF: 2.871

J Neural Transm (2014) 121:725–738
DOI 10.1007/s00702-013-1146-2

NEUROLOGY AND PRECLINICAL NEUROLOGICAL STUDIES - ORIGINAL ARTICLE

Pre-treatment with new kynurenic acid amide dose-dependently prevents the nitroglycerine-induced neuronal activation and sensitization in cervical part of trigemino-cervical complex

Annamária Fejes-Szabó · Zsuzsanna Bohár · Enikő Vámos · Gábor Nagy-Grócz · Lilla Tar · Gábor Veres · Dénes Zádori · Márton Szentirmai · János Tajti · István Szatmári · Ferenc Fülöp · József Toldi · Árpád Párdutz · László Vécsei

Received: 3 October 2013 / Accepted: 22 November 2013 / Published online: 3 January 2014
© Springer-Verlag Wien 2014

Abstract The systemic administration of nitroglycerine induces attacks in migraineurs and is able to activate and sensitize the trigeminal system in animals involving glutamate and α7-nicotinic acetylcholine receptors, among others. Kynurenic acid is one of the endogenous glutamate receptor antagonists, and exerts inhibitory action on the α7-nicotinic acetylcholine receptors. Since kynurenic acid penetrates the blood–brain barrier poorly, therefore a newly synthesized kynurenic acid amide, *N*-(2-*N*-pyrrolidinyl-ethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride ($KYNA_a$) was used with such a side-chain substitution to facilitate brain penetration in our study. We evaluated its modulatory effect on kynurenic acid concentration in the cervical part of trigemino-cervical complex (C1–C2) and in the model of nitroglycerine-induced trigeminal activation using male Sprague–Dawley rats. One hour after 1 mmol/kg bodyweight $KYNA_a$ administration, the kynurenic acid level increased significantly in C1–C2, which returned to the basal level at 300 min measured by high-performance liquid chromatography. $KYNA_a$ pre-treatment had dose-dependent, mitigating action on nitroglycerine-induced decrease in calcitonin gene-related peptide and increase in c-Fos, neuronal nitric oxide synthase and calmodulin-dependent protein kinase II alpha expression in the C1–C2. $KYNA_a$ also mitigated the behavioural changes after nitroglycerine. Thus, in this model $KYNA_a$ is able to modulate in a dose-dependent manner the changes in neurochemical markers of activation and sensitization of the trigeminal system directly and indirectly—via forming kynurenic acid, possibly acting on peripheral and central glutamate or α7-nicotinic acetylcholine receptors. These results suggest that application of kynurenic acid derivatives could be a useful therapeutic strategy in migraine headache in the future with a different mechanism of action.

Keywords Cervical part of trigemino-cervical complex · Kynurenic acid · Nitroglycerine · Trigeminal activation · Trigeminal sensitization · *N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride

Á. Párdutz, L. Vécsei contributed equally to this work.

A. Fejes-Szabó · Z. Bohár · E. Vámos · G. Nagy-Grócz · L. Tar · G. Veres · D. Zádori · M. Szentirmai · J. Tajti · Á. Párdutz · L. Vécsei (✉)
Department of Neurology, Faculty of Medicine, Albert Szent-Györgyi Clinical Centre, University of Szeged, Semmelweis u. 6, 6725 Szeged, Hungary
e-mail: vecsei.laszlo@med.u-szeged.hu

Z. Bohár · L. Vécsei
Neuroscience Research Group of the Hungarian Academy of Sciences and University of Szeged, Szeged, Hungary

I. Szatmári · F. Fülöp
Institute of Pharmaceutical Chemistry and Stereochemistry Research Group of the Hungarian Academy of Sciences, University of Szeged, Szeged, Hungary

J. Toldi
Department of Physiology, Anatomy and Neuroscience, University of Szeged, Szeged, Hungary

Introduction

Migraine is an intensively researched, very common neurological disease, with a partially known pathomechanism that includes activation and sensitization of the trigeminal system (Moskowitz 2008; D'Andrea and Leon 2010).

In most migraineurs, but not in healthy people (Sicuteri et al. 1987), systemic administration of the nitric oxide

donor nitroglycerine triggers a delayed migraine-like attack, which is similar to a spontaneous attack and is usually associated with symptoms that include nausea and photophobia (Christiansen et al. 1999). Moreover, nitroglycerine is able to sensitize the trigeminal system in humans (Di Clemente et al. 2009).

Various animal experiments have revealed that nitroglycerine can activate the trigeminal system, e.g. it is able to decrease the calcitonin gene-related peptide (CGRP) content of the primary trigeminal nociceptors in the cervical part of trigemino-cervical complex (C1–C2), suggesting the activation and transmitter release of first-order neurones (Pardutz et al. 2002). Nitroglycerine can also stimulate the second-order trigeminal neurones, increasing the c-Fos expression in the same area (Tassorelli and Joseph 1995). Moreover, nitroglycerine administration increases the neuronal nitric oxide synthase (nNOS) and calmodulin-dependent protein kinase II alpha (CaMKIIα) immunoreactivity in the C1–C2 of the rat (Pardutz et al. 2000, 2007), which may suggest a self-amplifying mechanism at the level of second-order trigeminal neurones relevant in the central sensitization process (Chacur et al. 2010; Fang et al. 2002). Taken together, these results indicate that the systemic administration of nitroglycerine is a valuable tool used to evaluate the activation and sensitization of the trigeminal system in animals.

Kynurenic acid is an endogenous end-product formed from L-kynurenine during the tryptophan metabolism. It is assumed that kynurenic acid plays an important role in the pathomechanism of several neurological disorders (Vecsei et al. 2013) and exerts a neuroprotective effect in certain neurodegenerative diseases (Nemeth et al. 2006; Fuvesi et al. 2012). There is evidence that it can also affect nociception (Mecs et al. 2009; Nasstrom et al. 1992; Pardutz et al. 2012), probably due to an antagonistic effect on *N*-methyl-D-asparate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and α7-nicotinic acetylcholine receptors (Pereira et al. 2002; Birch et al. 1988; Kessler et al. 1989) or an agonistic action on G-protein-coupled receptor-35 (Wang et al. 2006). Due to the poor ability of kynurenic acid to cross the blood–brain barrier (Fukui et al. 1991), its various derivatives with a better central nervous system action and a suggested similarity regarding their pharmacological effect were synthesized and successfully used under experimental conditions (Demeter et al. 2012; Gellert et al. 2012; Zadori et al. 2011). An earlier synthesized kynurenic acid derivative was more effective than the parent compound reducing the second-order trigeminal activation, as previously shown (Knyihar-Csillik et al. 2008) and experimental evidence suggests that this analogue has a similar pharmacological action as kynurenic acid with a presumed better blood–brain barrier penetrance (Marosi et al. 2010).

In the present study, we used a different, newly synthesized kynurenic acid amide ($KYNA_a$) which was prepared directly from kynurenic acid resulting in an altered side-chain.

Our first aim was to determine how peripheral $KYNA_a$ administration affects kynurenic acid concentrations in the peripheral blood and the central nervous system, in the cervical trigeminal complex by using high-performance liquid chromatography (HPLC).

Our next objective was to test whether the pre-treatment with this new $KYNA_a$ exerts a modulatory effect on the nitroglycerine-induced changes in CGRP, c-Fos, nNOS and CamKIIα expression. We also wanted to test if these morphological alterations reflecting trigeminal activation and sensitization are paralleled with behavioural changes of rats in the open field test.

Materials and methods

Animals

The procedures utilized in this study followed the guidelines for the Use of Animals in Research of the International Association for the Study of Pain and the directives of the European Economic Community (86/609/ECC). They were approved by the Committee of Animal Research at the University of Szeged (I-74-12/2012) and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XI./352/2012). Ninety adult male Sprague–Dawley rats weighing 200–250 g were used. The animals were maintained on a 12-h dark–12-h light cycle under standard laboratory conditions, with tap water and regular rat chow available ad libitum.

Drugs

The new kynurenic acid amide (Patent number #P0900281/PCT/HU2010/00050), *N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride ($KYNA_a$) (Fig. 1), was synthesized in the Department of Pharmaceutical Chemistry, University of Szeged by reacting kynurenic acid with 2-pyrrolidinoethylamine resulting in a side-chain containing tertiary nitrogen in pyrrole ring system with regard to

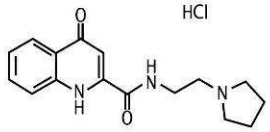

Fig. 1 The structure of the new kynurenic acid amide, *N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride ($KYNA_a$)

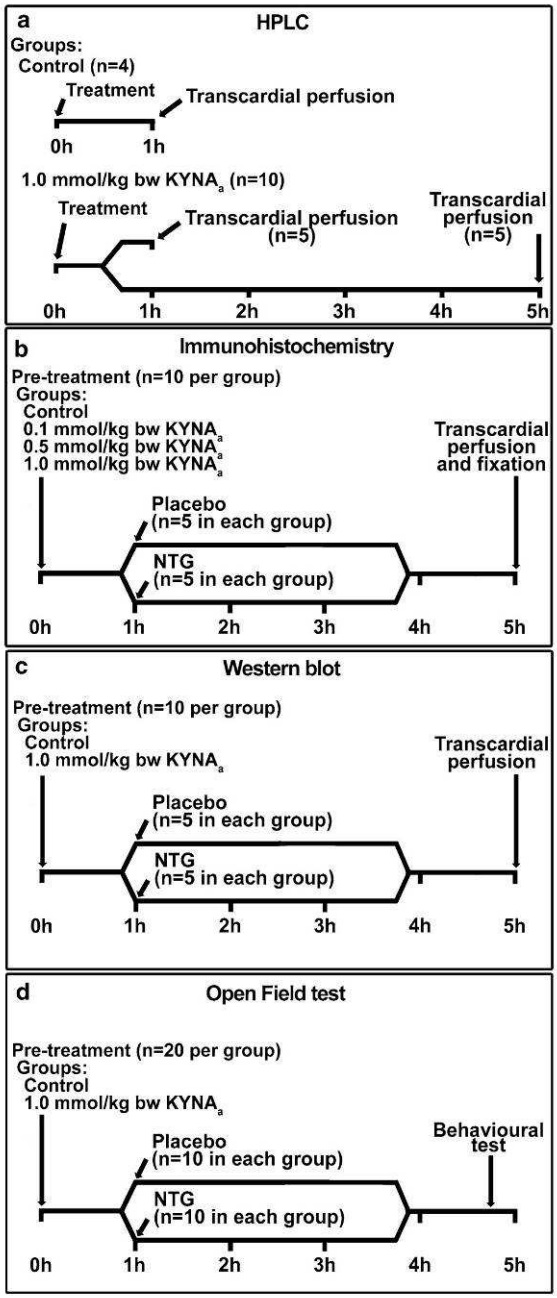

Fig. 2 Schematics of the experimental setup for high-performance liquid chromatography (HPLC) (**a**), immunohistochemistry (**b**), Western blot (**c**) and open field test (**d**). *kg bw* kg bodyweight

the following structural properties: (1) the presence of a water-soluble side-chain, (2) the inclusion of a new cationic centre, and (3) side-chain substitution to facilitate brain penetration (Fulop et al. 2012).

Nitroglycerine (Nitrolingual®) was purchased from Pohl-Boskamp and was administered intraperitoneally in a dosage of 10 mg/kg bodyweight (bw) as described before (Pardutz et al. 2000). Nitrolingual placebo was obtained from Pohl-Boskamp.

For the HPLC measurements The animals were divided into two groups. In the control group, the animals received only the intraperitoneal (i.p.) vehicle solution (physiological saline) as pre-treatment ($n = 4$), without drug. In the second group ($n = 10$), the rats were pre-treated with an i.p. injection of 1 mmol/kg bw $KYNA_a$ (diluted to 2 ml, pH 7.4) (Fig. 2a).

For immunohistochemistry The animals were divided into four groups ($n = 10$ per group). The animals of the control group received only the i.p. vehicle solution (physiological saline) as pre-treatment. In the remaining 3 groups, the rats were pre-treated with an i.p. injection of $KYNA_a$ at the dose of 0.1, 0.5 and 1 mmol/kg bw, respectively (diluted to 2 ml, pH 7.4) (Fig. 2b). One hour later in each group, half of the animals ($n = 5$) received an i.p. injection of nitroglycerine, while the other half of the rats ($n = 5$) received an i.p. injection of the placebo of nitroglycerine (Fig. 2b).

For Western blot The animals were divided into two groups ($n = 10$ per group). The animals in the control group received only the i.p. vehicle solution (physiological saline) as pre-treatment. In the second group, the rats were pre-treated with an i.p. injection of 1 mmol/kg bw $KYNA_a$ (Fig. 2c). The administration of nitroglycerine was performed as described above (Fig. 2c).

For the Open Field Test The animals were separated into two groups ($n = 20$ per group). In the control group, the animals received i.p. vehicle solution (physiological saline) as pre-treatment, without drug. In the second group, the rats were pre-treated with an i.p. injection of $KYNA_a$ at the dose of 1 mmol/kg bw (Fig. 2d). The administration of nitroglycerine was performed as described above (Fig. 2d).

Kynurenic acid detection with high-performance liquid chromatography (HPLC)

Sampling and sample preparation

At set time points (60 and 300 min, $n = 5$ per group) following the i.p. injection with the $KYNA_a$, the rats were deeply anaesthetized with chloral hydrate (0.4 g/kg bw, Sigma-Aldrich) and transcardially perfused with phosphate-buffered saline for 5 min. The animals of the control group ($n = 4$) underwent a similar procedure with one measurement point at 60 min. Then the brain samples with the corresponding upper cervical spinal cord segments (C1–C2) were removed. After dissection, the samples of C1–C2 were stored at −70 °C until analysis. The samples

were weighed and then sonicated in an ice-cooled solution (250 µl) comprising perchloric acid (2.5 % w/w, Scharlau), internal standard (3-nitro-L-tyrosine, 2 µM, Sigma-Aldrich,) and distilled water for 1.5 min in an Eppendorf tube. The content of the Eppendorf tube was centrifuged at 12,000 RPM for 10 min at 4 °C. From the supernatant 100 µl was transferred to a test vial.

Chromatographic conditions

The kynurenic acid concentrations of the samples were quantified based on the slightly modified method of Herve et al. (1996), with an Agilent 1100 HPLC system (Agilent Technologies). The system was equipped with a fluorescent and an UV detector, the former was applied for the determination of kynurenic acid and the latter for the determination of the internal standard. Chromatographic separations were performed on an Onyx Monolithic C18 column, 100 mm × 4.6 mm I.D. (Phenomenex Inc.) after passage through a Hypersil ODS pre-column, 20 × 2.1 mm I.D., 5 µm particle size (Agilent Technologies) with a mobile phase composition of 0.2 M zinc acetate (Sigma-Aldrich)/acetonitrile (Scharlau) = 95/5 (v/v), the pH of which was adjusted to 6.2 with acetic acid (VWR International), applying isocratic elution. The flow rate and the injection volume were 1.5 ml/min and 50 µl, respectively. The fluorescent detector was set at excitation and emission wavelengths of 344 and 398 nm. The UV detector was set at 365 nm wavelength.

HPLC method validation

Calibration curve and linearity Calibrants were prepared at six different concentration levels, from 1 to 100 nM and 0.5 to 5 µM for kynurenic acid and the internal standard, respectively. Three parallel injections of each solution were made under the chromatographic conditions described above. The peak area responses were plotted against the corresponding concentration, and the linear regression computations were carried out by the least square method with the freely available R program (R Foundation for Statistical Computing, R Development Core Team). Very good linearity was observed throughout the investigated concentration ranges for kynurenic acid and the internal standard when either fluorescence or UV detection was applied.

Selectivity The selectivity of the method was checked by comparing the chromatograms of kynurenic acid and the internal standard for a blank central nervous system sample and those for a spiked sample. All compounds could be detected in their own selected chromatograms without any significant interference.

LOD and LLOQ Limit of detection (LOD) and lower limit of quantitation (LLOQ) were determined via signal-to-noise ratio with threshold 3, according to the ICH guidelines (Validation of Analytical Procedures: Text and Methodology Q2 (R1), International Conference on Harmonization). The LOD was 0.4 nM, while the LLOQ was 1 nM for kynurenic acid, respectively.

Precision Replicate HPLC analysis showed that the relative standard deviation was ≤3.1 % for the concentration and ≤0.1 % for the retention time.

Recovery The relative recoveries were estimated by measuring spiked samples of kynurenic acid at two different concentrations with three replicates of each. No significant difference was observed for the lower and higher concentrations. The recoveries ranged from 82 to 92 % for kynurenic acid, respectively.

Immunohistochemistry

Histological procedure

Immunohistochemical procedures were performed as described earlier (Vamos et al. 2009, 2010) supplemented with the immunohistochemistry for c-Fos. Briefly, 4 h after treatment with placebo or nitroglycerine, the rats, anaesthetized with chloral hydrate, were perfused and fixed transcardially and the C1–C2 segment of the cervical spinal cord was removed, this is the area where most of trigeminal afferents involved in headache reach the second-order neurones (Strassman et al. 1994). After postfixation and cryoprotection, 30 µm cryostat sections were cut and treated as free-floating sections. After suppression of the endogenous peroxidase activity and several rinses in phosphate-buffered saline containing 1 % Triton X-100 (PBST, VWR International), sections were incubated in PBST containing (1) rabbit anti-rat CGRP polyclonal antibody (Sigma-Aldrich, C-8198, dilution: 1:20,000, incubation: overnight at room temperature) or (2) rabbit anti-rat c-Fos (H-125) polyclonal antibody (Santa Cruz Biotechnology, sc-7202, dilution: 1:1,000, incubation: overnight at room temperature) or (3) rabbit anti-rat nNOS polyclonal antibody (EuroProxima, 2263B220-1, dilution: 1:5,000, incubation: two nights at 4 °C) or (4) mouse anti-rat CaMKIIα monoclonal antibody (Sigma-Aldrich, C-265, dilution: 1:2,000, incubation: four nights at 4 °C). The immunohistochemical reaction was visualized by using Vectastain Elite avidin–biotin kits (Vector Laboratories; PK6101 for c-Fos, nNOS and CGRP; PK6102 for CaMKIIα) and stained with nickel ammonium sulphate (VWR International)-intensified 3,3′-diaminobenzidine (Sigma-Aldrich). The specificity of the immune reactions was checked by omitting the primary antiserum.

Data evaluation

All evaluations were performed by an observer blind to the experimental groups. The detailed methodologies were described previously (Vamos et al. 2009, 2010).

In brief, the area covered by CGRP-immunoreactive fibres was determined using Image Pro Plus 6.2® image analysis software (Media Cybernetics). Photomicrographs of sections were taken under the 10× objective of a Nikon Optiphot-2 light microscope fitted with an Olympus DP70 CCD camera (Olympus Corporation). After image acquisition, the laminae I–II in dorsal horn were determined manually as area of interest and a threshold grey level was established with the Image Pro Plus 6.2® image analysis software, as described in earlier studies (Vamos et al. 2009, 2010). The program expressed the area innervated by the immunoreactive fibres as the number of pixels with densities above the threshold. For the calibration, we measured formations with known area. The area covered by immunoreactive fibres per dorsal horn was calculated as the multiplication of the average number of pixels for the individual dorsal horn and the area of one pixel.

The c-Fos-, nNOS- and CaMKIIα-immunoreactive cells were counted under the 10× objective of a Nikon Optiphot-2 light microscope (Nikon Instruments) in laminae I–II of the C1–C2. The c-Fos-positive neurones were scored as those with obvious specific nuclear staining; the nNOS-immunoreactive neurones as those exhibiting cytoplasmic and dendritic staining and a nucleus; and the CaMKIIα-labelled cells with a clearly increased immunoreactivity relative to the background. The number of immunoreactive neurones per sections was calculated, i.e. the numbers of immunopositive neurones present on the two dorsal horns were summed. Thereafter, the results on the individual sections were averaged for each animal.

Photomicrographs were taken under the 40x objective of a Zeiss Axio Imager M2 Upright Microscope (Carl Zeiss MicroImaging) fitted with an AxioCam MRc camera (Carl Zeiss MicroImaging) and Adobe Photoshop CS2 9.0 graphics program was used to create the artwork.

Western blot

Sampling and sample preparation

In both groups 4 h after the nitroglycerine or placebo injections, the rats were deeply anaesthetized with chloral hydrate as described above, perfused transcardially with 100 ml ice-cold phosphate-buffered saline and the cervical (C1–C2) spinal cord was removed. Until measurements, the samples were stored at −70 °C. The C1–C2 segments were sonicated in ice-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-Aldrich). The lysates were cleared from cellular debris by centrifugation at 12,000 RPM for 10 min at 4 °C and supernatants were aliquoted and stored at −20 °C. Protein concentration was measured according to BCA protein assay method with BCA Protein Assay Kit (Novagen) using bovine serum albumin as a standard. Samples were cooled on ice during the whole procedure. Prior to loading, each sample was mixed with sample buffer, and denaturated by boiling for 3 min. Equal amounts of protein samples (20 μg/lane) were separated by standard SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 10 % (for nNOS and β-actin) and 15 % (for CGRP) Tris–Glycine gel and electrotransferred onto Amersham Hybond-ECL nitrocellulose membrane (0.45 and 0.2 μm pore size selectively, GE Healthcare). The Page Ruler Prestained Protein Ladder (Fermentas, 10–170 kDa) was used to determine approximate molecular weights. Following the transfer, membranes were blocked for 1 h at room temperature in Tris-buffered saline containing Tween 20 (TBST, MP Biomedicals) and 5 % non-fat dry milk powder and incubated in TBST containing 1 % non-fat dry milk and (1) rabbit anti-rat CGRP polyclonal antibody (Sigma-Aldrich, C-8198, dilution: 1:2,000, incubation: overnight at room temperature) or (2) mouse anti-rat nNOS monoclonal antibody (BD Biosciences, 610308, dilution: 1:2,500, incubation: overnight at room temperature) or (3) mouse anti-rat β-actin monoclonal antibody (Calbiochem, CP01, dilution: 1:10,000, incubation: overnight at room temperature). Next day after several rinses, membranes were incubated in TBST containing 1 % non-fat dry milk and horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology; sc-2030 and sc-2031) for 1 h at room temperature. Protein bands were visualized after incubation of membranes with the Super-Signal West Pico Chemiluminescent Substrate (Pierce) using Carestream Kodak BioMax Light film (Kodak).

Data evaluation

For densitometric analyses, films were scanned and quantified using Java ImageJ 1.47v analysis software (National Institutes of Health). The results were normalized to the absolute control animals. β-actin served as a control to ensure loading of equivalent amounts of sample proteins.

Open field test

Testing procedure

Before the open field test, animals were habituated in the dimly lit, quiet test room for at least 30 min. The

experiments were performed between 11 a.m. and 4 p.m. during the light cycle of rats. To avoid misinterpretation related to the acute cardiovascular effects of nitroglycerine and in parallel with earlier studies showing delayed behavioural alterations after systemic nitroglycerine in mice (Markovics et al. 2012), we tested the rats 3 h and 40 min after the nitroglycerine or placebo injections. The animals were placed in the open field box (48 × 48 × 40 cm, Experimetria Ltd., Hungary) and ambulation time, ambulation distance, local time and the number of rearings were registered for 15 min, and were evaluated using the Conducta 1.0 behaviour analysis program (Experimetria Ltd., Hungary). The rats did not receive any food or water during the observation period. The test box was cleaned and decontaminated after each animal.

Statistical analysis

Statistical analysis of the measurement data was carried out with IBM SPSS Statistics software (Version 20, IBM), using one-way analysis of variance followed by the Tukey or Tamhane post hoc test (except for the data of HPLC analyses), depending on the variances of the data, with $p < 0.05$ taken as statistically significant. Group values are reported as mean ± SEM. As in the treated groups the data of HPLC analysis showed non-Gaussian distribution, the non-parametric Kruskal–Wallis test was applied for group comparisons followed by Mann–Whitney U test for post hoc analysis, with $p < 0.05$ taken as statistically significant. The group values of HPLC analyses were reported as median and interquartile range.

Results

Kynurenic acid detection with HPLC

The HPLC measurements clearly indicated a significant ($^{*}p < 0.05$), more than twofold increase in kynurenic acid level in the C1–C2 60 min after 1 mmol/kg bw $KYNA_a$ administration (25.38 pmol/g wet weight, interquartile range: 23.26–40.80 pmol/g wet weight) compared to vehicle-treated samples (11.48 pmol/g wet weight, interquartile range: 8.85–15.00 pmol/g wet weight) (Table 1), while the concentration of the kynurenic acid decreased to baseline at 300 min (9.02 pmol/g wet weight, interquartile range: 6.43–30.92 pmol/g wet weight) (Table 1). Although it needs different validation methods, we screened the kynurenic acid concentration on the blood samples too. Preliminary results suggest a robust increase (approximately 50×) in the peripheral kynurenic acid levels reaching μmolar concentrations (data not shown).

Table 1 Results of HPLC measurements from the C1–C2 at different time points after $KYNA_a$ treatment

Groups		Kynurenic acid concentrations (pmol/g wet weight; median and interquartile range)
Control (saline) ($n = 4$)	60 min	11.48 (8.85–15.00)
1 mmol/kg bodyweight $KYNA_a$ ($n = 10$)	60 min ($n = 5$)	25.38 (23.26–40.80)*
	300 min ($n = 5$)	9.02 (6.43–30.92)

C1–C2 cervical part of trigemino-cervical complex, *$KYNA_a$* kynurenic acid amide, *N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride

* $p < 0.05$

Immunohistochemistry

The transverse sections of the cervical spinal cord (C1–C2) demonstrated abundant CGRP-positive fibres, and c-Fos-, nNOS- and CaMKIIα-immunoreactive neurones in the superficial layers (laminae I–II) of the dorsal horn. The area covered by immunoreactive fibres and the number of immunoreactive cells did not differ significantly between sections located at the various levels along rostrocaudal axis or between the right and left dorsal horns of the cervical segments.

In the control group (pre-treated with saline, $n = 10$), reduced CGRP staining can be observed on the sections in I–II laminae after nitroglycerine treatment compared to placebo-treated rats (Fig. 3). This decrease is reflected in area covered by CGRP as well, i.e. significant difference can be measured in nitroglycerine-treated rats as compared with placebo-treated animals ($^{**}p < 0.01$; Fig. 7a). In the same group, increased c-Fos, nNOS and CaMKIIα immunoreactivity (Figs. 4, 5, 6) can be observed in the superficial layers of the C1–C2 after treatment with nitroglycerine compared to placebo. The differences in the numbers of immunoreactive neurones proved to be statistically significant for all three immunostainings ($^{**}p < 0.01$, $^{***}p < 0.001$; Fig. 7b–d).

The pre-treatment with $KYNA_a$ affected dose-dependently the nitroglycerine-induced changes in the immunoreactivity of all studied markers. The administration of $KYNA_a$ in a dosage of 0.1 mmol/kg bw did not influence any of the alterations caused by nitroglycerine, whereas the pre-treatment with higher dosages of $KYNA_a$ (0.5 and 1 mmol/kg bw) was able to significantly reduce the effects of nitroglycerine on the CGRP-, c-Fos-, nNOS- and CaMKIIα-related changes in the C1–C2 ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; Figs. 3, 4, 5, 6, 7a–d).

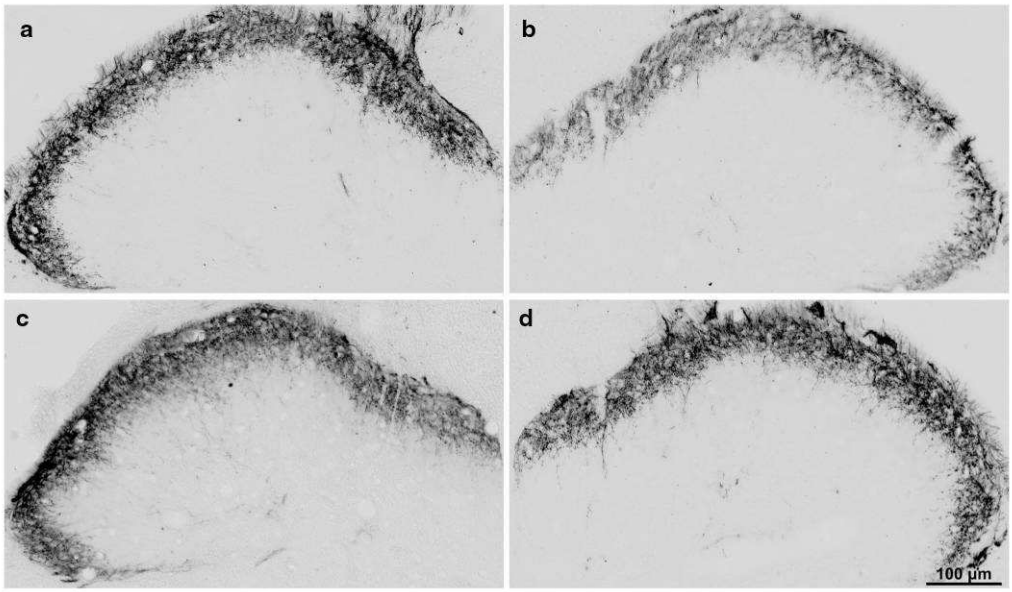

Fig. 3 Calcitonin gene-related peptide (CGRP) immunoreactivity. Representative photos of the cervical part of trigemino-cervical complex (C1–C2) in the rat after CGRP immunohistochemistry. In the control group (**a**, **b**), the immunoreactive staining of the CGRP fibres is decreased after the nitroglycerine injection (**b**) as compared with the placebo-treated animals (**a**). After 1 mmol/kg bodyweight $KYNA_a$ pre-treatment (**c**, **d**), the change otherwise induced by nitroglycerine cannot be observed (**d**) relative to the animals treated with placebo (**c**). *Scale bar* 100 µm

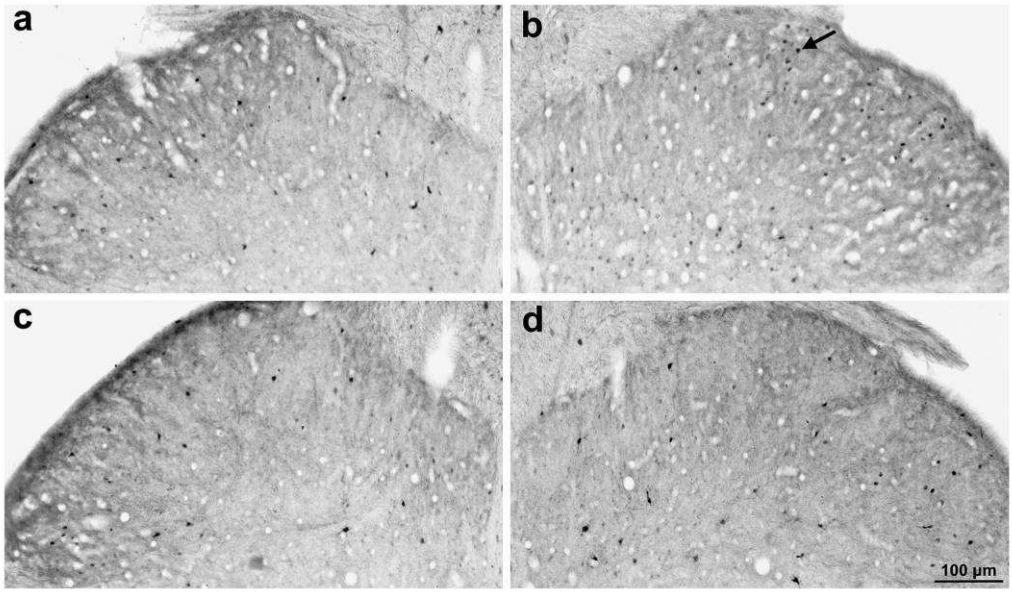

Fig. 4 c-Fos immunoreactivity. Representative photos of c-Fos-immunoreactivity in the cervical part of trigemino-cervical complex (C1–C2) in the rat. In the control group (**a**, **b**), more c-Fos-immunoreactive cells can be observed in the superficial laminae of the C1–C2 after nitroglycerine injection (**b**) as compared with the placebo-treated animals (**a**). This nitroglycerine-induced change is not observed after 1 mmol/kg bodyweight $KYNA_a$ pre-treatment (**c**, **d**). The black arrow in "**b**" indicates a c-Fos-positive cell. *Scale bar* 100 µm

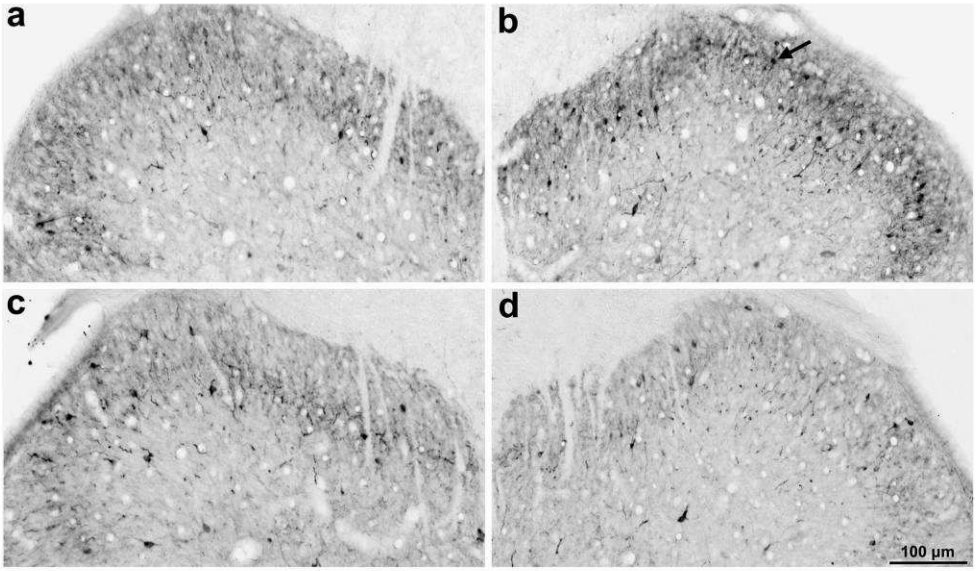

Fig. 5 Neuronal nitric oxide synthase (nNOS) immunoreactivity. Histological photos of nNOS-stained neurones in the cervical part of trigemino-cervical complex (C1–C2) in the rat. In the control group (**a**, **b**), an increased nNOS immunoreactivity can be observed in the superficial laminae of the C1–C2 after nitroglycerine injection (**b**) as compared with the placebo-treated animals (**a**). In the group pre-treated with 1 mmol/kg bodyweight $KYNA_a$ (**c**, **d**), the nitroglycerine-induced change is not observed. The *black arrow* in "**b**" points to an nNOS-immunoreactive neurone. *Scale bar* 100 µm

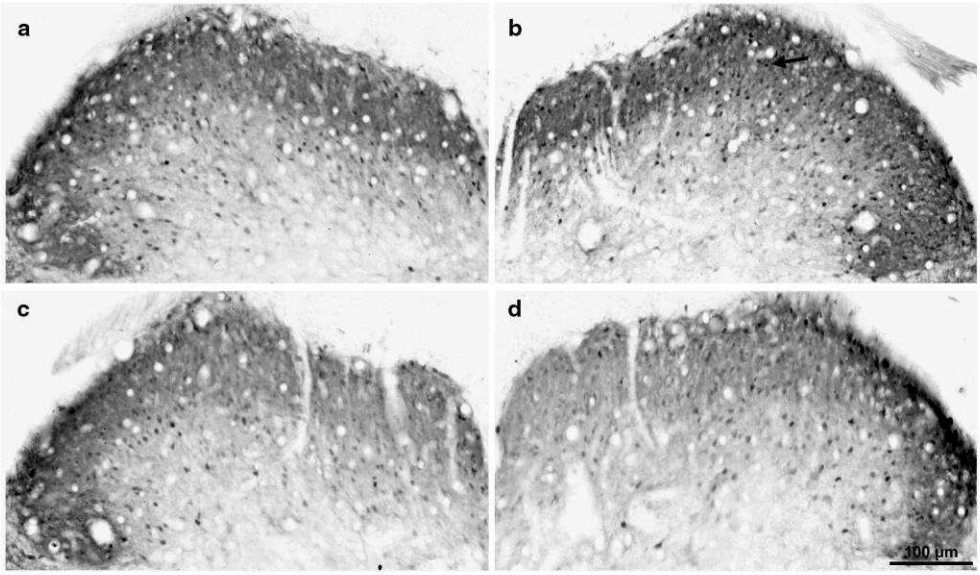

Fig. 6 Calmodulin-dependent protein kinase II alpha (CaMKIIα) immunoreactivity. Typical photos showing CaMKIIα-immunoreactive cells in the cervical part of trigemino-cervical complex (C1–C2) in the rat. Increased CaMKIIα staining can be observed in the superficial laminae of the C1–C2 in the control group (**a**, **b**) after nitroglycerine injection (**b**) as compared with the placebo-treated animals (**a**). In the 1 mmol/kg bodyweight $KYNA_a$ pre-treated group (**c**, **d**), this enhancement induced by nitroglycerine is not visible after nitroglycerine injection (**d**) relative to the placebo treatment (**c**). The *black arrow* in "**b**" points to a CaMKIIα-immunopositive neurone. *Scale bar* 100 µm

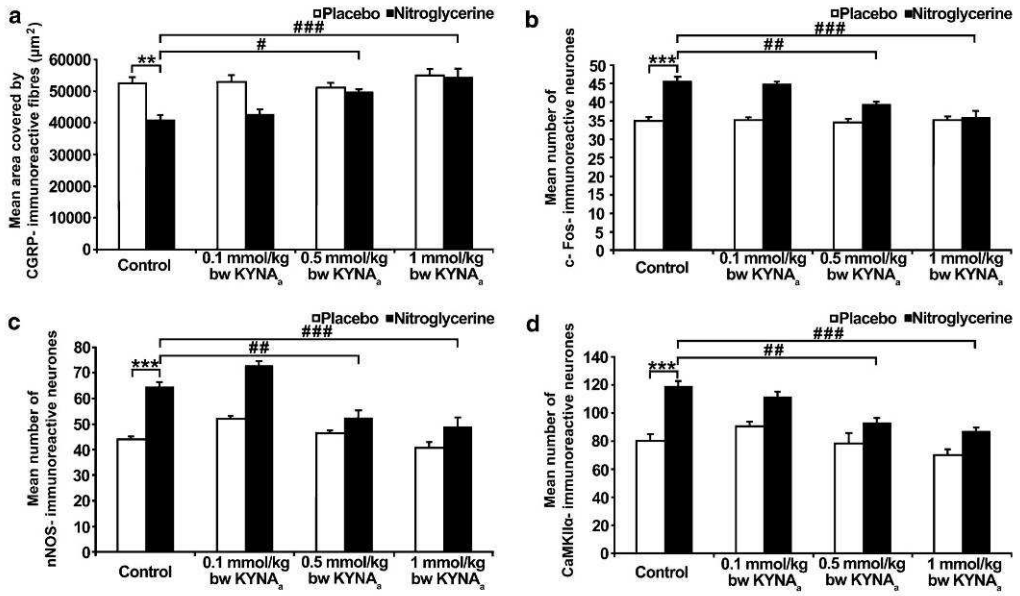

Fig. 7 *Diagrams* illustrating the measurement data in the control, 0.1, 0.5 and 1 mmol/kg bodyweight (bw) $KYNA_a$ groups after various immunohistochemical stainings. Within the groups, the differently *coloured columns* relate to the rats treated with placebo (*white bars*) or nitroglycerine (*black bars*) (means + SE of mean, $n = 5$ per column). **a** The mean area covered by calcitonin gene-related peptide (CGRP)-immunoreactive fibres. In the control group, nitroglycerine injection significantly decreased the mean area covered by CGRP-immunoreactive fibres in the cervical part of trigemino-cervical complex (C1–C2) as compared with the placebo-treated animals ($^{**}p < 0.01$). 0.1 mmol/kg bw dose of $KYNA_a$ did not significantly reduce the nitroglycerine-induced changes, while 0.5 and 1 mmol/kg bw doses of $KYNA_a$ were able to significantly decrease the effect of nitroglycerine ($^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$). **b** The mean number of c-Fos-immunoreactive neurones. Nitroglycerine injection significantly increased the mean number of c-Fos-immunoreactive cells in the C1–C2 in the control group as compared with the placebo-treated rats ($^{***}p < 0.001$). We could not detect significant difference between nitroglycerine-treated rats of control group and of group receiving 0.1 mmol/kg bw dose of $KYNA_a$, while there was significant difference between nitroglycerine-treated animals of control group and of groups pre-treated with 0.5 and 1 mmol/kg bw dose of $KYNA_a$ ($^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$). **c** The average number of neuronal nitric oxide synthase (nNOS)-immunoreactive cells. Nitroglycerine injection significantly enhanced the mean number of nNOS-immunoreactive neurones in the C1–C2 in the controls as compared with the rodents treated with placebo ($^{***}p < 0.001$). There was no difference in results of animals receiving nitroglycerine between the control and 0.1 mmol/kg bw $KYNA_a$ group. Both 0.5 and 1 mmol/kg bw dose of $KYNA_a$ reduced the effect of nitroglycerine as compared with the rats of the control group ($^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$). **d** The mean number of calmodulin-dependent protein kinase II alpha (CaMKIIα)-immunoreactive neurones. The mean number of CaMKIIα-immunoreactive cells was significantly increased after nitroglycerine injection in the C1–C2 in the control group as compared with the placebo treatment ($^{***}p < 0.001$). 0.1 mmol/kg bw dose of $KYNA_a$ was not, while 0.5 and 1 mmol/kg bw dose of $KYNA_a$ were able to decrease the nitroglycerine-induced changes in CaMKIIα-expression as compared with the animals of the control group ($^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$)

Western blot

Western blot analysis of the C1–C2 region confirmed the results obtained by CGRP and nNOS immunohistochemistry. A band characteristic of the CGRP peptide was identified at 15 kDa and nNOS protein was identified at 155 kDa (Fig. 8a, c). Densitometric analyses confirmed that the CGRP bands were significantly decreased ($^{*}p < 0.05$) and nNOS bands were significantly enhanced ($^{***}p < 0.001$) in segments C1–C2 after nitroglycerine administration as compared with the placebo-treated animals (Fig. 8b, d). This effect of nitroglycerine on CGRP and nNOS was attenuated by pre-treatment with 1 mmol/kg bw $KYNA_a$ (Fig. 8a–d).

Open field test

Treatment with nitroglycerine significantly decreased the ambulation distance of the animals compared to placebo-treated rats ($^{*}p < 0.05$; Fig. 9). Pre-treatment with 1 mmol/kg bw $KYNA_a$ attenuated this difference but a tendency for a lower ambulation distance was observed (Fig. 9). There were no significant changes in ambulation time, local time or in the number of rearing between the subgroups (data not shown).

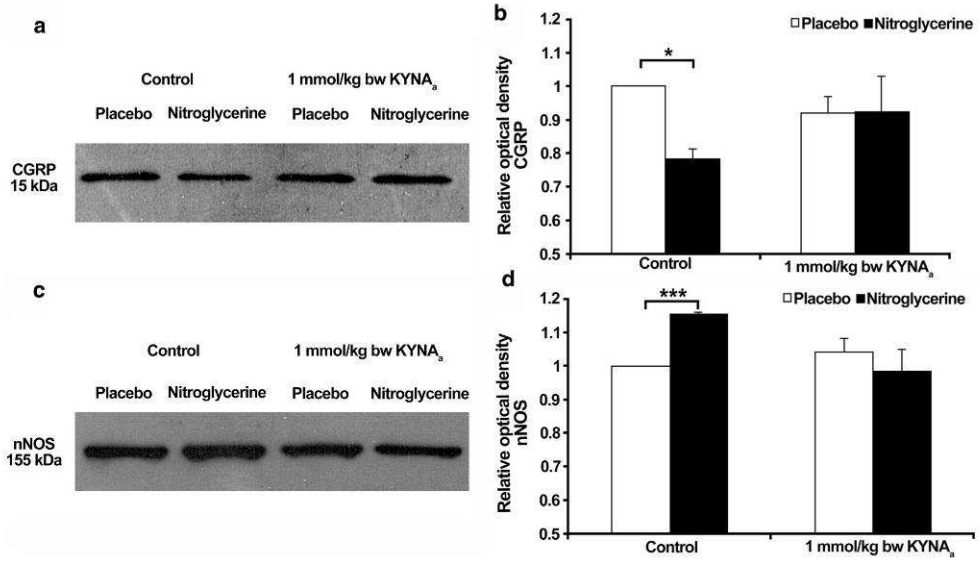

Fig. 8 Western blot of calcitonin gene-related peptide (CGRP) and neuronal nitric oxide synthase (nNOS) in the C1–C2 segments of the spinal cord. Density of CGRP band decreased (**a**–**b**) and density of nNOS band increased (**c**–**d**) significantly after nitroglycerin administration compared to the placebo in the control group ($*p < 0.05$; $***p < 0.001$), which was attenuated by pre-treatment with 1 mmol/kg bodyweight (bw) $KYNA_a$

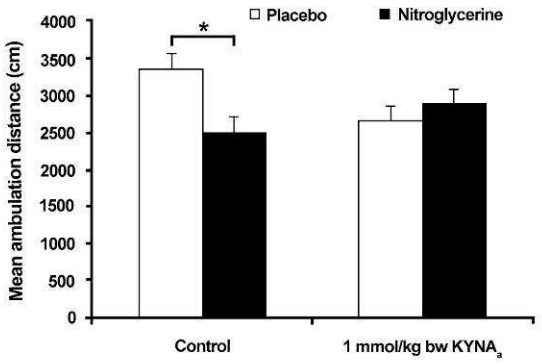

Fig. 9 *Diagram* showing the results of open field test. In the control group, treatment with nitroglycerine decreased the ambulation distance significantly compared to placebo-treated rats ($*p < 0.05$). Pre-treatment with 1 mmol/kg bodyweight (bw) $KYNA_a$ attenuated this effect of nitroglycerine, however, a tendency for a lower ambulation distance can be observed as compared with placebo-treated rats of control group as well

Discussion

In the present work, we confirmed earlier experimental findings showing that systemic administration of nitroglycerine exerts a stimulatory effect on the trigeminal neurones, inducing the reduction of CGRP and the up-regulation of c-Fos, nNOS and CaMKIIα in the C1–C2. The nitroglycerine-induced decrease in the area covered by CGRP-immunoreactive fibres and in the density of Western blot bands in cervical segments 1–2 of spinal cord indicates the release of CGRP from the central terminals of the primary trigeminal afferents, which is a general event after the activation of first-order trigeminal neurones (Zhang et al. 1994), and is in accordance with our own previous observations (Pardutz et al. 2002). Nitroglycerine-related activation also involves the second-order trigeminal neurones, as reflected by the elevated c-Fos expression observed now and previously by other researchers (Tassorelli and Joseph 1995). Furthermore, it can lead to a sensitization process in the C1–C2, marked by the enhancement of nNOS and CaMKIIα, both of these enzymes playing an important role in the development of pain-related central sensitization (Chacur et al. 2010; Fang et al. 2002).

The nitroglycerine-related activation of the C1–C2 neurones probably involves indirect, central action. The nitric oxide generated from nitroglycerine activates the Aδ and C fibres of the primary trigeminal neurones (Pardutz et al. 2000) which causes activation of the second-order trigeminal neurones leading to an increase in the c-Fos, nNOS and CaMKIIα expressions there. Supporting this theory, the increased c-Fos expression after nitroglycerine in the second-order trigeminal neurones can

be blocked by the destruction of capsaicin-sensitive fibres (Tassorelli et al. 1997). The relevance of the nitroglycerine model is also underlined by observations that these effects appear to be selective for the trigeminal system, as no changes were detected in most of these markers of activation and sensitization of the trigeminal system, i.e. in the number of nNOS- and CaMKIIα-immunoreactive neurones as well as in the area innervated by CGRP and serotonin immunoreactive fibres at the level of upper thoracic segments (Pardutz et al. 2000, 2002, 2007). In humans, administration of nitroglycerine induces a delayed migraine-like attack in migraineurs (Sicuteri et al. 1987) with typical migraine symptoms (Christiansen et al. 1999), which cannot be observed in healthy volunteers and is able to sensitize the trigeminal system (Di Clemente et al. 2009). Based on results from animal experiments, glutamate and α7-nicotinic acetylcholine receptors can play important role in this process. The activation of the first-order trigeminal neurones leads to an increased level of glutamate in the caudal part of spinal trigeminal nucleus, which correlates with the changes in sensory thresholds on the face of the rat (Oshinsky and Luo 2006). Probably via the NMDA receptors (Wang and Mokha 1996), the activation of the first-order trigeminal neurones leads to activation of the second-order neurones (Burstein et al. 1998). Experimental results suggest that NMDA receptors are involved in the nitric oxide synthesis (Entrena et al. 2005) and that there is an interaction between the NMDA and the nitric oxide/cGMP system via the activation of nNOS (Bredt et al. 1990). Additionally, the NMDA-induced excitotoxicity depends on the excess nitric oxide produced by the activation of nNOS (Garthwaite et al. 1988). The role of NMDA receptors in trigeminal pain processing is also supported by human data, whereas ketamine, an NMDA receptor antagonist was able to decrease migraine pain (Nicolodi and Sicuteri 1995). Furthermore, other glutamate receptors are also present in the caudal part of spinal trigeminal nucleus (Tallaksen-Greene et al. 1992), and can also contribute to this process. This concept is confirmed by that their antagonists are able to block the enhancement in the number of c-Fos-immunoreactive neurones (Mitsikostas et al. 1999) and the evoked potential responses (Storer and Goadsby 1999) in the caudal part of spinal trigeminal nucleus. The activation of second-order neurones can be modified by α7-nicotinic acetylcholine receptors as well, which presynaptically can influence the transmission of nociceptive information to the central nervous system (McGehee et al. 1995; Gray et al. 1996).

The results of the open field testing also showed a marked effect of nitroglycerine. The ambulation distance of the treated rats decreased significantly which may also reflect a pain condition (Denenberg 1969).

In the present experiments, the $KYNA_a$ proved to be able to attenuate dose-dependently the effects of nitroglycerine. 0.1 mmol/kg bw dose of $KYNA_a$ has not, while 0.5 and 1 mmol/kg bw doses of $KYNA_a$ have significantly reduced the decrease in CGRP immunoreactivity and the increases in c-Fos, nNOS and CaMKIIα expression, suggesting a blockade of trigeminal activation and sensitization. This effect was also supported by the Western blot results for CGRP and nNOS showing that employing the maximal dosage pre-treatment with $KYNA_a$ attenuated the nitroglycerine-induced changes. HPLC measurements suggesting a robust increase in kynurenic acid concentration in the blood and a more than twofold increase in central nervous system 1 h after $KYNA_a$ treatment supports the theory that $KYNA_a$ is transformed, at least partially, to kynurenic acid which might be also responsible for the observed effects. Since the increase of kynurenic acid in the central nervous system was observed in a much lesser extent, although the central effect cannot be fully excluded, the witnessed modulatory effects might be related to the peripheral action of kynurenic acid derived from $KYNA_a$. Meanwhile the direct effect of the $KYNA_a$ on the peripheral and central arm of the trigeminal system is also possible with a theoretical effect similar to kynurenic acid. This is underlined by the similar pharmacological action of an older kynurenic acid derivative with a presumed better blood–brain barrier penetrance (Marosi et al. 2010).

Attenuation of CGRP release from activated primary nociceptors by $KYNA_a$ also suggests a marked peripheral effect, an inhibition of the receptors involved in trigeminal activation. Similarly, peripheral, topical intra-articular administration of kynurenic acid reduced mechanical allodynia dose-dependently during pain conditions (Mecs et al. 2009). Glutamate receptors are present on the peripheral arm of the primary trigeminal nociceptors (Quartu et al. 2002; Watanabe et al. 1994) and their inhibition reduces the release of CGRP (Garry et al. 2000). The α7-nicotinic acetylcholine receptors are also present in the trigeminal ganglion (Liu et al. 1998). Their blockade may contribute to the reduction of glutamate release (Carpenedo et al. 2001), and it mitigates the facial vasodilatation induced by CGRP released from the primary nociceptors (Just et al. 2005). G-Protein-coupled receptor-35 is expressed within nociceptive pathways, including the dorsal root ganglion and spinal cord, at the mRNA and protein levels (Ohshiro et al. 2008), and is negatively coupled to adenylate cyclase—cyclic adenosine monophosphate signalling in the dorsal root ganglion neurones, which can modulate nociceptive signalling (Ohshiro et al. 2008).

The modulatory effect of $KYNA_a$ on the activation of second-order trigeminal neurones, reflected by c-Fos changes, may be a consequence of inhibition of the peripheral nociceptors, but it may also arise from the action

on receptors located in the central part of trigeminal system. Glutamate receptors are present postsynaptically on the second-order trigeminal neurones (Tallaksen-Greene et al. 1992) playing an important role in the pathomechanism of trigeminal activation related to noxious stimuli. Likewise, the inhibition of glutamate receptors reduces the increase in c-Fos expression in pain conditions (Mitsikostas et al. 1998, 1999). α7-Nicotinic acetylcholine receptors are located on primary trigeminal nociceptors (Liu et al. 1998) and their inhibition at the presynaptic level with kynurenic acid may contribute to the reduction of glutamate release (Carpenedo et al. 2001) and reduce the activation of second-order trigeminal neurones (Carstens et al. 2000). The possibility of central action of kynurenic acid and its derivatives is supported by results demonstrating that intrathecally injected kynurenic acid and 7-chlorokynurenic acid display dose-dependent and reversible analgesic effects in the hot-plate, tail-flick and formalin tests in mice (Nasstrom et al. 1992) and in the hot-plate and tail-flick tests in rats (Kristensen et al. 1993). Intrathecally administered 5,7-dichlorokynurenic acid dose-dependently reversed the hyperalgesia in hyperalgesic Mg-deficient rats (Begon et al. 2001). Moreover, in the nitroglycerine model of trigeminal activation, the i.p. administration of another kynurenic acid derivative and combined pre-treatment with L-kynurenine and probenecid prevented the activation of second-order trigeminal neurones (Knyihar-Csillik et al. 2007, 2008), whereas kynurenic acid alone was less effective in this model, probably because of its inferior level of blood–brain barrier penetrance (Knyihar-Csillik et al. 2008).

The inhibition of nNOS and CaMKIIα by $KYNA_a$ in the present study and by another kynurenic acid derivative in earlier works (Vamos et al. 2009, 2010) points to the attenuation of the nitroglycerine-induced central sensitization phenomena, both nNOS and CaMKIIα appearing to be essential in this process (Chacur et al. 2010; Fang et al. 2002). The central sensitization is a consequence of changes ongoing in the central nervous system, during which the neurones undergo anatomical and functional plastic changes after strong noxious stimulation, primarily the activation of AMPA and NMDA receptors being involved (Latremoliere and Woolf 2009). Both AMPA and NMDA receptor antagonists effectively reduce the established long-term potentiation of the C-fibre-mediated response in wide dynamic range neurones in the lumbar dorsal horn, which is thought to be related to central sensitization (Svendsen et al. 1998). In a behavioural study, another competitive NMDA antagonist, LY235959, administered either intrathecally or subcutaneously, significantly reduced the number of formalin-induced flinches in phase 2, i.e. the central sensitization (Davis and Inturrisi 2001). Taken together these results suggest that besides acting on the periphery, $KYNA_a$ might attenuate the increases in expression of nNOS and CaMKIIα, i.e. the nitroglycerine-induced central sensitization, probably through the inhibition of AMPA and/or NMDA receptors. However, it is also possible that blockade of the α7-nicotinic acetylcholine receptors could contribute to the inhibition of central sensitization indirectly, since glutamate release can be reduced by the inhibition of these receptors by kynurenic acid at the presynaptic level (Carpenedo et al. 2001).

The results of open field behavioural test show that the nitroglycerine- and placebo-treated group in $KYNA_a$ pre-treated animals did not differ from each other significantly, which may suggest antinociceptive effect of $KYNA_a$. However, the $KYNA_a$ pre-treated groups showed a tendency of lower ambulation distance, which may refer to a direct central action of $KYNA_a$. This concept is also supported by the fact that only glycine-site NMDA antagonists passing the blood–brain barrier had modulatory effect on ambulation distance in open field test (Christoph et al. 2005).

In conclusion, the new kynurenic acid derivative used in the present study, $KYNA_a$, mitigated the trigeminal activation at the level of the peripheral branches and second-order neurones. Moreover, at the level of the C1–C2, it abolished the alterations related to central sensitization. Besides an effect related to the transformation of $KYNA_a$ to kynurenic acid in the periphery, direct and indirect central actions might be responsible for this phenomenon. Since all of these events play a key part in certain headache conditions, $KYNA_a$ may have a possible future role in the treatment of these disorders with a different mechanism of action.

Acknowledgments This work was supported by OTKA KI05077, TÁMOP-4.2.2.A-11/1/KONV-2012-0052 and the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4.A/2-11-1-2012-0001 "National Excellence Program". Dr. Árpád Párdutz was supported by the Bolyai Scholarship Programme of the Hungarian Academy of Sciences. We acknowledge Mrs. Valéria Vékony for the histotechnical assistance, Péter Klivényi and István Ilisz for their valuable technical advices and David Durham for the linguistic correction of the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

References

Begon S, Pickering G, Eschalier A, Mazur A, Rayssiguier Y, Dubray C (2001) Role of spinal NMDA receptors, protein kinase C and nitric oxide synthase in the hyperalgesia induced by magnesium deficiency in rats. Br J Pharmacol 134(6):1227–1236. doi:10.1038/sj.bjp.0704354

Birch PJ, Grossman CJ, Hayes AG (1988) Kynurenate and FG9041 have both competitive and non-competitive antagonist actions at

excitatory amino acid receptors. Eur J Pharmacol 151(2):313–315 (pii:0014-2999(88)90814-X)
Bredt DS, Hwang PM, Snyder SH (1990) Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature 347(6295):768–770. doi:10.1038/347768a0
Burstein R, Yamamura H, Malick A, Strassman AM (1998) Chemical stimulation of the intracranial dura induces enhanced responses to facial stimulation in brain stem trigeminal neurons. J Neurophysiol 79(2):964–982
Carpenedo R, Pittaluga A, Cozzi A, Attucci S, Galli A, Raiteri M, Moroni F (2001) Presynaptic kynurenate-sensitive receptors inhibit glutamate release. Eur J Neurosci 13(11):2141–2147 (pii: ejn1592)
Carstens E, Simons CT, Dessirier JM, Carstens MI, Jinks SL (2000) Role of neuronal nicotinic-acetylcholine receptors in the activation of neurons in trigeminal subnucleus caudalis by nicotine delivered to the oral mucosa. Exp Brain Res 132(3):375–383. doi:10.1007/s002210000351
Chacur M, Matos RJ, Alves AS, Rodrigues AC, Gutierrez V, Cury Y, Britto LR (2010) Participation of neuronal nitric oxide synthase in experimental neuropathic pain induced by sciatic nerve transection. Braz J Med Biol Res 43(4):367–376. doi:10.1590/S0100-879X2010007500019
Christiansen I, Thomsen LL, Daugaard D, Ulrich V, Olesen J (1999) Glyceryl trinitrate induces attacks of migraine without aura in sufferers of migraine with aura. Cephalalgia 19 (7):660–667. doi:10.1046/j.1468-2982.1999.019007660.x (discussion 626)
Christoph T, Reissmuller E, Schiene K, Englberger W, Chizh BA (2005) Antiallodynic effects of NMDA glycine(B) antagonists in neuropathic pain: possible peripheral mechanisms. Brain Res 1048(1–2):218–227. doi:10.1016/j.brainres.2005.04.081
D'Andrea G, Leon A (2010) Pathogenesis of migraine: from neurotransmitters to neuromodulators and beyond. Neurol Sci 31(Suppl 1):S1–S7. doi:10.1007/s10072-010-0267-8
Davis AM, Inturrisi CE (2001) Attenuation of hyperalgesia by LY235959, a competitive N-methyl-D-aspartate receptor antagonist. Brain Res 894(1):150–153 (pii:S0006-8993(00)03325-4)
Demeter I, Nagy K, Gellert L, Vecsei L, Fulop F, Toldi J (2012) A novel kynurenic acid analog (SZR104) inhibits pentylenetetrazole-induced epileptiform seizures. An electrophysiological study: special issue related to kynurenine. J Neural Transm 119 (2):151–154. doi:10.1007/s00702-011-0755-x
Denenberg VH (1969) Open-field bheavior in the rat: what does it mean? Ann N Y Acad Sci 159(3):852–859. doi:10.1111/j.1749-6632.1969.tb12983.x
Di Clemente L, Coppola G, Magis D, Gerardy PY, Fumal A, De Pasqua V, Di Piero V, Schoenen J (2009) Nitroglycerin sensitises in healthy subjects CNS structures involved in migraine pathophysiology: evidence from a study of nociceptive blink reflexes and visual evoked potentials. Pain 144(1–2):156–161. doi:10.1016/j.pain.2009.04.018
Entrena A, Camacho ME, Carrion MD, Lopez-Cara LC, Velasco G, Leon J, Escames G, Acuna-Castroviejo D, Tapias V, Gallo MA, Vivo A, Espinosa A (2005) Kynurenamines as neural nitric oxide synthase inhibitors. J Med Chem 48(26):8174–8181. doi:10.1021/jm050740o
Fang L, Wu J, Lin Q, Willis WD (2002) Calcium-calmodulin-dependent protein kinase II contributes to spinal cord central sensitization. J Neurosci 22(10):4196–4204 (pii: 2002634322/10/4196)
Fukui S, Schwarcz R, Rapoport SI, Takada Y, Smith QR (1991) Blood-brain barrier transport of kynurenines: implications for brain synthesis and metabolism. J Neurochem 56(6):2007–2017. doi:10.1111/j.1471-4159.1991.tb03460.x
Fulop F, Szatmari I, Toldi J, Vecsei L (2012) Modifications on the carboxylic function of kynurenic acid. J Neural Transm 119(2):109–114. doi:10.1007/s00702-011-0721-7
Fuvesi J, Rajda C, Bencsik K, Toldi J, Vecsei L (2012) The role of kynurenines in the pathomechanism of amyotrophic lateral sclerosis and multiple sclerosis: therapeutic implications. J Neural Transm 119(2):225–234. doi:10.1007/s00702-012-0765-3
Garry MG, Walton LP, Davis MA (2000) Capsaicin-evoked release of immunoreactive calcitonin gene-related peptide from the spinal cord is mediated by nitric oxide but not by cyclic GMP. Brain Res 861(2):208–219 (pii: S0006-8993(99)02448-8)
Garthwaite J, Charles SL, Chess-Williams R (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature 336(6197):385–388. doi:10.1038/336385a0
Gellert L, Varga D, Ruszka M, Toldi J, Farkas T, Szatmari I, Fulop F, Vecsei L, Kis Z (2012) Behavioural studies with a newly developed neuroprotective KYNA-amide. J Neural Transm 119(2):165–172. doi:10.1007/s00702-011-0692-8
Gray R, Rajan AS, Radcliffe KA, Yakehiro M, Dani JA (1996) Hippocampal synaptic transmission enhanced by low concentrations of nicotine. Nature 383(6602):713–716. doi:10.1038/383713a0
Herve C, Beyne P, Jamault H, Delacoux E (1996) Determination of tryptophan and its kynurenine pathway metabolites in human serum by high-performance liquid chromatography with simultaneous ultraviolet and fluorimetric detection. J Chromatogr B Biomed Appl 675(1):157–161 (pii: 037843479500341X)
Just S, Arndt K, Doods H (2005) The role of CGRP and nicotinic receptors in centrally evoked facial blood flow changes. Neurosci Lett 381(1–2):120–124. doi:10.1016/j.neulet.2005.02.012
Kessler M, Terramani T, Lynch G, Baudry M (1989) A glycine site associated with N-methyl-D-aspartic acid receptors: characterization and identification of a new class of antagonists. J Neurochem 52(4):1319–1328. doi:10.1111/j.1471-4159.1989.tb01881.x
Knyihar-Csillik E, Toldi J, Mihaly A, Krisztin-Peva B, Chadaide Z, Nemeth H, Fenyo R, Vecsei L (2007) Kynurenine in combination with probenecid mitigates the stimulation-induced increase of c-fos immunoreactivity of the rat caudal trigeminal nucleus in an experimental migraine model. J Neural Transm 114(4):417–421. doi:10.1007/s00702-006-0545-z
Knyihar-Csillik E, Mihaly A, Krisztin-Peva B, Robotka H, Szatmari I, Fulop F, Toldi J, Csillik B, Vecsei L (2008) The kynurenate analog SZR-72 prevents the nitroglycerol-induced increase of c-fos immunoreactivity in the rat caudal trigeminal nucleus: comparative studies of the effects of SZR-72 and kynurenic acid. Neurosci Res 61(4):429–432. doi:10.1016/j.neures.2008.04.009
Kristensen JD, Post C, Gordh TJ, Svensson BA (1993) Spinal cord morphology and antinociception after chronic intrathecal administration of excitatory amino acid antagonists in the rat. Pain 54(3):309–316 (pii: 0304-3959(93)90030-S)
Latremoliere A, Woolf CJ (2009) Central sensitization: a generator of pain hypersensitivity by central neural plasticity. J Pain 10(9):895–926. doi:10.1016/j.jpain.2009.06.012
Liu L, Chang GQ, Jiao YQ, Simon SA (1998) Neuronal nicotinic acetylcholine receptors in rat trigeminal ganglia. Brain Res 809(2):238–245 (pii: S0006-8993(98)00862-2)
Markovics A, Kormos V, Gaszner B, Lashgarara A, Szoke E, Sandor K, Szabadfi K, Tuka B, Tajti J, Szolcsanyi J, Pinter E, Hashimoto H, Kun J, Reglodi D, Helyes Z (2012) Pituitary adenylate cyclase-activating polypeptide plays a key role in nitroglycerol-induced trigeminovascular activation in mice. Neurobiol Dis 45(1):633–644. doi:10.1016/j.nbd.2011.10.010
Marosi M, Nagy D, Farkas T, Kis Z, Rozsa E, Robotka H, Fulop F, Vecsei L, Toldi J (2010) A novel kynurenic acid analogue: a comparison with kynurenic acid. An in vitro electrophysiological study. J Neural Transm 117(2):183–188. doi:10.1007/s00702-009-0346-2

McGehee DS, Heath MJ, Gelber S, Devay P, Role LW (1995) Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors. Science 269(5231):1692–1696. doi:10.1126/science.7569895

Mecs L, Tuboly G, Nagy E, Benedek G, Horvath G (2009) The peripheral antinociceptive effects of endomorphin-1 and kynurenic acid in the rat inflamed joint model. Anesth Analg 109(4):1297–1304. doi:10.1213/ane.0b013e3181b21c5e

Mitsikostas DD, Sanchez del Rio M, Waeber C, Moskowitz MA, Cutrer FM (1998) The NMDA receptor antagonist MK-801 reduces capsaicin-induced c-fos expression within rat trigeminal nucleus caudalis. Pain 76(1–2):239–248 (pii: S0304-3959(98)00051-7)

Mitsikostas DD, Sanchez del Rio M, Waeber C, Huang Z, Cutrer FM, Moskowitz MA (1999) Non-NMDA glutamate receptors modulate capsaicin induced c-fos expression within trigeminal nucleus caudalis. Br J Pharmacol 127(3):623–630. doi:10.1038/sj.bjp.0702584

Moskowitz MA (2008) Defining a pathway to discovery from bench to bedside: the trigeminovascular system and sensitization. Headache 48(5):688–690. doi:10.1111/j.1526-4610.2008.01110.x

Nasstrom J, Karlsson U, Post C (1992) Antinociceptive actions of different classes of excitatory amino acid receptor antagonists in mice. Eur J Pharmacol 212(1):21–29 (pii: 0014-2999(92)90067-E)

Nemeth H, Toldi J, Vecsei L (2006) Kynurenines, Parkinson's disease and other neurodegenerative disorders: preclinical and clinical studies. J Neural Transm Suppl 70:285–304. doi:10.1007/978-3-211-45295-0_45

Nicolodi M, Sicuteri F (1995) Exploration of NMDA receptors in migraine: therapeutic and theoretic implications. Int J Clin Pharmacol Res 15(5–6):181–189

Ohshiro H, Tonai-Kachi H, Ichikawa K (2008) GPR35 is a functional receptor in rat dorsal root ganglion neurons. Biochem Biophys Res Commun 365(2):344–348. doi:10.1016/j.bbrc.2007.10.197

Oshinsky ML, Luo J (2006) Neurochemistry of trigeminal activation in an animal model of migraine. Headache 46(Suppl 1):S39–S44. doi:10.1111/j.1526-4610.2006.00489.x

Pardutz A, Krizbai I, Multon S, Vecsei L, Schoenen J (2000) Systemic nitroglycerin increases nNOS levels in rat trigeminal nucleus caudalis. NeuroReport 11(14):3071–3075. doi:10.1097/00001756-200009280-00008

Pardutz A, Multon S, Malgrange B, Parducz A, Vecsei L, Schoenen J (2002) Effect of systemic nitroglycerin on CGRP and 5-HT afferents to rat caudal spinal trigeminal nucleus and its modulation by estrogen. Eur J Neurosci 15(11):1803–1809 (pii: 2031)

Pardutz A, Hoyk Z, Varga H, Vecsei L, Schoenen J (2007) Oestrogen-modulated increase of calmodulin-dependent protein kinase II (CamKII) in rat spinal trigeminal nucleus after systemic nitroglycerin. Cephalalgia 27(1):46–53. doi:10.1111/j.1468-2982.2006.01244.x

Pardutz A, Fejes A, Bohar Z, Tar L, Toldi J, Vecsei L (2012) Kynurenines and headache. J Neural Transm 119(2):285–296. doi:10.1007/s00702-011-0665-y

Pereira EF, Hilmas C, Santos MD, Alkondon M, Maelicke A, Albuquerque EX (2002) Unconventional ligands and modulators of nicotinic receptors. J Neurobiol 53(4):479–500. doi:10.1002/neu.10146

Quartu M, Serra MP, Ambu R, Lai ML, Del Fiacco M (2002) AMPA-type glutamate receptor subunits 2/3 in the human trigeminal sensory ganglion and subnucleus caudalis from prenatal ages to adulthood. Mech Ageing Dev 123(5):463–471 (pii: S0047637401003 58X)

Sicuteri F, Del Bene E, Poggioni M, Bonazzi A (1987) Unmasking latent dysnociception in healthy subjects. Headache 27(4):180–185. doi:10.1111/j.1526-4610.1987.hed2704180.x

Storer RJ, Goadsby PJ (1999) Trigeminovascular nociceptive transmission involves N-methyl-D-aspartate and non-N-methyl-D-aspartate glutamate receptors. Neuroscience 90(4):1371–1376 (pii: S0306-4522(98)00536-3)

Strassman AM, Mineta Y, Vos BP (1994) Distribution of fos-like immunoreactivity in the medullary and upper cervical dorsal horn produced by stimulation of dural blood vessels in the rat. J Neurosci 14(6):3725–3735 (pii: 0270-6474/94/143725-11$05.00/O)

Svendsen F, Tjolsen A, Hole K (1998) AMPA and NMDA receptor-dependent spinal LTP after nociceptive tetanic stimulation. NeuroReport 9(6):1185–1190. doi:10.1097/00001756-199804200-00041

Tallaksen-Greene SJ, Young AB, Penney JB, Beitz AJ (1992) Excitatory amino acid binding sites in the trigeminal principal sensory and spinal trigeminal nuclei of the rat. Neurosci Lett 141(1):79–83 (pii: 0304-3940(92)90339-9)

Tassorelli C, Joseph SA (1995) Systemic nitroglycerin induces Fos immunoreactivity in brainstem and forebrain structures of the rat. Brain Res 682(1–2):167–181 (pii: 0006-8993(95)00348-T)

Tassorelli C, Joseph SA, Nappi G (1997) Neurochemical mechanisms of nitroglycerin-induced neuronal activation in rat brain: a pharmacological investigation. Neuropharmacology 36(10):1417–1424 (pii: S0028390897001226)

Vamos E, Pardutz A, Varga H, Bohar Z, Tajti J, Fulop F, Toldi J, Vecsei 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(4):425–429. doi:10.1016/j.neuropharm.2009.06.033

Vamos E, Fejes A, Koch J, Tajti J, Fulop F, Toldi J, Pardutz A, Vecsei L (2010) Kynurenate derivative attenuates the nitroglycerin-induced CamKIIalpha and CGRP expression changes. Headache 50(5):834–843. doi:10.1111/j.1526-4610.2009.01574.xHED1574

Vecsei L, Szalardy L, Fulop F, Toldi J (2013) Kynurenines in the CNS: recent advances and new questions. Nat Rev Drug Discov 12(1):64–82. doi:10.1038/nrd3793nrd3793

Wang XM, Mokha SS (1996) Opioids modulate N-methyl-D-aspartic acid (NMDA)-evoked responses of trigeminothalamic neurons. J Neurophysiol 76(3):2093–2096

Wang J, Simonavicius N, Wu X, Swaminath G, Reagan J, Tian H, Ling L (2006) Kynurenic acid as a ligand for orphan G protein-coupled receptor GPR35. J Biol Chem 281(31):22021–22028. doi:10.1074/jbc.M603503200

Watanabe M, Mishina M, Inoue Y (1994) Distinct gene expression of the N-methyl-D-aspartate receptor channel subunit in peripheral neurons of the mouse sensory ganglia and adrenal gland. Neurosci Lett 165(1–2):183–186. doi:10.1016/0304-3940(94)90740-4

Zadori D, Nyiri G, Szonyi A, Szatmari I, Fulop F, Toldi J, Freund TF, Vecsei L, Klivenyi P (2011) Neuroprotective effects of a novel kynurenic acid analogue in a transgenic mouse model of Huntington's disease. J Neural Transm 118(6):865–875. doi:10.1007/s00702-010-0573-6

Zhang RX, Mi ZP, Qiao JT (1994) Changes of spinal substance P, calcitonin gene-related peptide, somatostatin, Met-enkephalin and neurotensin in rats in response to formalin-induced pain. Regul Pept 51(1):25–32. doi:10.1016/0167-0115(94)90131-7

II.

Kynurenate derivative attenuates the nitroglycerin-induced CamKIIalpha and CGRP expression changes.

Vámos E, Fejes A, Koch J, Tajti J, Fülöp F, Toldi J, Párdutz Á, Vécsei L.

Kynurenate derivative attenuates the nitroglycerin-induced CamKIIalpha and CGRP expression changes.

Headache 2010 May;50(5):834-43.

IF: 2.642

Headache
© 2009 the Authors
Journal compilation © 2009 American Headache Society

ISSN 0017-8748
doi: 10.1111/j.1526-4610.2009.01574.x
Published by Wiley Periodicals, Inc.

Research Submission

Kynurenate Derivative Attenuates the Nitroglycerin-Induced CamKIIα and CGRP Expression Changes

Enikő Vámos, MSc; Annamária Fejes, MSc; Júlia Koch, MD; János Tajti, MD, PhD; Ferenc Fülöp, PhD, DSc; József Toldi, PhD, DSc; Árpád Párdutz, MD, PhD; László Vécsei, MD, PhD, DSc

Objective.—To examine the efficacy of L-kynurenine and a novel kynurenic acid derivative on the nitroglycerin-induced calmodulin-dependent protein kinase II alpha (CamKIIα) and calcitonin gene-related peptide (CGRP) expression changes in the rat caudal trigeminal nucleus.

Background.—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 the CamKIIα and decreases that of the CGRP there. As glutamatergic mechanisms may be crucial in trigeminal pain processing, the aim of our study was to examine the effects of L-kynurenine, a metabolic precursor of the *N*-methyl *D*-aspartate receptor antagonist kynurenic acid, on the nitroglycerin-induced changes in CamKIIα and CGRP immunoreactivity.

Methods.—One hour before the nitroglycerin (10 mg/kg bodyweight, s.c.) injection, the animals were pretreated with L-kynurenine (300 mg/kg bodyweight, i.p.) or 2-(2-*N*,*N*-dimethylaminoethylamine-1-carbonyl)-1*H*-quinolin-4-one hydrochloride (300 mg/kg bodyweight, i.p.), a novel kynurenic acid derivative. Four hours later, the rats were perfused transcardially and the cervical spinal cord segments were removed for immunohistochemistry.

Results.—L-kynurenine and 2-(2-*N*,*N*-dimethylaminoethylamine-1-carbonyl)-1*H*-quinolin-4-one hydrochloride pretreatment attenuated the nitroglycerin-induced changes in CamKIIα and CGRP immunoreactivity in the rat caudal trigeminal nucleus.

Conclusions.—These findings suggest a mechanism by which the inhibition of the excitatory amino acid receptors by kynurenic acid and its derivatives can alter trigeminal nociception.

Key words: calmodulin-dependent protein kinase II alpha, calcitonin gene-related peptide, caudal trigeminal nucleus, L-kynurenine, 2-(2-N,N-dimethylaminoethylamine-1-carbonyl)-1H-quinolin-4-one hydrochloride

Abbreviations: AMPA α-amino-3-hydroxyl-5-methyl-4-isoxazole propionate, CamKIIα calmodulin-dependent protein kinase II alpha, CGRP calcitonin gene-related peptide, HPLC high-performance liquid chromatography, i.p. intraperitoneal, IR immunoreactive, KYNA kynurenic acid, L-KYN L-kynurenine, nNOS neuronal nitric oxide synthase, NMDA *N*-methyl *D*-aspartate, NTG nitroglycerin, PBS phosphate-buffered saline, PROB probenecid, s.c. subcutaneous, TNC caudal trigeminal nucleus

(*Headache* 2010;50:834-843)

From the Department of Neurology, Albert Szent-Györgyi Clinical Centre, University of Szeged, Szeged, Hungary (E. Vámos, A. Fejes, J. Koch, J. Tajti, Á. Párdutz, and L. Vécsei); Department of Pharmaceutical Chemistry, University of Szeged, Szeged, Hungary (F. Fülöp); Department of Physiology, Anatomy and Neuroscience, University of Szeged, Szeged, Hungary (J. Toldi).

Financial supports: ETT-215/2006, NKTH 08/2004, OMFB 00449/2008.

Address all correspondence to Á. Párdutz, Department of Neurology, Albert Szent-Györgyi Clinical Centre, University of Szeged, Hungary, Semmelweis u. 6. H-6725 Szeged, Hungary.

Accepted for publication September 17, 2009.

Conflict of Interest: None

Systemic administration of the nitric oxide donor nitroglycerin (NTG) triggers a typical migraine attack without aura after a delay of several hours in many migraine patients, but it indicates only a mild, immediate headache in healthy volunteers.[1-3] The exact mechanism of NTG-induced migraine is not fully understood, but because of the similarity to the normal migraine attack, the role of the trigeminovascular system is implicated in the NTG model.[4] In the rat, systemic administration of NTG activates neurons in the caudal trigeminal nucleus (TNC).[5] Moreover, nitric oxide from NTG increases the number of neuronal nitric oxide synthase (nNOS) immunoreactive (IR) neurons in the same area suggesting a self-amplifying process, causing central sensitization.[6] This is present in migraineurs, too,[7] suggesting that NTG administration in rats can be considered as a model of trigeminal activation and sensitization.

After 4 hours, systemic NTG selectively increases the expression of calmodulin-dependent protein kinase II alpha (CamKIIα) in the spinal TNC, probably by stimulating its peripheral Aδ and C afferents.[8] CamKII is expressed in many tissues, but it is found in high abundance in the central nervous system and regulates calcium signalling in the synaptic transmission.[9] Animal experiments suggest that CamKII has an important role in nociceptive processing and contributes to central sensitization.[8]

In the lower TNC of the rat, systemically administered NTG can also cause a decrease in the area covered by calcitonin gene-related peptide (CGRP)-IR fibers, probably because of an increased release.[10,11] CGRP is a key transmitter in primary nociceptive afferents, and both basic research and clinical studies have provided evidence of the role of CGRP in the migraine pathomechanism.[12] The plasma concentrations of CGRP in the jugular venous blood are elevated during the headache phase of migraine,[12] with a correlation between the plasma CGRP level and the headache intensity.[13]

Glutamate is the main excitatory neurotransmitter in the central nervous system. Both human and animal studies have demonstrated its important role in migraine pathogenesis.[14-16] In migraine patients, higher glutamate levels have been measured in the plasma.[16] The glutamate-induced excitability is mediated via the ionotropic *N*-methyl *D*-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), kainate and metabotropic glutamate receptors.[17] 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.[18]

Kynurenic acid (KYNA), transaminated from L-kynurenine (L-KYN), is one of the few known endogenous antagonists of excitatory amino acid receptors.[19] Recent studies have revealed its possible protective role in migraine headache.[20,21] In the present study, our aim was to determine the effects of L-KYN in combination with probenecid (PROB) – a known inhibitor of organic acid transport from the cerebrospinal fluid, enhancing the KYNA levels in the central nervous system[22] – and 2-(2-*N*,*N*-dimethylaminoethylamine-1-carbonyl)-1*H*-quinolin-4-one hydrochloride, a newly synthesized KYNA derivative, on the NTG-induced immunoreactivity changes of CamKIIα and CGRP in the rat TNC.

METHODS

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 weighing 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-hour dark–light cycle.

Drugs.—L-kynurenine sulphate and PROB were from Sigma (Steinheim, Germany). The new compound – is covered by Patent Reference Number #P0900281 – was synthesized in the Department of Pharmaceutical Chemistry, University of Szeged. The doses of drugs were chosen on the basis of earlier works.[23,24]

Drug Administration.—For immunohistochemistry, the animals were divided into 3 groups (n = 12 per group). The animals in the first group received only the vehicle solution as pretreatment, without drug.

The rats in the second group received an intraperitoneal (i.p.) injection of L-KYN (300 mg/kg bodyweight, diluted to 2 mL, pH 7.4) combined with PROB (200 mg/kg bodyweight, diluted to 1.5 mL, pH 7.4). In the third group the rats were injected i.p. with the KYNA derivative (2-(2-*N*,*N*-dimethylaminoethylamine-1-carbonyl)-1*H*-quinolin-4-one hydrochloride) (300 mg/kg bodyweight, diluted to 2 mL, pH 7.4). One hour later, half of the animals received a subcutaneous (s.c.) injection of NTG (10 mg/kg bodyweight; Pohl-Boskamp GmbH, Hohenlockstedt, Germany), while the other half received a s.c. injection of the vehicle (Pohl-Boskamp GmbH, Hohenlockstedt, Germany). For high-performance liquid chromatography (HPLC) measurements, the animals were divided into 2 groups (n = 4 per group). The rats in the first group, were injected with vehicle solution. In the second group, the rats received an i.p. injection of L-KYN sulphate (300 mg/kg bodyweight diluted to 2 mL, pH 7.4) and PROB (200 mg/kg bodyweight diluted to 1.5 mL, pH 7.4).

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 phosphate-buffered saline (PBS, 0.1 M, pH 7.4), followed by 500 mL 4% paraformaldehyde in PBS. The cervical spinal cord segments between –5 and –11 mm from the obex were removed and postfixed overnight for immunohistochemistry in the same fixative. After cryoprotection, 30 µm cryostat sections were cut and serially collected in 16 wells containing cold PBS. Each well received sections at a 0.5-mm distance throughout the rostrocaudal extent of the C1 and C2 spinal cord segments. The free-floating sections were rinsed in PBS and immersed in 0.3% H_2O_2 in PBS for 30 minutes to suppress endogenous peroxidase activity. After several rinses in PBS containing 1% Triton X-100, sections were kept 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 1:20,000. The immunocytochemical reaction was visualized by using the avidin-biotin kit of Vectastain (Vector Laboratories Inc., Burlingame, CA, USA, PK6102 for CamKIIα and PK6101 for CGRP), and stained with nickel ammonium sulphate-intensified 3,3′-diaminobenzidine. The specificity of the immune reaction was controlled by omitting the primary antiserum.

KYNA Detection in the TNC.—One hour after drug or vehicle treatment, the rats were deeply anesthetized 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 minutes, 24°C). The supernatant were filtered with a syringe-driven filter unit (Millipore, Bedford, MA, USA, SLHV 013 NL). The KYNA contents 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, MA, USA, 30105-154030), and chromatographed isocratically at a flow rate of 1 mL/minute 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 minutes.

Data Evaluation and Statistical Analysis.—Transverse sections of the cervical spinal cord demonstrated numerous CamKIIα-IR neurons in dorsal horn laminae I-II. Stained sections were analyzed under a Nikon Phase Contrast (085 59762, Tokyo, Japan) light microscope and photomicrographs were taken using Olympus DP70 (Tokyo, Japan) camera equipment. In 3 different series of sections in each animal, the CamKIIα-IR cells in laminae I-II of the cervical spinal cord were counted by an observer blinded to the procedures. The individual sections in these series were taken at 0.5-mm intervals along the rostrocaudal axis.

The CGRP-IR fibers in laminae I and II of the cervical dorsal horns were determined by video imaging, using 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 4× 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 1600×1200 pixels. After image acquisition, a threshold grey level was established in order to detect IR fibers in the digitalized microscopic image. 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 on 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 fibers as the number of pixels with densities above the threshold. For the calibration, we measured known areas of different shapes. 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 CamKIIα and CGRP immunohistochemistry were taken using a Zeiss AxioImager microscope (Carl Zeiss Micro-Imaging, Thornwood, NY, USA) supplied with a PixeLINK CCD camera (PixeLINK, Ottawa, ON, Canada).

Group values are reported as means ± SEM Statistical comparisons between the control and NTG-treated groups at each pretreatment dose were made by using analysis of variance (ANOVA) followed by the Scheffe test. The independent Student *t*-test was used to analyse the results of HPLC measurements in the vehicle-treated and the L-KYN-PROB-treated groups.

Both analyses were implemented in SPSS (Version 11.0 for Windows, SPSS Inc.), with $P < .05$ taken as statistically significant.

RESULTS

The HPLC measurements of KYNA concentration clearly indicated a significantly increased KYNA level in the TNC 1 hour after L-KYN and PROB administration ($P \leq .001$).

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 2 sides of the TNC. NTG produced a significant increase in the number of CamKIIα-positive cells in the superficial layers of the TNC in the non-pretreated rats ($P \leq .01$, Fig. 1A,B; Fig. 2). L-KYN with PROB ($P \leq .001$, Fig. 1C-D; Fig. 2) or KYNA derivative ($P \leq .001$, Fig. 1E,F; Fig. 2.) attenuated the CamKIIα immunopositivity increase.

On transverse sections of the cervical spinal segments, there were abundant CGRP-positive fibers in the superficial layers of the dorsal horn. The area covered by these fibers was not significantly different at the various rostro-caudal levels, nor on the 2 sides of segments C1 and C2. The CGRP-innervated area in the NTG-treated group was significantly smaller than that in the placebo-treated group ($P \leq .05$, Fig. 3A,B; Fig. 4). The immunopositivity decrease was successfully attenuated by pretreatment with L-KYN-PROB ($P \leq .01$, Fig. 3C,D; Fig. 4) or the novel KYNA derivative ($P \leq .05$, Fig. 3E,F; Fig. 4).

DISCUSSION

Our HPLC data confirm earlier findings and observations,[23] we found that the administration of L-KYN in combination with PROB caused a robust increase in the level of KYNA in the rat TNC. KYNA penetrates the blood–brain barrier only poorly,[25] but under experimental conditions exogenously administered L-KYN has been demonstrated to increase the KYNA level in the brain dose-dependently.[23,26] Another approach is to use derivatives of KYNA which can readily cross the blood–brain barrier and display a similar effectiveness of KYNA. Recent data have shown that the co-administration of L-KYN with PROB and a KYNA analogue, SZR72, was able to reduce the *c-fos* activation in the animal models of migraine.[20,27] Further, 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.[24]

CamKII plays an important role in nociception; its native and phosphorylated forms in the superficial dorsal horns are enhanced by s.c. formalin and intradermal capsaicin.[28,29] It also can regulate glutamate

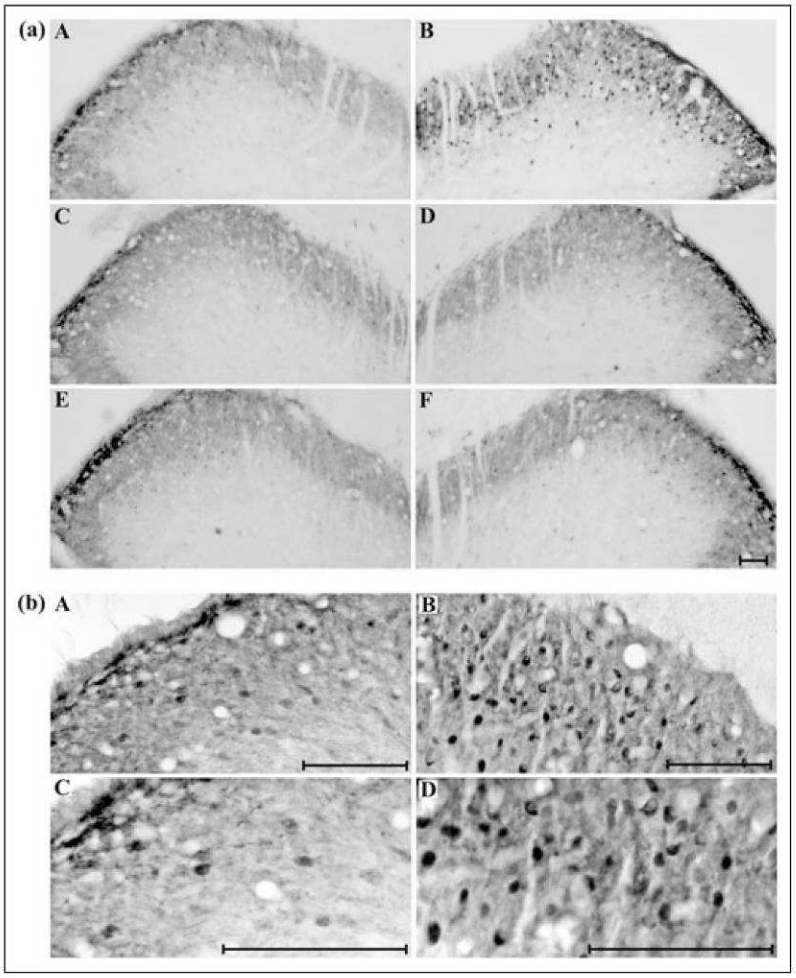

Fig 1.—(a) CamKIIα-immunopositivity in the upper cervical spinal cord in control (A, B), L-KYN-PROB-pretreated (C, D) and the KYNA derivative-pretreated (E, F) rats after placebo (A, C, E) or NTG (B, D, F) injections. (b) CamKIIα-immunoreactive neurons under 40× objective (upper series) and under 63× objective (lower series) in placebo (A, C) and NTG-treated (B, D) animals. Scale bar = 50 μm. CamKIIα = calmodulin-dependent protein kinase II alpha; L-KYN = L-kynurenine; KYNA = kynurenic acid; NTG = nitroglycerin; PROB = probenecid.

receptors, such as AMPA receptor, in the area of second-order trigeminal nociceptors.[30] Moreover, CamKII is capable of autophosphorylation, which increases its binding affinity for NMDA receptors in the rat. Its alpha subunit increases the ion currents through the AMPA receptor and the NMDA receptors,[31] suggesting its involvement in central sensitization of the sensory system. Previous studies have demonstrated increases in the expression of CamKIIα in the TNC after NTG administration.[8] Overall, it seems that nitric oxide can initiate a self-amplifying process, possibly leading to sensitization in the trigeminal system, which can be paralleled by the sensitization events occurring in migraineurs.[7] These NTG effects to the trigeminal system seemed to be selective as no changes were detected in upper thoracic segments, suggesting that CamKIIα may play a crucial role in the pathogenesis of migraine.[8]

The release or the increased turnover of CGRP in the spinal dorsal horns of appropriate segments has been reported after localized peripheral inflammation, capsaicin administration or s.c. formalin injections.[32-34] The decrease in the area occupied by CGRP-IR afferents is probably a consequence of an

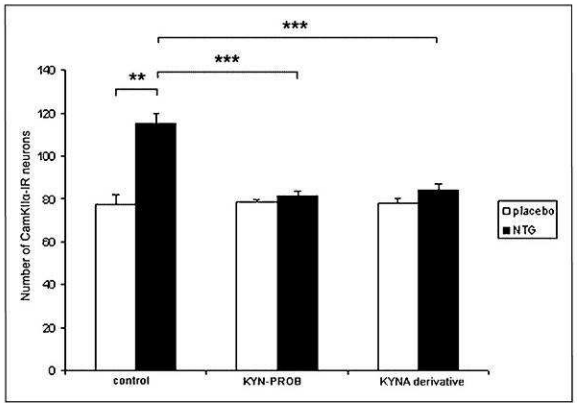

Fig 2.—Histogram showing the mean number of CamKIIα-IR cells in the L-KYN-PROB-pretreated and KYNA derivative-pretreated animals after vehicle (light bars) or NTG (dark bars) injections (means ± SEM, n = 6 per group). There was a significant increase in the CamKIIα-IR cells after the NTG injection in the control group, but not in the L-KYN-PROB- and the KYNA derivative-pretreated animals. CamKIIα = calmodulin-dependent protein kinase II alpha; IR = immunoreactive; L-KYN = L-kynurenine; KYNA = kynurenic acid; NTG = nitroglycerin; PROB = probenecid. **$P \leq .01$; ***$P \leq .001$.

increased release of CGRP because of the nitric oxide-mediated stimulation of trigeminal nociceptive Aδ and C afferents.[10]

Our data revealed that L-KYN or 2-(2-*N*,*N*-dimethylaminoethylamine-1-carbonyl)-1*H*-quinolin-4-one hydrochloride pretreatment mitigated the NTG-induced CamKIIα and CGRP immunoreactivity changes in the TNC, modulating the trigeminal activation at this level. The experimental data point to glutamate being strongly implicated at different sites of migraine pathogenesis.[35] Indeed, glutamate excites the TNC neurons when applied locally, and the extracellular levels of glutamate rise following a noxious stimulus along the trigeminal nerve.[36,37] Research findings suggest that the injection of glutamate antagonists in the trigeminocervical complex causes an inhibition of neuronal firing triggered by stimulation of the superior sagittal sinus.[38] A further interesting aspect is the role of glutamate transmission in pain generation. Animal studies have identified all the different glutamate receptors in the trigeminal system. Moreover, glutamate is involved in neuronal sensitization at the level of the trigeminal nucleus, a process involved in allodynia.[15] NMDA receptor antagonists are able to block sensitization of the dorsal horn neurons after stimulation of the peripheral nerve C-fibers or chemical stimulation of the skin.[39,40] MK-801, a non-selective NMDA receptor antagonist, mitigated capsaicin-evoked *c-fos* activation in the TNC.[41] Moreover, there is evidence of presynaptic NMDA receptors in the terminals to the spinal cord regulating nociceptive input.[42] Thus, the activation of NMDA receptors may play a pivotal role not only in the activation, but also in the development of central sensitization in the dorsal horn.[43,44]

In human cerebrospinal fluid, the concentration of KYNA is in low nanomolar range.[45] Besides the NMDA antagonistic effect, which may clearly modulate trigeminal nociception, KYNA at higher concentration, is able to act on the AMPA and kainate receptors, synthetic antagonists of which effectively blocked trigeminovascular nociception and may be effective in the acute treatment of migraine.[17,19,46,47] Interestingly, Prescott et al reported that KYNA in nanomolar to micromolar concentrations is able to facilitate AMPA receptor responses.[48] However, the concentration ranges appear to be controversial, because it was recently demonstrated that KYNA in micromolar concentrations still exerts a neuroinhibitory effect, whereas in nanomolar concentrations it behaves as a facilitator.[49] It is also known that KYNA inhibits the α7-nicotinic acetylcholine receptors non-competitively,[50] activation of these receptors at the presynaptic site taking part in the regulation of glutamate release.[51] Thus, KYNA can act on multiple targets (possibly via glutamate receptors) present both on the central projections of peripheral afferents and on the second-order trigeminal neurons in the TNC, attenuating the self-amplifying process and hence the sensitization triggered by nitric oxide. This can be paralleled by clinical data where the administration of ketamine, an NMDA receptor antagonist, proved effective against NO-induced headache.[52]

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

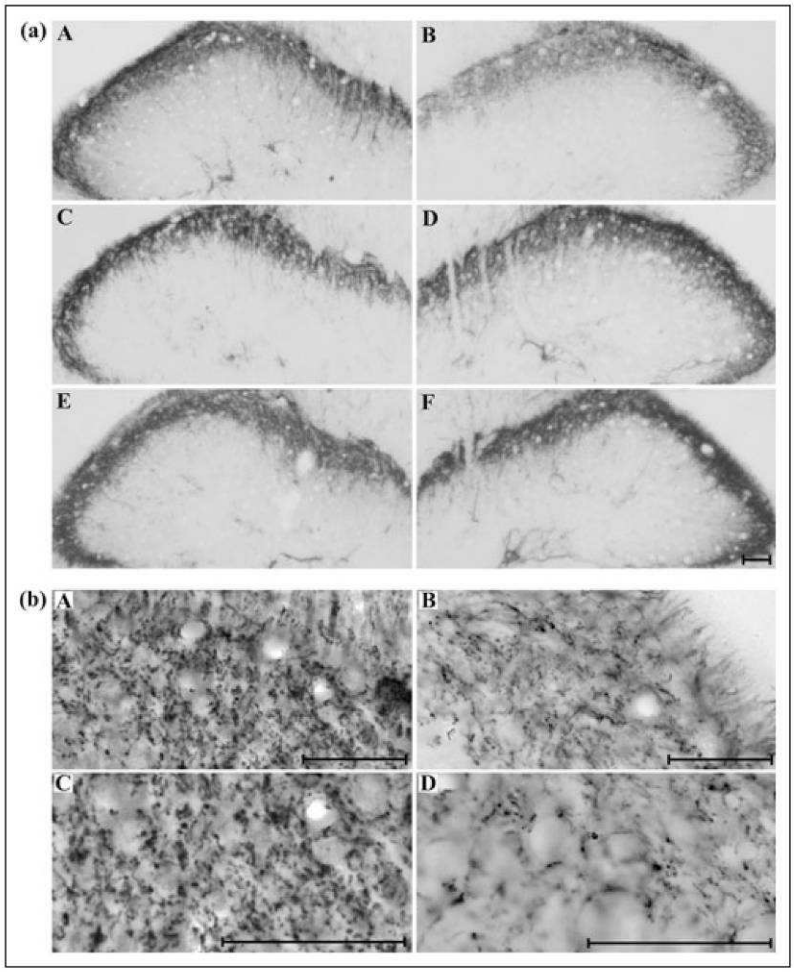

Fig 3.—(a) CGRP-immunoreactivity in the upper cervical spinal cord in control (A, B), L-KYN-PROB-pretreated (C, D), and KYNA derivative-pretreated (E, F) rats after placebo (A, C, E) or NTG (B, D, F) injections. (b) CGRP-IR fibers and buttons under 40× objective (upper series) and under 63× objective (lower series) in placebo (A, C) and NTG-treated (B, D) rats. Scale bar = 50 μm. CGRP = calcitonin gene-related peptide; IR = immunoreactive; L-KYN = L-kynurenine; KYNA = kynurenic acid; NTG = nitroglycerin; PROB = probenecid.

trigeminal activation and sensitization in the NTG model of migraine, and it may therefore open up novel therapeutic opportunities in headache management.

Acknowledgments: This work was supported by grants ETT-215/2006, NKTH 08/2004, and OMFB 00449/2008. We thank Attila Bebes for his image preparation.

STATEMENT OF AUTHORSHIP

Category 1

(a) Conception and Design
Árpád Párdutz; Enikő Vámos; László Vécsei

(b) Acquisition of Data
Enikő Vámos; Árpád Párdutz; Annamária Fejes; Júlia Koch

(c) Analysis and Interpretation of Data
Enikő Vámos; Árpád Párdutz; János Tajti; József Toldi; Ferenc Fülöp; László Vécsei

Category 2

(a) Drafting the Manuscript
Árpád Párdutz; Enikő Vámos

(b) Revising It for Intellectual Content
János Tajti; József Toldi; Ferenc Fülöp; László Vécsei

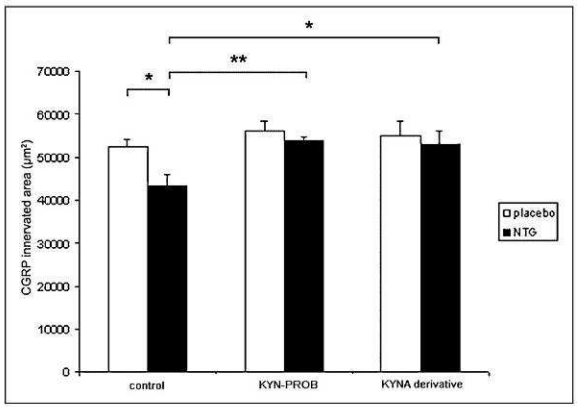

Fig 4.—Histogram showing the area in µm² covered by CGRP-IR fibers in superficial laminae I and II of the TNC in segments C1 and C2 in the 3 animal groups after vehicle (light bars) or NTG (dark bars) (means ± SEM, n = 6 per group). The combined treatment and 2-(2-*N*, *N*-dimethylaminoethylamine-1-carbonyl)-1*H*-quinolin-4-one hydrochloride (a novel KYNA derivative) significantly attenuated the NTG-induced changes. CGRP = calcitonin gene-related peptide; IR = immunoreactive; KYNA = kynurenic acid; NTG = nitroglycerin; TNC = caudal trigeminal nucleus. *$P \leq .05$; **$P \leq .01$.

Category 3

(a) Final Approval of the Completed Manuscript

Enikő Vámos; Annamária Fejes; Júlia Koch; János Tajti; Ferenc Fülöp; József Toldi; Árpád Párdutz; László Vécsei

REFERENCES

1. Sicuteri F, Del BE, Poggioni M, Bonazzi A. Unmasking latent dysnociception in healthy subjects. *Headache*. 1987;27:180-185.
2. Olesen J, Iversen HK, Thomsen LL. Nitric oxide supersensitivity: A possible molecular mechanism of migraine pain. *Neuroreport*. 1993;4:1027-1030.
3. Afridi SK, Kaube H, Goadsby PJ. Glyceryl trinitrate triggers premonitory symptoms in migraineurs. *Pain*. 2004;110:675-680.
4. Olesen J, Thomsen LL, Lassen LH, Olesen IJ. The nitric oxide hypothesis of migraine and other vascular headaches. *Cephalalgia*. 1995;15:94-100.
5. Tassorelli C, Joseph SA. Systemic nitroglycerin induces Fos immunoreactivity in brainstem and forebrain structures of the rat. *Brain Res*. 1995; 682:167-181.
6. Pardutz A, Krizbai I, Multon S, Vecsei L, Schoenen J. Systemic nitroglycerin increases nNOS levels in rat trigeminal nucleus caudalis. *Neuroreport*. 2000;11:3071-3075.
7. Burstein R, Cutrer MF, Yarnitsky D. The development of cutaneous allodynia during a migraine attack clinical evidence for the sequential recruitment of spinal and supraspinal nociceptive neurons in migraine. *Brain*. 2000;123:(Pt 8):1703-1709.
8. Pardutz A, Hoyk Z, Varga H, Vecsei L, Schoenen J. Oestrogen-modulated increase of calmodulin-dependent protein kinase II (CamKII) in rat spinal trigeminal nucleus after systemic nitroglycerin. *Cephalalgia*. 2007;27:46-53.
9. Hudmon A, Schulman H. Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. *Biochem J*. 2002;364:593-611.
10. Pardutz A, Multon S, Malgrange B, Parducz A, Vecsei L, Schoenen J. Effect of systemic nitroglycerin on CGRP and 5-HT afferents to rat caudal spinal trigeminal nucleus and its modulation by estrogen. *Eur J Neurosci*. 2002;15:1803-1809.
11. Arulmani U, Maassenvandenbrink A, Villalon CM, Saxena PR. Calcitonin gene-related peptide and its role in migraine pathophysiology. *Eur J Pharmacol*. 2004;500:315-330.
12. Goadsby PJ, Edvinsson L, Ekman R. Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. *Ann Neurol*. 1990;28:183-187.
13. Durham PL. CGRP receptor antagonists: A new choice for acute treatment of migraine? *Curr Opin Investig Drugs*. 2004;5:731-735.
14. Andreou AP, Goadsby PJ. Therapeutic potential of novel glutamate receptor antagonists in migraine. *Expert Opin Investig Drugs*. 2009;18:789-803.
15. Oshinsky ML, Luo J. Neurochemistry of trigeminal activation in an animal model of migraine. *Headache*. 2006;46(Suppl. 1):S39-S44.
16. Ferrari A, Spaccapelo L, Pinetti D, Tacchi R, Bertolini A. Effective prophylactic treatments of migraine lower plasma glutamate levels. *Cephalalgia*. 2009; 29:423-429.
17. Sang CN, Ramadan NM, Wallihan RG, et al. LY293558, a novel AMPA/GluR5 antagonist, is efficacious and well-tolerated in acute migraine. *Cephalalgia*. 2004;24:596-602.
18. Tallaksen-Greene SJ, Young AB, Penney JB, Beitz AJ. Excitatory amino acid binding sites in the

trigeminal principal sensory and spinal trigeminal nuclei of the rat. *Neurosci Lett*. 1992;141:79-83.

19. Kessler M, Terramani T, Lynch G, Baudry M. A glycine site associated with N-methyl-D-aspartic acid receptors: Characterization and identification of a new class of antagonists. *J Neurochem*. 1989;52:1319-1328.
20. Knyihar-Csillik E, Toldi J, Krisztin-Peva B, et al. Prevention of electrical stimulation-induced increase of c-fos immunoreaction in the caudal trigeminal nucleus by kynurenine combined with probenecid. *Neurosci Lett*. 2007;418:122-126.
21. Kiss C, Shepard PD, Bari F, Schwarcz R. Cortical spreading depression augments kynurenate levels and reduces malonate toxicity in the rat cortex. *Brain Res*. 2004;1002:129-135.
22. Cunningham RF, Israili ZH, Dayton PG. Clinical pharmacokinetics of probenecid. *Clin Pharmacokinet*. 1981;6:135-151.
23. Vecsei L, Miller J, MacGarvey U, Beal MF. Effects of kynurenine and probenecid on plasma and brain tissue concentrations of kynurenic acid. *Neurodegeneration*. 1992;1:17-26.
24. Vamos E, Pardutz A, Varga H, et al. l-kynurenine combined with probenecid and the novel synthetic kynurenic acid derivative attenuate nitroglycerin-induced nNOS in the rat caudal trigeminal nucleus. *Neuropharmacology*. 2009;doi:10.1016/j.neuropharm.2009.06.033.
25. Fukui S, Schwarcz R, Rapoport SI, Takada Y, Smith QR. Blood-brain barrier transport of kynurenines: Implications for brain synthesis and metabolism. *J Neurochem*. 1991;56:2007-2017.
26. Santamaria A, Rios C, Solis-Hernandez F, et al. Systemic DL-kynurenine and probenecid pretreatment attenuates quinolinic acid-induced neurotoxicity in rats. *Neuropharmacology*. 1996;35:23-28.
27. Knyihar-Csillik E, Mihaly A, Krisztin-Peva B, et al. The kynurenate analog SZR-72 prevents the nitroglycerol-induced increase of c-fos immunoreactivity in the rat caudal trigeminal nucleus: Comparative studies of the effects of SZR-72 and kynurenic acid. *Neurosci Res*. 2008;61:429-432.
28. Liang DY, Li X, Clark JD. Formalin-induced spinal cord calcium/calmodulin-dependent protein kinase II alpha expression is modulated by heme oxygenase in mice. *Neurosci Lett*. 2004;360:61-64.
29. Fang L, Wu J, Lin Q, Willis WD. Calcium-calmodulin-dependent protein kinase II contributes to spinal cord central sensitization. *J Neurosci*. 2002;22:4196-4204.
30. Ogawa A, Dai Y, Yamanaka H, Iwata K, Niwa H, Noguchi K. Ca(2+)/calmodulin-protein kinase IIalpha in the trigeminal subnucleus caudalis contributes to neuropathic pain following inferior alveolar nerve transection. *Exp Neurol*. 2005;192:310-319.
31. Kolaj M, Cerne R, Cheng G, Brickey DA, Randic M. Alpha subunit of calcium/calmodulin-dependent protein kinase enhances excitatory amino acid and synaptic responses of rat spinal dorsal horn neurons. *J Neurophysiol*. 1994;72:2525-2531.
32. Sluka KA, Dougherty PM, Sorkin LS, Willis WD, Westlund KN. Neural changes in acute arthritis in monkeys. III. Changes in substance P, calcitonin gene-related peptide and glutamate in the dorsal horn of the spinal cord. *Brain Res Brain Res Rev*. 1992;17:29-38.
33. Garry MG, Walton LP, Davis MA. Capsaicin-evoked release of immunoreactive calcitonin gene-related peptide from the spinal cord is mediated by nitric oxide but not by cyclic GMP. *Brain Res*. 2000;861:208-219.
34. Zhang RX, Mi ZP, Qiao JT. Changes of spinal substance P, calcitonin gene-related peptide, somatostatin, Met-enkephalin and neurotensin in rats in response to formalin-induced pain. *Regul Pept*. 1994;51:25-32.
35. Vikelis M, Mitsikostas DD. The role of glutamate and its receptors in migraine. *CNS Neurol Disord Drug Targets*. 2007;6:251-257.
36. Hill RG, Salt TE. An ionophoretic study of the responses of rat caudal trigeminal nucleus neurones to non-noxious mechanical sensory stimuli. *J Physiol*. 1982;327:65-78.
37. Bereiter DA, Benetti AP. Excitatory amino release within spinal trigeminal nucleus after mustard oil injection into the temporomandibular joint region of the rat. *Pain*. 1996;67:451-459.
38. Goadsby PJ, Classey JD. Glutamatergic transmission in the trigeminal nucleus assessed with local blood flow. *Brain Res*. 2000;875:119-124.
39. Haley JE, Sullivan AF, Dickenson AH. Evidence for spinal N-methyl-D-aspartate receptor involvement in prolonged chemical nociception in the rat. *Brain Res*. 1990;518:218-226.
40. Davies SN, Lodge D. Evidence for involvement of N-methylaspartate receptors in "wind-up" of class 2

neurones in the dorsal horn of the rat. *Brain Res.* 1987;424:402-406.
41. Mitsikostas DD, Sanchez del RM, Waeber C, Moskowitz MA, Cutrer FM. The NMDA receptor antagonist MK-801 reduces capsaicin-induced c-fos expression within rat trigeminal nucleus caudalis. *Pain*. 1998;76:239-248.
42. Liu S, Ruenes GL, Yezierski RP. NMDA and non-NMDA receptor antagonists protect against excitotoxic injury in the rat spinal cord. *Brain Res.* 1997;756:160-167.
43. Woolf CJ. Pain. *Neurobiol Dis*. 2000;7:504-510.
44. Hardingham GE, Bading H. The Yin and Yang of NMDA receptor signalling. *Trends Neurosci.* 2003;26:81-89.
45. Erhardt S, Blennow K, Nordin C, Skogh E, Lindstrom LH, Engberg G. Kynurenic acid levels are elevated in the cerebrospinal fluid of patients with schizophrenia. *Neurosci Lett*. 2001;313:96-98.
46. Storer RJ, Goadsby PJ. Trigeminovascular nociceptive transmission involves N-methyl-D-aspartate and non-N-methyl-D-aspartate glutamate receptors. *Neuroscience*. 1999;90:1371-1376.
47. Perkins MN, Stone TW. Actions of kynurenic acid and quinolinic acid in the rat hippocampus in vivo. *Exp Neurol*. 1985;88:570-579.
48. Prescott C, Weeks AM, Staley KJ, Partin KM. Kynurenic acid has a dual action on AMPA receptor responses. *Neurosci Lett*. 2006;402:108-112.
49. Rozsa E, Robotka H, Vecsei L, Toldi J. The Janus-face kynurenic acid. *J Neural Transm*. 2008;115:1087-1091.
50. Hilmas C, Pereira EF, Alkondon M, Rassoulpour A, Schwarcz R, Albuquerque EX. The brain metabolite kynurenic acid inhibits alpha7 nicotinic receptor activity and increases non-alpha7 nicotinic receptor expression: Physiopathological implications. *J Neurosci*. 2001;21:7463-7473.
51. Marchi M, Risso F, Viola C, Cavazzani P, Raiteri M. Direct evidence that release-stimulating alpha7* nicotinic cholinergic receptors are localized on human and rat brain glutamatergic axon terminals. *J Neurochem*. 2002;80:1071-1078.
52. Roffey P, Mikhail M, Thangathurai D. NMDA receptor blockade prevents nitroglycerin-induced headaches. *Headache*. 2001;41:733.