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

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Pre-treatment with new kynurenic acid amide dose-dependently prevents the nitroglycerine-induced neuronal activation and sensitization in cervical part of trigemino-cervical complex.
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II. Vámos E, **Fejes A**, Koch J, Tajti J, Fülöp F, Toldi J, Párdutz Á, Vécsei L.
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II. Tajti J, Párdutz A, Vámos E, Tuka B, Kuris A, Bohár Z, **Fejes A**, Toldi J, Vécsei L.
Migraine is a neuronal disease.
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III. **Fejes A**, Párdutz Á, Toldi J, Vécsei L.
Kynurenine metabolites and migraine: experimental studies and therapeutic perspectives.
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Kynurenines and headache.
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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.
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Effect of probenecid on the pain-related behaviour and morphological markers in orofacial formalin test of the rat.
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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
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NTG	nitroglycerin
PROB	probenecid
TNC	spinal trigeminal nucleus pars caudalis

Introduction

Migraine

Migraine is an idiopathic neurological disorder, which is characterised by spontaneous, recurrent headache attacks worsening the quality of life followed by symptom free intervals. Its prevalence is very high, about 12% causing immense direct and indirect economic deficit.

Several theories have been put forward regarding the pathophysiology of migraine, but none of them can explain the full spectrum of the disorder. One of the common points in these different theories of migraine is the activation of the trigeminal system - responsible for most of the pain processing on the head - during the attack. The first-order trigeminal neurones contain numerous neurotransmitters such as glutamate and calcitonin gene-related peptide (CGRP), which are released during the activation. Consequently, enhanced CGRP level was measured in external jugular vein during the migraine attack, which contributes to the peripheral plasma extravasation and the neurogenic inflammation in the dura mater. The released inflammatory substances rapidly sensitize the trigeminal first-order neurones resulting in a throbbing head pain.

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 releasing mainly glutamate, which acts on its own ionotropic and metabotropic receptors located in the trigeminal system. In response to activation, the expression of c-Fos transcription factor increases in the neurones, which is usually used as a marker of neuronal activation induced by noxious stimulation. Continuous excitation induces plastic and long-term changes in the second-order neurones resulting in central sensitization, which manifests clinically in cutaneous allodynia of scalp and face and worsens the efficacy of acute attack treatment with triptans. In these plastic changes, calcium/calmodulin-dependent protein kinase II alpha (CaMKIIα) plays an important role.

Since the exact pathomechanism of migraine is unknown and the current preventive and attack therapies are only partially effective and often poorly tolerated, scientific research is crucial in this field. Using animal experimental models, we can have better insight of the underlying mechanisms and a possibility to develop new drugs to treat the disorder.

Nitroglycerin model of migraine headache

Nitroglycerin (NTG) – a widely used drug to treat angina pectoris and myocardial infarction - is converted to nitric oxide (NO) in the body. Endogenously, NO is synthesized by nitric oxide synthases (NOSs) and is involved in the regulation of vessel tone, inflammatory responses, cell communication, regulation of glutamate release in the spinal cord, pain transmission and the development of hyperalgesia.

One of the most common side-effects of NTG is headache due to vasodilatation induced by NO immediately after its administration. However in most of the migraineurs, a delayed, typical migraine headache without aura develops, which can not be attributed to NO's prompt vasodilator effect. In addition to the migraine-like headache, NTG evolves numerous changes, which can be observed during a migraine attack too. It can trigger reproducible migraine with aura, premonitory symptoms, activation in the dorsal rostral brain stem and dorsal lateral pons and changes in evoked cortical response. Furthermore, NTG can sensitize the trigeminal system as well, inducing typical changes which reflect sensitization of neurones in TNC. 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.

Data from humans led to the hypothesis that NTG administration can function as an animal experimental model of migraine too, which was supported by experimental results. In animals NTG was able to trigger scratching head reactions, cage climbing, red ear and photophobia and facilitate the face-grooming behaviour reflecting the nocifensive responses. NTG administration induces dural and pial artery dilation, leakage of plasma proteins from dural blood vessels and increased meningeal and cortical regional blood flow. NTG treatment can influence various transmitters present in the trigeminal system, e.g. CGRP-immunoreactive (IR) fibres at the cervical part of TNC (C1-C2) undergo morphological changes suggesting transmitter release, in the same area, the expression of pituitary adenylate cyclase-activating polypeptide-38 and -27 are increased, meanwhile a marked increase of CGRP and substance-P in plasma was also observed. NTG treatment activates the trigeminal system reflected by changes of c-fos mRNA and c-Fos protein expression in the trigeminal ganglion and in the TNC. Moreover, there is experimental evidence regarding that NTG can cause the sensitization of the trigeminal system, which phenomenon is also present in migraineurs during the attack resulting in hypersensitivity and allodynia. Behaviourally, NTG induced a

dose-dependent and prolonged thermal and mechanical allodynia in mice, a hyperalgesic response in the tail-flick test, hyperalgesia at the level of the spinal cord after its epidural injection and acute and chronic hyperalgesia after its chronic intermittent administration to mice. Electrophysiological results show, that infusions of NTG 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. Morphologically, significant increase of neuronal NOS- (nNOS) and CaMKIIα-IR neurones in the C1-C2 were detected after NTG suggesting the presence of phenomenon of central sensitization.

To summarize, numerous experimental results 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 and many of its metabolites have neuroactive properties. L-kynurenine (L-KYN) itself is not a neuroactive compound, but it is the precursor of kynurenic acid, which is the main neuroprotective end product of this pathway probably due to various receptorial actions, like N-methyl-D-asparate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and α7-nicotinic acetylcholine (α7-nACh) receptor antagonism and G-protein-coupled receptor-35 (GPR-35) agonism. Changes in its concentration in pathological circumstances were described suggesting its role in pathomechanism of various neurological disorders. Furthermore, emerging evidence support that kynurenic acid can be crucial in nociception too. Based on these observations, kynurenic acid could provide a therapeutic option in many neurological diseases especially in migraine. Unfortunately, it has only a very limited ability to cross the blood–brain barrier, therefore administration of L-KYN can be used in various experimental settings. Having a good blood-brain barrier penetrance, L-KYN causes a dose-dependent increase in the level of kynurenic acid in the central nervous system, which can be amplified by a co-administration with probenecid (PROB). Furthermore, recent experimental results raise the possibility that application of various kynurenic acid derivatives with a presumed improved central nervous

system action and a retained pharmacological effect can be appropriate providing a promising therapeutic option instead of kynurenic acid.

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, drugs and drug administration

The procedures followed the international and European guidelines (86/609/ECC) and were approved by Hungarian committees of animal research (I-74-12/2012, XI./352/2012). Adult male Sprague-Dawley rats weighing 200-250 g were used.

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), were synthesized in the Department of Pharmaceutical Chemistry, University of Szeged.

For the HPLC measurements, the animals received intraperitoneal (i.p.) physiological saline in the control group, i.p. L-KYN (300 mg/kg body weight (bw)) and PROB (200 mg/kg bw) in the second group and i.p. 1 mmol/kg bw KYNAa2 in the third group.

For immunohistochemistry, the animals received i.p. physiological saline in the control group, i.p. L-KYN (300 mg/kg bw) combined with PROB (200 mg/kg bw) in the second group, i.p. KYNAa1 (1 mmol/kg bw) in the third group and i.p. KYNAa2 at the dosage of 0.1, 0.5 and 1 mmol/kg bw, respectively, in the remaining 3 groups. One hour after the pre-treatment, half of the animals received an i.p. NTG (10 mg/kg bw) and other half an i.p. Placebo of NTG.

For Western blotting, the animals received i.p. physiological saline in the control group and i.p. KYNAa2 (1 mmol/kg bw) in the second group followed by i.p. NTG (10 mg/kg bw) or i.p. Placebo one hour after the pre-treatment.

For the Open Field Test, the animals received i.p. physiological saline in the control group and i.p. 1 mmol/kg bw KYNAa2 in the second group followed by i.p. NTG (10 mg/kg bw) or i.p. Placebo one hour after the pre-treatment.

Kynurenic acid detection with HPLC

At determined time points (60 minutes in the control and L-KYN-PROB groups and 60 and 300 minutes in the KYNAa2 group) following the i.p. injection, the C1-C2 segments were removed. After sample preparation, the kynurenic acid content was measured with fluorescent detector. The retention time of kynurenic acid was about 6 minutes.

Immunohistochemistry

Four hours after treatment with Placebo or NTG, the C1-C2 segments were removed and processed for CGRP, c-Fos, nNOS and CaMKIIα immunohistochemistry. In laminae I and II of C1-C2, the area covered by CGRP-IR fibres was measured and number of c-Fos-, nNOS- and CaMKIIα-IR cells were counted.

Western blotting

Four hours after the NTG or Placebo injections, the dorsal horn of C1-C2 segment was removed. After sample preparation, protein separation and transfer, the CGRP, nNOS and β-actin protein bands were indentified and their densities were measured.

Open Field Test

The rats were tested three hours and forty minutes after the NTG or Placebo injections in the Open Field box for 15 minutes and ambulation time, ambulation distance, local time and the number of rearings were registered and evaluated.

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 as compared with control group ($p<0.001$). Similarly, kynurenic acid level in the C1-C2 showed a significant ($p<0.05$), more than two-fold increase 60 minutes after 1 mmol/kg bw KYNAa2 administration compared to vehicle treated samples, while the concentration of kynurenic acid decreased to baseline at 300 minutes.

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 horn. 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.

In the control group, significantly reduced CGRP staining and significantly increased c-Fos, nNOS and CaMKIIα immunoreactivity can be observed on the C1-C2 sections in I-II laminae after NTG treatment compared to Placebo treated rats ($p<0.01$, $p<0.001$).

Both L-KYN-PROB and KYNAa1 pre-treatment successfully attenuated the decrease in CGRP and increase in CaMKIIα immunopositivity ($p<0.01$, $p<0.001$). The pre-treatment with KYNAa2 affected dose-dependently the NTG-induced changes in the immunoreactivity of all studied markers. The administration of KYNAa2 in a dosage of 0.1 mmol/kg bw did not influence any of the alterations caused by NTG, whereas the pre-treatment with higher dosages of KYNAa2 (0.5 and 1 mmol/kg bw) was able to significantly reduce the effects of NTG on the CGRP-, c-Fos-, nNOS- and CaMKIIα-related changes in the C1-C2 ($p<0.05$, $p<0.01$, $p<0.001$).

Western blotting

Western blotting analysis of the C1-C2 region confirmed the results obtained by CGRP and nNOS immunohistochemistry. Densitometric analyses confirmed that the CGRP bands were significantly decreased ($p<0.05$) and nNOS bands were significantly enhanced ($p<0.001$) in dorsal horns of C1-C2 segments after NTG administration as compared with the Placebo-treated animals. This effect of NTG on CGRP and nNOS 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 ($p<0.05$). Pre-treatment with 1 mmol/kg bw KYNAa2 attenuated this difference, but a tendency for a lower basic ambulation distance was observed. There were no significant changes in ambulation time, local time or in the number of rearings between the subgroups.

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 and supported by other studies. Kynurenic acid has a poor blood-brain barrier penetrance, 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, because it is a known inhibitor of organic acid transporters, which are involved in the transport of kynurenic acid from brain through the blood-brain barrier. Using kynurenic acid derivatives with a presumed better blood-brain barrier penetrance is an alternative option to increase kynurenic acid concentration or mimic its effect. After 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, transformation of KYNAa1 to kynurenic acid has been described after i.p. administration in mice serum, besides a robust increase of KYNAa1 level. 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 proves its importance in migraine pathogenesis. Systemic NTG was able to reduce the CGRP content of the C1-C2, which is in line with earlier experiments. 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 and increased release or turnover of CGRP was reported at the level of the spinal cord in various pain models. 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. In earlier studies, it was also proven that the size of the CGRP-positive buttons is smaller in the C1-C2 after NTG, which is also suggestive of transmitter release. These animal experimental results can

be paralleled by the observations showing increased CGRP concentration in the jugular vein of migraineurs during the attack. 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. Our results indicate that L-KYN-PROB, KYNAa1 and in a dose-dependent manner KYNAa2 can mitigate the decrease of CGRP 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 can be paralleled by the observation that kynurenic acid is able to reduce allodynia when administered topically in the joints. Glutamate receptors can be found on the peripheral arm of the trigeminal system and their inhibition mitigates CGRP release. α7-nACh receptors are also present on the soma of primary trigeminal neurones and glutamate release and CGRP induced facial vasodilation are reduced after their inhibition. GPR-35 can be found within the sensory ganglia and spinal cord too, activation of which can modulate nociceptive signalling.

Activation of second-order trigeminal neurones

The NO-induced c-Fos expression observed at the level of the C1-C2 reflects an activation of the second-order trigeminal nerve cells, which was already described in earlier studies and appears to be selective to the trigeminal system. 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. 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 and they are involved in nociceptive processing. Inhibition of these receptors can mitigate c-Fos increase in pain conditions. α7-nACh receptors can be found on primary trigeminal nociceptors and their inhibition by kynurenic acid may block glutamatre release from the nerve endings and thus reduce the activation of secondary trigeminal neurones. Central action of KYNAa2 can be suggested in these experimental settings as well, since intrathecally administrated kynurenic acid and 7-chlorokynurenic acid demonstrated a dose-dependent anti-nociceptive action in various animal models. Similarly, 5,7-

dichlorokynurenic acid showed anti-hyperalgesic properties in Mg-deficient rats. Moreover, in an earlier study i.p. KYNAa1 was more effective mitigating c-Fos activation in the TNC after NTG administration when compared to kynurenic acid alone, probably due to its better blood-brain barrier penetrance.

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. NOS inhibitors block the activation of dorsal horn neurones after nociceptive stimuli, they can inhibit the trigeminovascular system and might be effective treating migraine and tension type headache. It was demonstrated that systemic NTG was able to enhance nNOS expression at the level of the C1-C2 and similar changes were seen in the spinal cord after intradermal capsaicin administration and in the TNC after facial formalin injections. In our experiments, NO derived from NTG might act *via* the stimulation of Aδ and C fibres of the primary trigeminal afferents and 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. CaMKIIα is present in the superficial layers of the spinal dorsal horns. NTG increased CaMKIIα in the second-order neurones of the trigeminal system, which was also shown in previous studies. This phenomenon can be paralleled with the results showing increased expression of CaMKIIα in the spinal cord after subcutaneous formalin, capsaicin and intrathecal substance-P administration. Moreover CaMKIIα is able of autophosphorilation, which enhances its activity. It can attach to NMDA receptors in rats and its alpha subunit enhances ion currents through AMPA and NMDA receptors and activates adenylate cyclase related to spinal cord sensitization in animals. 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 NTG-induced central sensitization phenomena. Both nNOS and CaMKIIα seem to be key players in this process, where neurones undergo plastic anatomical and functional changes involving the activation of AMPA and NMDA receptors 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. LY235959 - a

competitive NMDA antagonist - given intrathecally or subcutaneously attenuated the behavioural responses of the second phase after formalin administration, which corresponds to the sensitization process. 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.

Behavioural effect of NTG

NTG administration, which causes the activation and sensitization of the trigeminal system, 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 produced similar behavioural changes. 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 glycine-site NMDA antagonists 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.

Conclusions

Kynurenic acid and its derivatives mitigated the NTG-induced trigeminal activation at the level of first- and second-order neurones and abolished changes related to the central sensitization process at the level of the C1-C2, where most of the second order 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.

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