

**Effect of a novel kynurenic acid derivate on a model
of trigeminovascular activation induced by chemical
stimulation of rat dura mater**

Summary of Ph.D. Thesis

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Lukács M, Warfvinge K, Kruse LS, Tajti J, Fülöp F, Toldi J, Vécsei L, Edvinsson L, KYNA analogue SZR72 modifies CFA-induced dural inflammation-regarding expression of pERK and IL-1 β in the rat trigeminal ganglion, *Journal of Headache and Pain*, 2016 Dec; 17(1):64.

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Lukács M, Warfvinge K, Tajti J, Fülöp F, Toldi J, Vécsei L, Edvinsson L, Topical dura mater application of CFA induces enhanced expression of c-fos and glutamate in rat trigeminal nucleus caudalis: attenuated by KYNA derivate (SZR72), *Journal of Headache and Pain*, 2017 Dec; 18 (1):39.

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Tajti J, Szok D, Tuka B, Csáti A, Kuris A, Majláth Z, **Lukács M**, Vécsei L, Botulinum neurotoxin-a therapy in migraine; Ideggyógyászati Szemle, 2012 Mar 30, 65 (3-4):77-82

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1. Introduction

Migraine is a severe, debilitating neurological disease, being ranked as the sixth most disabling condition worldwide, having a huge impact on individual and public health.

Clinically migraine starts with the premonitory phase, represented by depression, tiredness or irritability that are linked to the hypothalamus. Aura phenomenon (visual, olfactory, sensory or motor auras) might inaugurate migraine attacks. The headache is usually unilateral, throbbing, pulsating. The pain lasts 4-72 hours and is accompanied by nausea, vomiting, photo- or phonophobia. Postdrome symptoms are similar to the premonitory signs of migraine attack. We emphasise that in the new classification the International Headache Society (IHS) defines a new subtype, called chronic migraine where migraine headache occurs on more than 15 days per month, for 3 months, with 8 attacks showing migraine features.

Migraineous pain sensation is thought to be consequence of trigeminovascular activation. The trigeminal ganglion (TG) consisting of pseudounipolar neurons, surrounded by satellite glial cells (SGCs) provides nociceptive fibers that innervate the dural, arachnoideal and pial blood vessels including the sinus saggitalis superior and the middle meningeal arteries. These fibers are non-myelinated (C-fibers) or thinly myelinated fibers (A δ fibers), that form part of the ophthalmic branch (V1) but to lesser extent of the maxillar (V2) and mandibular (V3) division of the trigeminal nerve. Central projections of the TG form the spinal trigeminal tract (Sp5C) and terminate in the caudal part of the brainstem called trigeminal nucleus caudalis (TNC), extended to the C₁-C₂ region of the spinal cord, called the trigeminocervical complex (TCC). Ascending connections of the TCC to the thalamus contribute to the development of pain sensation and other brainstem and diencephalic connections induce the additional symptoms of migraine. The “pain matrix” including the thalamus, the primary and secondary somatosensory cortex, the insula, the anterior cingular cortex and the prefrontal area are thought to be involved in the integration of nociceptive, affective and cognitive responses, leading to all the neurological symptoms that occur during migraine attacks.

Despite a lot of studies made to reveal the pathomechanism of migraine, some aspects remain untangled regarding the initiation of migraine pain. One possible way of activation could be the neurogenic inflammation (NI), representing a sterile inflammation caused by the local release of different neuropeptides (CGRP, PACAP, SP), plasma protein leakage, activation of

pericytes and mast cells and blood-brain barrier dysfunction. It has also been demonstrated that chemical stimulation of the dural receptive fields by application of inflammatory substances onto the dura mater causes hypersensitivity to mechanical and thermal stimulation, leading to activation of the trigeminal system.

The European Federation of Neurological Society (EFNS) guideline divides migraine treatment into migraine attack treatment and prophylactic treatment. In case of migraine attack treatment the actual gold standard therapy are the triptans, 5-hydroxytryptamine (serotonin) $1B/1D$ receptor agonists ($5HT_{1B/1D}$). Other drugs of choice in case of acute therapy are NSAIDs, antiemetics to treat nausea and ergot alkaloids. Preventive treatment includes β -adrenergic blocking agents, calcium ion channel blockers, antiepileptic drugs and antidepressants. Chronic migraine represents a therapeutic challenge for clinicians and researchers as triptans cannot be used as they tend to elevate the risk of chronification and also the occurrence of clinically significant drug-drug interaction is high. Lately, intramuscular injection of Botulinum toxin A has proven to be efficient in chronic migraine but its way of administration represents a clear disadvantage.

Tryptophan (TRP), an essential α -amino-acid is the precursor of the neurotransmitter 5-HT. The major route for TRP metabolism is the kynurenine pathway (KP) having two important neuroactive metabolites: the neuroprotective kynurenic acid (KYNA) and the neurotoxic quinolinic acid (QUIN). Both neuroactive metabolites of the KP have been shown to play important roles in various CNS diseases, acting mainly on glutamate (Glu) receptors. As KYNA has a very low capacity to penetrate the BBB, new analogues are needed in order to take advantage of its anti-inflammatory and neuroprotective properties. During the last years, our research group has synthesized several different KYNA derivatives to facilitate BBB penetration. The new derivatives have proven to be effective in animal models of cerebral ischaemia, Huntington's disease, epilepsy and trigeminal activation. A possible interaction between KYNA and inflammatory cytokines suggests a link between the KP and the immune system, leading to the idea that one possible site of action for KYNA derivatives could be neurogenic inflammation.

2. Aims

The aims of our study are the following:

- I. Development of a new animal model for trigeminal activation using chemical stimulation of the dura mater with CFA, in comparison with a well-known inflammatory soup (IS).
- II. To test whether application of CFA on the surface of the dura mater is able to cause long-term activation of the TG, serving as a model of migraine pain chronification.
- III. To evaluate whether activation of TNC and central sensitization occurs following CFA induced activation.
- IV. To examine the effects of a new KYNA derivate in our animal model both in the TG and TNC.
- V. To clarify whether repeated treatment of KYNA analogue is more effective than one dose pre-treatment.
- VI. To provide a mapping of different inflammatory cytokines and neuropeptides in the TNC, other areas of the brainstem and C₁-C₂ region of the spinal cord.

3. Materials and methods

Synthesis of the novel KYNA derivate

The KYNA amide used in our experiments was designed in the Pharmaceutical Chemistry and Research Group for Stereochemistry, University of Szeged Hungary. This novel KYNA analogue (N-(2-N,N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride) has the following structural properties: the presence of a water-soluble side-chain, the inclusion of a new cationic centre, and side-chain substitution. All these changes in the molecular structure facilitates BBB penetration.

Animal procedure

Primarily we have set up the animal model for our future experiments, regarding the chemical substance used for the trigeminal stimulation and the optimal time-points for the activation. Adult male Sprague-Dawley rats (220-320 g) were used (n=72 for IHC, n=67 for Western blot (WB) and n=5 for myograph study). The animals were raised and maintained under

standard laboratory conditions. Before the interventions, the animals were anesthetized with an intraperitoneal (i.p.) injection of 4% chloral hydrate (0.01 ml/g body weight, Sigma-Aldrich, St. Louis, MO, USA). The head was fixated in a stereotaxic frame and a handheld drill was used to remove a 3x3 mm portion of the skull. For 30 rats IS (containing 10 μ M bradykinin, 10 μ M serotonin, 10 μ M prostaglandin E2 and 100 μ M histamine, pH 5.0; the recipe was adapted from Strassman et al); for 30 animals CFA (Sigma-Aldrich, St. Louis, MO, USA) and for 21 animals (controls) physiological saline was applied onto the dural surface. As absolute controls, 9 unoperated rats (fresh) were used. Same amount of IS, CFA or saline was used (10 μ l) and was left on the dura for 20 min. Afterwards the substances were washed away with saline, the hole was covered with bone wax and the wound was sutured. The animals were transcardially perfusion-fixated after 4 h, 24 h and 7 days. TG from both sides, TNC brainstem region and C₁-C₂ region of the spinal cord were removed (-1, +5 mm from the obex). For the myographic studies, 5 unoperated (fresh) rats CO₂ anaesthesia was used and rats were decapitated. Middle meningeal artery (MMA) was dissected free, using dissection microscope.

Treatment with KYNA analogue

The test the effect of KYNA derivate treatment on a model of migraine pain chronification, we opted for the 7 days animal model using CFA application, as this model led to the most intense long-term activation of the TG. The animals were divided into 4 groups: acute treatment (pre-treatment with KYNA analogue 1h before operation), acute saline (saline 1 h before operation), repeated treatment (treatment with KYNA analogue every 12 hrs, for 7 days) and repeated saline (saline every 12 hrs, for 7 days). The KYNA analogue (1 mmol/kg bodyweight dissolved in 1 ml saline) or saline (1 ml) was administrated i.p.

Tissue analysis

For immunohistochemistry (IHC) specimens were post-fixated in 4% paraformaldehyde solution followed by cryoprotection and freezing. Afterwards the samples were transported to Lund University, Sweden, where the IHC studies were performed. Sections of 12 μ m were used for hematoxylin-eosin (HE) and IHC staining for pER1/2, IL-1 β and CGRP in the TG. To encompass the whole image of the TNC and C₁- C₂ region of the spinal cord, sections were collected from 6 different levels (100-120 sections in total per animal). Areas of the brainstem were identified using rat brain atlas. Sections of the TCC were used for IHC staining for Glu, c-fos, PACAP, SP, TNF α , IL-6 and IL-1 β .

For Western blot (WB) the TGs were homogenized in cell extract denaturing buffer. Following centrifugation the supernatants were collected. Protein concentrations were determined with a protein assay reagent and a microplate reader, followed by gel electrophoresis. WB studies were performed for pERK1/2, IL-1 β and CGRP. The band optical density ratio was quantified by using ImageJ software. As loading control protein we used β -actin and GAPDH. For each gel an absolute control was used as a point of reference for all measurements.

In case of myograph studies each ~2 mm long segment of the MMA was mounted in an arterial myograph, on a pair of 25 μ m metal wires. Following normalisation of the vessels, CFA and IS was applied on the segments.

Statistical analysis

For the WB and myograph studies SPSS 15.0 for Windows was used. The data was analysed with multivariate (one-way ANOVA test) with Bonferroni correction post-testing and for group comparisons Student's t-test was used. In case of the myograph studies for both substances (IS and CFA) a cumulative concentration vs. response curve was made. Levels of probability $p < 0.05$ were considered significant.

Ethical approval

The study followed the guidelines of the European Communities Council (86/609/ECC). Experiments were approved by the Committee of Animal Research at the University of Szeged (I-74-14-16/2008; I-74-12/2012) and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XI./15.1/02384/001/2007; XXIV/352/2012).

4. Results

Immunohistochemistry-Trigeminal ganglion

a. Phospho extracellular signal-regulated kinase (pERK1/2)

pERK1/2 immunoreactivity was observed in a few neuronal nuclei and nucleoli of the in the fresh TGs. In the CFA and IS treated rats, pERK1/2 immunopositivity was observed in the SGCs at all three time points (4 h, 24 h and 7 days). At 4 h we couldn't note any significant difference between the IS, CFA or the saline groups. The SGC activation was missing in the

24 h and 7 days saline specimens. No difference was seen between left and right side. In the 7 days CFA model, showing the most intense immunoreactivity we used a specific SGC marker (GS) and performed double IHC to determine the exact localisation of pERK1/2. Co-localisation of the two markers demonstrates that pERK1/2 is present in the SGCs. KYNA analogue treatment resulted in abolished pERK1/2 immunoreactivity in SGC, no difference was seen following repeated treatment. The negative control, i.e. when the primary antibody was omitted, displayed no immunoreactivity.

b. Interleukin 1 β (IL-1 β)

IL-1 β immunoreactivity was observed in the cytoplasm (in a granular manner) and few nuclei of the neurons and in the outer layer of the nerve fibers. The same staining pattern was seen in the saline-treated animals. At 7 days both in the CFA and IS groups, homogeneous, condense and intensely stained IL-1 β immunoreactivity was detected. This homogenous staining was seen close to the neuronal cell membrane, which differed obviously from the granular pattern observed in the fresh and saline treated TGs. Following i.p. treatment with KYNA analogue prior the operation, the staining returned to the granular pattern observed in fresh TGs and the condensed immunoreactivity close to the cell membrane disappeared. Repeated treatment did not show any difference compared to one-dose usage of KYNA derivate.

c. Calcitonin-gene related peptide (CGRP)

In fresh TGs CGRP immunoreactivity was noted in the neurons and nerve fibers. The immunopositivity was seen in the neuronal cytoplasm, without any nuclear staining. The staining ranged between homogeneous staining and a granular immunoreactive pattern surrounding the nucleus. The fiber staining appeared as a thin, pearl-like immunopositivity. In the CFA and IS treated rats, CGRP immunoreactivity was observed in the neurons and nerve fibers at all three time points. As compared with the control specimens (fresh and saline-treated rats) an increase of immunoreactive fibers were found. As no change in the staining pattern was detected no CGRP staining was performed for KYNA derivate treatment groups.

Immunohistochemistry -Trigemincervical complex

a. Glutamate (Glu)

In case of fresh (unoperated) rats, Glu immunopositivity was seen in fibers of the trigeminal tract on every level of the TNC. A few homogeneously stained glial cells and some neurons in the caudal part of the TNC could also be detected. Following application of CFA on the dura mater, an obvious increase in the intensity and amount of Glu positive neurons in the TNC.

The aspect of the staining is specific for the medial part of the spinal trigeminal nucleus: irregularly arranged, triangular or multipolar shaped, medium-sized cells. No difference in the fiber staining was noted. Following i.p. treatment with the KYNA derivate, the intensity and amount of immunoreactive cells decreased, close to the level observed in healthy, intact animals. No clear difference was noted in the fiber and glial cell staining and neither between pre-treatment and repeated-treatment groups. I.p. treatment with saline was not able to lower CFA induced activation.

b. C-fos

In unoperated (fresh) rats, few c-fos positive neuronal nuclei, but no nucleolei, were observed in the caudal part of Sp5C and TNC mainly in the gelatinous layer. After application of CFA, an increased amount of c-fos positive nuclei could be detected, especially in the caudal areas of the TNC. No increased immunoreactivity was visualised following saline use instead of CFA. Administration of KYNA derivate was able to reduce the CFA-induced activation in neuronal nuclei at every level of the TNC. No change was noted following repeated treatment. I.p. saline was not able to diminish CFA induced activation.

c. Pituitary adenylate cyclase-activating polypeptide (PACAP)

PACAP immunoreactivity was found in fibers of the trigeminal tract, both in fresh and CFA treated animals. In addition, PACAP immunoreactivity was found in different areas of the brainstem, in the large neurons of the anterior horn and the ependymal cells of the central canal. PACAP immunopositive fibers could be observed in almost every tract of the spinal cord (dorsal corticocerebellar tract, spinocerebellar tracts, medial longitudinal tract, and pyramidal tract). Following CFA application PACAP positive fibers were found in the fasciculus cuneatus and gracilis.

d. Substance P (SP)

SP immunoreactivity was limited to nerve fibers of the Sp5C and to the gelatinous layer. No difference was noted between different levels of TNC and a slight increased intensity of the fiber staining could be detected following the CFA induced activation, but not in the gelatinous layer.

e. Tumor necrosis factor α (TNF α), Interleukin-6 (IL-6) and Interleukin-1 β (IL-1 β)

Immunoreactivity was detected as a dense fiber staining in the spinal trigeminal tract, but no glial or neuronal staining was detected neither at the cranial, nor at the caudal level of the TNC. In the spinal cord few, small sized neurons were detected, mainly surrounding the

central canal. Some immunopositive fibers were observed in different other tracts of the spinal cord (dorsal cortico-cerebellar tract, spino-cerebellar tracts, medial longitudinal tract and pyramidal tract). No difference was noted following application of CFA on the dura mater or pre-treatment with KYNA derivate.

Western blot – Trigeminal ganglion

In order to investigate quantitatively the proteins expressed in the TG, WB was performed for pERK1/2, IL-1 β and CGRP. The only significant increase was present in pERK1/2 24 hours models compared to fresh rats. In case of IL-1 β the same tendency was detected as in the IHC studies, but the difference didn't reach the level of significance. For CGRP we saw no significant difference.

For the i.p. treatment groups we only checked pERK1/2 and IL-1 β . Following i.p. treatment with KYNA derivate the same tendency was observed as in the IHC studies but the difference didn't reach a significant level.

Myography

To investigate whether the activation caused by the IS and CFA seen in the trigeminal system could be due to the vasomotor effects of the substances, as suggested by the vascular theory, myography studies were performed. IS caused a strong contraction of the MMAs, whereas CFA did not result in any vasomotor response.

5. Discussion

To the best of our knowledge the present investigation is the first study to examine whether application of algescic substances onto the dural receptive field causes long-term inflammatory response in the trigeminal system. Local effects of the inflammatory substances on the MMA and neuro-immuno-glial activation in the trigeminal ganglion were followed. Also the effect of a novel KYNA derivate in the present chronification model of trigeminal activation was detected. The study addressed the question whether repeated, daily treatment of KYNA analogue would show more efficacy in abolishing the activation. Additionally we present the immunostaining pattern of several neuronal messengers and cytokines, potentially involved in migraine pathophysiology, in the TNC, other areas of the brainstem and C₁-C₂ region of the spinal cord. As one inflammatory substance we used an IS, a cocktail of bradykinin, histamine, serotonin and prostaglandin E₂, which are found to be released endogenously

during inflammatory states and they might play a role in neurogenic inflammation. CFA has been used for animal models of inflammatory and autoimmune disease. Although it has been used for more than 50 years some aspects of its way of action is still unknown. Following application of CFA on the dura mater we did not notice any sign of inflammation. We explain the lack of inflammatory signs (typically: tumor, calor, rubor, dolor) by the fact that inflammation in the CNS occurs in a different way due to multiple factors. It is well known that the CNS doesn't contain dendritic cells, their function is overtaken by macrophages and perivascular pericytes. The function of microglia, astrocytes and mast cells is depressed under physiological conditions, they get activated during pathological states. The presence of the BBB also changes the immune response in neurogenic inflammation, as the BBB permeability is low for large cells, like leukocytes. T cells can penetrate the BBB but they are not as efficient in immune response as the dendritic cells of other tissues. We presume that central sensitization might be the explanation for long-term potentiation. CFA has proven to cause the same activation as the IS, therefore we opted for CFA application in our further work. With reference to the time-points, we saw activation in the TG even after 4 hours but no change was noted between inflammatory agent-treated and saline-treated groups neither in pERK1/2 staining. This might be explained by the fact that there are numerous extracranial pain-sensitive structures including the skin, muscles and periosteum. We presume that the early activation is due to the operation itself, therefore we opted for the 7 days models for our future experiments.

Our findings support the theory of the immuno-neuro-glial interaction in the TG related to migraine. The pERK1/2 activation in the TG following activation emphasizes the role of SGCs in the pathophysiology of inflammation and pain. The condensed, homogenous material in the IL-1 β staining, close to the neuronal cell-membrane also represents a higher packing density of IL-1 β in the immediate neighbourhood of the SGCs.

In the second part of our work we asked the question whether application of CFA on a defined area of the dura mater could cause long-term activation of second-order neurons. Therefore we have studied various cytokines suggested being involved in the pathophysiology of migraine. Glu has proven to play a pivotal role in the CSD phenomenon and has been shown to be co-released with CGRP from the neurons of the TG upon activation. Increased Glu immunoreactivity suggests the long-term activation of the second-order neurons, leading to central sensitization. C-fos is a widely used proto-oncogene in neuronal activation of the TCC. We detected increased Glu and c-fos immunopositivity after CFA application. In case

of TNF α , IL-6 or IL-1 β staining we did not see any change. This might correlate with the temporary elevated concentration of these cytokines during migraine attacks that returns to normal after 1-2 hours. Our results also suggest the limitation of IHC studies as we cannot rule out an increased release from the trigeminal fibers that did not give any morphological alteration in the staining pattern. In PACAP staining slight activation was detected in the fasciculus cuneatus and cuneiformis. Further studies are needed to elucidate the role of this potential activation. We saw no obvious increase in SP staining, supporting the contradictory role of SP in migraine pathophysiology.

As presented in the introductory part, modulation of the kynurenine pathway might represent an appropriate therapeutic tool in migraine treatment and is currently investigated in preclinical animal studies. Although KYNA derivatives have proven to be effective in trigeminal activation models, some aspects still remain untangled regarding the exact site of action. In our study we detected abolished activation both in the TG and the TNC following treatment with KYNA analogue. We suggest two possible explanations: 1. In animal models of trigeminal activation KYNA derivatives act peripherally. We presume that the TG might be a possible site of action, as it plays a key role in trigeminal activation and it has proven to be placed outside the BBB. 2. Under pathological conditions, like neurogenic inflammation the BBB gets opened for various molecules which might explain the central effect of the substances upon trigeminal activation, without them being present in the CNS under physiological conditions. We designed our study to answer to the question whether daily, repeated treatment with KYNA analogue would be more potent than one dose prior the operation. We couldn't detect a more diminished reaction following chronic daily treatment, suggesting that KYNA derivatives might act as preventive treatment. Further pharmacokinetic and animal studies are planned to elucidate the site and mechanism of action of the KYNA analogues.

Our WB studies show the same tendency as the IHC without reaching the level of significance. This might be explained by a technical problem. In the IHC methodology the microscope study allowed us to identify different regions of the TG, whereas in case of the WB studies the whole TG was used for the protein measurements as it is impossible macroscopically to localize the exact area of V1 region.

While testing the model the myograph studies were done in order to test vasomotor effects of both substances. The vasogenic hypothesis of Wolff has proven to be obsolete due to the lack of evidence of major vasodilatation during migraine attacks with fMRI studies. Our myograph

studies also support this view as the IS caused a strong vasocontraction, whereas no change on the artery tone was noted following diluted or direct CFA administration on the MMA.

6. Original statements of the thesis

- I. To our knowledge this is the first study using chemical stimulation of rat dural receptive field with application of CFA on the dura mater. We demonstrate that CFA has the same ability to induce trigeminal activation as IS, which was widely used in previous studies.
- II. We found increased pERK and IL-1 β immunopositivity after 7 days following CFA induced activation, demonstrating that our experiment serves as a model of migraine pain chronification.
- III. We detected increased Glu and c-fos immunoreactivity in the TNC and C₁-C₂ region of the spinal cord after 7 days following CFA application on the dura, demonstrating activation of second-order neurons.
- IV. The novel KYNA derivate was able to diminish pERK and IL-1 β immunopositivity in the TG and Glu and c-fos immunoreactivity in the TNC and C₁-C₂ region.
- V. Daily treatment with KYNA derivate was not found to be more effective than one dose pre-treatment.
- VI. PACAP, SP, TNF α , IL-6 and IL-1 β immunoreactivity was detected in the fibers of Sp5C. PACAP positive fibers were seen in different areas of the brainstem, in almost every tract of the spinal cord and in fasciculus cuneatus and gracilis. TNF α , IL6 and IL-1 β immunoreactivity was noted in the large neurons of the anterior and posterior horn, close to the central canal and also in fibers of different spinal tracts.

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