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

Ph.D. Thesis

LUKÁCS MELINDA

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1. List of abbreviations:

3HK	3-hydroxykynurenine
5-HT	5-hydroxytriptamine (serotonin)
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BBB	Blood-brain barrier
CaMKII	Calcium-calmodulin-dependent protein kinase
CFA	Complete Freund's adjuvant
CGRP	Calcitonine gene related peptide
CNS	Central nervous system
CSD	Cortical spreading depression
CSF	Cerebro-spinal fluid
DAPI	4,6- diamino-2-phenylindole
EFNS	European Federation of Neurological Society
Glu	Glutamate
Gly	Glycine
GS	anti-glutamine synthetase
HE	Hematoxylin-Eosin (HE) staining
ICHD	International Classification for Headache Disorders
IDO1	Indolamine 2,3-dioxygenase 1
IHC	Immunohistochemistry
IHS	International Headache Society
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IS	Inflammatory soup
KAT	Kynurenine aminotransferase
KMO	Kynurenine 3-monooxygenase
KP	Kynurenine pathway
KYN	L-kynurenine
KYNA	Kynurenic acid
LC	Locus ceruleus
MMA	Middle meningeal artery
NI	Neurogenic inflammation
NKA	Neurokinin A
NMDA	*N*-methyl-D-aspartate
NO	Nitric oxide

NRM	Nucleus raphe magnus
NSAID	Non-steroid inflammatory drugs
NTG	Nitroglycerin
PACAP	Pituitary adenylate cyclase-activating polypeptide
PAG	Periaqueductal grey mater
PBS	Phosphate buffered saline
PPE	Plasma protein extravasation
QUIN	Quinolinic acid
SGC	Satelite glial cell
SP	Substance P
Sp5C	Spinal trigeminal tract
SuS	Superior salivator nucleus
TCC	Trigeminocervical complex
TG	Trigeminal ganglion
TNC	Trigeminal nucleus caudalis
TNFα	Tumor necrosis factor α
TRP	Tryptophan
VPM	Ventroposteromedial thalamus
WB	Western blot

2. Introduction

2.1. Migraine-epidemiology and general features

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 (1). It has a one year prevalence of 15-18% worldwide. Migraine has a huge effect on life quality (2) and also has high socio-economic costs (3). Due to the fact that migraine affects mostly young women (4), during the peak years of social productivity, migraine costs 19.6 billion $ in the United States (5) and 18.4 billon € in the European Union annually (3).

The International Headache Society (IHS) offers standardized diagnostic criteria for primary and secondary headaches, the International Classification for Headache Disorders (ICHD). This represents a great tool in improving the diagnostic accuracy leading to a more focused and specific research both in preclinical and clinical phase.

Diagnostic criteria for migraine without aura (6):

A. At least five attacks fulfilling diagnostic criteria B.-D.
B. Headache attacks lasting 4-72 hours (untreated or unsuccessfully treated)
C. Headache has at least two of the following four characteristics:
    - Unilateral location
    - Pulsating character
    - Moderate or severe pain intensity
    - Aggravation by or causing avoidance of routine physical activity (e.g. walking or climbing stairs)
D. During headache at least one of the following:
    - Nausea and/or vomiting
    - Photophobia and phonophobia
E. Not better accounted for by another ICHD-3 diagnosis.

The IHS also defines a new subtype, called chronic migraine where migraine headache occurs on more than 15 days per month per month, having the characteristics of migraine on at least 8 days per month for at least 3 months (6).

Although a lot has been done in order to understand the pathophysiology of migraine, some aspects still remain untangled. In 1940 Ray and Wolf put forward the vasogenic theory of migraine, caused by short period of vasoconstriction and a reactive vasodilatation of the cranial arteries (7). More recent studies have questioned this theory, suggesting that migraine pain originates in the central nervous system, with underlying importance of the phenomenon called cortical spreading depression (8, 9).

2.2. Clinical manifestation

Clinically migraine starts with the premonitory phase, represented by depression, tiredness or irritability that are linked to the hypothalamus (10). Aura phenomenon might inaugurate migraine attacks (11, 12). The most common signs are visual auras (scotomas, flashing lights) (13). Non-visual auras might be represented by olfactory, sensory (paraesthesia), motor (temporary paresis for example in familiar hemiplegic migraine) symptoms (11, 14) or speech disturbances (dysarthria) (15). The next phase is the headache itself with the features and accompanying symptoms mentioned above. Few studies have been focused on the last period, the postdrome phase of migraine. Prospective, diary-studies show that postdrome symptoms are similar to the premonitory signs of migraine attack, represented by tiredness, mood disturbance, concentration difficulties, neck stiffness (16).

1.3. Anatomy of the pain pathways

Migraineous pain sensation is thought to be consequence of trigeminovascular activation. The anatomy of the trigeminal system was in details described in the last decades, helping to understand the pathophysiology of migraine (17). 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 (18, 19). Mechanical, chemical or electrical stimulation of the dura mater proximate to these large blood vessels, has proven to give rise to different symptoms of migraine: headache, nausea and photophobia (20-22). These fibers are non-myelinated (C-fibers) or thinly myelinated fibers (Aδ 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 (23). Dural afferents also

terminate in the cervical dorsal root ganglion (24). These nociceptive nerve terminal contain calcitonine gene-related peptide (CGRP), substance P (SP), neurokinin A (NKA) and pituitary adenylate cyclase-activating polypeptide (PACAP) that are released upon activation (24-26). 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 C_1-C_2 region of the spinal cord (27). TNC and C_1-C_2 region is called trigeminocervical complex (TCC). Nociceptive information from the dural vasculature are relayed through the TCC, therefore stimulation of the dura mater in animal models leads to activation of the TCC (20, 28). 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 (29, 30). Dural nociceptive information are transmitted via the TCC mainly to the ventroposteromedial (VPM) thalamus (31). Somatosensory and viscerosensory inputs from the head and orofacial zone are conveyed to the hypothalamic nuclei (32), leading to the premonitory symptoms of migraine (sleep disturbance, mood changes, food craving) (30). Other afferents from the TCC are responsible for the pain processing and modulation like the superior salivator nucleus (SuS) (33), the locus ceruleus (LC), the nucleus raphe magnus (NRM) (34) and the periaqueductal grey mater (PAG) (35, 36). 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 (37, 38). A recent fMRI study has shown a cyclic behaviour in the activation of the spinal trigeminal neurons: interictally they are less responsive to trigeminal nociceptive stimuli compared to healthy controls, prior the attack activation of the trigeminal neurons normalizes and signal intensity becomes reduced again during migraine attacks. This change in the activation of the spinal trigeminal neurons might represent an important element in the generation of migraine attacks (39).

2.4. Theories of migraine

Despite a lot of studies made to reveal the pathomechanism of migraine, some aspects remain untangled. In 1940 Wolff and colleagues put op the vasogenic theory, stating that mechanical irritation of the perivascular nerve fibers, caused by a short vasoconstriction, followed by vasodilation might induce the pain in migraine (40). Recent imaging studies have demonstrated

that meningeal vessels do not dilate during migraine attacks and the modest vasodilatation in the intracranial arteries is not affected by triptan therapy, proving that the vascular theory is inappropriate (41). The importance of perivascular nerve endings is still undebatable and the headache is still believed to be mediated by these nociceptive fibers that terminate in the TG (42, 43). 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 (44-46). Clinical data regarding neurogenic inflammation is still contradictory: the effect of sumatriptan and non-steroid inflammatory drugs (NSAIDs) blocking plasma protein extravasation (PPE) (47, 48) supports the occurrence of NI, but the fact that the main migraine marker CGRP has failed to produce PPE (49) could question this theory. The prodromal symptoms and the role of cortical spreading depression (CSD) in migraine suggested that the starting point of the attack is the central nervous system (CNS). Cortical spreading depression is a depolarization wave, followed by hyperpolarization, starting from the occipital lobe (50). There is a strong belief that CSD can be correlated with the aura phenomenon (51, 52) and altered cortical switch between hyper- and hypoexcitability states are present in the CNS of migraine patients (53, 54). Another hypothesis emphasizes the role of the above mentioned brainstem and diencephalic nuclei. They are presumed to be “migraine-generators” or “migraine-modulators”, as they normally gate sensory inputs and their dysfunction leads to pain sensation (43). An important feature would be the spectrum of actions that arise during peripheral and central sensitization, leading to chronification of pain sensation. In case of peripheral sensitization dural nerve endings become sensitized for mechanical and chemical agents, leading to the throbbing character of migraine pain, aggravated by physical activity (21). Central sensitization of the trigeminal neurons are likely to account for the allodynia (55). All these data support the crucial role of understanding the pathophysiology of migraine in order to reassess the mechanisms that exist in migraine. To our concept migraine is a neuronal disease that originates in central mechanisms that causes hypersensitivity of the nociceptive, dural perivascular nerve endings.

2.5. Migraine therapy

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-hydroxytriptamine (serotonin) $_{1B/1D}$ receptor agonists ($5HT_{1B/1D}$). Their way of administration are: subcutaneous, oral, suppository and intranasal (56). Large clinical trials have proven the efficacy of triptans, although they are effective only in 28-59% (57-59). They should be taken very early, in the beginning of the headache, therefore repeated administration might be necessary leading to drug overuse. The administration of triptans is restricted to 9 days/month as they tend to elevate the risk of chronification (60). Therefore they do not represent a therapeutic strategy in case of chronic migraine. Due to their rare but severe cardiovascular side-effects triptans are contraindicated in stroke, coronary disease, hypertension and pregnancy (61, 62). Other drugs of choice in case of acute therapy are NSAIDs, antiemetics to treat nausea and ergot alkaloids. 2-4 hours following treatment with NSAIDs pain restarts in 80% of the patients and the analgesics do not represent migraine specific therapy (63-65). In case of ergot alkaloids very few randomized, placebo-controlled trials are available (56). Triptans have proven superiority in efficacy compared to ergot alkaloids (66, 67) but lower recurrent rates of headache was noted in case of ergot alkaloid usage, therefore they can be used for patients having long attacks or regular frequency (56). Prophylactic treatment is needed when days with headache exceeds 8-10 days/month (68). Preventive treatment includes β-adrenergic blocking agents, calcium ion channel blockers, antiepileptic drugs and antidepressants (56, 69). Chronic migraine represents a therapeutic challenge for clinicians and researchers. As mentioned above, triptans cannot be used in case of chronification, and due to multiple drug therapy the occurrence of clinically significant drug-drug interaction is high (70, 71). Lately, intramuscular injection of Botulinum toxin A has proven to be efficient in chronic migraine but its way of administration represents a clear disadvantage (72-74).

2.6. Animal models

All the above mentioned suggest that a lot has been done to understand the pathophysiology of migraine but some aspects still need to be untangled. Beside the central role of the trigeminal system, other brain structures and phenomenons like NI or CSD seem to be involved. All these highlight the necessity for further studies in order to understand the pathomechanism of migraine and develop migraine-specific new therapeutic targets. To study the mechanisms involved in migraine animal models are used. To our knowledge, no animal model has been developed so far

which would be able to completely reflect the clinical manifestations of human migraine, therefore various animal models of trigeminal activation are available.

In case of the orofacial formalin test formalin is injected subcutaneously in the whisker pad of rats, causing the activation of the trigeminal pathway (75). Electrical stimulation of the TG represents another animal model widely used in preclinical studies. Usually a hole is drilled through the skull using specific coordinates and a bipolar electrode is introduced in the TG, causing PPE, release of specific neuropeptides (49) and activation of the TNC (76). Also increased level of CGRP in extracerebral circulation was detected following electrical stimulation of the TG both in animal models and human (77, 78). Nitroglycerin (NTG), a lipophilic molecule that is able to penetrate through the blood-brain barrier (BBB), is known to be a strong vasodilator due to release of nitric oxide (NO) (79). Following (intraperitoneal) i.p. administration of NTG in rats increased synthesis on neuronal nitric oxide synthase (nNOS) was detected in the TG and TNC (80). 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 (20, 21, 81).

We consider that chemical stimulation of the dura mater with algesic or inflammatory substances might represent a suitable method for *in vivo* examination, therefore we opted for this animal model in our research work. Freund's adjuvant is an antigen emulsion in mineral oil used as immunpotentiator. The complete form of the solution, called Complete Freund's adjuvant (CFA) contains dried and inactivated Mycobacterium tuberculosis. CFA injected in the hind paw of rats is widely used as a model for acute and chronic peripheral pain (82, 83). CFA was also used before in a model of trigeminal stimulation via injection in the temporomandibular joint, causing long-term activation (84).

2.7. The kynurenine pathway

Tryptophan (TRP), the precursor of the neurotransmitter 5-HT is an essential α-amino-acid, is the precursor of the neurotransmitter 5-HT (85). The major route for TRP metabolism is the kynurenine pathway (KP), having NAD^+ and $NADP^+$ as end products. In this metabolic pathway, L-kynurenine (KYN) can be metabolized through two branches: one branch providing the neuroprotective kynurenic acid (KYNA) and another branch providing the neurotoxic quinolinic acid (QUIN) (86-88). Both neuroactive metabolites of the KP have been shown to play important roles in various

CNS diseases (89, 90) acting mainly on the glutamate (Glu) receptors. *N*-methyl-D-aspartate (NMDA) receptor consists of three subunits (NR1, NR2, and NR3) and is activated by binding of both Glu and glycine (Gly). Gly is essential for the proper functioning of the NMDA receptor with the glycine binding site being located on the NR2 subunit (91, 92). KYNA acts on the NMDA receptors binding to the Gly-binding site (91) and also on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-sensitive (AMPA) receptors with a dual action: in low concentrations it enhances, whereas in higher concentrations it decreases the activity of the receptor (93, 94). This leads to the conclusion that modulation of the kynurenine pathway might represent an appropriate therapeutic tool in migraine treatment. As KYNA has a very low capacity to penetrate the BBB, different strategies are needed in order to take advantage of its anti-inflammatory and neuroprotective properties. One possibility could be the use of a prodrug such as L-kynurenine or its derivates that can cross the BBB (95-97); whereas shifting the KP towards the production of KYNA using different enzyme inhibitors would represent another possible therapeutic strategy (98, 99). During the last years, our research group has synthesized several different KYNA derivates to help BBB penetration. These derivatest have proven to be effective in animal models of cerebral ischemia (100), Huntington's disease (101), epilepsy (102) and trigeminovascular activation (69). The new KYNA analogues have proved their efficacy in the TNC following nitroglycerin-induced c-fos activation (96, 103). A possible interaction between KYNA and inflammatory cytokines (IFNα, IFNγ, TNFα, TGF-β, IL-1β, IL-4, IL-6, IL-23) suggests an interaction between the KP and the immune system, leading to the idea that one possible site of action for KYNA derivates could be neurogenic inflammation (104, 105). The possible interaction between the KP and inflammation is presented on Figure 1.

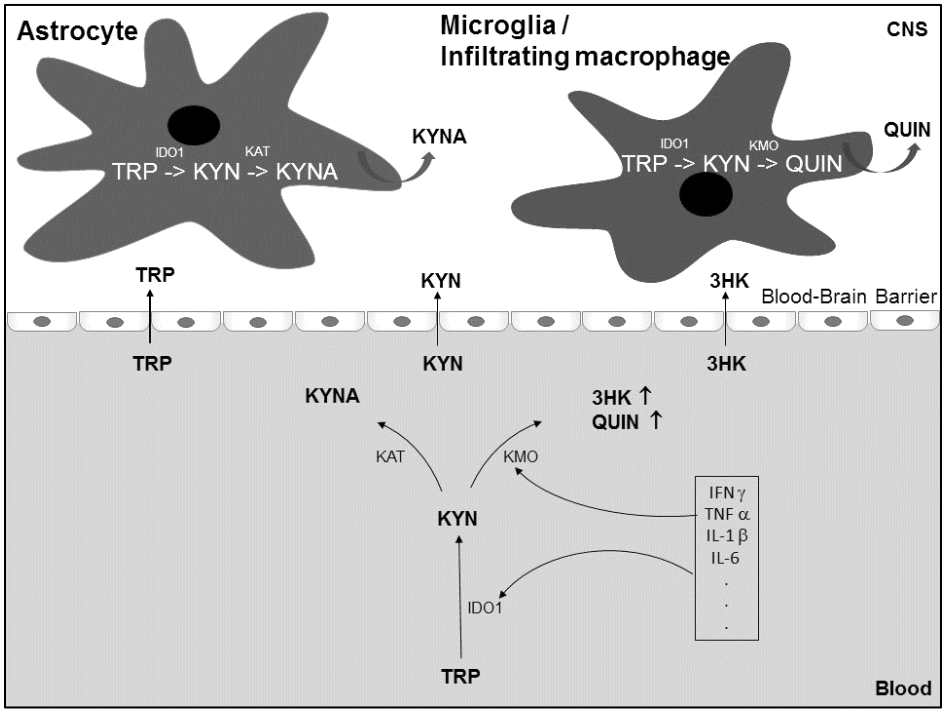


Figure 1. The kynurenine pathway (KP) and inflammation. The figure presents a simplified version of the KP. In the first metabolic step, tryptophan (TRP) is converted to L-kynurenine (KYN) in a process mediated by indolamine 2,3-dioxygenase 1 (IDO1). Kynurenine 3-monooxygenase (KMO) mediates the metabolism of KYN into 3-hydroxykynurenine (3HK) and quinolinic acid (QUIN), a neurotoxic metabolite. The other, neuroprotective branch of the KP results in the production of kynurenic acid (KYNA) and is mediated by kynurenine aminotransferase (KAT). Inflammatory mediators increase the activity of IDO1 and KMO, leading to elevated levels of 3HK and QUIN, whereas they have no effect or even decrease the activity of KAT. 3HK and KYN can be transported across the BBB and can be used for further QUIN production in the CNS as QUIN cannot pass the BBB. Microglia and macrophages, cells that under inflammatory conditions can penetrate the BBB, express the KMO branch of the KP, leading to elevated levels of toxic 3HK and QUIN. On the other hand, astrocytes convert TRP to KYNA, as they contain KAT and they are unable to produce QUIN, as they lack KMO.

3. Aims

The aims of our study are the following:

I. Development of a new animal model for trigeminal activation using chemical stimulation if 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.

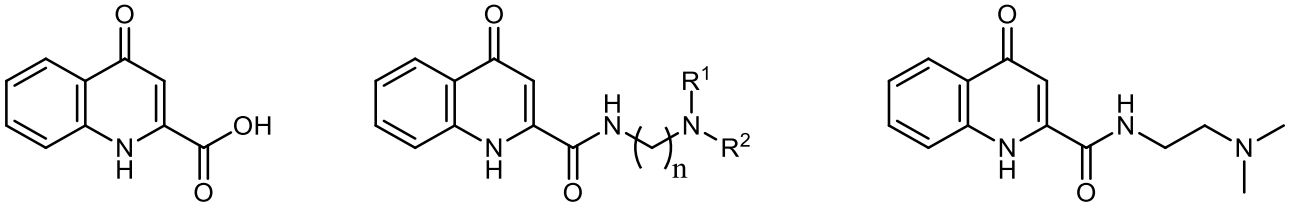
V. To clarify whether repeated treatment of KYNA analogue is more effective than one dose pre-treatment.

VI. To provide a mapping of different inflammatory cytokines and neuropeptides in the TNC, other areas of the brainstem and C_1-C_2 region of the spinal cord.

4. Materials and methods

4.1. 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 (106).



1

4-oxo-1,4-dihydroquinoline-2-carboxylic acid

2

3

N-(2-(dimethylamino)ethyl)-4-oxo-1,4-dihydroquinoline-2-carboxamide

Figure 2. Chemical structure of KYNA and KYNA derivates. 1. Chemical structure of KYNA. 2. General chemical formula for the KYNA derivates, containing a cationic side-chain. 3. Chemical structure of the KYNA derivate used in our study (106).

4.2. Animal procedures

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 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. The

craniectomy was done on the left side, postero-laterally to the bregma with caution not to penetrate the dura (Figure 3).

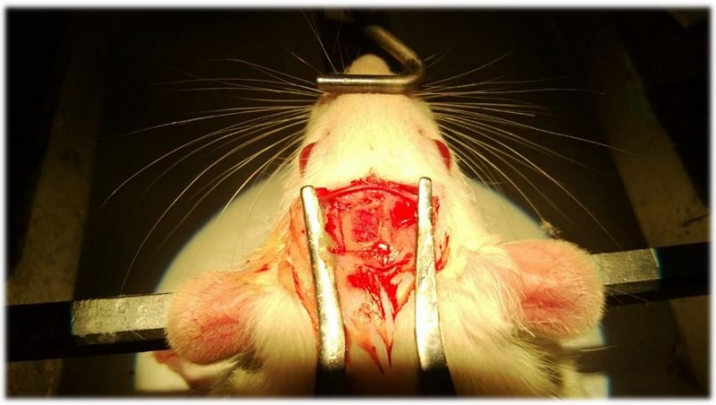


Figure 3. Craniectomy. The animal was deeply anesthetized with chloral hydrate. The head of the animal was fixated in a stereotaxic frame to prevent the substances from spreading. The craniectomy was done on the left side, postero-laterally to the bregma.

For 30 rats inflammatory soup (IS), containing 10 µM bradykinin, 10 µM serotonin, 10 µM prostaglandin E2 and 100 µM histamine, pH 5.0; the recipe was adapted from Strassman et al. (21); 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 (Table 1). Same amount of IS, CFA or saline was used (10 µl) and left on the dura for 20 min. In order to prevent the substances from spreading, the head of the animal was fixated in the stereotaxic frame so that the dural surface covered with the liquid was completely horizontal. After 20 min, the IS or CFA was washed out with saline, the hole was covered with bone wax and the wound was sutured. The effect of IS, CFA and saline were examined after 4 h, 24 h and 7 days. At the end-points the animals were transcardially fixation-perfused with 4% paraformaldehyde in 0.1 M phosphate-buffer (PB) for immunohistochemistry. TG from both sides, TNC brainstem region and C_1-C_2 region of the spinal cord were removed (-1, +5 mm from the obex).

		CFA	Soup	Saline	Fresh
IHC					
	4 hours	6	6	3	
	24 hours	6	6	3	
	7 days	6	6	3	
	Unoperated (fresh)				3
WB					
	4 hours	0	0	0	
	24 hours	6	6	6	
	7 days	6	6	6	
	Unoperated (fresh)				6

Table 1. Number of animals used for testing the animal model.

For the myographic studies, 5 unoperated (fresh) rats CO_2 anaesthesia was used and rats were decapitated. Middle meningeal artery (MMA) was dissected free, using dissection microscope. The arteries were isolated in 119 mM NaCl, 15 mM $NaHCO_3$, 4.6 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 5.5 mM glucose and 26 μM EDTA. Following the dissection, a similarly composed buffer, also including 1.5 mM $CaCl_2$ and without EDTA, was used. All solutions were aerated with gaseous mixture composed of 95% O_2 and 5% CO_2, to maintain a pH of 7.4.

4.3. I.p. treatment with KYNA analogue

The test the effect of KYNA derivate treatment, 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) (Table 2). The KYNA analogue (1 mmol/kg bodyweight dissolved in 1 ml saline) or saline (1 ml) was administrated i.p. prior the operation.

Groups	1 hour before the operation	Every 12 hours for 7 days	No. of animals for IHC	No. of animals for WB
Acute treatment KYNA-an	KYNA derivate	-	6	5
Acute treatment saline	saline	-	4	5
Repeated treatment KYNA derivate	KYNA derivate	KYNA derivate	6	5
Repeated saline	saline	saline	4	5
Unoperated	-	-	4	5

Table 2. Number of animals used for different treatment methods.

4.4. Tissue analysis

For IHC studies specimens were post-fixated for one day in 4% paraformaldehyde in 0.1 M PB, followed by cryoprotection using 10%, 20% and 30% glucose in 0.1 M PB. Samples were frozen on dry ice, stored at -80 ºC and transported to Lund University were the IHC studies were performed. Samples were embedded in gelatine medium (30% egg albumin, 3% gelatin), cryosectioned at 12 µm and stored at -20 ºC until use. To encompass the whole image of the TNC and C_1-C_2 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 (107). As the preliminary immunohistochemical studies revealed no difference between the treatment groups at 4 hours this time point was not investigated with WB.

Animals for WB were perfused transcardially with PBS after 24 hours or 7 days. As healthy controls unoperated (fresh) rats were used. TG were immediately frozen on dry ice and stored at -80 ºC until assay.

4.5. Hematoxylin-Eosin (HE) staining

For orientation and examination of tissue conditions HE staining was performed using the standard protocol (Htx 4 min, Eosin 30 s).

4.6. Immunohistochemistry

Immunofluorescence staining was performed to demonstrate the localisation of different mediators and neurotransmitters in the TG, TNC and C_1-C_2 region. Sections were thawed at room temperature, then rehydrated in PBS containing 0.25% Triton X-100 (PBS-T) for 15 min, followed by exposure to primary antibodies in PBS-T containing 1% bovine serum albumin (BSA) and incubated overnight at 4 ºC. Sections were subsequently equilibrated at room temperature, washed in PBS-T for 3x15 min, followed by incubation with the secondary antibody for 1 h in a dark room, at room temperature. Sections were rinsed again in PBS-T for 3x15 min and mounted with anti-fading mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA). For nucleus staining we used Vectashield medium containing 4,6- diamino-2-phenylindole (DAPI; Vector Laboratories). Sections in which the primary antibody was omitted served as negative controls. For localisation of pERK1/2 immunopositivity double staining was performed using primary antibodies against pERK1/2 and anti-glutamine synthetase (GS) separately, not mixed in a cocktail. Stainings were repeated two times and analysed blinded by two separate examiners.

Name	Product code	Host	Dilution	Company
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	4376	Rabbit	1:50	Cell Signaling Technology, Danvers, MA, USA
Anti IL-1 beta antibody	ab 9787	Rabbit	1:100	Abcam; Cambridge, UK
Calcitonin-gene related peptide, polyclonal	B-47-1	Rabbit	1:800	Europroxima, Arnhem, Netherlands
Phospho-p38 MAPK (Thr180/Tyr182)	9216	Mouse	1:200	Cell Signaling Technology, Danvers, MA, USA
Phospho-SAPK/JNK (Thr183/Tyr185)	9252	Mouse	1:200	Cell Signaling Technology, Danvers, MA, USA
Anti TNF-alpha antibody	ab66579	Rabbit	1:200	Abcam; Cambridge, UK
Anti IL-6 antibody	ab6672	Rabbit	1:100	Abcam; Cambridge, UK
Anti-Glutamine Synthetase Antibody, Clone GS-6	MAB302	Mouse		Merck Milipore, Darmstadt, Germany
Anti c-fos	PC38	Rabbit	1:100	Merck Millipore, Darmstadt, Germany
Anti PACAP-38	B57-1	Rabbit	1:100	Europroxima, Arnhem, Netherlands
Anti Glutamate	G9282	Mouse	1:100	Sigma-Aldrich, St-Luis, MO, USA

Anti Glutamate	AB5018	Rabbit	1:100	Merck Millipore, Darmstadt, Germany
Anti Substance P	B 45-1	Rabbit	1:200	Europroxima, Arnhem, Netherlands
Anti IL-1β	ab 9787	Rabbit	1:100	Abcam, Cambridge, UK
Anti IL-6	ab6672	Rabbit	1:200	Abcam, Cambridge, UK
Anti-TNF α	ab66579	Rabbit	1:400	Abcam, Cambridge, UK

Table 3. Details of primary antibodies used for IHC.

Conjugate and host	Against	Dilution	Company
FITC (goat)	anti-rabbit	1:100	Cayman Chemical, Ann Arbor, MI, USA
Alexa 488 (goat)	anti-mouse	1:100	Invitrogen, CA, USA
Alexa 594 (donkey)	anti-rabbit	1:100	Jackson Immuno Research, West Baltimor, PA, USA

Table 4. Details of secondary antibodies used for IHC.

4.7. Microscopic analysis

Sections were examined and images were obtained by using a light- and epifluorescence microscope (Nikon 80i, Tokyo, Japan), coupled to a Nikon DS-2 MV camera. For image analysis we used FITC (480 nm), TRITC (540 nm) and DAPI (360 nm) filters. Adobe Photoshop CS3 (v.8.0; Adobe Systems, Mountain View, CA, USA) was used to visualize co-labelling by superimposing the digital images.

4.8. Western blot

The TGs were homogenized in cell extract denaturing buffer (BioSource, Vacaville,CA, USA), containing phosphatase and protease inhibitor cocktails (Sigma, St. Louis, MO, USA). Following centrifugation (12,000 rpm, 4 ºC, 10 min) the supernatants were collected. Protein concentrations were determined with a protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) and a Tecan Infinite M200 microplate reader. Protein samples were mixed with Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA, USA) and heated (95 ºC, 4 min). Afterwards equal amounts (40 µg) of protein were loaded onto 4–15% Ready Gel Precast Gels (Bio-Rad Laboratories,

Hercules, CA, USA) with a molecular weight marker (Precision Plus Protein Standard, Bio-Rad Laboratories, Hercules, CA, USA). Following gel electrophoresis, blocking in 5% non-fat milk or BSA and incubation with primary antibodies (4 ºC, overnight) and secondary antibodies (1 hour, room temperature) were performed. Details of the primary and secondary antibodies used are given in Tables 5 and 6. The membranes were visualized with Fujifilm LAS-1000 Luminescent Image Analyzer (Fujifilm, Stamford, CT, USA) and finally 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.

Name	Product code	Host	Dilution	Company
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	4376	Rabbit	1:1000	Cell Signaling Technology, Danvers, MA, USA
Anti IL-1 beta antibody	ab 9787	Rabbit	1:500	Abcam; Cambridge, UK
Anti-Calcitonin Gene Related Peptide	C8198	Rabbit	1:250	Sigma-Aldrich, St. Louis, MO, USA
Anti-CGRP antibody	ab47027	Rabbit	1:900	Abcam; Cambridge, UK
Anti β-actin	Sc-477778	Mouse	1:5000	Santa Cruz Biotech, Santa Cruz, CA, USA
GAPDH (D16H11) XP mAb	5174	Rabbit	1:1000	Cell Signaling Technology, Danvers, MA, USA

Table 5. Details of primary antibodies used for WB.

Conjugate and host	Against	Dilution	Company
HRP-conjugated	Anti-rabbit	1:2000	Cell Signaling Technology, Danvers, MA, USA
HRP-conjugated	Anti-mouse	1:2000	Cell Signaling Technology, Danvers, MA, USA

Table 6. Details of secondary antibodies used for WB.

4.9. Myography

Each ~2 mm long segment of the MMA was mounted in an arterial myograph, on a pair of 25 μm metal wires. One of the wires was connected to a micrometer screw where the vascular tone could be adjusted and the other wire to a force displacement transducer, paired with an analog/digital converter (ADInstruments, Oxford, UK). Data were recorded on a computer through use of a PowerLab unit. For recording and calculations LabChart (ADInstruments, Oxford, UK) was used. All experiments were done at 37 ºC. The first step was to do the normalisation of the vessels to 90% of the internal circumference a vessel would have at 50 mmHg. A reference value was created by replacing part of the NaCl with KCl (60 mM K^+); any vessel with a maximum contractile capacity of less than 0.1 mm was excluded. Segments were stably precontracted with 30 mM K^+. CFA and IS was applied on the segments.

4.10. 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.

4.11. 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). The animals were raised and maintained under standard laboratory conditions, on a 12h light-dark cycle, with free access to tap water and rat chow. The number of animals used and their suffering were kept at minimum.

5. Results

5.1. HE staining

By keeping the orientation of the TG while preparing the samples, we could identify the peripheral (cortical) and the central (medullary) zones, and also the V1, V2 and V3 regions of the TG. HE staining was performed to identify the typical structure if the TG: neurons of different sizes, surrounded by a single layer of SGCs. These neuron/SGC units were intermingled between fibers. The morphology of the different TGs was in general good, though tissue shrinkage was observed in some of the TGs.

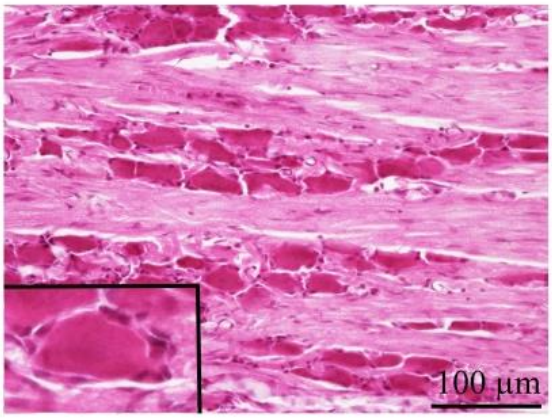


Figure 4. HE staining of the TG. Different sized neurons, surrounded by SGC and intermingled between fibers.

5.2. Immunohistochemistry

5.2.1. Trigeminal ganglion

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

pERK1/2 immunoreactivity was observed in a few neuronal nuclei and nucleoli in the fresh TGs. We could not detect any immunoreactivity in the cytoplasm of the neurons or SGCs. The negative control, i.e. when the primary antibody was omitted, displayed no immunoreactivity.

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, only few SGCs were activated in the medullar zone of the V1 (anteromedial) region, compared to the peripheral zone. We couldn't note any significant difference between the IS, CFA or the saline groups. At 24 h and 7 days high-intensity pERK1/2 immunoreactivity was detected in the anteromedial part of the TG in the SGCs in both IS and CFA groups. At 7 days CFA caused activation was still present in the TG, whereas IS samples have shown decreased immunopositivity compared to the 24 hours samples. This activation was missing in the saline specimens. No difference was seen between left and right side (Figure 5).

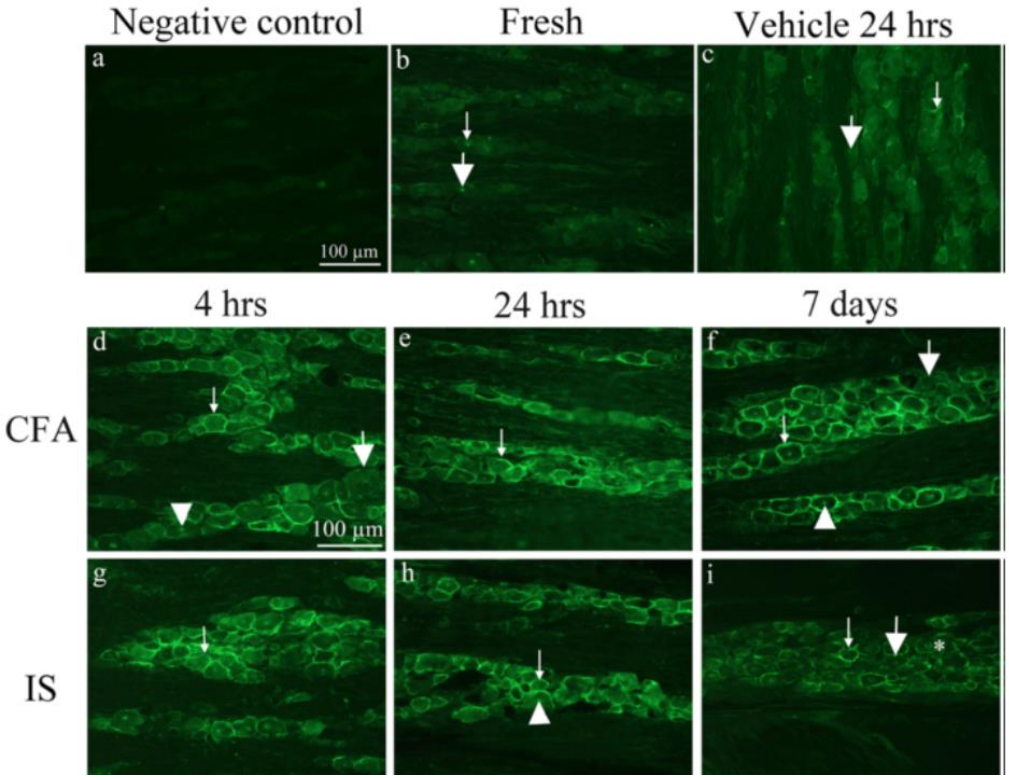


Figure 5. pERK1/2 immunostaining in the TG a. Negative control, showing no immunoreactivity. b. pERK1/2 staining in fresh animals with a few positive nuclei (thin arrow) and nucleolei (thick arrow). c. 24 hrs saline group showing similar aspect with a few positive SGCs (thin arrow). pERK1/2 immunopositivity was observed following application of CFA and IS at different time-points (d-i). Positive SGCs (thin arrow) could be detected at all three time-points. Also some negative SGCs were seen (thick arrow). In the 7 days CFA model only few negative SGCs (thick arrow) were observed with a lot of intensely stained SGCs (thin arrow).

Positive nuclei (arrow head) were also seen at all three time-points, more remarkably at the 7 days CFA model. Some positive neuronal nuclei with no nucleoli could also be detected (asterics). Immunopositive fibers were detected in all the samples.

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 (Figure 6).

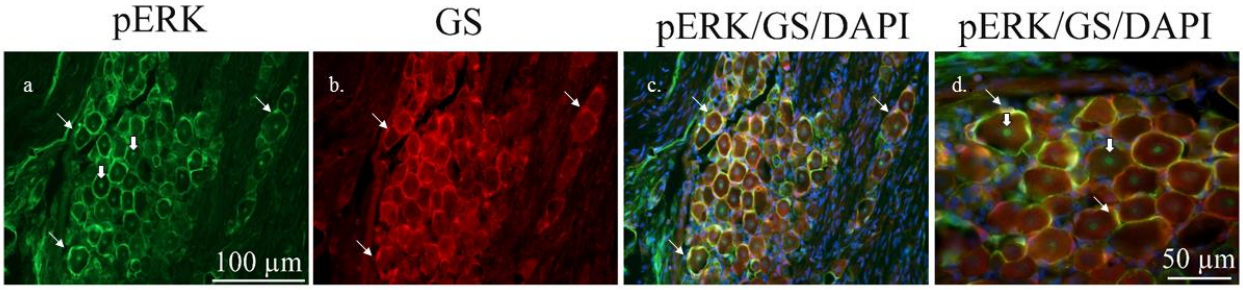


Figure 6. pERK1/2 and GS double IHC staining in the TG in 7 days CFA model. a. Intense p-ERK1/2 immunoreactivity in the SGCs (thin arrow) and in the neuronal nucleoli (thick arrow). b. GS staining is specific for the SGCs (thin arrow). c-d. Colocalisation of pERK1/2 and GS in the SGCs (thin arrows) is shown on the merged images. Thick arrows point to the positive nucleoli. Nuclear DAPI staining is also included in the images.

In contrast KYNA analogue treatment resulted in abolished pERK1/2 immunoreactivity in SGC. Repeated treatment with the KYNA derivate mitigated the SGC activation compared to saline treatment; both positive and negative SGC were detected. No difference was noted following repeated treatment with KYNA analogue.

A summary of the results for pERK1/2 immunostaining in different treatment groups, using a semi-quantitative method (+/-) is presented in Table 7.

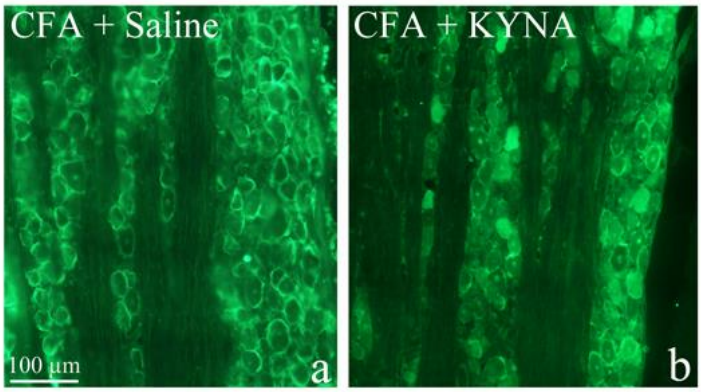


Figure 7. pERK 1/2 immunoreactivity. Treatment with KYNA derivate following CFA induced activation in the TG. High intensity staining can be detected in the SGCs following application of CFA on the dura mater (a). KYNA derivate pretreatment was able to diminish CFA induced activation of the SGCs (b).

	pERK			IL-1β		
	Neurons (nucleoli)	**Fibers**	**SGC**	**Neurons**	**Fibers**	**SGC**
Acute treatment KYNA derivate	+	+	+/-	+/-	+	-
Acute treatment saline	+	+	+++	+++	+	-
Repeated treatment KYNA derivate	+	+	++	+/-	+	-
Repeated treatment saline	+	+	+++	+++	+	-
Unoperated (fresh)	+	+	+/-	-	+	-

Table 7. Summary of a semi-quantitative evaluation of pERK1/2 and IL-1β immunopositivity in different treatment groups.

b. Interleukin 1β (IL-1β)

IL-1β immunoreactivity was observed in the cytoplasm (in a granular manner) (Figure 8) 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 (Figure 8).

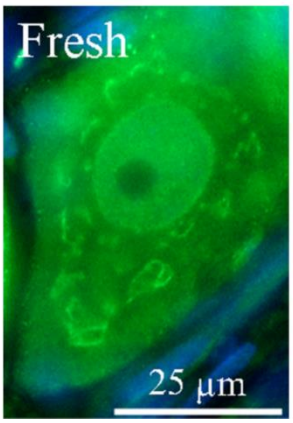


Figure 8. IL-1β IHC staining in fresh (unoperated) animals. IL-1β immunopositivity was detected in the cytoplasm of the neurons, in a granular manner, close to the neuronal nucleus. Neuronal nuclei were also stained, without the nucleoli. Nuclear DAPI staining was also included in the merged image.

In the CFA and IS treated rats, increased IL-1β immunoreactivity was observed intracellularly in the neurons and in the fibers at all three time points compared to saline treatment specimens. However, at 7 days both in the CFA and IS groups, homogeneous, condense and intensely stained IL-1β 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 (Figure 9). We could not detect any immunopositivity in the SGCs and no difference was noted between left and right side.

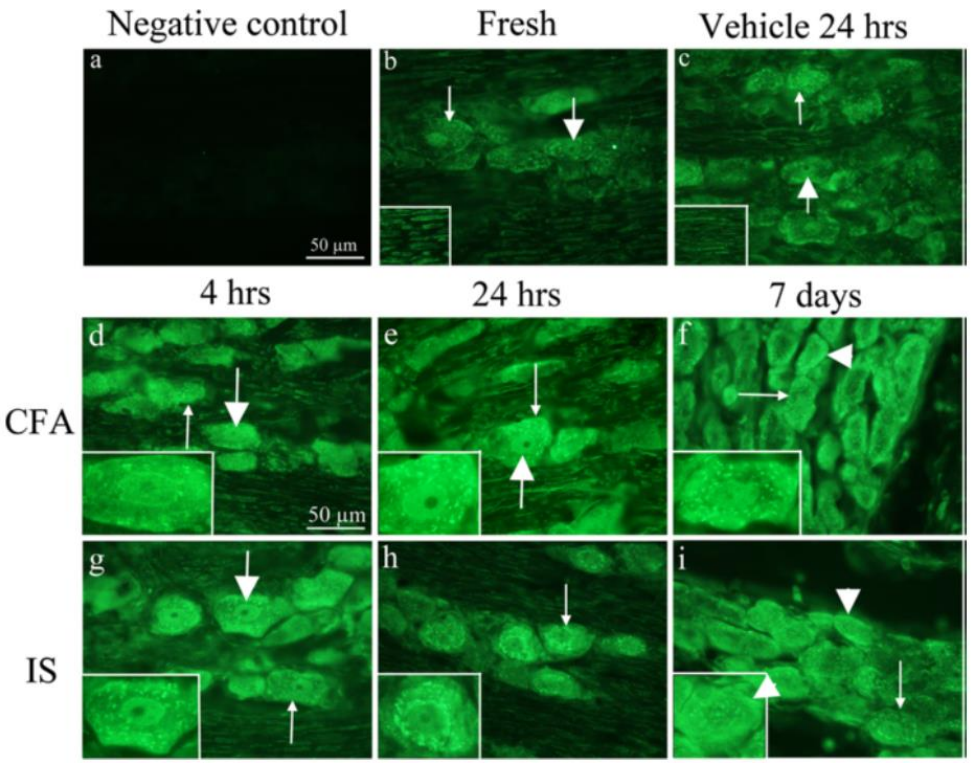


Figure 9. IL-1β immunopositivity in the TG. a. Negative control (omitting the primary antibody) showed no immunopositivity. b. In case of fresh (unoperated) rats positive neuronal nuclei (thick arrow) and granular cytoplasmatic staining (thin arrow) was detected. c. Following application of saline (vehicle) on the dura mater no change was noted. d-i. IL-1β immunopositivity was observed following application of CFA and IS at different time-points. In the 7 days models increased immunoreactivity was observed as a homogenous, condensed material close to the cell-membrane (arrow-head). In all the samples positive fibers were seen.

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. We considered this result as an abolished reaction induced by the KYNA treatment as it was missing following i.p. treatment with saline. In case of i.p. saline treatment the same homogenous arrangement was present. No difference was noted in the staining of the neuronal nuclei and the fibers, and also no immunoreactivity was seen in the SGC (Figure 10).

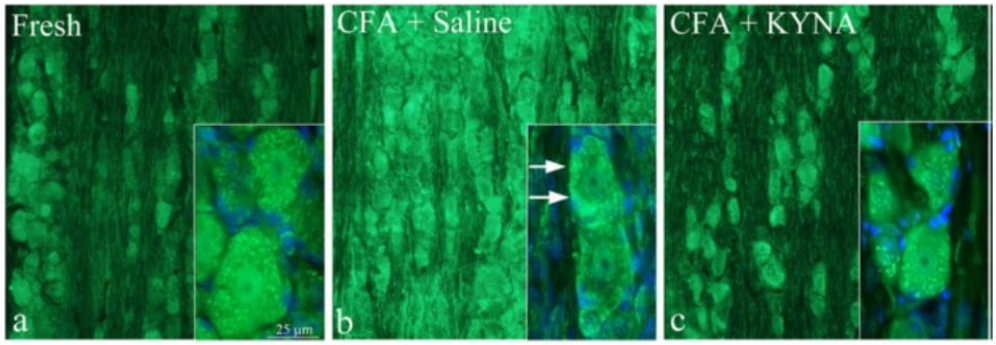


Figure 10. IL-1β immunoreactivity. Treatment with KYNA derivate following CFA induced activation in the TG. a. IL-1β immunoreactivity in fresh (un-operated) animals, showing positive neuronal nuclei, fibers and granular cytoplasmatic staining. b. Following i.p. pre-treatment with saline and application of CFA on the dura mater an intense, homogenous staining appeared, close to the neuronal cell membrane (arrows). c. I.p. pre-treatment with KYNA derivate was able to abolish CFA induced activation and the image was similar to the fresh samples. No condensed, homogenous material was observed in the neuronal cytoplasm.

A summary of the results for IL-1β immunostaining in different treatment groups, using a semi-quantitative method (+/-) is presented in Table 7.

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. No immunoreactivity was seen in the SGCs. The negative control did not exhibit immunoreactivity (Figure 12 a-c).

In the CFA and IS treated rats, CGRP immunopositivity 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, no change was seen in neuronal of SGC staining. No difference was observed between the different time-points or between the findings for IS or CFA (Figure 11). As no change in the staining pattern was detected, no CGRP staining was performed for KYNA derivate treatment groups.

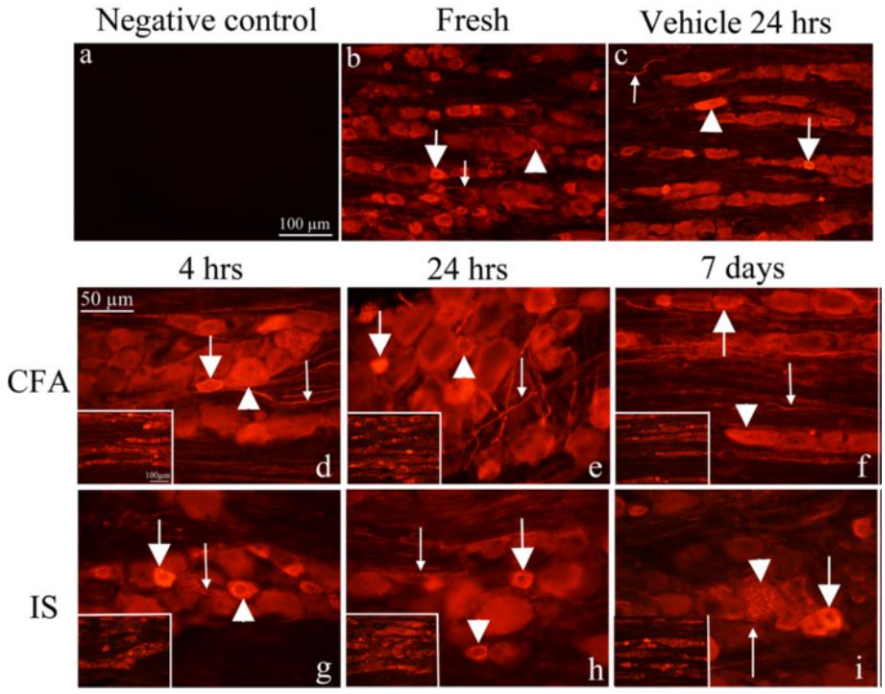


Figure 11. CGRP immunoreactivity in the TG. The negative control show no immunoreactivity. b.-i. Immunoreactivity in fresh (unoperated) animals and application of saline (vehicle), CFA and IS at different time-points. Pear-like fiber staining could be detected in all the samples, a slight increase in the staining intensity was noted in CFA and IS groups (thin arrow). Small, homogenously stained neurons were observed, with empty nuclei (thick arrow). Granular cytoplasmatic staining in the medium- and large-sized neurons was seen (arrow head).

5.2.2. Trigeminal nucleus caudalis and C_1-C_2 region of the spinal cord

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 could also be detected. Also some neurons, showing the same staining pattern were seen in the caudal part of the TNC. Following application of CFA on the dura mater, an obvious increase in the intensity and amount of Glu positive neurons was noted in the TNC, showing the same staining pattern. In the gelatinous layer a clearly increased intensity was observed and with higher magnification, cells with intensely stained cytoplasm were identified. 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 and the CFA induced activation was diminished, 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 (Figure 12). A summary of the results for Glu immunostaining in different treatment groups, using a semi-quantitative method (+/-) is presented in Table 8.

On C_1-C_2 level of the spinal cord immunopositivity was seen in the anterior and dorsal horn (lamina I, lamina II) and the areas surrounding the central canal. In this region no difference was noted between the animal groups.

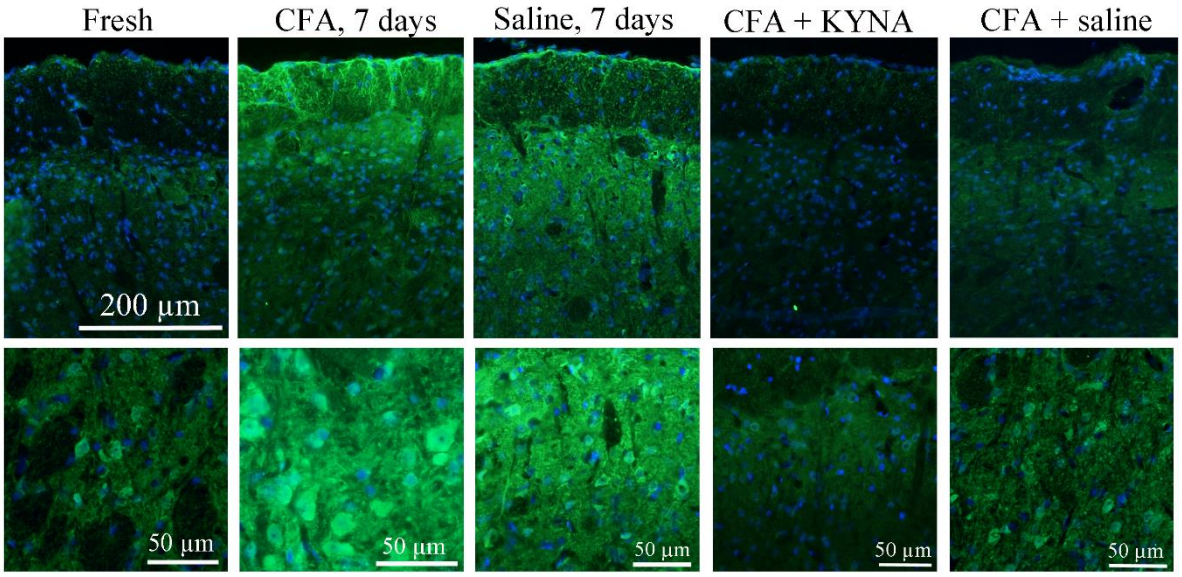


Figure 12. Glu immunoreactivity in TNC. In case of fresh (unoperated) animals a few homogenously stained neurons were detected in the TNC. Following CFA application on the dura a clear increase was observed both in the staining intensity and in the amount of Glu positive cells. After saline application on the dura a slight increase was detected compared to the fresh samples. I.p. treatment with KYNA derivate diminished the amount of Glu positive cells following CFA induced activation, whereas i.p. saline treatment could not abolish CFA induced activation. With

higher magnification homogenously stained neurons, with empty nucleoli could be observed. Glu positive fibers were detected in all the samples.

Group	Neurons	Fibers	Glial cells
CFA+no treatment	+++	++	-\+
Saline+no treatment	+	++	-\+
CFA+acute treatment KYNA derivate	-\+	++	-\+
CFA+repeated treatment KYNA derivate	-\+	++	-\+
CFA+acute saline treatment	+++	++	-\+
CFA+repeated saline treatment	+++	++	-\+
Fresh (unoperated)	-\+	++	-\+

Table 8. Summary of a semi-quantitative evaluation of Glu immunopositivity in different treatment groups.

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. No variation was detected between different parts of the TNC.

After application of CFA an increased amount of c-fos positive nuclei could be detected, especially in the caudal areas of the TNC, close to the spinal cord. 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. The aspect was similar to the low expression seen in fresh rats. No significant difference could be observed following repeated-treatment with KYNA derivate. After i.p. administration of saline, we noted no change in c-fos expression, showing that treatment with saline did not have effect on the CFA-induced TNC activation (Figure 13).

A summary of the results for c-fos immunostaining in different treatment groups, using a semi-quantitative method (+/-) is presented in Table 9.

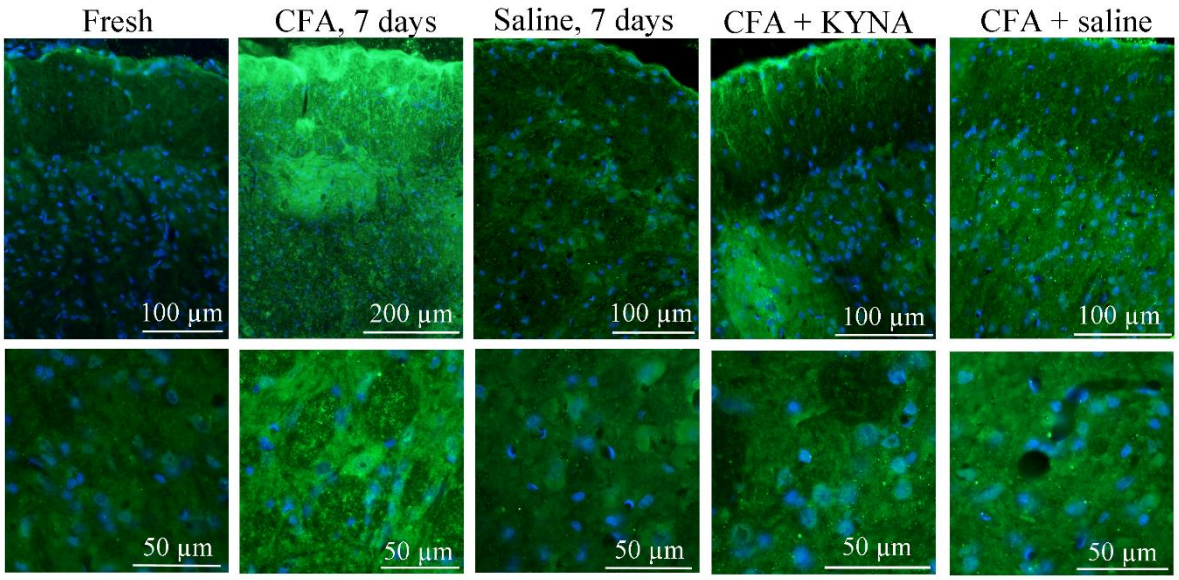


Figure 13. C-fos immunoreactivity in TNC. In case of fresh rats few c-fos positive neuronal nuclei but no nucleolei were seen. As sign of CFA induced activation increased amount of c-fos positive nuclei were detected. Saline application on the dura mater caused no increase in immunoreactivity compared to fresh samples. I.p. treatment with KYNA derivate was able to abolish the CFA induced activation, whereas i.p. saline administration had no such effect.

Group	Neurons
CFA+no treatment	+++
Saline+no treatment	+
CFA+acute treatment KYNA derivate	+
CFA+repeated treatment KYNA derivate	+
CFA+acute saline treatment	+++
CFA+repeated saline treatment	+++
Fresh (unoperated)	-\+

Table 9. Summary of a semi-quantitative evaluation of c-fos immunopositivity in different treatment groups.

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 (Figure 14) 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 (Figure 14) and to the gelatinous layer. No difference was noted between different levels of TNC and a slightly 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 (TNFα)

TNFα immunoreactivity was detected as a dense fiber staining in the Sp5C 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 (Figure 14). 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.

f. Interleukin-6 (IL-6)

IL-6 immunopositivity was detected in the nerve fibers and some homogeneously stained glial cells of the Sp5C were observed. Also some neurons of the TNC, showing homogenously stained cytoplasm were detected. Some positive neurons could be seen in the caudal part of the spinal

trigeminal nucleus, in the large neurons of the anterior horn, in the dorsal horn and the ependymal cells of the central canal. Intensely positive fibers were observed in the cuneate and gracile fasciculus and different other tracts of the spinal cord (Figure 14). After application of CFA, similar staining patterns as for the non-CFA treated groups were found.

g. IL-1β

IL-1β showed the same staining pattern as for IL6 and TNFα, with no change after CFA induced activation (Figure 14). The immunoreactivity observed showed a granular cytoplasmatic stainig, previously described in the TG

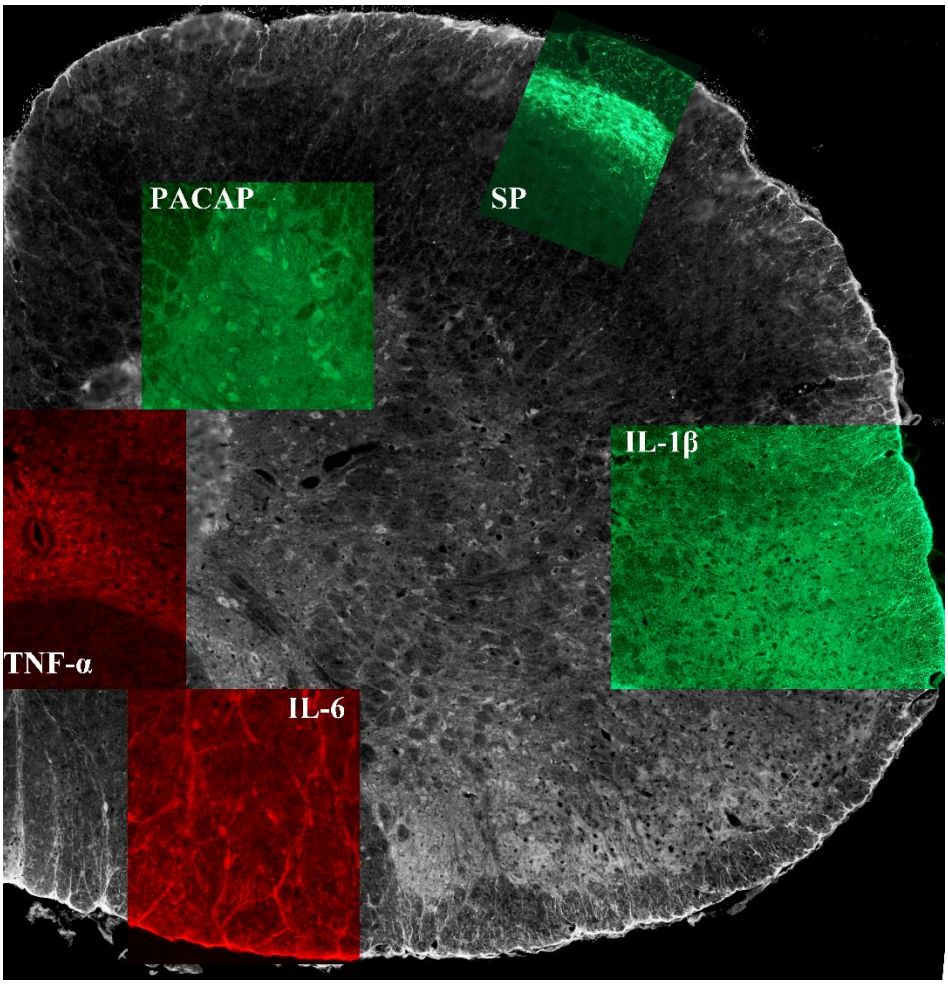


Figure 14. Overview of SP, PACAP, TNFα, IL-1β and IL-6 immunoreactivity in C_1-C_2 region of the spinal cord. Intensely stained SP positive fibers were detected in Sp5C. The giant cells of the anterior horn were found PACAP positive. Large number of TNFα positive cells were detected surrounding the central canal. IL-1β and IL-6 immunopositivity is shown in the nerve fibers and glial cells of different tracts of the spinal cord.

5.3. Western blot

In order to investigate quantitatively the proteins expressed in the TG WB was performed for pERK1/2, IL-1β and CGRP. The only significant increase was present in pERK1/2 in the 24 hours models compared to fresh rats. Additionally, increased tendency was seen in IL-1β, particularly in the 24 hours models both for CFA and IS. In case of IL-1β 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 (Figure 15).

As in case of CGRP no difference was found with WB, for the i.p. treatment groups we only checked pERK1/2 and IL-1β. 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 (Figure 16).

5.4. 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. As CFA consists largely of mineral oil and is not water soluble, we made measurements adding CFA directly on the vessels. No vasomotor effects were observed following direct application of CFA on the MMA (Figure 17).

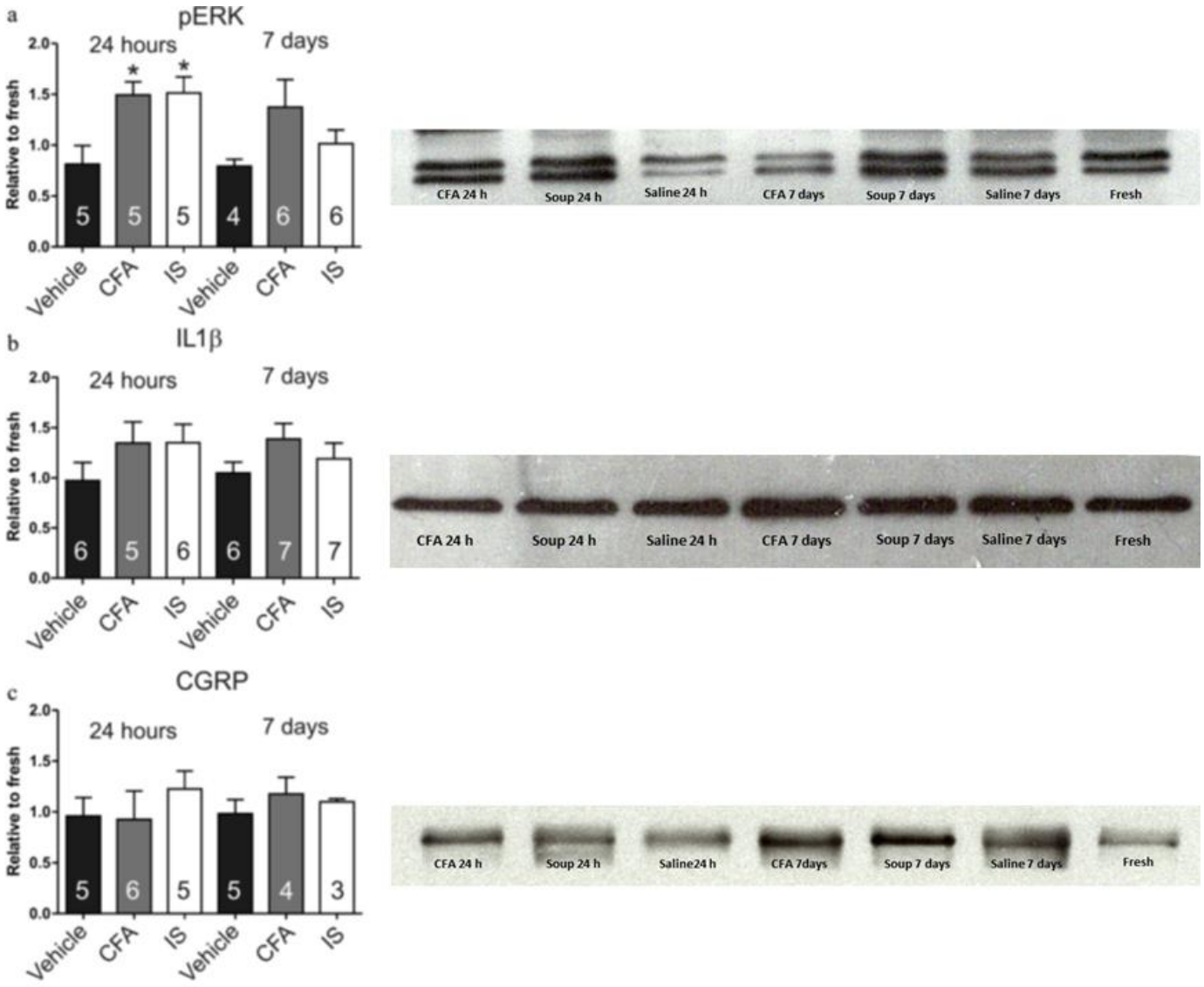


Figure 15. WB in the TG for pERK, IL-1β and CGRP at different time-points following CFA and IS application on the dura. a. pERK was found increased ($p<0.05$, one-way ANOVA, with Bonferroni post-test). Increasing tendency can be observed in IL-1β, without reaching significance. No significant change could be observed in CGRP following CFA and IS activation.

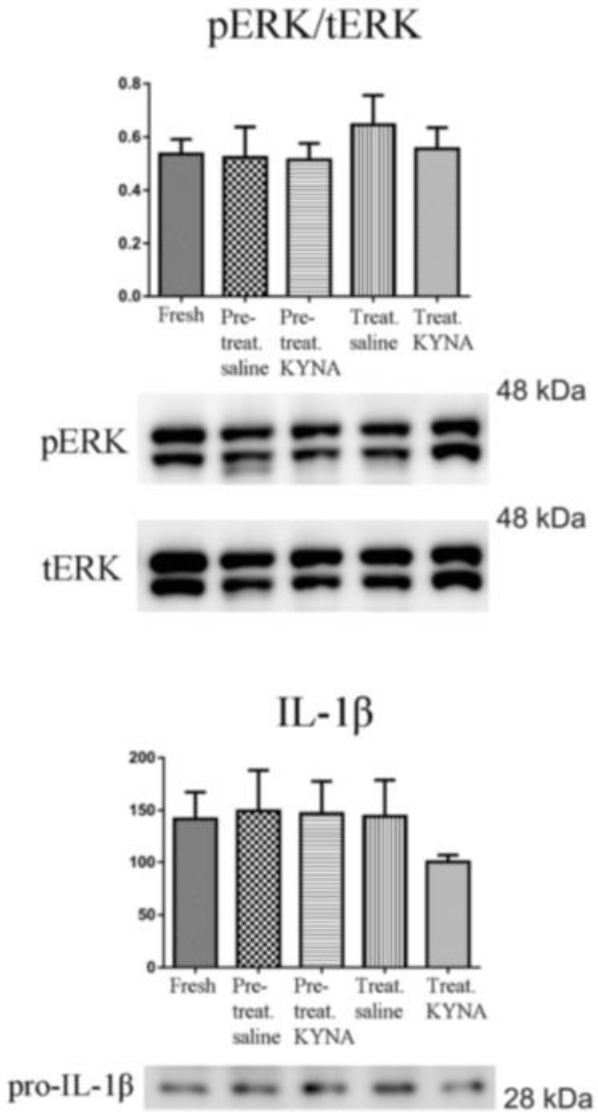


Figure 16. WB in the TG for pERK, IL-1β for different treatment groups following CFA induced activation. In case of i.p. treatment with KYNA derivate the same tendency was found as in IHC studies, without reaching a significant level.

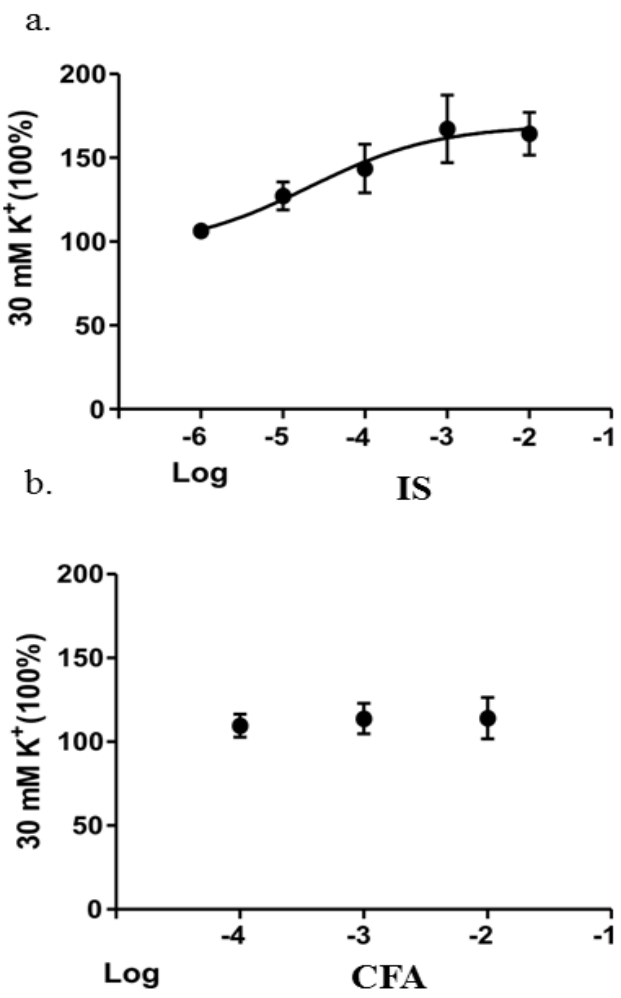


Figure 17. Myography studies on MMA of fresh rats. a. IS caused vasocontraction of the MMA. B. CFA did not show any significant vasomotor change.

6. Discussion

To the best of our knowledge, the present investigation is the first study to examine whether application of algesic 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. We also planned to study the the effect of a novel KYNA derivate in the present chronification model of trigeminal activation. We 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 C_1-C_2 spinal region of the spinal cord.

IS, one of the inflammatory substances that we have used 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 (20, 21). Although CFA has been used for more than 50 years for animal models of inflammatory and autoimmune diseases, some aspects of its way of action is still unknown. It is presumed that in the early phase, the immunological reaction is mediated by macrophages and dendritic cells via phagocytosis and in the later phase proliferation of T lymphocytes occurs (108). Studies show that CFA increases permeability of BBB for various small molecules and induces perivascular extravasation but no sign for T cell infiltration was detected (109). 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 know 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 (110, 111) . 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 (112) but they are not as efficient in immune response as the dendritic cells of other tissues (45). We assume that neither

does the long-term activation occur in the “regular way”. Instead of the involvement of T-cells which is still questionable in the CNS following CFA application, 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.

Regarding the location of the activation we opted for an area close to the MMA as previous studies have shown that focal stimulation, distant from major arteries was not capable of inducing pain. We also used a relatively long-term stimulation (20 min) compared to previous studies (18, 113).

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 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 (113, 114). Nociceptive fibers also undergo activation during removal off skull bones (115). As pERK1/2 is thought to be a robust and early marker of noxious stimulation (116), we presume that the early activation is due to the operation itself. Sustained pERK1/2 activation was detected in the IS and CFA groups but not in case of saline application, leading to the conclusion that the long-term activation in the TG is caused by the peripheral sensitisation induced by the inflammatory agents.

It is also worth mentioning that the stimulation was detected bilaterally both in the TG and the TNC. This might be a methodological problem, as it is very difficult to avoid the spreading of the inflammatory substances although the head of the animal is fixated. Previous tracing studies that characterizing the innervation of the dura mater have shown that in case of application of the labelling substances on a caudo-medial region of the dura mater, labelled neurons appeared bilaterally. This might be explained by the spreading of out inflammatory substances to the superior sagital sinus that has bilateral innervation from the TG (117).

Our WB studies show the same tendency as the IHC with an elevated level of pERK1/2, IL-1β and CGRP after CFA or IS but the differences were statistically not significant. This might be explained by a technical problem. In the IHC methodology the microscope study allowed us to identify different regions of the TG. We were able to focus our results mainly on the V1 region 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. As our major purpose was to present a detailed staining pattern of different markers and cytokines in different areas of the brainstem and the spinal cord upon activation, WB studies were not performed in case of the TNC.

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; however, the diameter of the MMA has not been measured previously (43, 118, 119). 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. An earlier study regarding the vascular theory found that a short period of vasoconstriction occurs, which triggers the release of vasodilator agents, such as CGRP (120). Vasodilatation has been suggested not to be the initiator of migraine attack, could rather be a consequence or side phenomena (41). We show that IS and CFA causes similar activation in the TG, acting differently on the vessels, leading to the conclusion that the vasodilatation is not a trigger of the changes observed in our model.

Our findings support the theory of the imuno-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 (121, 122). The condensed, homogenous material in the IL-1β staining, close to the neuronal cell-membrane represents a higher packing density of IL-1β in the immediate neighbourhood of the SGCs. As previous studies have detected IL-1β receptors on the surface of glial cells conditions (123), an autocrine-paracrine stimulation (proinflammatory-activated) might explain the importance of the increased immunoreactivity close to neuronal cell membrane. We presume that the granular staining pattern of IL-1β immunoreactivity, detected under basal conditions might be localised in the Golgi-apparatus or the endoplasmatic reticulum, involved in the protein synthesis (124). Further studies are needed to investigate the exact localisation of IL-1β immunoreactivity. Previous cell culture studies have shown increased release of CGRP while incubation with IL-1β (125, 126), mediated by the MAPK pathways (127, 128). In comparison with the unoperated animals, the number and intensity of CGRP-positive neurons were increased even after 7 days, although no change in the staining pattern was noted. Here we need to emphasize the limitations of the IHC studies. IHC is not the best method for quantification, it is suitable for detecting changes that occur in expression and in location. We consider that in case of no change in location and no obvious difference that can be detected in the staining intensity, we cannot state any sign of activation based only on IHC. For quantification WB is a more suitable method. As we saw no obvious, qualitative change in the staining, CGRP was omitted from the further experiment on the TG.

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 (129) and has been shown to be co-released with CGRP from the neurons of the TG upon activation (130). Elevated levels of Glu were found in the serum of migraine patients (131) and in the cerebrospinal fluid (CSF) of chronic migraine patients (132). Our findings are consistent with these studies as increased amount of Glu positive cells were detected in the TNC. Increased Glu immunoreactivity suggests long-term activation of the second-order neurons, leading to central sensitization. Increased c-fos immunopositivity also supports this finding, as c-fos is a widely used proto-oncogene in neuronal activation of the TCC (97, 133).

PACAP is another neuromodulator having various biological functions such as inhibitory effect on neurogenic inflammation (134). PACAP has been suggested to be involved in the trigeminovascular activation as PACAP infusion caused headache in healthy volunteers (135) and serum PACAP levels were altered in ictal compared to interictal phase of migraine and in cluster headache (136, 137). It is suggested that the effect of PACAP is biphasic: lower concentration increasing, higher concentration inhibiting the NMDA receptor activation (138). We have found PACAP positive fibers and cells in the fasciculus cuneatus and gracilis, showing a slight increase following CFA induced activation. Recent PET and fMRI studies revealed activation of nucleus cuneiformis in chronic migraine and other pain conditions (139, 140), that is supposed to play a role in pain modulation like tactile sensation hypersensitivity and muscle tenderness, integrating somatosensory input with other stimuli. (141). Whether this somatosensory hypersensitivity could be caused by PACAP release in the fasciculus gracilis and cuneiformis might represent a new interesting perspective for future studies.

Earlier studies have shown release of SP from the areas of TNC after electrical stimulation of the TG in rat (142). In spite of this, human studies did not support a key role of SP in migraine pathophysiology as no marked change in CSF or plasma SP levels were noted neither in migraine, nor in cluster headache (143, 144). Same contradictory results were found regarding the plasma levels of proinflammatory-cytokines (145-147). The SP immunopositivity in Sp5C, with a slight increase in the staining intensity following CFA application was not considered a sign of activation. In case of TNFα, IL-6 or IL-1β 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 (145, 147) and also suggests 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.

As presented in the introductory part, modulation of the KP might represent an appropriate therapeutic tool in migraine treatment. Lower levels of KYNA were detected in the serum of patients suffering from chronic migraine and cluster headache (90, 148), suggesting that the neuroprotective KYNA could represent a therapeutic target in migraine treatment. As KYNA has a very low capacity to penetrate the BBB and undergoes rapid clearance from the body (149), our research group has synthetized various KYNA derivates to facilitate BBB penetrance.

Although KYNA derivates have proven to be effective in trigeminal activation models (103, 150), some aspects still remain untangled. As only few pharmacokinetic studies were performed, their exact way of action is unknown. They might either act as an intact structure, having similar effects to KYNA or they might serve as prodrugs, dissociating into KYNA (151, 152). One study was based on measuring the concentration of two KYNA analogues and KYNA in rat serum and TNC following i.p. treatment with the KYNA derivates. The study found a sharp increase followed by a sudden decrease in serum levels and the fifth hours the derivates were still present in the serum, whereas levels of KYNA returned to the baseline, suggesting that only a small amount of the derivate dissociate into KYNA. Surprisingly, in the CNS, the amount of the derivates was found below the lower limit of detection (151). 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 derivates act peripherally. We presume that the TG might be a possible sight of action, as it plays a key role in trigeminal activation and it has proven to be placed outside the BBB (153). 2. Under pathological conditions, like neurogenic inflammation the BBB gets opened for various molecules (45) which might explain the central effect of the substances upon trigeminal activation, without them being present in the CNS under physiological conditions. It is still a question of debate whether therapies in migraine need to have central effects or not. The anti-CGRP monoclonal antibodies representing the most promising therapy in migraine are already in phase 3 clinical trials and due to their high molecular weight are unable to pass the BBB, they act on the periphery (154). Nevertheless, further pharmacokinetic and animal studies are planned to elucidate the site and mechanism of action of the KYNA analogues.

Another import aspect of KYNA treatment is the dosage. A previous study from our research group demonstrated that 0.1 mmol/kg dose was not able to be effective on CGRP, nNOS, c-fos and CaMKII immunoreactivity, whereas a dose of 0.5 mmol/kg and 1 mmol/kg has proven efficacy (103). As our main purpose was to test long-term activation, we opted for the higher dose (1 mmol/kg).

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 derivates might act as preventive treatment.

7. Conclusion

To our knowledge, our work is the first study using application of CFA on rat dura mater as a model of trigeminal activation. Chemical activation using CFA has proven the same efficacy as the IS. Both CFA and IS caused long-term activation in the TG regarding pERK and IL-1β immunoreactivity. The WB study showed the same tendency, without reaching a significant level. We were able to detect increased Glu and c-fos immunopositivity in the TNC and C_1-C_2 region of the spinal cord, demonstrating activation of second-order trigeminal neurons.

Only few studies are available to evaluate the effect of the novel KYNA derivates on long-term trigeminal activation models. In our work we demonstrate the efficacy of the KYNA analogue both in the TG and TNC. We couldn't report a more efficient action following chronic treatment with KYNA derivate, suggesting that one-dose pre-treatment is sufficient to prevent the trigeminal activation. We plan further studies in order to answer the question whether the new KYNA derivates act via peripheral or central mechanisms.

We also offer detailed mapping of different cytokines and pro-inflammatory mediators potentially involved in the pathophysiology of migraine in the TNC, other areas of the brainstem and the spinal cord. This would lead to a better understanding of the complex phenomenon that occurs during migraine attacks and especially in case of migraine pain chronification.

8. 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-1β 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 C_1-C_2 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-1β immunopositivity in the TG and Glu and c-fos immunoreactivity in the TNC and C_1-C_2 region.

V. Daily treatment with KYNA derivate was not found to be more effective than one dose pre-treatment.

VI. PACAP, SP, TNFα, IL-6 and IL-1β 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. TNFα, IL6 and IL-1β 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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Dural administration of inflammatory soup or Complete Freund's Adjuvant induces activation and inflammatory response in the rat trigeminal ganglion

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Abstract

Background: Migraine is a painful disorder with a huge impact on individual and public health. We hypothesize that migraine pain originates from a central mechanism that results secondarily in hypersensitivity in peripheral afferents associated with the cerebral and cranial blood vessels. It has previously been shown that application of inflammatory or algesic substances onto the dura mater or chemical stimulation of the dural receptive fields causes hypersensitivity to mechanical and thermal stimulation together with direct activation of the TG. We asked whether local inflammation of dura mater induces inflammatory activation in the trigeminal ganglion.

Methods: We performed topical administration of inflammatory soup (IS) or Complete Freund's Adjuvant (CFA) onto an exposed area of the rat dura mater *in vivo* for 20 min. The window was closed and the rats were sacrificed after 4 h and up to 7 days. Myography was performed on middle meningeal arteries. The trigeminal ganglia were removed and processed for immunohistochemistry or Western blot.

Results: Both CFA and IS induced enhanced expression of pERK1/2, IL-1β and CGRP in the trigeminal ganglia. The pERK1/2 immunoreactivity was mainly seen in the satellite glial cells, while IL-1β reactivity was observed in the neuronal cytoplasm, close to the cell membrane, seemingly as sign of neuro-glial interaction. The CGRP expression in the neurons and nerve fibres was enhanced after the application of either inflammatory agent. Myography resulted in a strong vasoconstrictor response to IS, but not to CFA.

Conclusions: These results suggest that the application of IS or CFA onto the dura mater causes long-term activation of the TG and demonstrate the importance of the neuro-glial interaction in the activation of the trigeminovascular system.

Keywords: Inflammatory soup; Complete Freund's Adjuvant; Dura mater; Trigeminal ganglion; pERK1/2; IL-1β; CGRP

Background

Migraine is a painful, debilitating neurological disorder with a huge impact on individual and public health. In a survey about years lived with disability, migraine was ranked third [1].

Great progress has been made towards understanding the pathophysiology of migraine; however some questions still remain regarding the origin of migrainous pain and its chronification. There are two contrasting theories of migraine pathogenesis: the neurogenic theory suggests that migraine is a CNS disorder involving a genetic change in the central ion channels, with underlying importance of the phenomenon of cortical spreading depression (CSD). This may lead to activation of the nociceptive afferents causing alteration in the diameter of intracranial vessels, serving as a trigger or modifier in the trigeminovascular system [2]. On the other hand, Ray and Wolff in the 40's put forward the vasogenic theory, suggesting that migraine is a vascular disorder, caused by a short period of

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vasoconstriction and a reactive vasodilatation of the cranial arteries (2–5). We hypothesize that migraine pain originates from a central mechanism that secondarily results in hypersensitivity in peripheral afferents associated with the cerebral and cranial blood vessels, thus underscoring the crucial role of trigeminal perivascular nociceptive afferents [3, 4]. The dural afferents might be activated further during dural exposure to inflammatory substances or when the dural mast cells are degranulated [5].

The trigeminal ganglion (TG) with its bipolar neurons, surrounded by satellite glial cells (SGCs), forms the converging point between the CNS and peripheral structures such as the cranial arteries. We are interested in whether local inflammation can induce alterations in the TG neurons and SGCs. Previous experiments involving the use of cell culture [6] or TG organ culture [7] demonstrate that activation of intracellular protein kinases could be rapidly induced with an inflammatory response within the TG. It has also been shown that application of inflammatory or algesic substances onto the dura mater or chemical stimulation of the dural receptive fields causes hypersensitivity to mechanical and thermal stimulation and also activation in the TG and in the brainstem trigeminal neurons [8–11]. We consider that this may serve as a suitable method for *in vivo* examination. In support of this view, infusion of nitroglycerine or local capsaicin onto the dura mater elicits increased extracellular signal-regulated kinase (ERK) phosphorylation in the meningeal arteries and TG [12, 13].

An important component in the inflammatory response is IL-1β, which has been shown to be implicated in neuropathic pain. Overall previous findings reveal the importance and complexity of interaction between neurons, glial and immune cells, playing an important role in the maintenance of neuropathic pain after nerve injuries [14–16].

Neuroscientists ascribe major importance to calcitonin gene-related peptide (CGRP) in the pathophysiology of migraine, in view of its vasodilatory effect and role in the transmission of nociception. CGRP receptor antagonist could offer an approach in the anti-migraine therapy [17, 18]. *In vitro* studies using inflammatory cocktails (histamine, bradykinin, serotonin, prostaglandin E2) on cultured trigeminal neurons have shown large increase of CGRP release [19].

The aim of the present study was to observe the activation of rat TG neurons and SGCs *in vivo* after dural application of inflammatory soup (IS) or Complete Freund's Adjuvant (CFA).

Methods

Animal procedures

Adult male Sprague–Dawley rats (220–320 g) were used ($n = 95$, 48 for immunohistochemistry, 42 for Western blot and 5 for myography). The animals were raised and maintained under standard laboratory conditions. The study followed the guidelines of the European Communities Council (86/609/ECC) and was approved by the Committee of the Animal Research of 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).

Prior to interventions, the animals were deeply anesthetized with an intraperitoneal injection of 4 % chloral hydrate (0.01 ml/g body weight, Sigma-Aldrich, St. Louis, MO, USA). The head of the animal was fixated in a stereotaxic frame and a handheld drill was used to remove a 3x3 mm portion of the skull. The hole was made postero-laterally to the bregma (5 mm), on the left side, care being taken not to penetrate the dura mater.

In 18 animals IS was applied onto the dural surface, in 18 animals CFA (inactivated and dried Mycobacterium tuberculosis in mineral oil; Sigma-Aldrich, St. Louis, MO, USA) and in 9 animals (controls) physiological saline was applied as vehicle. As absolute controls, three unoperated rats (fresh) were used. IS contained 10 μM bradykinin, 10 μM serotonin,10 μM prostaglandin E2 and 100 μM histamine, pH 5.0; the recipe was from Strassman et al. [9]. Ten μl of IS, CFA or vehicle was applied, and left on the dural surface for 20 min. To prevent the substances from spreading, the head of the animal was adjusted in the stereotaxic frame so that the dural surface covered with the liquid was completely horizontal. After 20 min, the IS or CFA was washed out with saline, bone wax was used to cover the hole and the wound was sutured with three stitches. The effect of IS, CFA and vehicle were examined after 4 h, 24 h and 7 days. The animals did not receive any analgesic after the surgery. At the end-points the animals were fixation-perfused transcardially with 4 % paraformaldehyde in 0.1 M phosphate-buffer (PB) for immunohistochemistry. TG was removed from both sides. After post-fixation for one day in 4 % paraformaldehyde in 0.1 M phosphate buffer (PB), the specimens were cryoprotected using 10 %, 20 % and 30 % glucose in 0.1 M PB. Specimens were frozen on dry ice, stored at −80 °C and subsequently embedded in gelatine medium (30 % egg albumin, 3 % gelatin), cryosectioned at 12 μm and stored at −20 °C until use.

Hematoxylin-Eosin (HE) staining and immunohistochemistry

Sections were HE stained, using standard protocol (Htx 4 min, Eosin 30 s) for orientation and examination of tissue conditions.

Immunofluorescence staining was performed to demonstrate the localisation of pERK1/2, IL-1β and CGRP in the TG. Sections were thawed at room temperature, then rehydrated in phosphate buffer saline (PBS)

containing 0.25 % Triton X-100 (PBS-T) for 15 min. Sections were exposed to primary antibodies in PBS-T containing 1 % bovine serum albumin (BSA) and incubated overnight at 4 °C. After incubation with the primary antibody (see Table 1), sections were equilibrated at room temperature, rinsed in PBS-T for 3x15 min, followed by incubation with the secondary antibody for 1 h in a dark room, at room temperature (see Table 2). Sections were washed with PBS-T for 3x15 min and mounted with anti-fading mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA). For nucleus staining Vectashield medium containing 4,6-diamino-2-phenylindole (DAPI; Vector Laboratories) was used. Samples in which the primary antibody was omitted served as negative controls. Double immunohistochemistry with primary antibodies against pERK1/2 and anti-glutamine synthetase (GS) were used separately, not mixed in a cocktail.

Microscopic analysis

Sections were examined and images were obtained by using a light- and epifluorescence microscope (Nikon 80i, Tokyo, Japan), coupled to a Nikon DS-2 MV camera. For image analysis FITC (480 nm), TRITC (540 nm) and DAPI (360 nm) filters were used. Adobe Photoshop CS3 (v.8.0; Adobe Systems, Mountain View, CA, USA) was used to visualize co-labelling by superimposing the digital images.

Western blot

Animals for Western blot (12 for IS, 12 for CFA and 12 for vehicle) were perfused transcardially with PBS after 24 h or 7 days. Six fresh rats were used as controls. TG were immediately frozen on dry ice and stored at −80 °C until assay.

TG were homogenized in cell extract denaturing buffer (BioSource, Vacaville,CA, USA) containing phosphatase and protease inhibitor cocktails (Sigma, St. Louis, MO, USA). After centrifugation (12,000 rpm, 4 °C, 10 min) the supernatants were collected. Protein concentrations were measured with a protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) and a Tecan Infinite M200 microplate reader. Protein samples were mixed with Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA, USA) and heated (95 °C, 4 min). Equal amounts (40 μg) of protein were loaded onto 4–15 % Ready Gel Precast Gels (Bio-Rad Laboratories, Hercules, CA, USA) with a molecular weight marker (Precision Plus Protein Standard, Bio-Rad Laboratories, Hercules, CA, USA). Gel electrophoresis was followed by blocking in 5 % non-fat milk or BSA and incubation with primary antibodies (see Table 1, 4 °C, overnight) and secondary antibodies (see Table 2, 1 h, room temperature). Finally, the membranes were visualized with Fujifilm LAS-1000 Luminescent Image Analyzer (Fujifilm, Stamford, CT, USA) and the band optical density ratio was quantified by using ImageJ software. Data were calculated relative to GAPDH as a loading control and further normalized to the fresh control (set to be 1) for each individual run.

Myography

For myography studies, fresh rats ($n = 5$) were decapitated under CO_2 anaesthesia. Middle meningeal artery (MMA) was dissected free, using dissection microscope, for details see Haanes and Edvinsson [20]. MMAs were

Table 1 Details of primary antibodies used for IHC and WB

	Name	Product code	Host	Dilution	Company
IHC					
	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	4376	Rabbit	1:50	Cell Signaling Technology, Danvers, MA, USA
	Anti IL-1 beta antibody	ab 9787	Rabbit	1:100	Abcam; Cambridge, UK
	Calcitonin-gene related peptide, polyclonal	B-47-1	Rabbit	1:800	Europroxima, Arnhem, Netherlands
	Phospho-p38 MAPK (Thr180/Tyr182)	9216	Mouse	1:200	Cell Signaling Technology, Danvers, MA, USA
	Phospho-SAPK/JNK (Thr183/Tyr185)	9252	Mouse	1:200	Cell Signaling Technology, Danvers, MA, USA
	Anti TNF-alpha antibody	ab66579	Rabbit	1:200	Abcam; Cambridge, UK
	Anti IL-6 antibody	ab6672	Rabbit	1:100	Abcam; Cambridge, UK
	Anti-Glutamine Synthetase Antibody, Clone GS-6	MAB302	Mouse	1:100	Merck Milipore, Darmstadt, Germany
WB					
	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	4376	Rabbit	1:1000	Cell Signaling Technology, Danvers, MA, USA
	Anti IL-1 beta antibody	ab 9787	Rabbit	1:500	Abcam; Cambridge, UK
	Anti-Calcitonin Gene Related Peptide	C8198	Rabbit	1:250	Sigma-Aldrich, St. Louis, MO, USA
	Anti β-actin	Sc-477778	Mouse	1:5000	Santa Cruz Biotech, Santa Cruz, CA, USA
	GAPDH (D16H11) XP mAb	5174	Rabbit	1:1000	Cell Signaling Technology, Danvers, MA, USA

Table 2 Details of secondary antibodies used for IHC and WB

	Conjugate and host	Against	Dilution	Company
IHC				
	FITC (goat)	Anti-rabbit	1:100	Cayman Chemical, Ann Arbor, MI, USA
	Alexa 594 (goat)	Anti-mouse	1:100	Invitrogen, CA, USA
WB				
	HRP-conjugated	Anti-rabbit	1:2000	Cell Signaling Technology, Danvers, MA, USA
	HRP-conjugated	Anti-mouse	1:2000	Cell Signaling Technology, Danvers, MA, USA

isolated in 119 mM NaCl, 15 mM $NaHCO_3$, 4.6 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 5.5 mM glucose and 26 μM EDTA. After dissection, a buffer with similar composition, but including 1.5 mM $CaCl_2$ and without EDTA was used. All solutions were aerated with gaseous mixture composed of 95 % O_2 and 5 % CO_2, to maintain a pH of 7.4.

Each ~2 mm long segment of the MMA ($n = 5$) from fresh rats was mounted in an arterial myograph, on a pair of 25 μm metal wires. One wire was connected to a micrometer screw where the vascular tone could be adjusted and the other to a force displacement transducer, paired with an analogue/digital converter (ADInstruments, Oxford, UK). Data were recorded on a computer through use of a PowerLab unit and LabChart (ADInstruments, Oxford, UK) was used for recording and calculations.

All experiments were conducted at 37 °C. The segments were normalized to 90 % of the internal circumference a vessel would have at 50 mmHg. A reference value for each segment was created by replacing part of the NaCl with KCl (60 mM K^+); any segment with a maximum contractile capacity of less than 0.1 mm was excluded. Segments were stably precontracted with 30 mM K^+. For both substances a cumulative concentration vs. response curve was made.

Results

HE staining

HE staining of the TG is shown in Fig. 1. By keeping the orientation of the TG, we could identify the peripheral (cortical) and the central (medullary) zones, and also the V1, V2 and V3 regions of the TG. The staining revealed neurons of different sizes, enveloped by a single layer of SGCs. These neuron/SGC units were intermingled between fibers. The morphology of the different TGs was in general good, though tissue shrinkage was observed in some of the TGs.

Immunohistochemistry

pERK1/2

pERK1/2 can be used as a dynamic, rapid and robust marker of noxious stimulation, as ERK1/2 phosphorylation can occur within a minute [21]. We were interested in the long term activation of pERK, as it could be related to permanent activation of the TG. pERK1/2 immunoreactivity was observed in a few nuclei and nucleoli of the neurons in the fresh TGs. No immunoreactivity was detected in the cytoplasm of the neurons or SGCs. In the 24 h vehicle group, some pERK1/2 immunoreactivity was found in the SGCs. The negative control, i.e. when the primary antibody was omitted, displayed no immunoreactivity (Fig. 2a-c).

In the CFA (Fig. 2d-f) and IS (Fig. 2g-i) treated rats, pERK1/2 immunoreactivity was observed in the SGCs at all three time points (4 h, 24 h and 7 days). At 4 h, only few SGC were activated in the V1 (anteromedial) region of the ganglion, compared to the peripheral zone (data not shown). No significant difference could be seen between the IS, CFA or the vehicle groups. At 24 h, high-intensity pERK1/2 immunoreactivity was detected in the anteromedial part of the TG in the SGCs in both IS and CFA specimens. A slight increase in pERK1/2 immunoreactivity was observed in the neuronal nucleoli in the 7 days specimens, in both the CFA and IS groups. No difference was seen between left and right side (data not shown).

To assess whether the pERK1/2 co-localized with the specific SGC marker GS, double immunohistochemistry was performed. Indeed, the markers co-localized (Fig. 3a-d), demonstrating that pERK1/2 is present in the SGCs.

IL-1β

IL-1β plays a pivotal role in inflammation and has been shown to be present in the spinal cord, following the injection of CFA into the rat hind paw [22]. We were therefore interested if similar changes could occur in the TG after inflammatory activation. The negative control displayed no IL-1β immunoreactivity (Fig. 4a). In fresh TG, IL-1β immunoreactivity was observed in the neuronal cytoplasm (in a granular manner), in a few nuclei and in the nerve fibres (Fig. 4b). The same pattern was found in the vehicle-treated animals (Fig. 4c).

The most interesting finding is the activation pattern of IL-1β. Firstly we do not see any activation in the vehicle control compared to fresh, which illustrates that this is a pure effect of the inflammatory substances

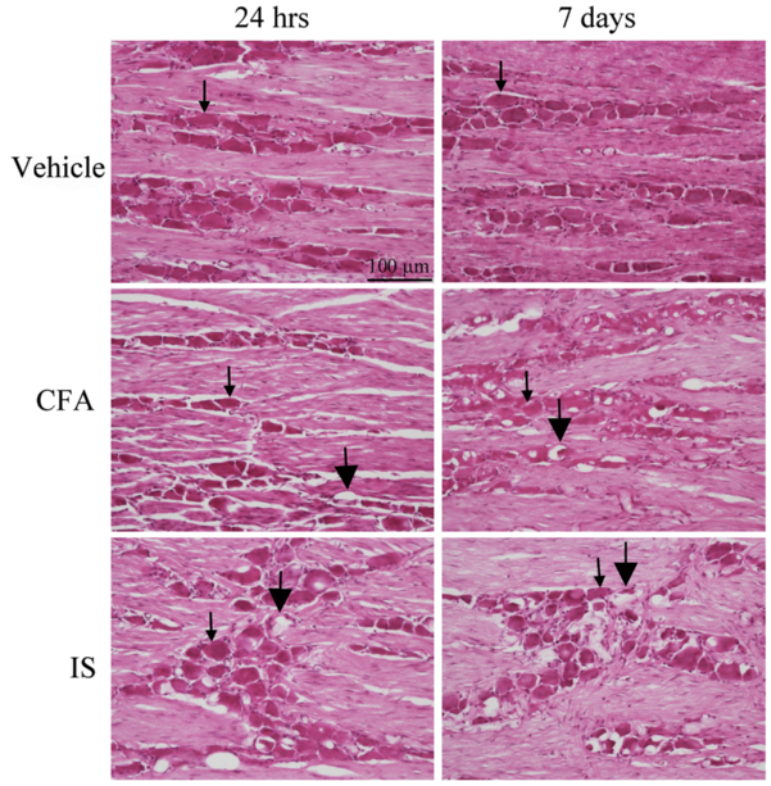

Fig. 1 Hematoxylin-Eosin staining of TG from animals treated with vehicle (control), CFA and IS. The ganglia consists of bipolar neurons sorrounded by a single layer of SGCs (*thin arrows*). In the CFA and IS treated groups vacuoles (*thick arrows*) can be seen, as sign of tissue shinkrage and cell damage

added on the dura. In the CFA (Fig. 4d-f) and IS (Fig. 4g-i) treated rats, IL-1β immunoreactivity was observed intracellularly in the neurons and in the fibres at all three time points. However, we do see a difference in the pattern of staining at the different time points. At 4 h there is more of the granular staining in the IS compared to the CFA, suggesting a fast response/activation from the IS. Similar increase in the granular pattern is observed after 24 h post the CFA stimulation, at this time point there is no big difference between CFA and IS. At day seven a "ring" of IL-1β immunoreactivity below or close to the neuronal cell membrane was evident, which differed from the granular pattern observed in the fresh TGs (Fig. 4b). This is clearly stronger in the CFA compared to the IS treated rats. No immunoreactivity was observed in the SGCs. No difference was detected between the left and right sides.

CGRP

Previous experiments have revealed a strong relationship between the release of IL-1β and CGRP in the neurons. Pre-exposure of trigeminal neurons to an IL-1β conditioned medium led to an increased CGRP release in the neurons after stimulation with capsaicin. CGRP is also the main peptide believed to be involved in migraine pathophysiology.

Our negative control did not exhibit immunoreactivity (Fig. 5a). In fresh TG, neurons and nerve fibers were CGRP immunoreactive (Fig. 5b). The immunoreactivity was observed in the neuronal cytoplasm. The staining ranged between homogeneous staining of the cytoplasm to a granular immunoreactive pattern around the nucleus, possibly in the ER. A limited number of thin, CGRP-positive pearl-like fibers were also observed in the TG. No immunoreactivity was seen in the SGCs. The same pattern as for the fresh TG was seen in the vehicle-treated animals (Fig. 5c).

In the CFA (Fig. 5d-f) and IS (Fig. 5g-i) treated rats, CGRP immunoreactivity was observed in the neurons and nerve fibres at all three time points. As compared with the control specimens (fresh and vehicle-treated rats, Fig. 5b-c), we observed an increase in immunoreactive fibres (Fig. 5d-i), particularly in the CFA treated animals, this increased staining of the fibres is persistent at all time-points (Fig. 5d-i).

Other antibodies tested

We additionally studied the immunoreactivity of TNF-α, IL-6, pp38 and pJNK, but in our hands these proved negative (data not shown).

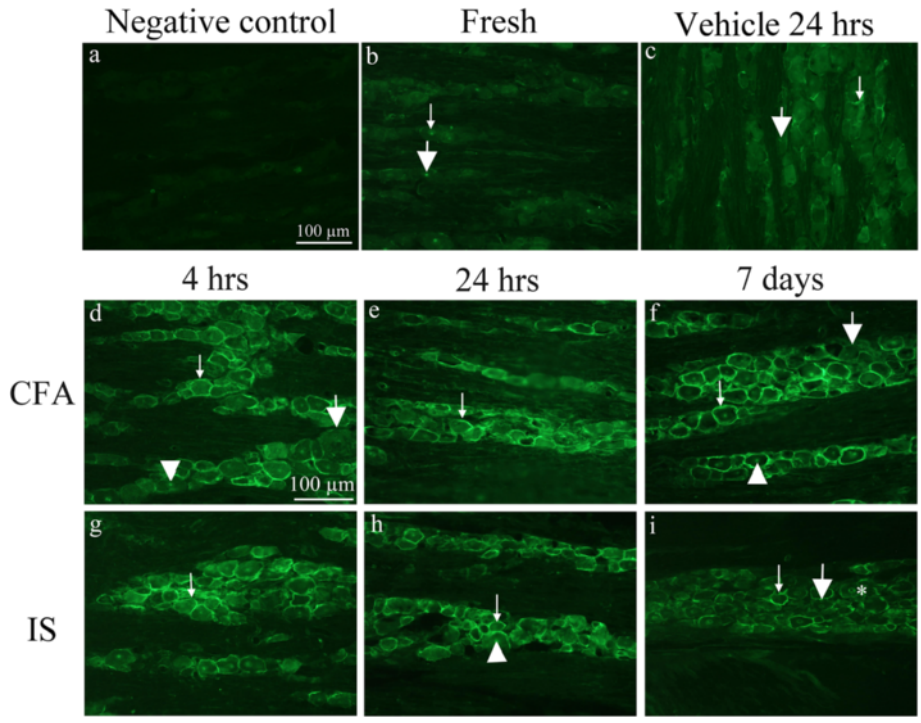

Fig. 2 pERK immunohistochemistry on the TG. **a**. Negative control, showing no immunoreactivity. **b**. pERK1/2 staining in fresh animals. pERK positive nuclei (*thin arrow*) and nucleolei (*thick arrow*) can be detected. **c**. 24 h vehicle group shows weakly increased pERK1/2 immunoreactivity in the SGCs (*thin arrow*) and in the nucleolei (*thick arrow*). The TGs were studied at various time points (**d-i**). Positive immunoreactivity with different intensity can be detected in SGC (*thin arrows*) in all specimens. Negative SGC can also be seen (*thick arrows*). At 7 days CFA model, only few negative SGC were observed. Intensely stained nucleolei were also detected (*arrow heads*), more obvious at day 7 CFA. In the 7 days IS group, a lot of positive neuronal nuclei but no nucleolei were observed (*asterisk*)

Western blot

Since immunohistochemistry is generally better for observing "ON/OFF" changes in expression together with protein localization, we aimed to more quantitatively investigate the protein expression in the TG. Western blots therefore performed for pERK1/2, IL-1β and CGRP (representative blot can be found in Additional file 1: Figure S1). The only significant increase was seen for pERK1/2 after stimulation with both CFA and IS for 24 h (Fig. 6a). We did not see significance IL-1β or CGRP (Fig. 6b-c). We did, however, see a tendency for an increase in IL-1β, particularly after 24 h of stimulation with CFA or IS. Significance was not reached, which could be due to the complete ganglion was used for Western blot. However, we do observe the same tendencies as for our immunostainings.

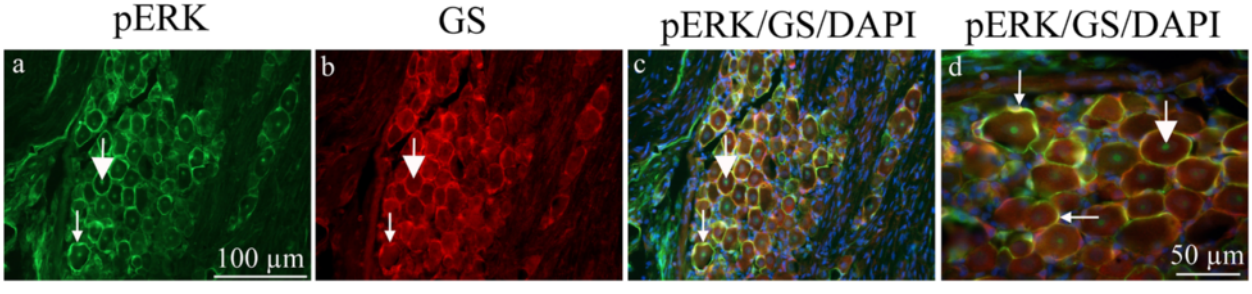

Fig. 3 pERK1/2 and GS double immunohistochemistry on the TG 7 days after CFA. **a**. All SGCs seem to be immunoreactive. Thin arrow point at pERK1/2 immunoreactivity in SGCs, thick arrow at positive neuronal nucleolei. **b**. GS immunoreactivity was found in the SGCs. Thin arrow point at GS immunoreactivity. **c**. and **d**. Co-localization of pERK1/2 and GS is demonstrated in the merged images. Nuclei DAPI staining is included. Thin arrows point at pERK1/2/GS co-localization and thick arrows at positive neuronal nucleolei

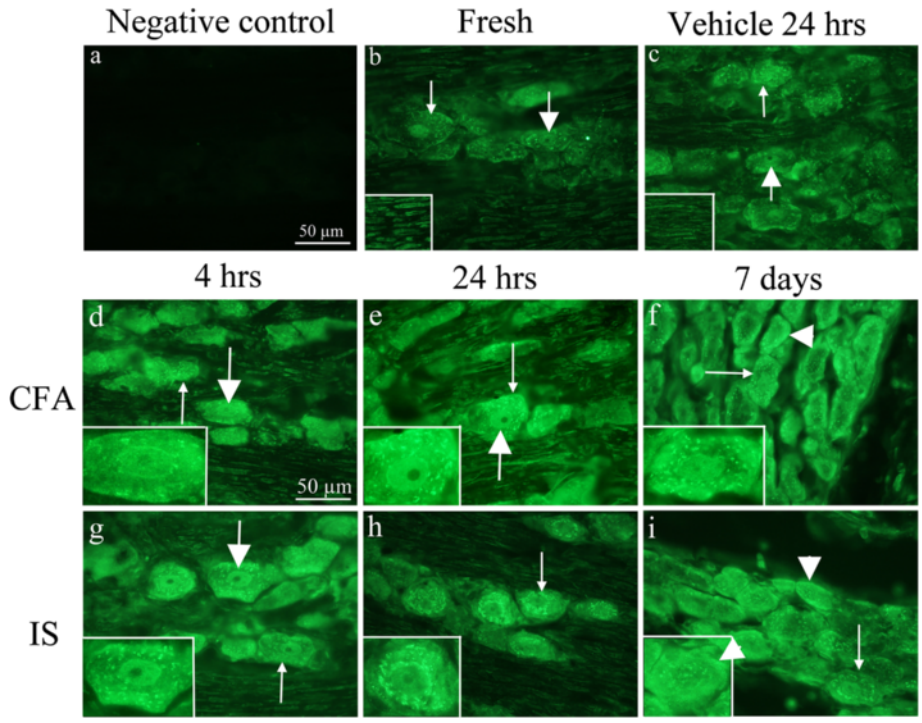

Fig. 4 IL-1β immunohistochemistry on the TG **a**. Negative control showing no immunoreactivity. **b**. IL-1β staining in fresh animals. Immunoreactive neuronal nuclei could be detected (*thick arrow*). In addition, an intracytoplasmatic granular staining was seen (*thin arrow*). **c**. 24 h vehicle group showed a similar pattern as for fresh animals. Inserts immunoreactive nerve fibers. **d-i**. Immunoreactivity was detected in the neuronal nuclei, but no nucleolei in the CFA and IS treated groups (*thick arrows*, **d-i**). In the cytoplasm immunoreactivity was detected in a granular manner (*thin arrows*, **d-i**). In the 7 days CFA and IS groups, increased immunoreactivity was seen as a condensed, homogeneous material close to the cell membrane (**i**, *arrow heads*)

Myography

In our model, we observe activation of the trigeminal ganglion using the inflammatory substances CFA and IS. Based on the vascular theory of migraine, we wanted to see if the agents caused vasodilation or vasocontraction when applied to the middle meningeal artery. MMAs from fresh rats were isolated and mounted in a wire myograph. IS caused a strong contraction of the MMAs (Fig. 7a), whereas CFA did not result in any vasomotor response (Fig. 7b). It is worth mentioning that CFA consists largely of mineral oil and is not water soluble. No vasomotor effects occurred when CFA was added directly onto the MMA in the myograph (data not shown).

Discussion

To the best of our knowledge the present study is the first study to examine the inflammatory response in the TG after the application of algesic substances onto the dural receptive field. The local effects of inflammatory agents on the MMA, and neuro-glial activation in the TG, were followed.

The vasogenic theory has recently been questioned in consequence of the lack of evidence of major cranial vasodilatation during migraine attacks [23, 24]. It is currently conceived that the activation of the trigeminovascular system after chemical stimulation of the dura is explained by activation of perivascular sensory fibres, leading to hypersensitivity to mechanical and thermal stimuli [8, 9]. Previous studies of the onset of this hypersensitivity [8] have indicated that peripheral sensitization occurs rapidly, within 20 min after the application of inflammatory agents onto the dural surface and central sensitization becomes established after 120–240 min [11]. Based on these previous findings we speculated that hypersensitivity and trigeminovascular activation could be maintained for a longer period of time (7 days) and that application of algesic substances onto the dura mater could be used as an animal model for long term activation of the trigeminovascular system.

The inflammatory soup consisted of bradykinin, histamine, serotonin and prostaglandin E2, which are found to be released endogenously during inflammation and have a role in neurogenic inflammation [4, 8, 9, 25]. CFA is considered to be an important immune-potentiator which is widely used in peripheral pain models [26, 27]. Interestingly, we see a similar pattern between the right and left TG, even though the inflammatory agents were added on only one side. This

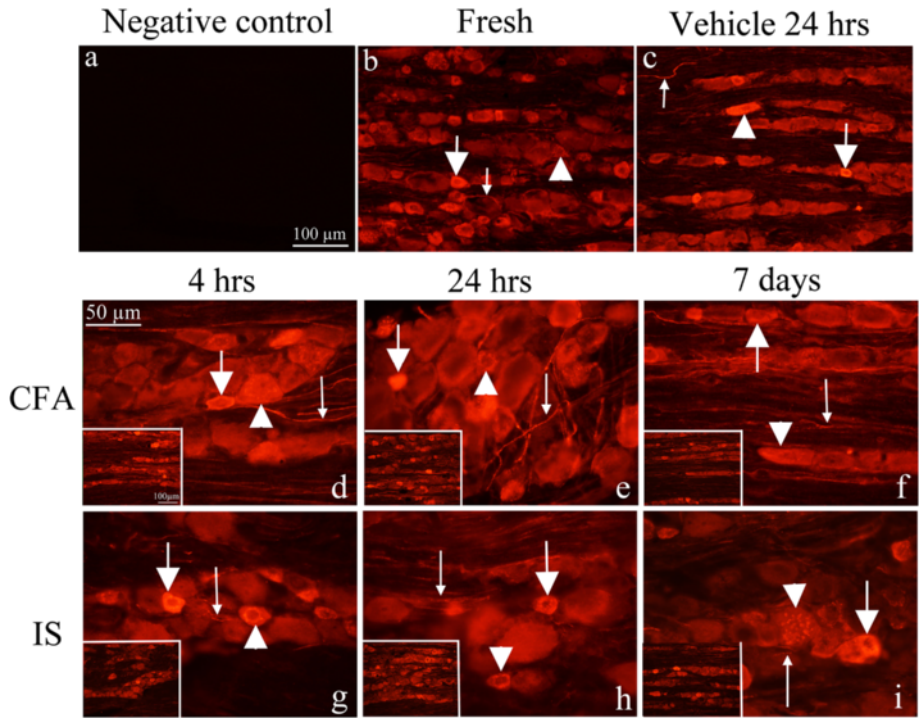

Fig. 5 CGRP immunohistochemistry on the TG. **a**. No immunoreactivity was observed in negative controls. **b**. In fresh animals pearl like staining was observed in thin fibres (*thin arrow*). Homogeneous staining was detected in small neurons (*thick arrow*) as well as intracytoplasmatic granular staining (*arrow head*). **c**. A similar immunoreactivity as for fresh animals was detected in the vehicle group, with thin fibre (*thin arrow*), small neurons (*thick arrow*) and granular cytoplasmatic (*arrow head*) staining. **d-i**. A pearl like staining was observed in thin fibres (*thin arrow*), homogeneous staining was detected in the small neurons (*thick arrow*) as well as intracytoplasmatic granular staining (*arrow head*). No difference could be found between the different algesics and time points. Inserts, smaller magnification

could be explained by earlier tracing studies that characterize the innervation of the dura mater where the application of the labelling substances on a caudomedial region of the dura mater, labelled neurons appeared bilaterally [28]. In fresh ganglia, only minor pERK1/2 immunoreactivity was seen (Fig. 2). The immunoreactivity increased at the early time points and was extended up to 7 days. There was no significant difference between the CFA and IS groups. We observed a pERK1/2 immunoreactivity in the vehicle treated SGCs as compared to fresh TG. This might be explained by the surgical procedure itself. There are numerous extracranial pain-sensitive structures including skin, muscles and periosteum [29, 30]. Furthermore, it should be taken into consideration that nociceptive fibres also undergo activation during removal of skull bones [13]. Previous studies have indicated a decrease in pERK1/2 activation, with return to basal levels within 2 h after stimulation [13, 31, 32]. In our experimental setup, increased pERK1/2 immunoreactivity was detected at both 24 h and 7 days after stimulation. This might suggest that pERK1/2 activation depends on the stimulated dural area and the exposure time of the inflammatory agents.

Prolonged activation of pERK1/2, participates in the regulation of nuclear proteins and transcription factors, and might be a critical determinant in sustained activation of V1 region in the TG. Our findings suggest an interplay between neurons and SGCs, were SGCs could serve an role in the pathophysiology of inflammation and pain. After the activation of the dural-sensitive trigeminal neurons, it has previously been shown that the neurons release CGRP which stimulates the SGCs to release pro-inflammatory cytokines which can further stimulate the TG neurons [31, 33].

In a previous study involving a transfected rat sciatic nerve model, the upregulation of IL-1β was found 35 days after surgery, suggesting that it may have a role in maintained neuropathic pain [34]. In our results we show an increase in the IL-1β as early as 4 h for the IS and at 24 h for both IS and CFA. Interestingly, at 7 days, we observed a dense staining close to the cell membrane. We cannot fully explain this staining, but it might suggest a higher packing density of IL-1β

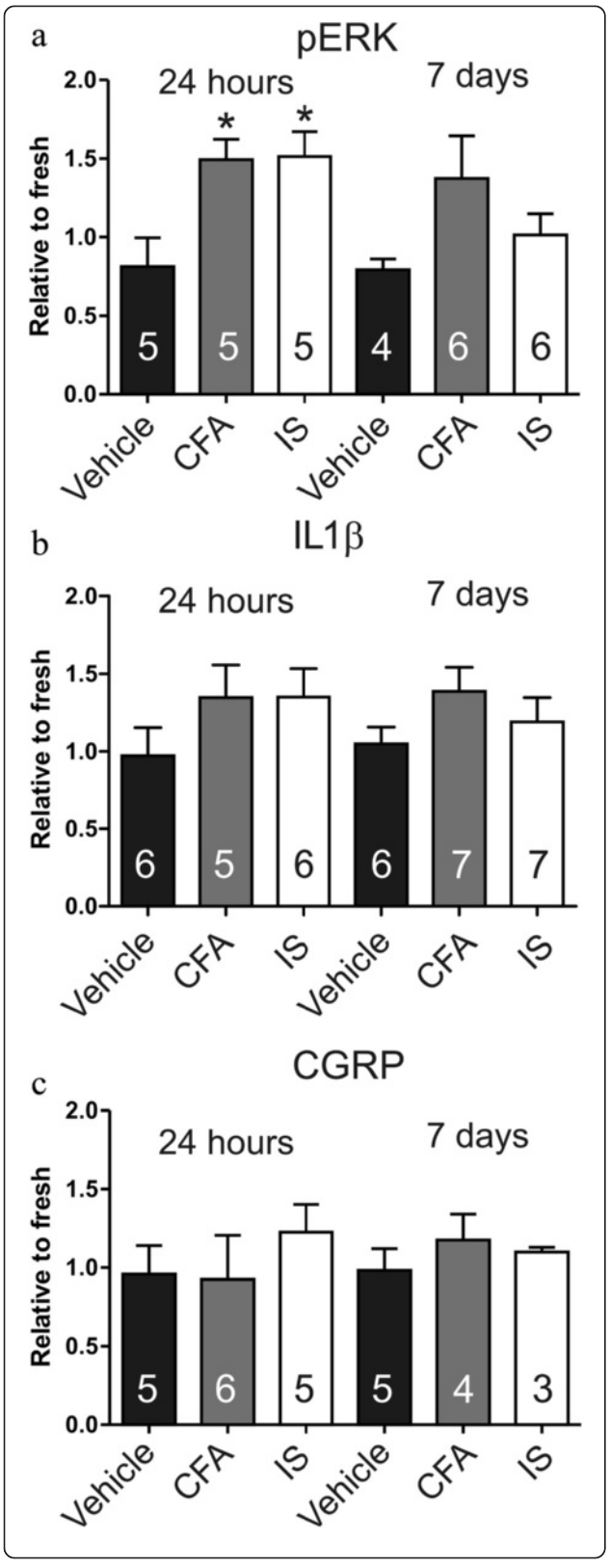

Fig. 6 Average data from Western blots of the TG. **a** Western blot for pERK. The level of pERK is higher in CFA and IS treated groups. *, $P < 0.05$, one-way ANOVA, with Bonferroni post-test. **b** Western blot for IL-1β. There is a tendency of increased IL-Iβ activation following CFA or IS application on the dura. No significant difference was seen between the groups. **c** Western blot for CGRP. No significant changes in CGRP activation following CFA or IS application on the dura

close to the cell membrane which could indicate a potentially increased release. Importantly, earlier studies have detected IL-1β receptors on the surface of SGCs [35].

Following stimulation with IL-1β, SGCs have been shown to be activated, which in turn may influence the neuronal responses [36]. Previous cell culture studies have demonstrated an elevated release of CGRP while incubation with IL-1β [36, 37], mediated by the MAPK activation [38–40]. Our findings of increased levels of pERK1/2, CGRP fibres and IL-1β support the view of a possible interaction between these three molecules after induced local inflammation. A key role has recently been

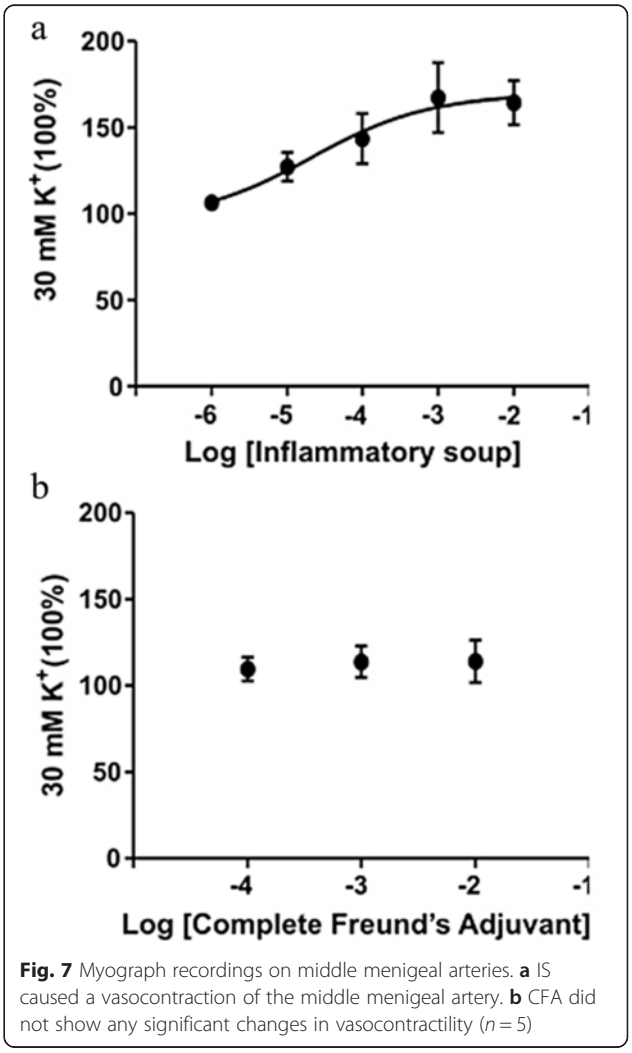

Fig. 7 Myograph recordings on middle menigeal arteries. **a** IS caused a vasocontraction of the middle menigeal artery. **b** CFA did not show any significant changes in vasocontractility ($n = 5$)

ascribed to increased CGRP release in the TG neurons [41] and CGRP receptor antagonists have a therapeutic effect in migraine and other pain syndromes [27, 42] that could to act on the TG [43]. Indeed, also in this inflammation activated TG, we observe an increase in the CGRP after 7 days.

Our Western blot studies revealed the same tendency with significantly elevated level of pERK1/2 and a tendency of elevated IL-1β and CGRP after CFA or IS but where the differences were statistically not significant. It is worth pointing out that for the immunohistochemistry, detailed microscopy, allowed us to focus on the V1 region of the TG, whereas in the Western blot, the protein measurements were made on whole TG as it is impossible macroscopically to localize the exact area to analyse. In addition, the TG for Western blot is not fixed with perfusion, thus some trigeminal activation can occur during the tissue dissection.

Based on the vascular theory of migraine, we wanted to see if the agents caused vasodilation or vasocontraction when applied to the middle meningeal artery. Interestingly, the IS caused a strong contraction of the artery, whereas nothing occurred with the artery tone following the CFA administration. Vasodilatation has been suggested to be the initiator of migraine, but could also be a consequence or a side phenomenon [44] and it has previoulsy been shown that not all migraine attacks are associated with vasodilation [45]. An earlier study supporting the vascular hypothesis found that a short period of vasoconstriction triggers the release of vasodilator agents, such as CGRP, and could explain some of the findings presented here [42]. Overall, we show that the contractile IS and non-vasoactive CFA causes activation of the TG suggesting that vasodilation of the MMA is not the trigger the TG activation.

Conclusion

The present study has demonstrated that the application of IS or CFA onto the dural surface of rats can be used as a method to induce changes in the expression of pERK1/2, IL-1β and CGRP positive nerve fibres in the TG. These findings suggest a possible immuno-neuro-glial interaction. This opens up an interesting line for further investigations on the central effects of inflammatory agents and their relation to migraine and pain.

Additional file

Additional file 1: Figure S1. Representative Western blot. The figure shows a representativce Western blot for the data that are presented in Fig. 6. (TIFF 2843 kb)

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ML, developed the practical method, carried out the surgeries, immunostainings, Western blot and drafted the manuscript. KAH developed the practical method, carried out the myograph studies, and drafted the manuscript. ZM contributed on WB studies. JT and LV participated in the design of the study, coordination and helped to draft the manuscript. KW performed image anaysis, participated in the design of the study and drafted the manuscript. LE conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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SHORT REPORT Open Access

KYNA analogue SZR72 modifies CFA-induced dural inflammation- regarding expression of pERK1/2 and IL-1β in the rat trigeminal ganglion

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Abstract

Background: Neurogenic inflammation has for decades been considered an important part of migraine pathophysiology. In the present study, we asked the question if administration of a novel kynurenic acid analogue (SZR72), precursor of an excitotoxin antagonist and anti-inflammatory substance, can modify the neurogenic inflammatory response in the trigeminal ganglion.

Methods: Inflammation in the trigeminal ganglion was induced by local dural application of Complete Freunds Adjuvant (CFA). Levels of phosphorylated MAP kinase pERK1/2 and IL-1β expression in V1 region of the trigeminal ganglion were investigated using immunohistochemistry and Western blot.

Findings: Pretreatment with one dose of SZR72 abolished the CFA-induced pERK1/2 and IL-1β activation in the trigeminal ganglion. No significant change was noted in case of repeated treatment with SZR72 as compared to a single dose.

Conclusions: This is the first study that demonstrates that one dose of KYNA analog before application of CFA can give anti-inflammatory response in a model of trigeminal activation, opening a new line for further investigations regarding possible effects of KYNA derivates.

Keywords: Complete Freund's Adjuvant, Dura mater, Trigeminal ganglion, pERK1/2, IL-1β, KYNA

Background

Neurogenic inflammation (NI) has for decades been considered an important part of migraine pathophysiology [1]. Basic studies of NI show that it is characterized by proinflammatory responses, caused by the stimulation of peripheral terminals of the primary sensory neurons located in the trigeminal ganglion [2], ultimately involved in sensitization and allodynia. Despite growing interest on the role of neuro-immune interactions in migraine, studies show controversial results regarding serum cytokine levels [3–5].

An interaction between the kynurenine pathway and the immune system has been suggested [6]; the kynureninine system by itself can be activated by inflammatory agents and kynurenic acid has a clear antiinflammatory effect [7]. One of the first studies demonstrating that the kynurenine pathway has a central role in migraine, was performed by Knyihár-Csillik and coworkers, revealed that electrical stimulation of the trigeminal ganglion decreased kynurenine-aminotransferase immunoreactivity in rat dura mater [8]. Recent studies strengthen the importance of the kynurenine system in case of primary headaches, showing significant reduction in levels kynurenic acid in patients with chronic migraine [9–11].

In order to advance our understanding we have developed a method to study inflammation in the trigeminal

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Table 1 Animal groups used for immunohistochemistry and Western blot

Groups	Treatment 1 h before operation	Treatment every 12 hrs, for 7 days	No of animals, IHC	No of animals, WB
Pre-treatment SZR72	KYNA derivate	-	6	5
Pre-treatment saline	saline	-	4	5
Repeated treatment SZR72	KYNA derivate	KYNA derivate	6	5
Repeated saline	saline	saline	4	5
Intact control	-	-	4	5

ganglion induced by local dural application of Complete Freund's Adjuvant (CFA) [12].

In the present study we administered a novel kynurenic acid analogue (SZR72), a glutamate antagonist, to demonstrate its ability to modify this trigeminal ganglion response and might therefore represent a future approach to migraine treatment.

Methods

The present study is based on the animal model of inducing inflammatory response in the trigeminal ganglion via activation of the peripheral branches in the dura mater of the trigeminal neurons [12].

Synthesis of novel KYNA derivative

The KYNA amide was designed in the Department of Pharmaceutical Chemistry and MTA-SZTE Research Group for Stereochemistry, University of Szeged Hungary. The synthesis was performed by coupling of KYNA and 2-dimethylaminoethylamine, afterwards treatment of ethanolic hydrogen chloride, resulting *N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride. The structural properties of SZR72 are the following: presence of a water-soluble side-chain, the inclusion of a new cationic center, and side-chain substitution in order to facilitate brain penetration [6, 13].

Animals

Adult male Sprague-Dawley rats (220–300 g) ($n = 49$, 24 for immunohistochemistry, 25 for Western blot) were used. The animals were maintained under standard laboratory conditions with free access to food and tap water. The study followed the guidelines of the European Communities Council (86/609/ECC) and approved by the Ethics Committee of The Faculty of Medicine, University of Szeged, Hungary.

Operations

We have recently described the method in detail [12].

Treatments

The animals were divided into 5 groups: (i) pre-treatment KYNA (KYNA analog 1 h before CFA administration), (ii) pre-treatment saline (saline 1 h before CFA), (iii) repeated treatment (KYNA analog every 12 h, for 7 days), (iv) repeated saline (saline every 12 h, for 7 days) and (v) fresh (intact, control rats) (Table 1). The KYNA analog (300 mg/kg body weight was dissolved in 1 ml saline) or saline (1 ml) were given intraperitoneally.

As shown before [12] the "inflammatory" response to dural CFA was studied after 1 week, left trigeminal ganglion was removed and the specimens were prepared for immunohistochemistry or Western blot.

Immunohistochemistry and microscopic analysis

Immunohistochemistry was performed to demonstrate the localization of pERK1/2 and IL-1β, and semi-quanitatively evaluate the alterations in their expression in the trigeminal ganglion. Details of the antibodies are given in Table 2. The immunohistochemistry method and the microscopic analysis were described in our previous study [12].

Table 2 Details of primary and secondary antibodies used for IHC and WB

	Name	Product code	Host	Dilution	Company
IHC					
	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	4376	Rabbit	1:50	Cell Signaling Technology, Danvers, MA, USA
	Anti IL-1 beta antibody	ab 9787	Rabbit	1:100	Abcam; Cambridge, UK
WB					
	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	4376	Rabbit	1:1000	Cell Signaling Technology, Danvers, MA, USA
	Anti IL-1 beta antibody	ab 9787	Rabbit	1:500	Abcam; Cambridge, UK

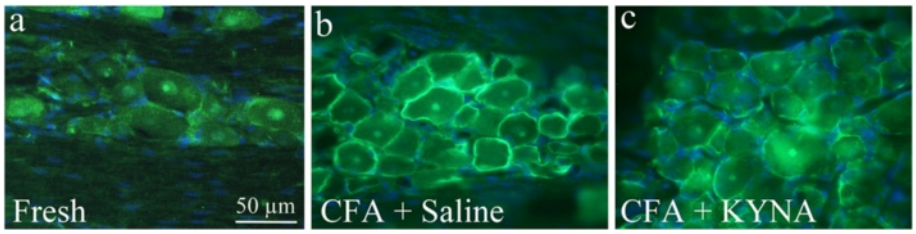

Fig. 1 pERK1/2 in the trigeminal ganglion. **a** In fresh animals, pERK1/2 immunoreactivity was detected in a few nuclei of the trigeminal ganglia, including nucleoli, in fresh animals. No immunoreactivity was found in the neuronal cytoplasm. A few SGC were considered as positively stained. **b** In CFA animals repeated treatment with saline i.p., high-intensity pERK1/2 immunoreactivity was observed in SGCs. **c** Animals treated with KYNA for 7 days, diminished immunoreactivity to pERK1/2 in SGC

Western blot

The method used for Western blot is described in one of our studies [14]. Data were normalised to an internal loading control sample to adjust for gel-to-gel variation and both pERK and pro-IL1beta calculated relative to t-ERK.

Findings

Immunohistochemistry

In evaluating the immunohistochemical results, the medullary zone of the trigeminal ganglion and the V1 region were chosen.

pERK1/2

As described earlier [12], pERK1/2 immunoreactivity was detected in a few nuclei of the neurons, including nucleoli, in fresh animals. A few satellite glial cells (SGC) were considered as positively stained (Fig. 1a). In CFA treated animals i.p. saline revealed high-intensity pERK1/2 immunoreactivity in SGCs, especially in the anteromedial zone of the ganglion (Fig. 1b). No significant difference was noted in case of repeated use of saline i.p.

In contrast, animals treated with the novel KYNA derivate, showed abolished pERK1/2 immunoreactivity in SGC (Fig. 1c). Repeated treatment with the KYNA analog resulted in mitigation of the SGC activation compared to saline treatment; both positive and negative SGC were detected.

Blinded analysis of the SGCs fluorescence intensity showed significant difference between the control groups and the pre-treatment group with KYNA derivate ($p < 0.05$). In contrast, no significant difference between the control groups and repeated treatment with KYNA analog was found ($p = 0.069$), neither between the pre-treatment and repeated treatment groups with KYNA analog ($p = 0.567$).

IL-1β

As described before [12], IL-1β immunoreactivity was observed in the neuronal cytoplasm (in a granular manner), in a few nuclei and in the nerve fibers of

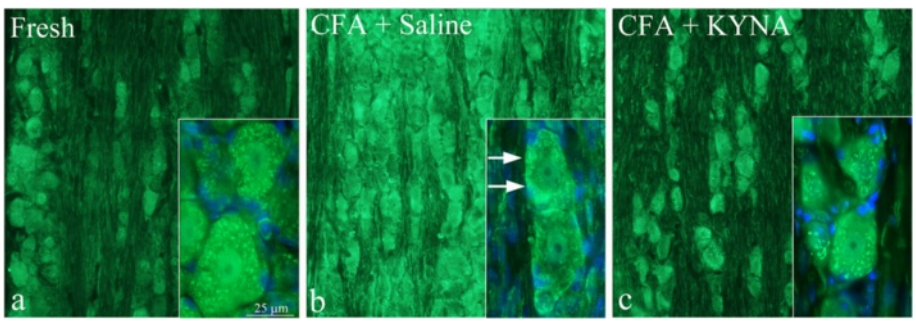

Fig. 2 IL-1β in the trigeminal ganglion. **a** IL-1β immunoreactivity was observed in the neuronal cytoplasm (in a granular manner), in a few nuclei and in the nerve fibers of fresh animals. No immunoreactivity was detected in the SGC. **b** After i.p. treatment with saline for 7 days, increased IL-1β immunoreactivity was observed both intracellularly in the neurons, and in the fibers. In addition, a "ring" IL-1β immunoreactivity close to the neuronal cell membrane was evident. **c** Following i.p. treatment with KYNA for 7 days, the homogenous immunoreactivity close to the cell membrane disappeared, returning to the granular cytoplasmatic pattern observed in fresh animals. No difference could be noted in the neuronal nuclei and in fibers, and no immunoreactivity was detected in the SGC

fresh animals. No immunoreactivity was detected in the SGC (Figs. 2a and 3). After i.p. treatment with saline and application of CFA, increased IL-1β immunoreactivity was observed both intracellularly in the neurons, and in the fibers. In addition, a "ring" IL-1β immunoreactivity close to the neuronal cell membrane was evident, which differed from the granular pattern seen in fresh animals (Fig. 2b).

Following i.p. treatment with KYNA analogue, the homogenous immunoreactivity close to the cell membrane disappeared, returning to the granular cytoplasmatic pattern observed in fresh animals. Repeated treatment with KYNA derivate showed no difference compared to the pretreatment with KYNA. No difference could be noted in the neuronal nuclei and in fibers, and no immunoreactivity was detected in the SGC (Fig. 2c).

Blinded analysis of the neuronal fluorescence in general, but also the presence of homogenous immunofluorescence close to the cell membrane, showed significant difference between control groups (pre-treatment and repeated treatment with saline) and pre-treatment with KYNA analog ($p < 0.05$). IL-1β immunoreactivity was significantly decreased in the repeated treatment KYNA group compared to control groups treated with saline ($p < 0.001$). Between the pre-treated and repeated treatment groups, no significant difference could be noted ($p = 0.969$).

A schematic drawing of the main results for the immunohistochemistry is shown in Fig. 4 and results are summarized in Table 3.

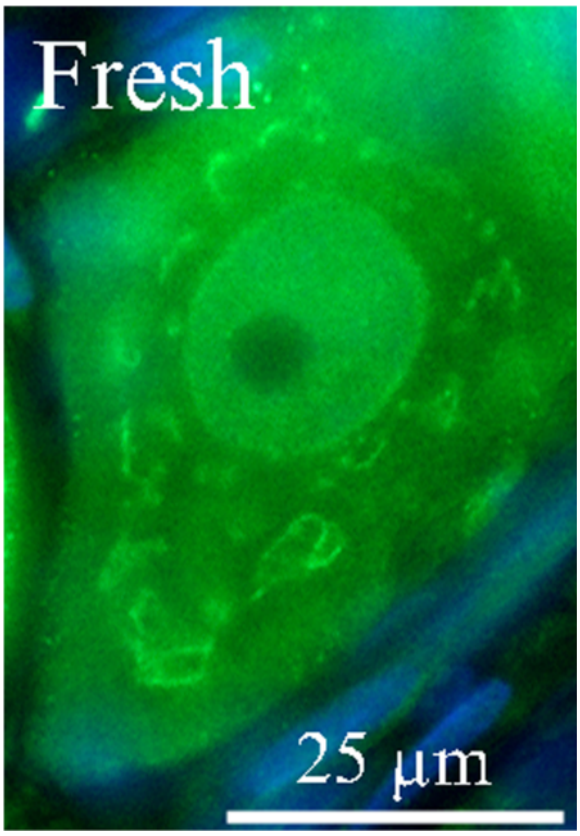

Fig. 3 A detailed distribution of IL-1β in a trigeminal ganglion neuron

Western blot

Staining for both pERK/tERK and pro-IL-1β using tissue lysates of complete left trigeminal ganglia did not reveal any significant changes in expression levels between treatment groups as analyzed by one-way ANOVA ($p = 0.8282$ and $p = 0.7461$, respectively) with Bonferroni multiple correction post-testing and Student's t-test (Fig. 5).

Discussion

The main question asked was if the KYNA derivate could modify the CFA-induced long-term inflammatory activation of the trigeminal ganglion, shown by increased expression of pERK1/2 and IL-1β [12].

The role of the kynurenine pathway in the CNS is very complex, modulating several neurotransmitters. In 1947 Beadle at al. [15] discovered that the major route for tryptophan metabolism to nictoniamide and its conjugates is the kynurenine pathway. Tryptophan represents a precursor of serotonin, a neurotransmitter playing an important role in the migraine pathophysiology [16]. The kynurenine pathway, having several neuroactive metabolites including kynurenic acid (KYNA) [17, 18], has an important role in various diseases of the CNS [6, 19]. Astrocytes represent one source of neuroprotective KYNA [6, 20]; KYNA was presumed to have protective effect in neuronal cell death [21, 22]. Studies have also suggested that an elevated extracellular KYNA level would be needed to act more effectively [23], leading to the idea of systemic administration of KYNA. This was not proved to be an ideal therapeutic option, as KYNA poorly penetrates the blood-brain barrier and it undergoes a rapid clearance from the brain and the circulation [24]. To overcome these difficulties new KYNA analogues were synthesized to facilitate blood-brain penetration [13]. It has been shown that the analogue SZR72 has similar neuroprotective effect as KYNA and cross more easily the BBB [25, 26].

In the present study SZR72 (1 mmol/kg bodyweight) reduced the CFA- elevated response on pERK1/2 and IL-1β activation in the trigeminal ganglion. The experiments were designed to answer the question if in case of chronification of migraineous mechanism (i) one dose of SZR72 is enough to attenuate the activation or if (ii) daily treatment would be needed.

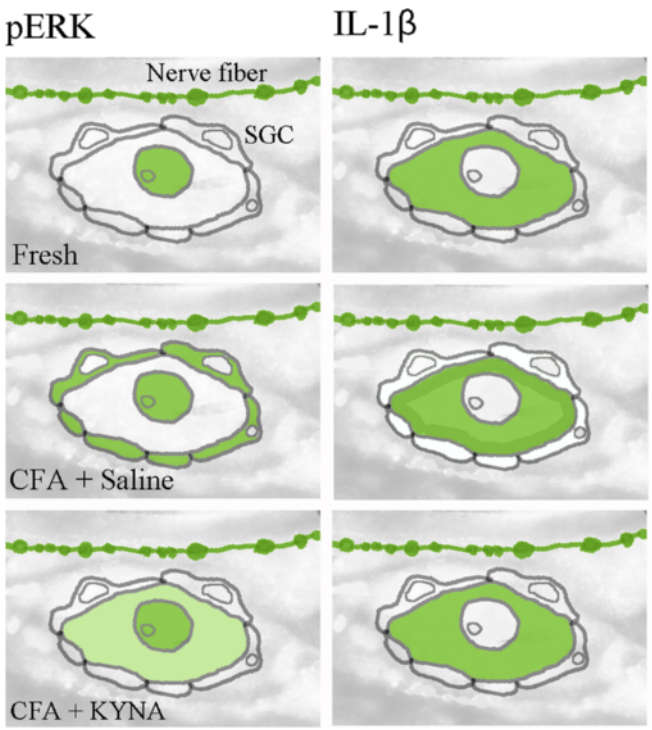

Fig. 4 A schematic drawing of the distribution of pERK1/2 and IL-1β

The pERK1/2 is suggested to represent a rapid and robust marker of activation and inflammation [27, 28], whereas IL-1β is a late marker of maintained neuropathic pain [29]. In case of using one dose of SZR72, pERK1/2 activation in the SGC was significantly diminished, whereas in case of repeated treatment the difference was not found to be significant. This finding might be explained by the property of pERK1/2 as being an early marker and not an optimal sign of prolonged activation.

The IL-1β activation was modified both after one dose of SZR72 and after repeated treatment with SZR72. Although no significant difference was observed between the pre-treatment and repeated treatments in case of IL-1β, there was a significant difference between the repeated SZR72 treated group and control groups. IL-1β might represent a more proper marker to examine long term effects of SZR72.

When it comes to our Western blot studies, the same tendency can be followed, without any significant difference between the groups. This might be explained by a methodological problem: to isolate macroscopically the V1 region is almost impossible therefore the whole ganglia were processed in case of WB studies. We might reasonably postulate that our WB studies are not specific enough for the V1 region of the trigeminal ganglion.

Table 3 Summary of results for IHC for pERK and IL-1β regarding neurons, fibers and satellite glial cells (SGC); intensity scale: "-" no staining, "+/-" very weak staining, "+"-weak staining, "++"-moderate staining, "+++" strong staining

	pERK 1/2			IL-1β		
Groups	Neurons (nucleolei)	Fibers	SGC	Neurons	Fibers	SGC
Pre-treatment SZR72 (dura exposed to CFA)	+	+	+/-	+/-	+	-
Pre-treatment saline (dura exposed to CFA)	+	+	+++	+++	+	-
Repeated treatment SZR72 (dura exposed to CFA)	+	+	++	+/-	+	-
Repeated treatment saline (dura exposed to CFA)	+	+	+++	+++	+	-
Intact control (unoperated)	+	+	+/-	-	+	-

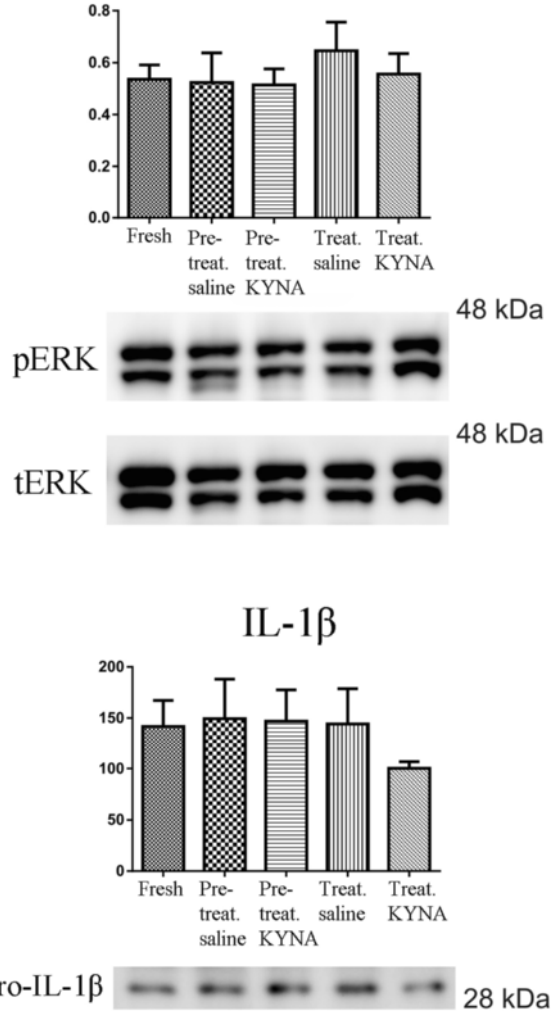

Fig. 5 Western blot of pERK, t-ERK and IL-1β in the whole trigeminal ganglion revealing no significant difference between the treatment groups

In conclusion, this is the first study to address the question whether daily use of the new KYNA analog would be more effective than one dose prior activation in a chronification model of trigeminal activation. Pretreatment with one dose was able to abolish pERK and IL-1β activation in the trigeminal ganglion. These findings open a new line for further investigations which could result in a new way to modulate inflammation in chronic migraine.

Competing interests
The authors declare that they have no competing interests.

Authors' contribution
ML, KW, JTajti, LV and LE designed the study. FF and JT synthesized the kynurenic acid amide 2. ML and KW performed the all the experiments except for Western blot that was performed by LSK. ML, KW, LE and JTajti analyzed the data and prepared the manuscript. KW, LV, LE and JTajti supervised all aspects of the project and revised the manuscript. All authors read and approved the final manuscript.

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RESEARCH ARTICLE Open Access

Topical dura mater application of CFA induces enhanced expression of c-fos and glutamate in rat trigeminal nucleus caudalis: attenuated by KYNA derivate (SZR72)

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Abstract

Background: Migraine is a debilitating neurological disorder where trigeminovascular activation plays a key role. We have previously reported that local application of Complete Freund's Adjuvant (CFA) onto the dura mater caused activation in rat trigeminal ganglion (TG) which was abolished by a systemic administration of kynurenic acid (KYNA) derivate (SZR72). Here, we hypothesize that this activation may extend to the trigeminal complex in the brainstem and is attenuated by treatment with SZR72.

Methods: Activation in the trigeminal nucleus caudalis (TNC) and the trigeminal tract (Sp5) was achieved by application of CFA onto the dural parietal surface. SZR72 was given intraperitoneally (i.p.), one dose prior CFA deposition and repeatedly daily for 7 days. Immunohistochemical studies were performed for mapping glutamate, c-fos, PACAP, substance P, IL-6, IL-1β and TNFα in the TNC/Sp5 and other regions of the brainstem and at the C_1-C_2 regions of the spinal cord.

Results: We found that CFA increased c-fos and glutamate immunoreactivity in TNC and C_1-C_2 neurons. This effect was mitigated by SZR72. PACAP positive fibers were detected in the fasciculus cuneatus and gracilis. Substance P, TNFα, IL-6 and IL-1β immunopositivity were detected in fibers of Sp5 and neither of these molecules showed any change in immunoreactivity following CFA administration.

Conclusion: This is the first study demonstrating that dural application of CFA increases the expression of c-fos and glutamate in TNC neurons. Treatment with the KYNA analogue prevented this expression.

Keywords: TNC, CFA, c-fos, Glutamate, KYNA analogue

Background

Migraine is among the leading causes of disability, having a huge impact on public health [1, 2]. Studies show that each year 2.5% of episodic migraine disease converts into chronic migraine [3] which appears as a distinct entity in the classification of the International Headache Society. Although numerous studies have been performed aiming to understand the pathophysiology of migraine and the chronification process, however this is still enigmatic. The trigeminal system plays a pivotal role in the genesis of migraine headache [4, 5]. The pseudo-unipolar nerve cells of the trigeminal ganglion (TG), provide sensory innervation of cranial structures and meningeal vessels while central projections terminate in trigeminal nucleus caudalis (TNC) and C_1-C_2 region of the spinal cord [6]. This trigeminovascular complex transmits pain signals from meningeal and cerebral vessels to the brainstem and second-order neurons terminate in the thalamus and cortical regions, where further transmission and modulation of

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pain sensation occur [6–8]. Following continous and repeated stimulation peripheral and central sensitisation of the primary-neurons might occur, leading to reduced activation treshold, represented clinically by allodynia [4, 9, 10].

Previous studies have shown that application of inflammatory substances on the dura mater causes central sensitisation of the neurons in TNC and at C_1-C_2 levels of the spinal cord [6, 10, 11]. Recent studies have demonstrated lower levels of kynurenic acid (KYNA) in serum of patients suffering from chronic migraine compared to controls [12, 13]. KYNA could be a new therapeutic line in migraine chronification, but KYNA can poorly cross the blood–brain barrier (BBB), while newer KYNA analogues have better BBB penetration characteristics [14, 15]. We have recently developed an animal model for long-term trigeminovascular activation following application of Complete Freund's Adjuvant (CFA) onto the surface of the dura mater [16]. We found activation of satellite glial cells and neurons of the trigeminal ganglion (IL-1, pERK1/2) that were abolished by the KYNA-analogue, SZR72 [17] possibly acting on peripheral and central gluatamate receptors (30). The present study was designed to examine whether dural application of CFA can cause activation of the central part of the trigeminalvascular system,: the TNC and C_1-C_2 regions of the spinal cord. We asked the question whether the CFA-induced activation might be mitigated by use of SZR72 intraperitoneally.

Methods

Synthesis of novel KYNA derivative

The KYNA amide reported here was designed in the Pharmaceutical Chemistry and Research Group for Stereochemistry, University of Szeged Hungary. The synthesis procedure has previously been presented [15, 17]. The KYNA analogue (SZR72, 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 in order to enhance brain penetration [17].

Animals

Adult male Sprague–Dawley rats (220–300 g) ($n = 30$) were used. The animals were raised and maintained under standard laboratory conditions with free access to food and tap water. The study followed the guidelines of the European Communities Council (86/609/ECC) and was approved by the Ethics Committee of The Faculty of Medicine, University of Szeged, Hungary (I-74-12/2012).

Treatments

The animals were divided into 7 groups: (i) CFA + saline application to the dura, (ii) saline application to the dura, (iii) pre-treatment KYNA (KYNA analog, 300 mg/kg body weight dissolved in 1 ml saline, 1 h before CFA administration), (iv) pre-treatment saline (saline, 1, ml 1 h before CFA), (v) repeated treatment (KYNA analog, 300 mg/kg body weight dissolved in 1 ml saline every 12 h, for 7 days), (vi) repeated saline (saline 1 ml every 12 h, for 7 days) and (vii) fresh (intact, control rats) (Table 1).

Operation

The operation has been described in details earlier [16, 17]. Briefly, animals were deeply anesthetized and a handheld drill was used to remove a 3x3 mm large portion of the parietal bone, cooled by saline irrigation to avoid local healing. The hole was made postero-laterally to the bregma (5 mm), on the left side, care being taken not to penetrate the dura mater. Ten μl of CFA (Sigma-Aldrich, St. Louis, MO, USA) or saline was applied on the dural surface, and washed with saline after 20 min.

Both treated and control animals were transcardially fixation-perfused with 4% paraformaldehyde in buffer after 7 days. As fresh control, intact rats were used.

Tissue analysis

After the perfusion-fixation the TNC brainstem region and C_1-C_2 region of the spinal cord were removed (−1, +5 mm from the obex). Specimens were frozen on dry ice, stored at −80 °C and prepeared for immunohistochemistry. To encompass TNC, sections were collected from 6 different levels from the central canal was visualized to the C_1 segment of the spinal cord. (100–120 sections in total per animal).

Immunohistochemistry and microscopic analysis

Immunohistochemical staining was performed to demonstrate the localization of glutamate, c-fos, TNF-α, IL-1β, IL-6, substance P and PACAP. Details of the primary and secondary antibodies are given in Table 2 and 3.

The immunohistochemical method and the microscopic analysis have been described earlier [16]. The areas of the brainstem were identified using rat brain atlas (Paxinos and Watson, second edition, 1986). Each procedure was repeated a minimum of three times to validate the results

Table 1 Groups of animals

Group	Dura application	Pre-treatment	Repeated treatment	No. of animals
CFA	CFA	-	-	6
Saline	saline	-	-	3
CFA + KYNA pre-treatment	CFA	KYNA	-	6
CFA + saline pre-treatment	CFA	saline	-	3
CFA + KYNA repeated	CFA	KYNA	KYNA	6
CFA + saline repeated	CFA	saline	saline	3
Fresh, control rats	-	-	-	3

Table 2 Details of primary antibodies used for immunohistochemistry

Name	Product code	Host	Dilution	Company
Anti c-fos	PC38	Rabbit	1:100	Merck Millipore, Darmstadt, Germany
Anti PACAP-38	B57–1	Rabbit	1:100	Europroxima, Arnhem, Netherlands
Anti Glutamate	G9282	Mouse	1:100	Sigma-Aldrich, St-Luis, MO, USA
Anti Glutamate	AB5018	Rabbit	1:100	Merck Millipore, Darmstadt, Germany
Anti Substance P	B 45–1	Rabbit	1:200	Europroxima, Arnhem, Netherlands
Anti IL-1β	ab 9787	Rabbit	1:100	Abcam, Cambridge, UK
Anti IL-6	ab6672	Rabbit	1:200	Abcam, Cambridge, UK
Anti-TNF α	ab66579	Rabbit	1:400	Abcam, Cambridge, UK

and minimize any experimental errors using the same antibody stock. Negative controls were performed for each set by omitting the primary antibody. One examinator was blinded. Any resulting immunofluorescence would suggest unspecific binding of the secondary antibodies.

Results

Immunohistochemistry

Glutamate

In intact (fresh) animals, glutamate immunoreactivity (GI) was detected in fibers of the trigeminal tract on every level of the TNC (Fig. 1a and b). A few homogeneously stained glial cells could also be found. The staining also displayed some homogenously labelled neurons, especially in TNC in the caudal part of the brainstem.

Application of CFA on the dura (group CFA 7days), similar staining pattern was observed, with an obvious increase in the intensity and amount of glutamate positive neurons in the TNC (Fig. 1). In the gelatinous layer a clearly increased intensity could be seen. With higher magnification, cells with intensely stained cytoplasm were identified in this region (Fig. 1). The aspect is specific to the medial part of the spinal trigeminal nucleus: triangular or multipolar shaped, medium-sized cells with an irregular arrangement. No difference in the fiber staining was noted. In the application of saline group, the same GI pattern was observed (Fig. 1).

Table 3 Details of secondary antibodies used for immunohistochemistry

Conjugate and host	Against	Diluation	Company
FITC (goat)	anti-rabbit	1:100	Cayman Chemical, Ann Arbor, MI, USA
Alexa 488 (goat)	anti-mouse	1:100	Invitrogen, CA, USA
Alexa 594 (donkey)	anti-rabbit	1:100	Jackson Immuno Research, West Baltimor, PA, USA

In group pretreated with SZR72, the increased expression was abolished. The intensity and number of glutamate immunoreactive cells remained at the level observed in healthy, intact animals. No clear difference could be visualized between pre-treatment and repeated-treatment of KYNA, and no difference was noted in the fibers and glial cells. In the application of saline group, the same GI pattern was observed as in fresh control (Fig. 1).

On the level of the C_1-C_2 region of the spinal cord GI was found in the anterior and dorsal horns (lamina I, lamina II) and the areas surrounding the central canal. In these areas no difference was found between the different groups (no cells, only fibers). A summary of these results is presented in Table 4.

C-fos

In intact (fresh) rats, few c-fos positive neuronal nuclei, but no nucleolei, were observed in the caudal part of the spinal trigeminal nucleus (Sp5C, TNC); these were mainly seen in the gelatinous layer (Fig. 2). No difference could be observed between cranial and caudal levels of the TNC.

After application of CFA onto the dura mater, an increase in the number of c-fos positve nuclei could be detected, especially in the caudal areas of the TNC, close to the spinal cord (Fig. 2). No increased immunoreactivity was visualised using saline application on the dura (Fig. 2).

Administration of SZR72 reduced the CFA-induced activation in neuronal nuclei at every level of the TNC, similar to the low expression seen in fresh rats. No significant difference could be shown between the pretreatment and repeated-treatment of SZR72. After treatment with saline, we noted no increase in the c-fos expression, showing that treatment with saline did not have effect on the CFA-induced TNC activation (Fig. 2i).

The results of the c-fos immunostaining are summarized in Table 5.

PACAP

PACAP immunoreactivity was found in fibers of the trigeminal tract, both in healthy and CFA inflammation-induced animals. PACAP immunoreactivity was found in the brainstem, in the spinal cord, especially in the large neurons of the anterior horn, in the dorsal horn, around the central canal and the ependymal cells of the central canal. PACAP immunoreactive fibers were observed in almost every tract in the spinal cord (dorsal corticocerebellar tract, spinocerebellar tracts, medial longitudinal tract, pyramidal tract). In these territories no difference was detected between different groups (SZR 72 had no effect).

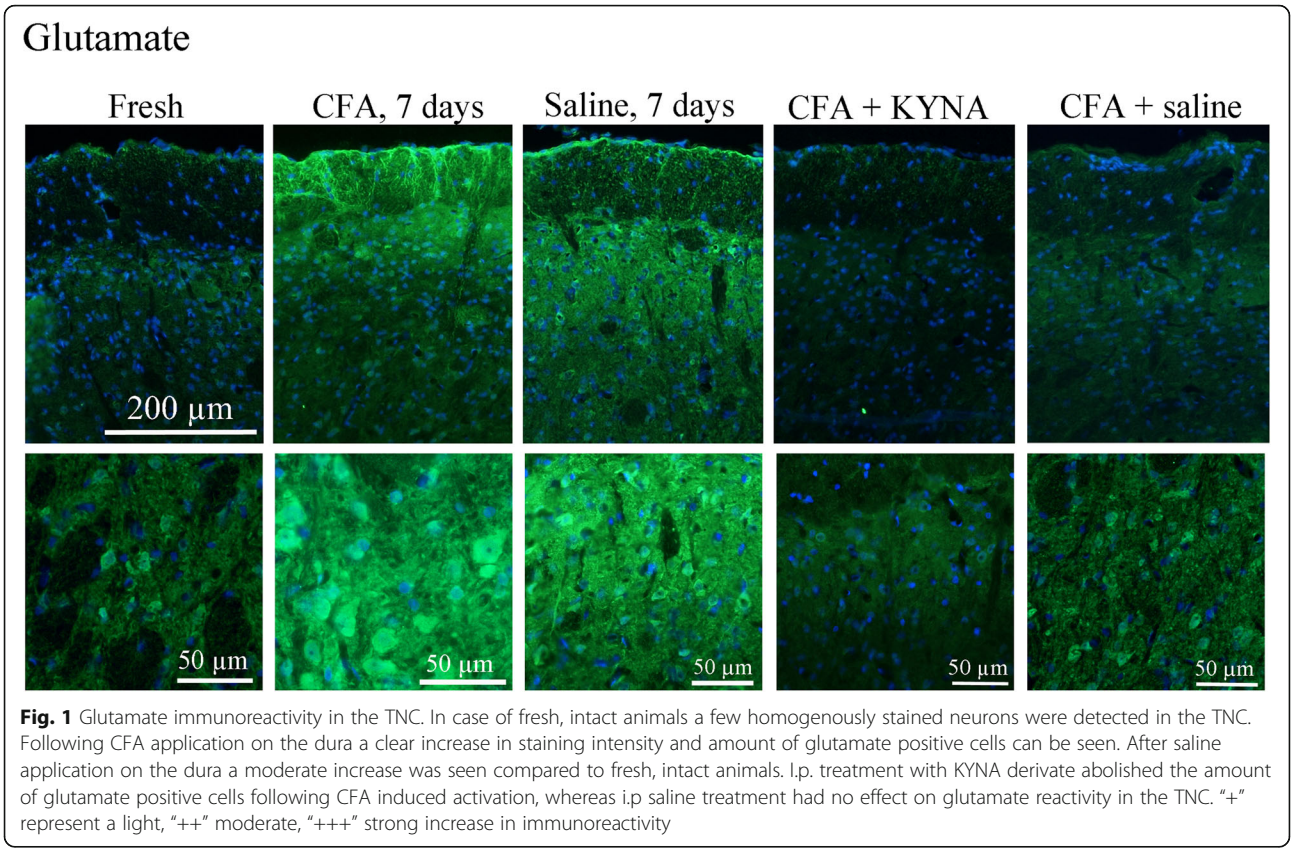

Fig. 1 Glutamate immunoreactivity in the TNC. In case of fresh, intact animals a few homogenously stained neurons were detected in the TNC. Following CFA application on the dura a clear increase in staining intensity and amount of glutamate positive cells can be seen. After saline application on the dura a moderate increase was seen compared to fresh, intact animals. I.p. treatment with KYNA derivate abolished the amount of glutamate positive cells following CFA induced activation, whereas i.p saline treatment had no effect on glutamate reactivity in the TNC. "+" represent a light, "++" moderate, "+++" strong increase in immunoreactivity

Substance P

Substance P immunoreactivity was limited to nerve fibers of the spinal trigeminal tract and to the gelatinous layer (Fig. 3). Some positive fibers, surrounding the SP5C were also visualized. No difference was noted between different levels of TNC and a slight increased intensity of the fiber staining could be detected after CFA application, but not in the gelatinous layer.

TNF-α

TNF-immunoreactivity was found in the trigeminal tract, with dense fiber staining in the spinal trigeminal tract, but no glial or neuronal staining was detected at either level of the TNC. In the spinal cord few, small sized neurons were detected, especially around the central canal (Fig. 3). Some TNF- α positive fibers were identified in other tracts of the spinal cord (dorsal cortico-cerebellar tract, spino-cerebellar tracts, medial longitudinal tract, pyramidal tract). No difference was noted between the groups.

Table 4 Summary of glutamate immunostaining in TNC for different treatment groups

Group	Neuronal staining	Fiber staining	Glial cell staining
Fresh	-\+	++	-\+
CFA 7d	+++	++	-\+
Saline 7d	+	++	-\+
CFA+ SZR72 one dose	-\+	++	-\+
CFA+ SZR72 repeated	-\+	++	-\+
CFA+ saline one dose	+++	++	-\+
CFA + saline repeated	+++	++	-\+

IL-6

IL-6 positivity was detected in the fibers and in the cytoplasm of some glial cells, showing a homogenous staining in the spinal trigeminal tract. Some homogeniously stained neurons were detected in the TNC. In the spinal cord, some positive neurons could be seen in the caudal part of the spinal trigeminal nucleus, in the large neurons of the anterior horn, in the dorsal horn and the ependymal cells of the central canal (Fig. 3). Intensely positive fibers were visualised in the cuneate and gracile fasciculus. After application of CFA, similar staining patterns as for the non-CFA groups were found.

IL-1β

IL-1β immunohistochemistry showed the same staining pattern as for IL6 and TNFα, with no change after CFA induced activation (Fig. 3). IL-1β immunoreactivity showed a granular cytoplasmatic stainig, previously described in the TG (14).

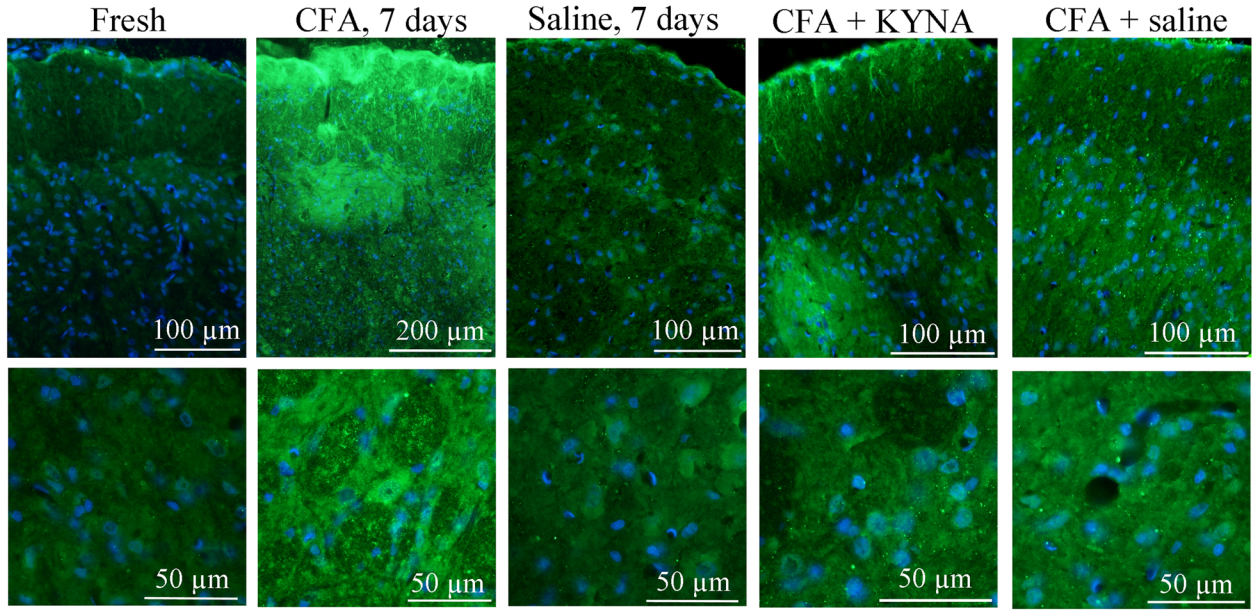

Fig. 2 C-fos immunoreactivity in the TNC. In case of fresh, intact rats few c-fos positive neuronal nuclei but no nucleolei could be observed. After appilcation of CFA on t he dura mater large amount of c-fos positive nuclei was detected. Saline application on the dura mater caused no increase in immunoreactivity compared to fresh, intact control. I.p. treatment with KYNA derivate was able to abolish the activation caused by application of CFA on the dura. I.p. saline had no such effect. "+" represents a light, "++" moderate, "+++" strong increase in immunoreactivity

Discussion

In this study we present the immunostaining pattern of several neuronal messengers and cytokines in the TNC/C_1-C_2 spinal region (11) that are indicated in migraine pathophysiology. CFA is a potent immun- potentiator, used in various peripheral pain model. (Spinal distribution of c-Fos activated neurons expressing enkephalin in acute and chronic pain models, 1st manu) We asked the question whether application of CFA on a defined area of the dura mater could cause activation of second-order neurons in the TNC and whether this activation can be mitigated by systemic adminstration of a KYNA analogue.

Gluatamate, the major excitatory neurotransmitter in CNS plays a key role in the trigeminovascular activation, especially in central sensitisation via activation of NMDA receptors [18, 19]. Glutamate appears to be involved in nociception since glutamate is expressed in the trigeminal ganglion and other sensory ganglia [20, 21]. Glutamate can be released from neurons following nociceptive stimuli putatively acting on satellite glial cells (SGC) [22] but is also expressed in the sensory Aδ – fibers (19).

Table 5 Summary of c-fos immunostaining in TNC for different treatment groups

Group	Neuronal staining
Fresh	-\+
CFA 7d	+++
Saline 7d	+
CFA + SZR72 one dose	+
CFA+ SZR72 repeated	+
CFA + saline	+++
CFA + saline repeated	+++

The kynurenine pathway, the major route of tryptophan metabolism to nicotinamide, has an important role in several diseases of the CNS [23–25]. Kynurenic acid (KYNA) is one of the neuroactive metabolits of the kynurenine pathway in human astrocytes [26] protecting against neuronal cell-death [27]. KYNA in low concentration enhances AMPA receptor activity [28, 29], while in high concentrations blocks the NR1 subunit of the NMDA receptors [19, 30]. NMDA receptor consists of NR1, NR2 and NR3 subunits, where NR1 subunit has a glycine- binding domain. Glycine is essential for the functioning of the NMDA receptor and KYNA acts as an antagonist on the gylcine-binding site (NR1) (18). High level of KYNA might have a neuroprotective effect and could act on glutamate receptors, exerting an inhibitory effect on glutamate release [23].

Here we asked the question whether the KYNA analogue might act on the fibers and cells in the TNC/C_1-C_2. Previous work has shown a positive effect in TG following CFA injection into the temporomandibular joint [31] while SZR 72 decreased c-fos activation in the TNC in nitroglycerin

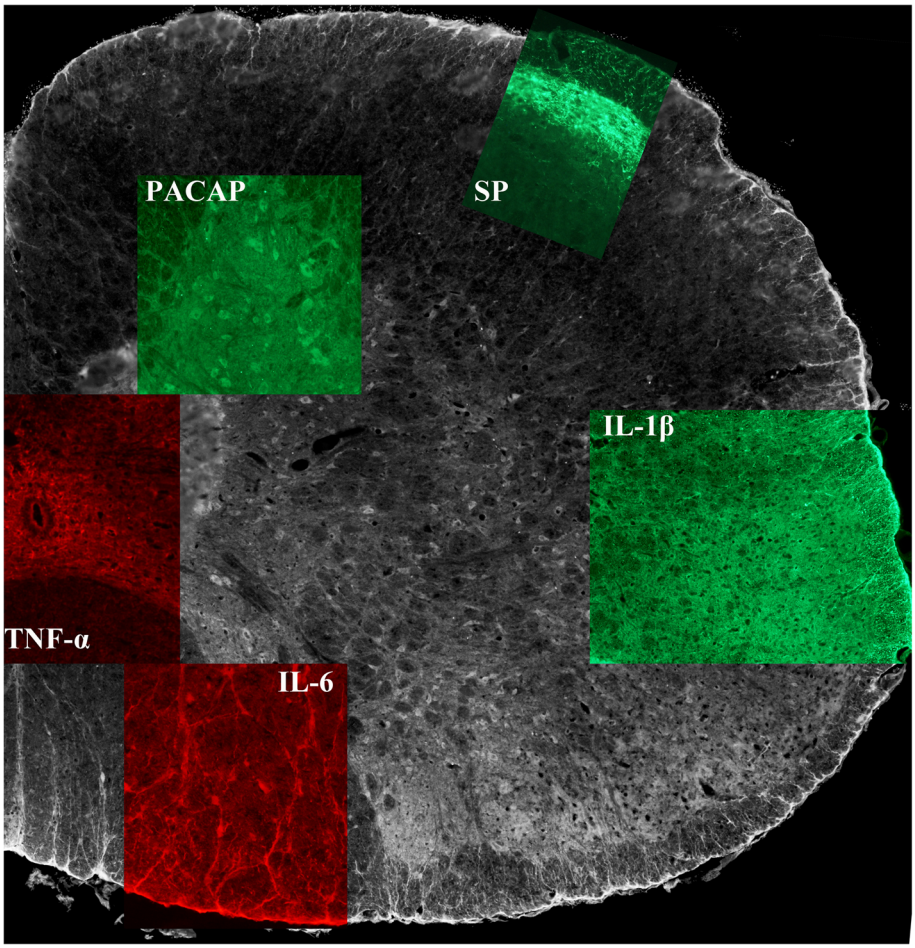

Fig. 3 Summary of PACAP, Substance P, TNF-α, IL-6 and IL-1β immunoreactivity in the C_1-C_2 region of the spinal cord. PACAP immunoreactivity was detected in the dorsal horn and in almost every tract of the spinal cord. Substance P immunoreactvity was limited to the fibers of the spinal trigeminal tract. TNF-α immunopositivity was mainy seen in the small cells surrounding the central canal. IL-6 immunopositivity was shown in the large neurons of the anterior horn and in nerve endings of these cells. IL-1 β immunopositvity was mainy seen in different tracts of the spinal cord, few positive cells, with granular intracytoplasmatic staining were also detected

induced trigeminal activation [32]. We have reported that one dose of SZR 72 is able to reduce dura mater applied CFA induced activation in the TG [16]. C-fos immunoreactivity is a widely used marker of neuronal activity in the TNC [33, 34]. In the present study we report increased c-fos immunoreactivity following dura mater application of CFA as a sign of neuronal activity of TNC neurons. This effect is attenuated by SZR72. Glutamate activation as a sign of central sensitization can be observed in the second-order neurons after use of CFA, this effect that is also mitigated by the KYNA analogue. Surprisingly repeated-treatment of SZR 72 was not seen to be more effective than pre-treatment with one dose prior CFA application neither in the TG [16] nor in the TNC. Therefore we postulate that early KYNA derivate intervention can block the development of central sensitization, whereas late, repeated treatment might not be able to further moderate mechanisms of central sensitization. Consequently, we assume that the action of the KYNA analogue seems to be exerted on the periphery that is conveyed to neurons of TNC, but an effect on central mechanisms cannot be surely excluded. Further studies are needed to elucidate the possible site of actions of the KYNA derivate.

In this study we examine a fair number of molecules suggested to play a role in migraine. Among these CGRP and PACAP 38 (PACAP) is currently of particular interest. CGRP plays an important role in migraine pathophysiology and localization of CGRP and its receptors (CLR and RAMP1) has already been described in TNC and C_1 region of the spinal cord [35, 36]. PACAP is a neuromodulator that has some common actions with CGRP, sharing the same receptors RAMP1 subunit [37]. PACAP might play a role in migraine having various neurobiological functions such as inhibitory effect on neurogenic inflammation [38]. PACAP has shown to be involved in trigeminovascular activation as PACAP-38

infusion caused headache in healthy volunteers [39] and PACAP-38-like immunoreactivity has proved to be altered in ictal compared to interictal phase of migraine and in cluster headache [40, 41]. It is suggested that the effect of PACAP is biphasic: lower concentration increasing, higher concentration inhibiting the NMDA receptor activation [42]. We have found PACAP positive but no change in TNC and C_1-C_2.

In addition, we examined several other molecules putatively involved in migraine pathophysiology: SP, IL-1β, IL-6 and TNF-α which have all been shown to be associated with activation of the trigeminovascular system [43–46]. While we could document their presesnce in the TNC and C_1-C_2 of the spinal cord, we did not observe a difference in expression between saline vehicle or CFA administration.

Conclusion

In conclusion, we report activation in neurons and fibers of TNC and C_1-C_2 following application of CFA on the dura mater that was mitigated by SZR 72. To our knowledge this is the first study that presents the cellular distribution of proinflammatory cytokines (IL-6, IL- 1β and TNF-α) in the TNC and in the C_1-C_2 region of the spinal cord. This might represent a further step in understanding the functional neuroanatomy of the trigeminal pathway. We found increased c-fos and glutamate immunoreactivity in the TNC following application of CFA on the dura mater that was abolished by the KYNA derivate. Further studies are needed to explore the possible mechanisms involved, which could result in a new therapeutic line in treatment of migraine.

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Authors' contribution

ML, KW, JTajti, JToldi LV, LE designed the study. FF synthesized the kynurenic acid derivate. ML and KW performed all the experiments. ML, KW, J Tajti and LE analyzed the data. KW, J Tajti, LV and LE supervised al aspects of the project and revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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