The role of endocannabinoid and kynurenine systems in the pathomechanism of migraine: animal experimental data

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Ph.D. Thesis

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LIST OF ABBREVIATIONS

AEA	anandamide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
C1-C2	upper cervical spinal cord
CB	cannabinoid receptor
CB1	cannabinoid receptor1
CB2	cannabinoid receptor2
CGRP	calcitonin gene–related peptide
COX-2	cyclooxygenase-2
CSD	cortical spreading depression
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
i.p.	intraperitoneal
IDO	indoleamine 2,3-dioxygenase
KAT-II	kynurenine aminotransferase-II
KMO	L-kynurenine 3-monooxygenase
KYNA	kynurenic acid
KYNU	L-kynurenine hydrolase
L-KYN	L-kynurenin
NF-κB	nuclear factor kappa B
NMDA	N-methyl-D-aspartate
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NSAIDS	non-steroidal anti-inflammatory drugs
NTG	nitroglycerin
PACAP	pituitary adenylate cyclase-activating peptide
PBS	phosphate-buffered saline
QUIN	quinolinic acid
TBST	Tris-buffered saline containing Tween 20
TDO	tryptophan 2,3-dioxygenase
TG	trigeminal ganglion

TNC	caudal trigeminal nucleus
TRPV1	transient receptor potential vanilloid 1

Summary

Primary headache disorders include migraine, which is one of the most frequent neurological diseases and affects more than 14% of the whole population. Despite the research efforts, its exact pathomechanism is not fully revealed, but evidence points to the role of glutamate and its receptors as key players. Kynurenic acid (KYNA) is an endogenous glutamate receptor antagonist produced by the kynurenine pathway. Tryptophan 2,3-dioxygenase (TDO2) and indoleamine 2,3-dioxygenase (IDO1,2) convert L-tryptophan to N-formyl-L-kynurenine, to be further transformed to L-kynurenine (L-KYN). Kynurenine aminotransferase-II (KAT-II), L-kynurenine hydrolase (KYNU) and L-kynurenine 3-monooxygenase (KMO) are key enzymes in the later steps of the kynurenine pathway.
Recent studies suggest that the endocannabinoid system is involved in nociceptive signal processing and cannabinoid receptor (CB) agonists are able to attenuate nociception in animal models of pain.

One of the human and animal models of migraine is the systemic administration of the nitric oxide donor (NO) nitroglycerin (NTG). NTG can provoke migraine like attacks in migraineurs and besides its activation it initiates a self-amplifying process in the trigeminal system, probably leading to central sensitization.

We used the NTG model of migraine in rats to study the role of endocannabinoids and kynurenines in the pathomechanisms of migraine.

Our results show that NTG is able to increase the markers of the sensitization process in the upper cervical spinal cord, namely transient receptor potential vanilloid type 1 (TRPV1), neuronal nitric oxide synthase (nNOS), nuclear factor kappa B (NF-κB) and cyclooxygenase-2 (COX-2) in the trigemino-cervical complex. On the other hand, we have found that AEA modulates the NTG-induced changes, thus it influences the activation and central sensitization process in the trigeminal system, probably via CBs.
It has been also demonstrated that NTG is able to down-regulate the kynurenine pathway, with a potential influence on the glutamatergic system as well, contributing to the development of trigeminal activation and sensitization in animals.

Introduction

I. Migraine

Migraine is a chronic neurological disorder characterized by recurrent headaches lasting for 4-72 hours and commonly accompanied by nausea, photophobia and phonophobia. The word derives form the Greek hemicrania-„pain on one side of the head", introduced by Galen in 200 AD. This syndrome affects 16% of the total population (Smitherman and Gadda, 2013) and its incidence is three times more common in women than in men. The total cost of healthcare for patients with migraine in Europe in 2010 was 18.4 billion € (Olesen et al., 2012).

It is well-known, that the activation and sensitization of the trigeminal system is essential during the attack (Edvinsson and Uddman, 2005). Continuous activation of peripheral trigeminal afferents leads to peripheral (first order) sensitization, which manifests as a throbbing pain worsening during physical activity (Burstein et al., 2011). Sustained nociceptive inputs can lead to sensitization of the second and ultimately to the third order neurons, so called central sensitization (Goadsby, 2005) manifested as allodynia when non painful stimuli are perceived as painful. Previous data have shown, that after the onset of central sensitization during the migraine attack, the acute treatment becomes less effective (Burstein et al., 2004). Although, this theory has been contested recently, stating that the severity of headache might be a better indicator, than the symptoms of sensitization (Diaz-Insa et al., 2011; Goadsby et al., 2008), it is generally accepted that the latter plays an essential role in the genesis of migraine (Burstein et al., 2000).

Besides the trigeminal system, the key players in the pathomechanism of migraine are the brainstem migraine generators including periaqueductal grey matter, locus coeruleus, dorsolateral pons, nucleus raphe magnus and the nucleus raphe dorsalis (Weiller et al., 1995).

Another important process in migraine is the cortical spreading depression (CSD), which is a self-propagating wave of depolarization that spreads across the cerebral cortex. It is widely accepted that CSD is the basis of the migraine aura (Lauritzen, 1994; Moskowitz, 2008). CSD can activate the trigeminal system (Moskowitz et al., 1993), enhance persistent blood flow and induce plasma protein extravasation in dura mater in rats (Bolay et al., 2002), which phenomena are also present under the migraine attack. The possible connection between CSD

and migraine may be glutamate and its receptors, since they have a relevant role in the propagation and generation of CSD (Lauritzen et al., 1988).

The role of glutamate seems pivotal in many other aspects of migraine. Glutamate receptors are found throughout in the trigeminal system. N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate receptors are present within the brainstem nuclei of the trigeminal system (Tallaksen-Greene et al., 1992) and NMDA receptor mRNA was found in the trigeminal ganglion (Watanabe et al., 1981), as well. NMDA, AMPA, kainate receptors can also be found in the superficial layers of the spinal cord (Furuyama et al., 1993), where the brainstem trigeminal nuclei extend. Activation of NMDA receptors is one of the most important step in initiating and maintaining the central sensitization (Latremoliere and Woolf, 2009), which can be blocked by competitive (D-CPP) and non-competitive (MK801) NMDA receptor antagonists in rats (Ma and Woolf, 1995). In addition, a conditional deletion of the NR2 subunit of NDMA receptors inhibits the synaptic inputs trough NMDA receptors and the central sensitization in rats (Hughes et al., 2003), as well. Besides NMDA, the metabotropic glutamate receptors also contribute to the mechanical allodynia (Soliman et al., 2005). Data from human studies showed, that the levels of glutamate were higher in the plasma, cerebrospinal fluid and platelets in migraine patients compared with non-migraineurs (D'Andrea et al., 1991; Martinez et al., 1993; Rothrock et al., 1995), which could indicate an increased activation of their receptors, thus hyperexcitability (Vecsei et al., 2015). One of the endogenous glutamate receptor antagonists is kynurenic acid (KYNA), which is produced by the kynurenine pathway.

II. The kynurenine system

KYNA is believed to be a neuroprotective metabolite of tryptophan (Vecsei et al., 2013) that interacts with glutamate receptors, aryl hydrocarbon receptor, G protein-coupled receptor 35 and elicits anti-glutamatergic actions. KYNA was discovered by Justus von Liebig in 1853 in the urine, and half a century later, the substance was identified as a byoproduct of tryptophan metabolism. Several lines of data confirm that KYNA and its analogues have anti-nociceptive effects in different migraine-models (Csati et al., 2015; Fejes-Szabo et al., 2014a; Lukacs et al., 2016), probably by attenuating the trigeminal activation and sensitization. The first step in the kynurenine pathway is the conversion of tryptophan to N-formyl-L-kynurenine by

tryptophan 2,3‑dioxygenase (TDO2) and indoleamine 2,3‑dioxygenase (IDO1,2): the rate-limiting enzymes of tryptophan metabolism. IDO was discovered in 1957 as heme protein (Hayaishi et al., 1957), which can be activated through IFN-γ (Heyes et al., 1997) and which is present in the central nervous system (Watanabe et al., 1981), while TDO occurs primarily in the peripheral tissues, especially in the liver (Knox and Mehler, 1950). IDO and TDO convert tryptophan to N-formyl-L-kynurenine by opening tryptophan ring in a reaction, which produces peroxides and highly reactive oxygen and hydroxyl radicals (Reyes Ocampo et al., 2014). N‑formyl-L-kynurenine is further degraded by formamidase to L-kynurenine (L-KYN), which is converted to KYNA by kynurenine aminotransferases (KAT)s, to 3-hydroxykynurenine by L-kynurenine 3-monooxygenase (KMO) and to anthranilic acid by L-kynurenine hydrolase (KYNU). The other metabolite of the kynurenine pathway is quinolinic acid (QUIN). In contrast to KYNA, QUIN is an agonist of the NMDA receptors and can provoke neuronal death and also causes lipid peroxidation and generates reactive oxygen species (Behan et al., 1999; Guidetti and Schwarcz, 1999). QUIN is then converted into NAD^+ in the final step of this branch of the kynurenine pathway. Figure 1.

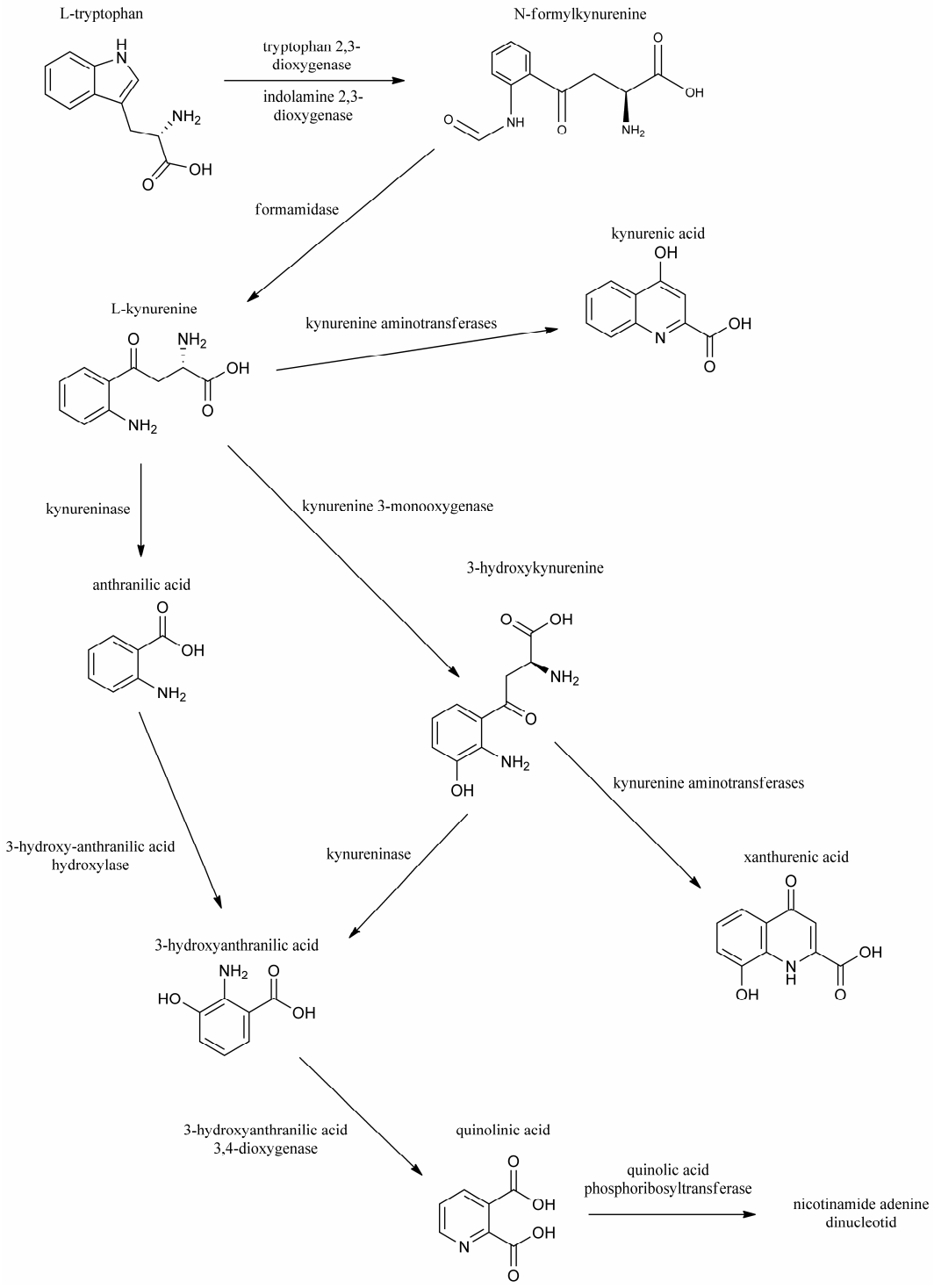

Figure 1. The kynurenine pathway (Nagy-Grocz et al., 2017)

III. The cannabinoid system and endocannabinoids

Cannabis has been used for a long time to reduce nausea and vomiting in chemotherapy and to treat pain, migraine and muscle spasticity (Borgelt et al., 2013). Cannabinoid receptor1 **(**CB1) is present in the central nervous system, liver, lung and cannabinoid receptor2 (CB2) is expressed primarily in the immune system (Pettit et al., 1996), but also can be found in the central nervous system specially in microglia (Van Sickle et al., 2005). Robbins and co-workers have demonstrated, that recreational cannabis and dronabinol (a synthetic tetrahydrocannabinoid) aborted cluster attacks in human patients (Robbins et al., 2009). Combinations of a small-dose cannabinoid with non-steroidal anti-inflammatory drugs (NSAIDs) have a more pronounced antinociceptive effect in mice than large-dose cannabinoid or NSAIDs alone, thus combinations of cannabinoids and NSAIDs may give a new perspective in the pharmacotherapy of pain (Ulugol et al., 2006).

Animal models of pain have shown that fluctuations in the endocannabinoid levels in the nervous system are related to pain processing and antinociception (Guindon and Hohmann, 2009). CB1 is present in the trigeminal ganglion (TG) and on the axon terminals of primary sensory neurons in the nociceptive areas in the spinal cord in rats (Pertwee, 2001). CB1 is able to inhibit the responses of trigeminal neurons with Aδ- and C-fiber inputs from the dura mater (Akerman et al., 2007), pointing to the importance of the endocannabinoid system in pain processing (Wilson and Nicoll, 2002).

Although the psychoactive properties of cannabinoids (Crawley et al., 1993), restrict their therapeutic application, the interactions between the endocannabinoid system and pain mediation is intensively studied in several laboratories.

N-Arachidonylethanolamide or anandamide (AEA) is the first discovered endocannabinoid, an agonist of CBs and transient receptor potential vanilloid type 1 (TRPV1), which is a nonselective cation channel activated by numerous stimuli, such as heat and vanilloids (Caterina et al., 1999; Caterina et al., 1997). AEA has vasodilatatory actions (Pertwee, 1997), which are not mediated by CBs (White and Hiley, 1998). It is well known that AEA is able to reduce nitroglycerin (NTG)-induced hyperalgesia and c-Fos expression in the caudal trigeminal nucleus (TNC) in rats (Greco et al., 2010), which means that AEA is capable of modulating the activation of the trigeminal system. Another nitric oxide (NO) donor, sodium nitroprusside is able to stimulate AEA transport (Maccarrone et al., 2000), suggesting a strong

interaction between NO and AEA. In the rat mesencephalon, NTG increased the activity of fatty acid amide hydrolase, degrading enzyme of AEA (Greco et al., 2010).

IV. Animal models of migraine: the nitroglycerin model

NTG or glyceryl trinitrate is a highly lipophilic organic nitrate, which has been used, for more than 150 years, for the treatment of angina pectoris and myocardial infarction (Chiariello et al., 1976). It readily crosses biological membranes, including the blood-brain barrier (Torfgard et al., 1989) and is converted to NO in the body mainly enzymatically probably by mitochondrial aldehyde dehydrogenase (Chen et al., 2005). The importance of NO is shown by the fact, that it was proclaimed Molecule of the Year in 1992.

One of the side-effects of NTG is headache (Iversen and Olesen, 1994) due to vasodilatation induced by NO, which occurs immediately after its administration, but about four hours later it is followed by a typical migraine without aura in migraine patients (Sicuteri et al., 1987), which can not be attributed to NO's prompt vasodilator effect (Di Clemente et al., 2009; Sicuteri et al., 1987). This observation and the results of a pilot study showing that treatment with a nitric oxide synthase (NOS) inhibitor attenuates spontaneous migraine headaches in 67% of subjects (Lassen et al., 1997), contributed to the implication of NO in migraine pathogenesis. An increasing amount of evidence suggests that NTG is able to activate and sensitize the trigeminal system in humans. NTG was able to trigger reproducible migraine with aura (Afridi et al., 2005; Afridi et al., 2004), during which activation in the primary visual area of the occipital cortex was demonstrated using positron emission tomography (Afridi et al., 2005). In healthy volunteers, sublingual NTG caused changes in trigeminal nociceptive blink reflex, pain thresholds and evoked cortical response, which are specifically due to the action NO on central nervous system structures (Di Clemente et al., 2009). Furthermore, similar changes to the first phenomenon has been reported during migraine attacks and thought to reflect sensitisation of neurons in TNC (Kaube et al., 2002), thus NTG seems to be able to sensitize the trigeminal system. The NTG-induced changes in evoked cortical response are comparable to those found immediately before and during a migraine attack (Afra et al., 2000; Siniatchkin et al., 1999). Based on above mentioned data NTG administration can be used as an animal experimental model of migraine. This was justified by experiments, since NTG can induce changes in animals similar to phenomena happening in humans during the NTG-induced attack.

Administration of NTG was able to trigger scratching head reactions, climbing cage, red ear and photophobia in rats (Gao et al., 2014; Markovics et al., 2012; Zhu et al., 2011). NTG injection induces dural and pial artery dilation (Gozalov et al., 2007; Read et al., 1997) and leakage of plasma proteins from dural blood vessels in rats (Reuter et al., 2001).

NTG is able to increase c-Fos-immunoreactivity (a marker of the neuronal activation process) in the TNC in rats suggesting the activation of the second order trigeminal neurons there (Tassorelli and Joseph, 1995). A significant upregulation of c-fos mRNA followed by an upregulation of Fos protein was observed also in the TNC after NTG infusion in rats, which were attenuated by pre-treatment with sumatriptan, demonstrating the specificity of this model for migraine (Ramachandran et al., 2012).

NTG treatment can trigger a release of neurotransmitters present in the trigeminal system. It caused a decrease in medullary and pontine levels of serotonin, which may result from the interaction of NTG-released NO and serotonin in central areas devoted to the modulation of nociception (Tassorelli et al., 2002). Systemic administration of NTG in male rats produced a significant increase of neuronal NOS (nNOS) and calmodulin-dependent protein kinase II immunoreactive neurons and the area covered by serotonin-immunoreactive fibres and a significant decrease of the area innervated by calcitonin gene-related peptide (CGRP)-immunoreactive afferents in the cervical part of TNC but not in the thoracic dorsal horn (Pardutz et al., 2007; Pardutz et al., 2000; Pardutz et al., 2002). These observations obviously show that the effect of NTG is specific to the trigeminal system, and confirm that NTG is really suitable model for examination of the trigeminal system.

Pituitary adenylate cyclase-activating peptide (PACAP)-38 and -27 immunoreactivity was also increased in the TNC of rats after NTG injection (Tuka et al., 2012), and similar tendency was observed in the plasma of migraineurs during the attack compared to the interictal period (Tuka et al., 2013). PACAP is present in the trigeminal system (Tajti et al., 1999) and it has a vasodilatatory effect (Chan et al., 2011). In addition, similar to NTG, PACAP-38 can induce headache in volunteers and migraine without aura in migraineurs (Schytz et al., 2009).

NO is also involved in the central sensitization process in the trigeminal system (Pardutz et al., 2000), probably acting *via* the activation of trigeminal Aδ and C fibres, since the destruction of the latter with capsaicin abolishes the effect of NTG (Tassorelli et al., 1997).

A dose-dependent and delayed NTG-induced thermal and mechanical allodynia was identified in mice, which were reduced by sumatriptan (Bates et al., 2010). NTG caused a hyperalgesic response to the tail-flick test (Greco et al., 2014; Greco et al., 2008), which was inhibited by treatment with non-peptide CGRP receptor antagonist MK-8825 (Greco et al., 2014). Chronic intermittent administration of NTG to mice caused acute and chronic hyperalgesia, where the acute but not the chronic one was significantly reduced by sumatriptan, whereas both the acute and chronic hyperalgesia was significantly attenuated by topiramate which is effective in migraine prophylaxis (Pradhan et al., 2014).

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

V. Markers of the sensitization process

TRPV1 is present in the spinal cord and is considered as a molecular integrator of chemical and physical stimuli that elicit pain (Tominaga et al., 1998). In addition, NO donors can activate TRPV1 resulting in an increase of intracellular calcium concentration in different cell types (Miyamoto et al., 2009; Pan et al., 2013), which suggests that TRPV1 may be modulated by NO.

NO is synthesized from arginine by nitric oxide synthase, neuronal isoform of which (nNOS) has an outmost importance in nociception and sensitization and it is present in the trigeminal system as described earlier (Dohrn et al., 1994). NO donors may trigger a self-amplifying process at the level of central projection site of the trigeminal system by increasing endogenous NO synthesis, which might be relevant in the central sensitization phenomenon (Burstein et al., 2000).

Nuclear factor kappa B (NF-κB) has a crucial role in inflammation process by controlling many genes including cytokynes. Several studies have shown that proinflammatory cytokynes contribute to the development of pain and hyperalgesia (Kress and Reeh, 1996). Cyclooxygenase-2 (COX-2) is present in the dorsal horn of spinal cord too and it has a substantial role in the processing of pain (Mazario et al., 2001). COX formally known as prostaglandin-endoperoxide synthase, is an enzyme that is responsible for formation of

prostanoids, including thromboxane and prostaglandins such as prostacyclin. Gao and Duan have found that COX-2 expression increased in the TNC after orofacial nociception (Gao and Duan, 2010).

Aims

1. The purpose of our studies was to investigate the modulatory effects of a CB agonist AEA on the NTG-induced expression of the markers of the sensitization process, namely TRPV1, nNOS**,** NF-κB and COX-2.

2. Furthermore, our aim was to examine the effect of NTG on the expression of the enzymes of the kynurenine pathway, as TDO2, IDO1, KAT-II, KYNU and KMO.

Materials and methods

I. Animals

The procedures utilized in this study followed the guidelines for the Use of Animals in Research of the International Association for the Study of Pain and the directive of the European Economic Community (86/609/ECC). They were permitted 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). 54 adult male Sprague-Dawley rats weighing 200-250 g were used. The animals were raised and maintained under standard laboratory conditions with tap water and regular rat chow available *ad libitium* on a 12 hour dark-12 hour light cycle.

II. Drug administration

Examination of the sensitization markers:

The animals were divided into four groups (n=6 per group for immunohistochemistry, n=5 per group for Western blot analysis).

The animals in the first group, called placebo group, received only the vehicle solution (physiological saline) as treatment. In the second group, the rats were treated with an intraperitoneal (i.p.) injection of NTG (10 mg/kg bodyweight, Pohl Boskamp). In the third and fourth group, animals received i.p. AEA (2x5 mg/kg bodyweight, Sigma Aldrich) half hour before and one hour after the placebo or NTG treatment. Greco and colleagues have demonstrated that single 20 mg/kg doses of AEA before the NTG administration are able to reduce NTG-induced c-Fos expression in the caudal part of spinal trigeminal nucleus (Greco et al., 2010). AEA was dissolved in physiological saline. In the case of the first and second groups, animals were treated with physiological saline instead of AEA.

Kynurenine pathway experiment:

The animals were separated into two groups (n = 5). The animals in the first group, called placebo group, received only the vehicle solution (physiological saline) as treatment. In the second group, the rats were treated with an i.p. injection of NTG (10 mg/kg bodyweight, Pohl Boskamp).

III. Immunohistochemistry

Four hours after the placebo/NTG injection, the rats were perfused transcardially. The upper cervical spinal cord (C1-C2) segments of the cervical spinal cord between (-5) and (-11) mm from the obex were removed and postfixed overnight for immunohistochemistry in the same fixative. After cryoprotection, 30 μm cryostat sections were cut and serially collected in wells containing cold phosphate-buffered saline (PBS). The free-floating sections were rinsed in PBS and immersed in 0.3% H_2O_2 in methanol or PBS for 30 minutes. After several rinses in PBS containing 1% Triton X-100, sections of C1-C2 were kept overnight at room temperature in anti-TRPV1 antibody (Santa Cruz, s.c.28759) at a dilution of 1:500, or for two nights at 4 °C in anti-nNOS antibody (EuroProxima, 2263B220-1) at a dilution of 1:5000, or for two nights at 4 °C in anti- NF-κB antibody (Abcam, ab97726) at a dilution of 1:100. The immunohistochemical reaction was visualized by the Vectastain avidin-biotin kit of Vector Laboratories (PK6101), and nickel ammonium sulphate-intensified 3,3'-diaminobenzidine. The specificity of the immune reaction was controlled by omitting the primary antisera.

IV. Western blot analysis

Four hours after the placebo/NTG injection, the animals were perfused transcardially with 100 mL PBS and the dorsal horns of C1-C2 and TNC between 1 and (-5) mm from the obex segments were extracted. Until the measurements, they were stored at −80 °C. The samples were sonicated in ice cold lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% igepal, 0.1% cholic acid, 2 μg/mL leupeptin, 2 mM phenylmethylsulphonyl fluoride, 1 μg/mL pepstatin, 2 mM EDTA and 0.1% sodium dodecyl sulphate. The lysates were centrifuged at 12 000 RPM for 10 minutes at 4 °C and supernatants were aliquoted and stored at -20 °C until use. Protein concentration was determined with BCA Protein Assay Kit using bovine serum albumin as a standard. Previous to loading, each sample was mixed with sample buffer, and denaturated by boiling for 3 minutes. Equal amounts of protein samples (20 μg/lane) were separated by standard SDS polyacrylamide gel electrophoresis on 10% Tris-Glycine gel and electrotransferred onto Amersham Hybond-ECL nitrocellulose membrane (0.45 μm pore size). We used the Page Ruler Prestained Protein Ladder (10-170 kDa) to define approximate molecular weights. Following the transfer, membranes were blocked for one hour at room temperature in Tris-buffered saline containing Tween 20 (TBST) and 5% non fat dry milk powder. Then, they were incubated in TBST containing 1% non fat dry milk and nNOS

antibody (BD Biosciences, 610308, dilution: 1:2000, incubation: overnight at room temperature), or COX-2 antibody (Proteintech, 12375-1-AP, dilution: 1:1000, incubation: overnight at room temperature), or β-actin antibody (Calbiochem, CP01, dilution: 1:100 000, incubation: overnight at room temperature) or TDO2 antibody (LifeSpan BioSciences, LS-C111058, dilution: 1:500, incubation: overnight at room temperature), or IDO1 antibody (Abcam, ab106134, dilution: 1:500, incubation: overnight at room temperature), or KAT-II antibody (Santa Cruz, sc-67376, dilution: 1:10 000, incubation: overnight at room temperature) or KYNU antibody (Abcam, ab96365, dilution: 1:500, incubation: overnight at room temperature) or KMO antibody (Abcam, ab83929, dilution: 1:4000, incubation: overnight at room temperature) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Cell Signaling Technologies, 8884, dilution: 1:1000, incubation: overnight at room temperature). Next day, membranes were incubated in TBST containing 1% non fat dry milk and horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology, sc-2030, sc-2031) for two hours at room temperature. Protein bands were visualized after incubation of membranes with the SuperSignal West Pico Chemiluminescent Substrate using Carestream Kodak BioMax Light film.

V. Data evaluation

All evaluations were implemented by an observer blind to the experimental groups. The detailed methods were described previously (Gundersen et al., 1988; Vamos et al., 2010; Vamos et al., 2009).

a. Immunohistochemistry (TRPV1, nNOS, NF-κB)

The photomicrographs of the stained sections of C1-C2 were taken using Zeiss AxioImager microscope supplied with an AxioCam MRc Rev.3 camera (Carl Zeiss Microscopy, Jena, Germany).

The area covered by TRPV1-immunoreactive fibres and nNOS-immunoreactive cells was determined by Image Pro Plus 6.2® image analysis software (Media Cybernetics). After image acquisition, the laminae I-II in dorsal horn were defined manually as area of interest and a threshold grey level was validated with the image analysis software, as described in an earlier study (Fejes-Szabo et al., 2014b; Vamos et al., 2010). The program calculated the area innervated by the immunoreactive fibres and cells as the number of pixels with densities

above the threshold, the data were expressed as area fractions (%) of the corresponding immunolabelled structures.

We used the unbiased optical dissector method to calculate the volume densities of the NF-κB-immunoreactive cells (Gundersen et al., 1988).

b. Western blot analysis (nNOS, COX-2, TDO2, IDO1, KAT-II, KYNU, KMO)

For densitometric analyses, films were scanned and quantified using Java ImageJ 1.47v analysis software (National Institutes of Health). The results were normalized to β-actin or GAPDH. In the case of nNOS, COX-2 and KAT-II, we used the C1-C2 segments of the animals, but in TDO, IDO, KYNU and KMO Western blots, we used TNC segments for the examinations.

VI. Statistical analysis

Statistical analysis of measurements were performed in SPSS Statistics software (Version 20.0 for Windows, SPSS Inc.) using one-way analysis of variance (ANOVA) followed by the Tukey or Tamhane or Fishers Least Significant Difference post hoc test depending on variances of data, with $P<0.05$ taken as statistically significant. Group values are reported as means ± SEM.

Results

I. NTG induced an increase in TRPV1 expression in the C1-C2 - AEA inhibited this phenomenon

On transverse sections of the C1-C2 segments, there were abundant TRPV1-positive fibres in the superficial layers of the dorsal horn. The TRPV1-immunoreactive area in the NTG-treated group was significantly higher compared to the placebo-treated group ($p<0.05$). The NTG-induced increase was attenuated by treatment with AEA ($p<0.05$). (Figure 2.)

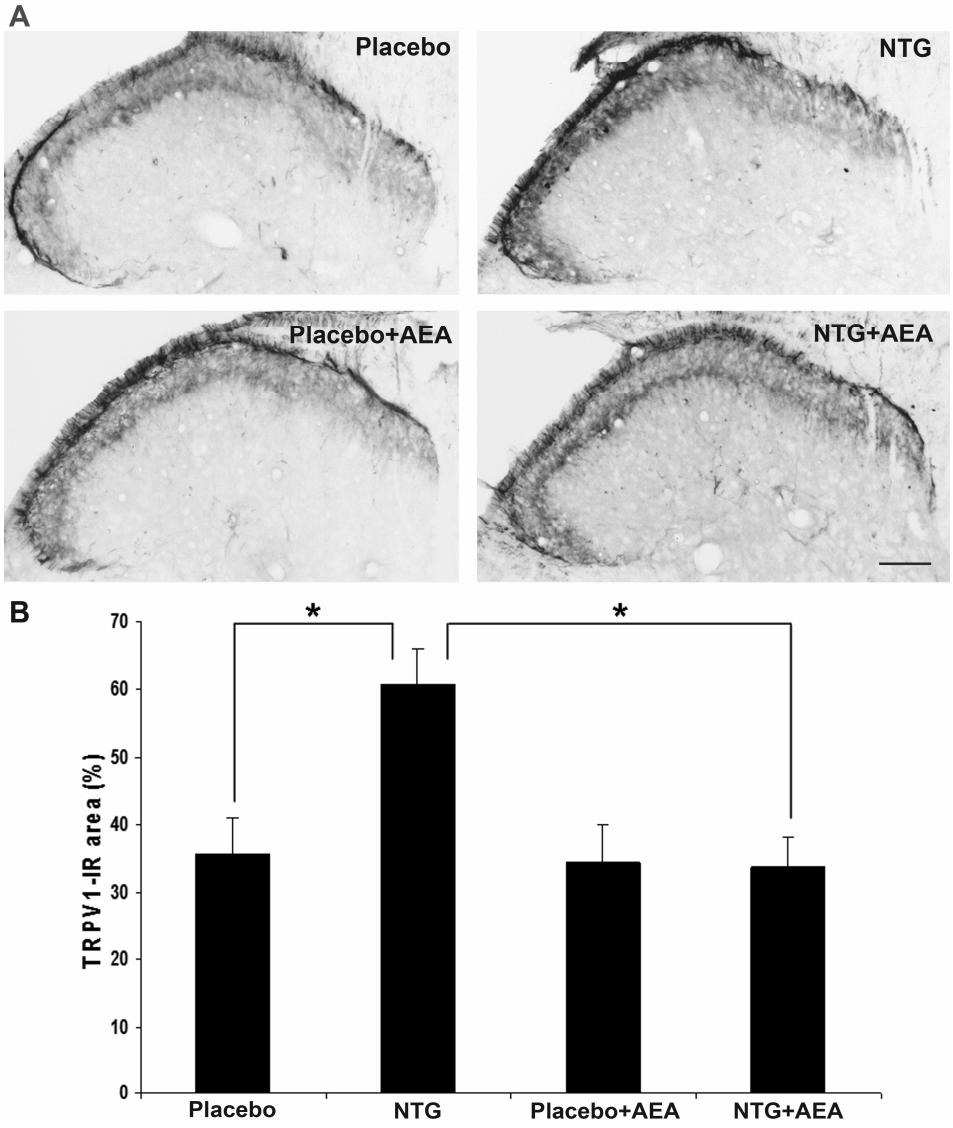

Figure 2. A. Representative photomicrographs of the TRPV1 expression in the C1-C2 segments. B. Changes in area fractions of TRPV1-immunoreactive fibers in superficial laminae I and II of the C1-C2 segments. In the NTG group, the area covered by TRPV1 was significantly higher than in the placebo group. AEA seemed to block this effect. Scale bar: 100 µm, *$p<0.05$; AEA: anandamide, C1-C2: upper cervical spinal cord, NTG: nitroglycerin, TRPV1-IR: transient receptor potential vanilloid 1 immunoreactive

II. NTG increased nNOS expression in the C1-C2 and AEA attenuated this effect

On transverse sections of the C1-C2 segments, nNOS-immunoreactive neurons and processes with cytoplasmic staining can be observed in the superficial layers of the dorsal horn. In the NTG group, the area fraction of nNOS-immunoreactive structures was significantly higher than in the placebo-treated group ($p<0.01$). AEA treatment resulted in a decrease of nNOS-immunopositive structures ($p<0.001$). (Figure 3.)

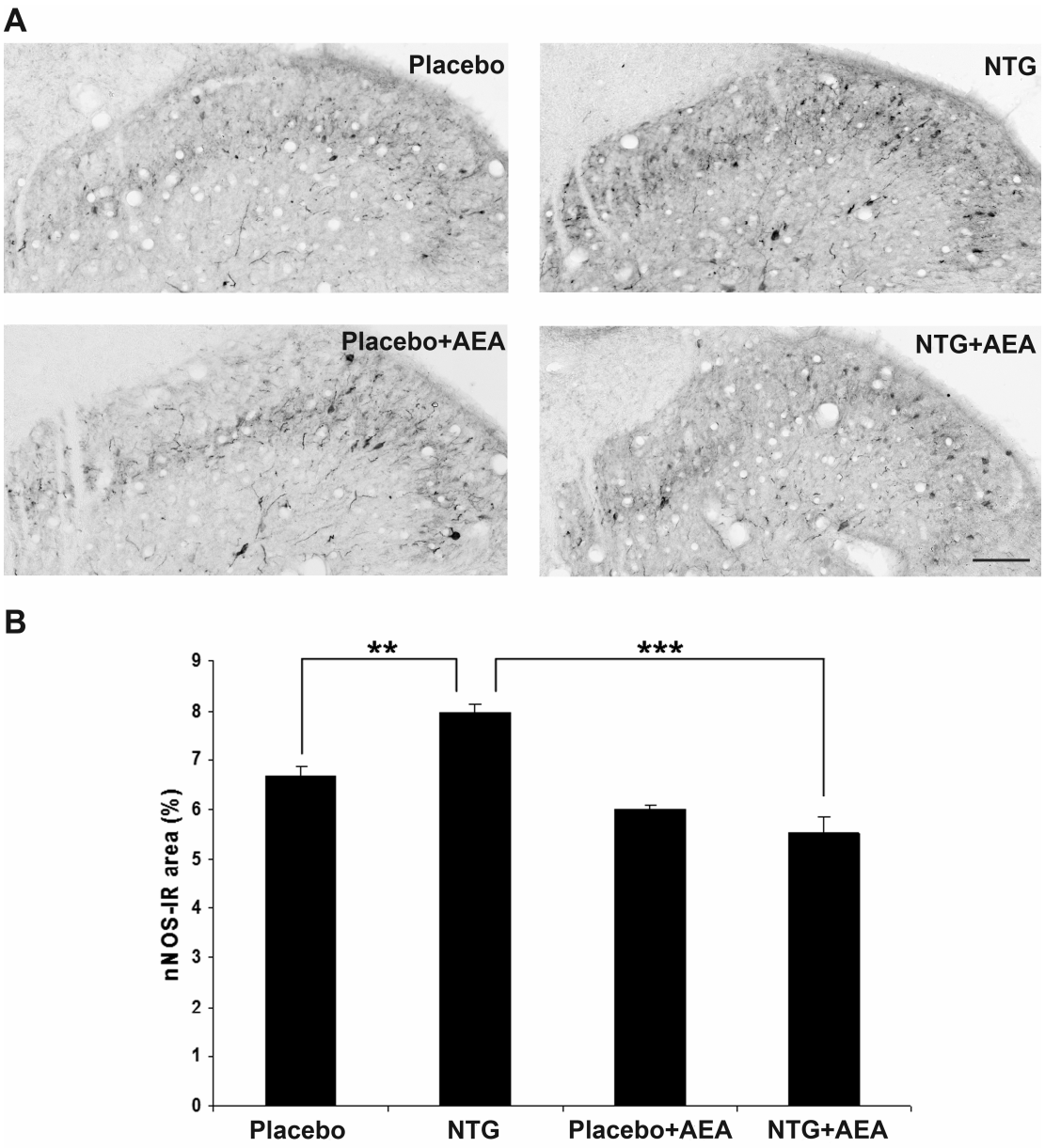

Figure 3. A. Representative photomicrographs of nNOS expression in the C1-C2 segments. B. Changes in the nNOS-immunoreactive area fractions. In the NTG group, the area fraction of nNOS-IR structures was increased compared to the placebo-treated group. AEA was able to attenuate this effect. Scale bar: 100 µm, **p<0.01; ***p<0.001; AEA: anandamide, C1-C2: upper cervical spinal cord, nNOS-IR: neuronal nitric oxide synthase immunoreactive, NTG: nitroglycerin

Western blot analysis of the C1-C2 region confirmed the results obtained by nNOS immunohistochemistry. A band characteristic of the nNOS protein was identified at 155 kDa. Densitometric analyses confirmed that the nNOS bands were significantly enhanced ($p<0.01$) in C1-C2 after NTG administration as compared with the placebo-treated animals. This effect was blocked by the treatment with AEA ($p<0.05$). (Figure 4.)

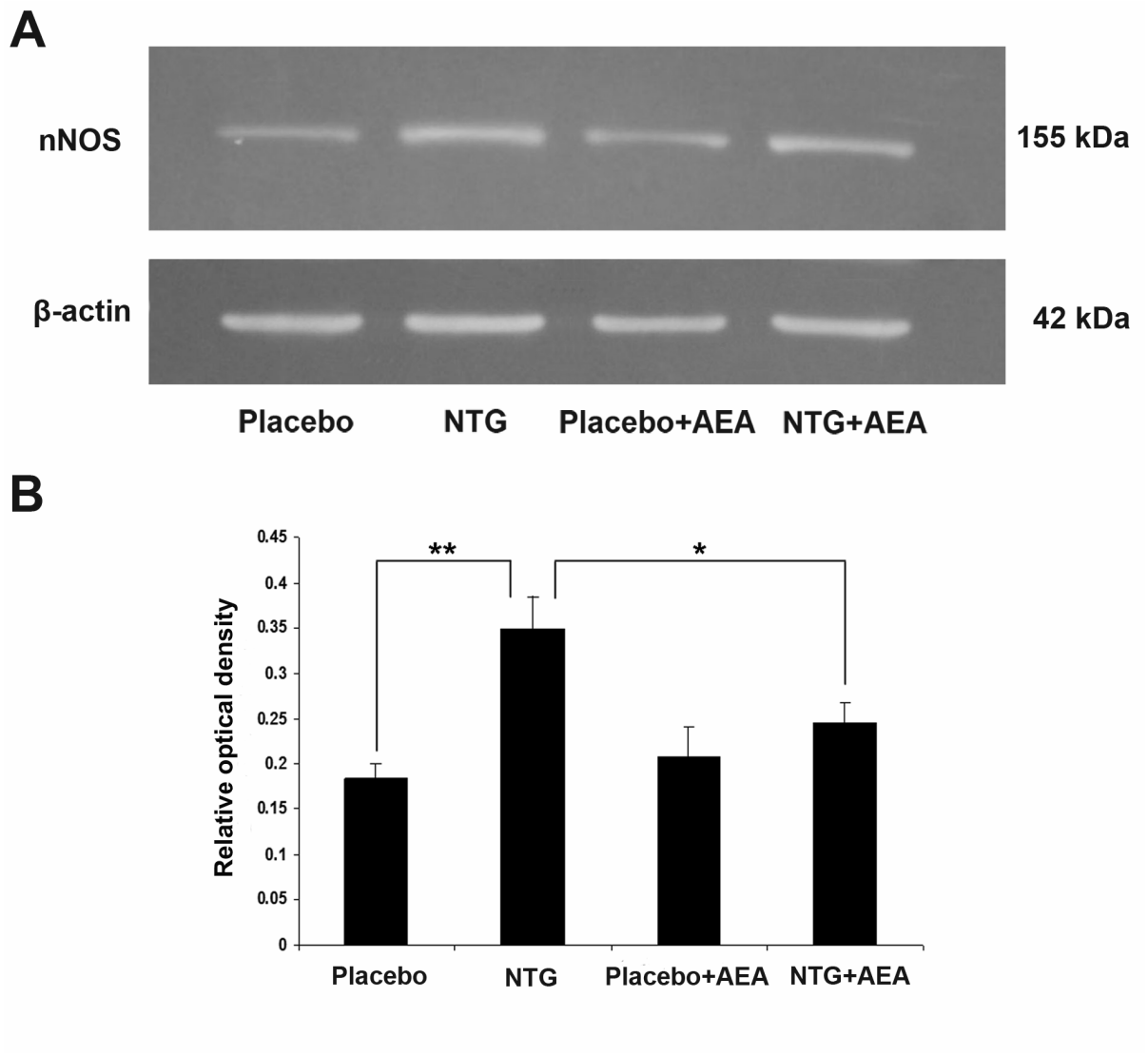

Figure 4. A. Western blot of nNOS and β-actin expression in the C1-C2. B. The quantitative analysis shows that in the NTG group, the relative optical density of nNOS specific band was significantly higher than in the placebo group. AEA treatment weakened this effect. *$p<0.05$; **$p<0.001$ AEA: anandamide, C1-C2: upper cervical spinal cord, NTG: nitroglycerin

III. NTG treatment enhanced NF-κB expression and AEA mitigated this phenomenon

In transverse sections of the C1-C2 region, high number of NF-κB-positive cells can be seen in the superficial layers of the dorsal horn. In the NTG treated animals the volume density of NF-κB-positive cells was significantly higher than in the placebo group ($p<0.05$). This value decreased in the AEA injected group ($p<0.05$). (Figure 5.)

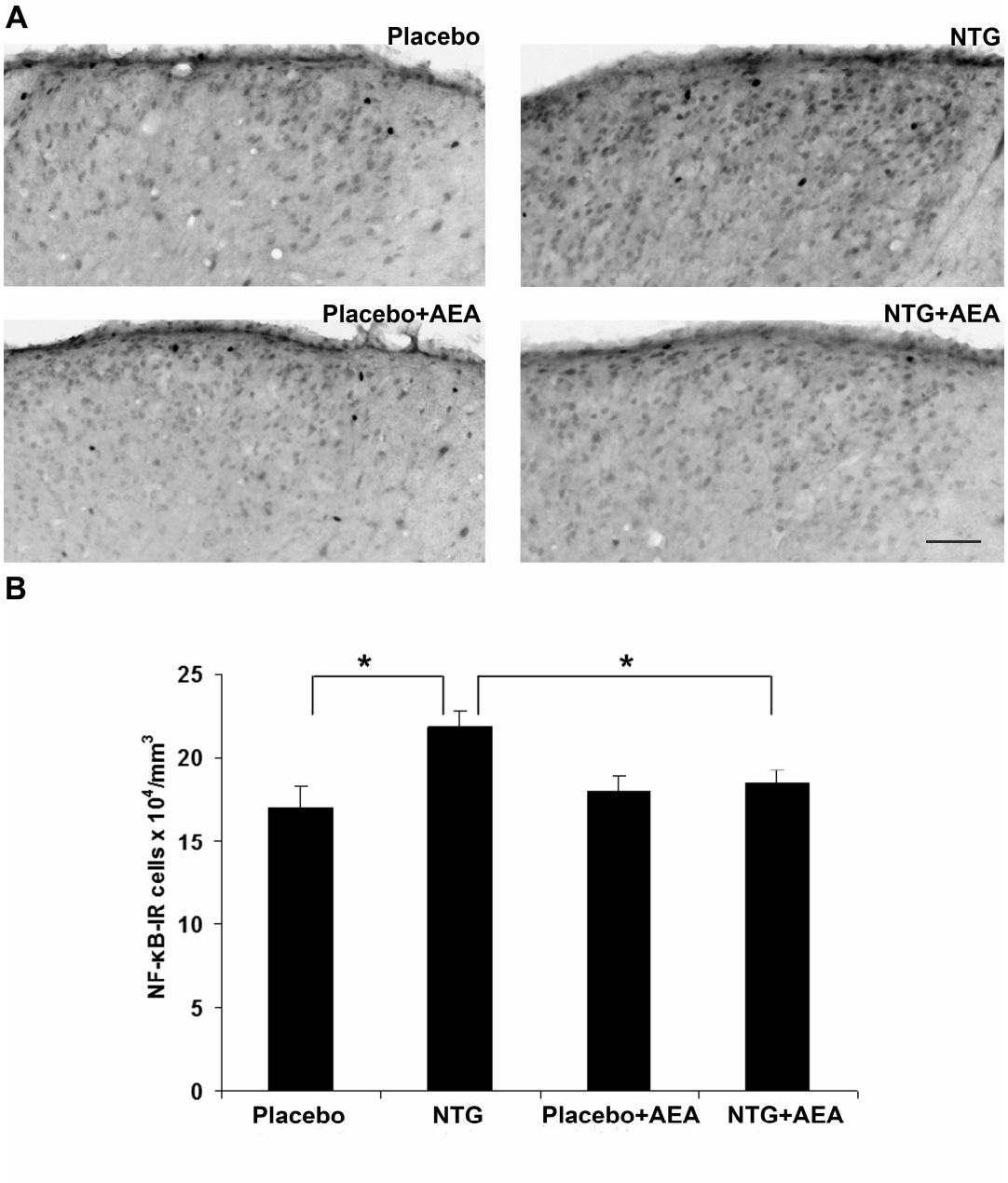

Figure 5. A. Photomicrographs of the NF-κB expression in the C1-C2 segment. B. Diagram shows that NTG treatment resulted in a significant increase in the volume density of NF-κB-immunoreactive cells. This effect was not observed in AEA-injected animals. Scale bar: 50 μm, *$p<0.05$, AEA: anandamide, C1-C2: upper cervical spinal cord, NF-κB-IR: nuclear factor kappa B-immunoreactive, NTG: nitroglycerin

IV. NTG enhanced expression of COX-2 enzyme and AEA inhibited this action

A band characteristic of the COX-2 protein was identified at 68 kDa in Western blot assay. Densitometric analyses showed that the COX-2 bands were significantly enhanced ($P<0.01$) in segments C1-C2 after NTG administration as compared with the placebo-treated animals. The effect of NTG was decreased by the AEA treatment ($p<0.01$). (Figure 6.)

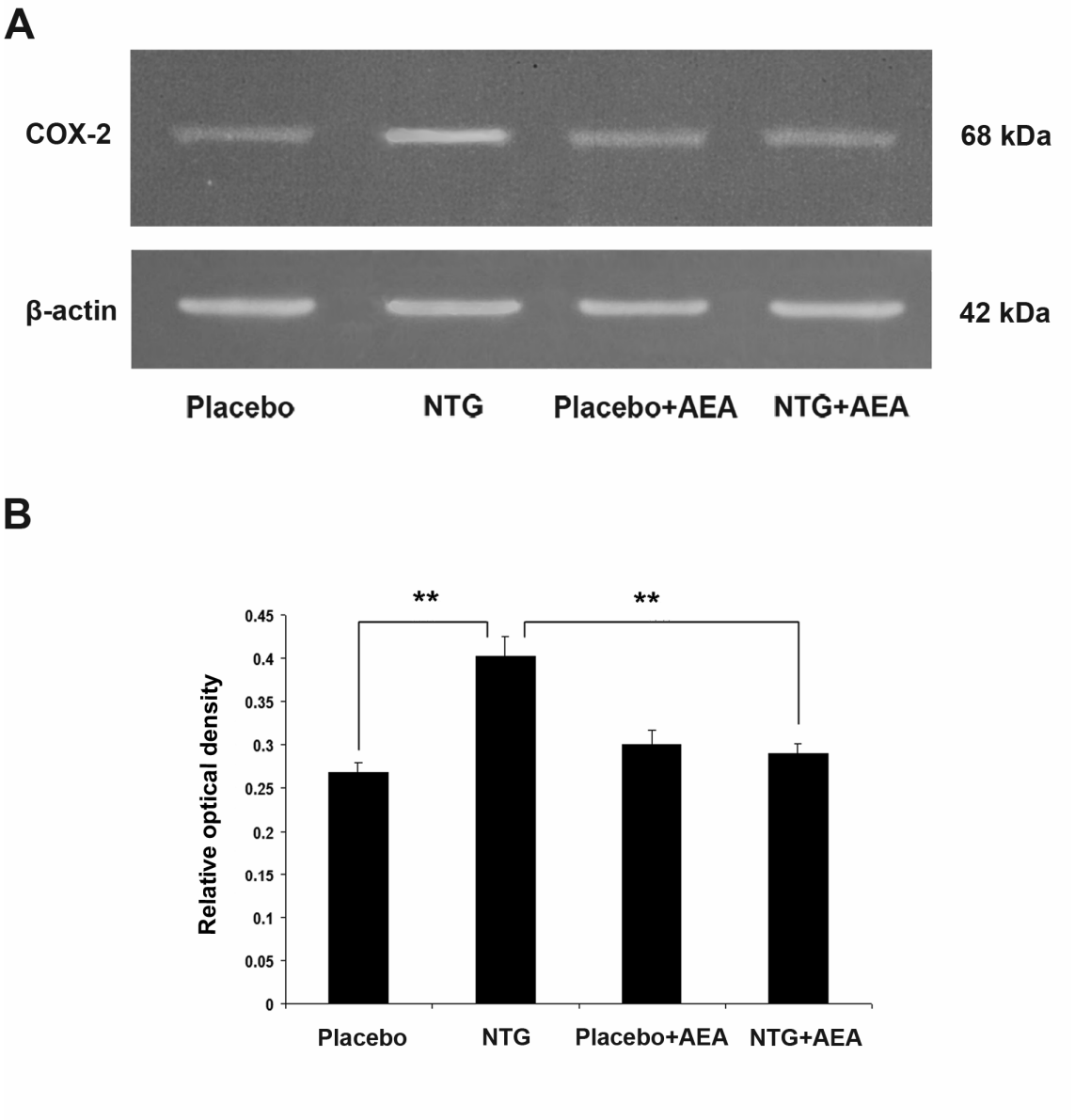

Figure 6. A. Western blot of COX-2 and β-actin expression in the C1-C2. B. Densitometry of the individual bands indicated that in the NTG-treated animals the expression of COX-2 was significantly higher than in the placebo group. No such effect was seen in the AEA-injected group. **p<0.01; AEA: anandamide, C1-C2: upper cervical spinal cord, COX-2: cyclooxygenase-2, NTG: nitroglycerin

V. NTG induced a decrease in TDO2 expression

A band characteristic of the TDO2 protein was identified at 50 kDa in Western blot assay. Densitometric analyses showed that the TDO2 bands were significantly decreased ($p < 0.05$) in the TNC after NTG administration as compared with the placebo-treated animals. (Figure 7.)

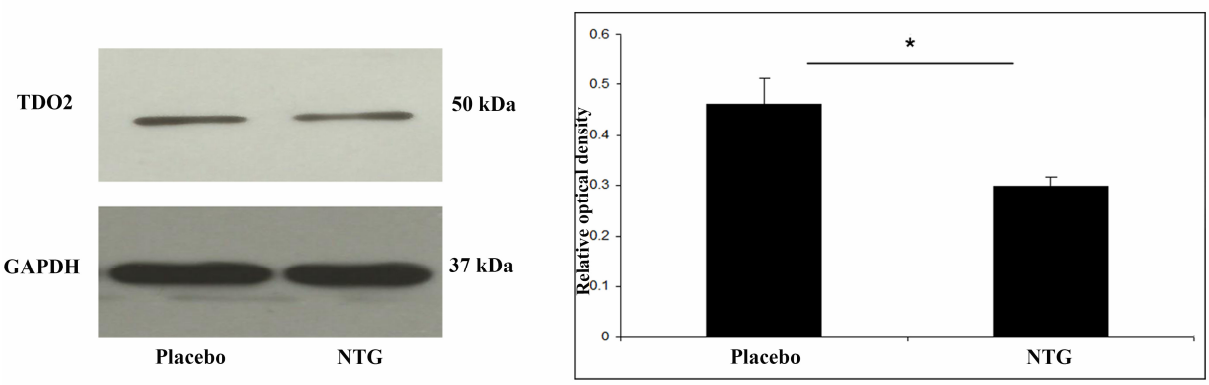

Figure 7. Western blot analysis of TDO2 and GAPDH protein from the TNC. The quantitative analysis shows that in the NTG group the relative optical density of TDO2 specific bands were significantly less pronounced compared with the placebo group. *$p < 0.05$; GAPDH: glyceraldehyde 3-phosphate dehydrogenase, NTG: nitroglycerin, TDO2: tryptophan 2,3-dioxygenase 2, TNC: caudal trigeminal nucleus

VI. NTG treatment resulted in a diminished IDO1 expression

A band characteristic of the IDO1 protein was referred at 45 kDa in Western blot assay. Densitometric analyses confirmed that the IDO1 bands were significantly weaker ($p < 0.05$) in the TNC after NTG administration as compared with the placebo-treated animals. (Figure 8.)

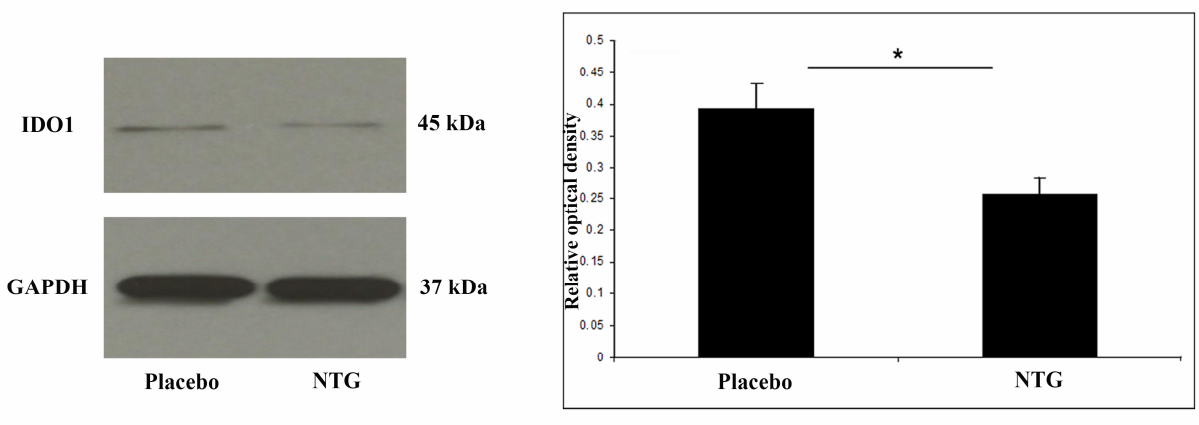

Figure 8. Western blot of IDO1 and GAPDH expression in the TNC. The quantitative analysis shows that in the NTG group, the relative optical density of IDO1 specific bands was significantly decreased compared with the placebo group. *$p < 0.05$; GAPDH: glyceraldehyde 3-phosphate dehydrogenase, IDO1: indoleamine 2,3-dioxygenase, NTG: nitroglycerin, TNC: caudal trigeminal nucleus

VII. NTG decreased KAT-II expression

A band characteristic of the KAT-II protein was referred at 60 kDa in Western blot assay. Densitometric analyses confirmed that the KAT-II bands were significantly weaker ($p<0.05$) in segments C1-C2 after NTG administration as compared with the placebo-treated animals. (Figure 9.)

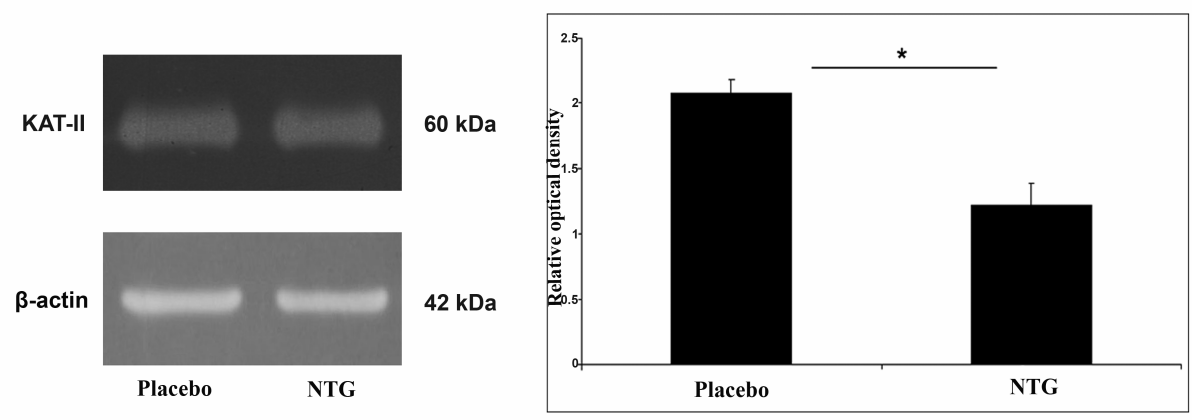

Figure 9. Western blot of KAT-II and β-actin expression in the C1-C2. Quantitative data demonstrate that in the NTG group, the relative optical density of KAT-II is significantly lower than in the placebo group. *$p<0.05$; C1-C2: upper cervical spinal cord, KAT-II: kynurenine-aminotransferase-II, NTG: nitroglycerin

VIII. NTG was able to reduce the expression of KYNU

We could identify a band at 35 kDa characteristic for the KYNU protein. In animals, which had received NTG, the density of KYNU protein bands was weaker in TNC segments ($p < 0.05$) as compared with the placebo-treated group. (Figure 10.)

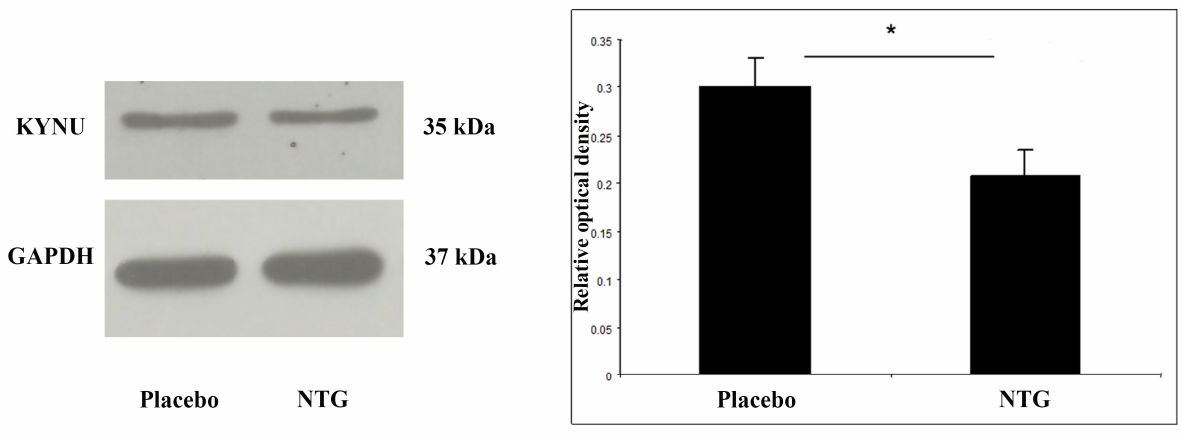

Figure 10. Representative Western blot bands and diagram of KYNU and GAPDH in the TNC. The quantitative analysis shows that in the NTG group, the relative optical density of KYNU specific bands were significantly smaller compared with the placebo group. *$p < 0.05$; GAPDH: glyceraldehyde 3-phosphate dehydrogenase, KYNU: kynurenine hydrolase, NTG: nitroglycerin, TNC: caudal trigeminal nucleus

IX. KMO expression was lower after NTG administration

A band characteristic of the KMO protein was identified at 56 kDa in Western blot assay. Densitometric analyses showed that the KMO bands were significantly decreased ($p < 0.05$) in segments TNC after NTG administration as compared with the placebo-treated animals. (Figure 11.)

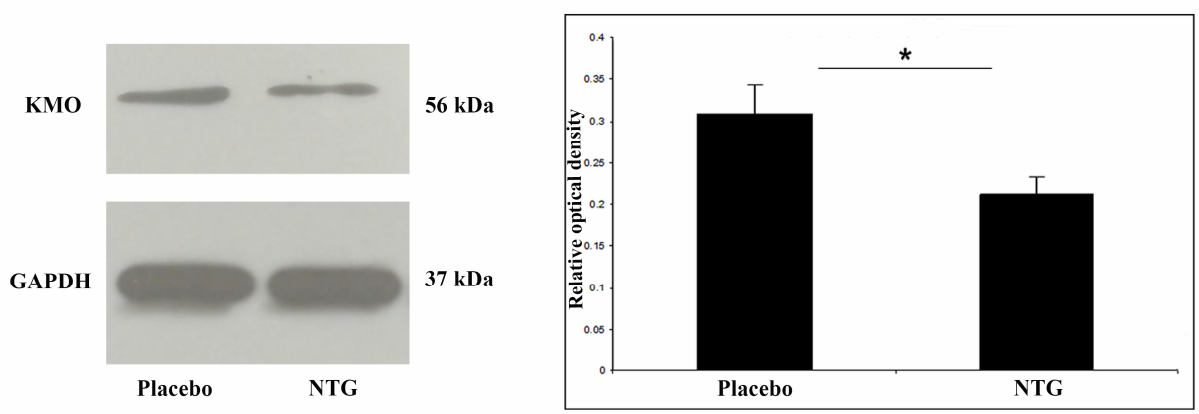

Figure 11. Illustrative Western blot bands and diagram of KMO and GAPDH in the TNC. The quantitative analysis shows that in the NTG group, the relative optical density of KMO specific bands was significantly weaker compared with the placebo group. *p < 0.05> GAPDH: glyceraldehyde 3-phosphate dehydrogenase, KMO: L-kynurenine 3-monooxygenase, NTG: nitroglycerin, TNC: caudal trigeminal nucleus

Discussion

I. Experiments with the sensitization markers

TRPV1 is present in the terminals of primary sensory neurons in the dorsal part of spinal cord (Valtschanoff et al., 2001) and co-expressed with CB1 (Morisset et al., 2001). Experimental data indicate that TRPV1 contributes to peripheral sensitization, allodynia and hyperalgesia (Saloman et al., 2013), central blockade of this receptor is able to attenuate central terminal sensitization (Kim et al., 2014). Our results show that NTG significantly increases TRPV1 expression in the C1-C2 segments of the rat, thus it may be the indicator of sensitization phenomena in the trigeminal system in our experimental setting.

It is well established that NO donors can activate TRPV1 in several cell types (Leonelli et al., 2013; Miyamoto et al., 2009) and in inflammatory pain models increased receptor expression was also reported (Ji et al., 2002; Kao et al., 2012). This indicates that the NTG effect on TRPV1 expression is indirect - it may cause neurogenic inflammation (Reuter et al., 2001), and the inflammatory mediators - like serotonin, bradykinin, etc. - can activate TRPV1 by stimulating trigeminal nociceptive neurons (Strassman et al., 1996). Pro-inflammatory mediators, such as tumor necrosis factor α, interleukin 1 (IL-1), interleukin 6 (IL-6) and bradykinin enhance TRPV1 (Malek et al., 2015). In the animal model of inflammation, Complete Freud's adjuvant increases TRPV1 mRNA expression in the dorsal root ganglion (Amaya et al., 2003), this also supports the idea that inflammation upregulates TRPV1 expression. To summarize, we may assume that NTG is able to activate TRPV1 mainly *via* inflammatory mediators. On the other hand, there are research data indicating that systemic administration of CGRP increases the expression of TRPV1 in the TG of rats (Chatchaisak et al., 2013), which might also play a role in this process. NTG can increase the release of CGRP by NO-mediated stimulation of Aδ and C fibers (Pardutz et al., 2002).

Our recent data also show that AEA, a CB receptor and TRPV1 agonist attenuates the effect of NTG on TRPV1 changes. Activation of ionotropic CBs can result in inhibition of nociceptors and antihyperalgesia and antinociception in certain pain models (Akopian et al., 2009). Intrathecal administration of AEA decreases thermal pain sensitivity and its effect can be altered with the TRPV1 antagonist capsazepine (Horvath et al., 2008). AEA may also

cause a desensitization of TRPV1 in skeletal muscle arterioles (Lizanecz et al., 2006), suggesting that AEA is able to mitigate TRPV1 activity. On the other hand, AEA can inhibit neurogenic, CGRP- and NO-induced dural vasodilatation, and this involves pre- and postsynaptic mechanisms (Akerman et al., 2004b). Recent report shows that the AEA level changes are able to modulate CGRP mRNA-expression after NTG-treatment in human peripheral blood mononuclear cells (Peng et al., 2014). We do not know exactly the role of TRPV1 in the AEA modulated sensitization process, but based on the available literature data (Akerman et al., 2007; Akerman et al., 2004a), we hypothesize that the role of CB1 is more pronounced than TRPV1 in this context.

It is known that nNOS is a key player in nociception (Lin et al., 1999) and its role in the sensitization cascade is intensively studied. In our present experiment administration of NTG increased the expression of nNOS in the C1-C2, which is in line with earlier results (Pardutz et al., 2000). The most probable explanation for this phenomenon is, that NO activates small caliber fibres in the trigeminal system and the increase of nNOS expression in the second order trigeminal neurons induces a self-amplifying mechanism (Pardutz et al., 2000; Tassorelli et al., 1997). The present results indicate that AEA is able to inhibit this effect. Several studies have shown that there is an interaction between NO and cannabinoid system e.g. nNOS and CB1 are co-localized in neurons in lamina II of the spinal cord (Salio et al., 2002). NTG-induced hyperalgesia is associated with a fluctuation of the activity of endocannabinoid system in various brain areas of rats (Greco et al., 2010). Our data are in line with Hillard and co-workers findings, who reported that CB1 agonists inhibit KCl-induced activation of nNOS in cultured cerebellar granule cells (Hillard et al., 1999). In addition, Carney and co-workers have detected that cannabinoid agonist downregulated nNOS protein and mRNA in neuronal cells (Carney et al., 2009). These data suggest that NTG is able to generate sensitization process and AEA inhibits this effect by blocking nNOS.

In our experiment NTG increased NF-κB expression in the superficial layers of the dorsal horn in the C1-C2 segments. Similar effect was reported by Reuter et al., who demonstrated that NTG infusion is able to trigger the activation of NF-κB in dura mater (Reuter et al., 2002). It is not clear how NTG can activate NF-κB-pathway, it might be related to a direct neuronal effect of NTG, or indirect effect *via* dural inflammation (Greco et al., 2005). Concerning the cellular mechanisms, it is important to note that both TRPV1 and nNOS

might play a role in this effect. It was shown that Ca^{2+} influx through TRPV1 may modulate the nuclear translocation and increased the activity of NF-κB (Sappington and Calkins, 2008). On the other hand the increase in nNOS expression is accompanied by increased NF-κB expression and activation (Parahova, 2009). Furthermore, Sancho and colleagues have noticed that AEA inhibits tumor necrosis factor-α-induced activation, by inhibition of a cytokine-induced cascade (Sancho et al., 2003). In addition, Nakajima and co-workers have found that AEA also blocked lipopolysaccharide-induced activation, suggesting that AEA inhibits proinflammatory mediators by blocking NF-κB activation (Nakajima et al., 2006). Tassorelli and her group reported that parthenolide (inhibitor of NF-κB) attenuated NTG-induced c-Fos activation in TNC (Tassorelli et al., 2005), which indicates that NF-κB may be important in the NTG-induced trigeminal activation and its inhibition is able to modulate the nociceptive process. Our data reconfirm, that NTG is able to activate NF-κB, thus can trigger neurogenic inflammation, which has a key role in sensitization phenomena. Furthermore, we detected that AEA is able to reduce this effect. Endocannabinoids might operate a negative feedback control over the proinflammatory process by suppressing the activation of transcription factors involved in the inflammatory action (Berdyshev et al., 2001).

NO may also cause neurogenic inflammation by increasing NF-κB levels, which may lead to the upregulation of COX-2 in inflammatory pain (Lee et al., 2004). It is well-known, that NSAIDs, which exert their effects through the inhibition of COX-enzymes (Yaksh et al., 2001), are effective in the treatment of migraine and tension-type headache (Lange et al., 2000). In animal studies, it has been shown that COX-2 is involved in the NTG-induced activation and sensitization process of the trigeminal system. Pre-treatment with indomethacin (non-selective COX inhibitor) and NS398 (selective COX-2 inhibitor) reduced the NTG-induced c-Fos (Tassorelli et al., 1997), nNOS and calmodulin-dependent protein kinase II alpha expression-changes in the TNC (Varga et al., 2009; Varga et al., 2007). Tassorelli and colleagues have demonstrated that COX-2 expression is increased in the hypothalamus and caudal brain stem after NTG injection (Tassorelli et al., 2007), thus COX is one of the mediators of NTG-induced neuronal activation. Furthermore, NO is able to activate COX-enzymes in fibroblasts, probably by an interaction with the iron-hem center of the enzyme (Salvemini et al., 1993). It is important to note, that AEA is one of the substrates of COX-2 producing prostaglandin and ethanolamids (Yu et al., 1997). In our study, we have found that

AEA is able to inhibit the NTG-induced COX-2 increase. Our assumption is that it may be associated with a negative feedback mechanism, but it is possible that after the cleavage of AEA some metabolites may downregulate COX-2 expression. Since AEA is able to reduce cytokine-induced cascade and proinflammatory mediators (Nakajima et al., 2006), it is also possible, that the reduction of the inflammatory process is able to downregulate the COX-2 expression.

II. Studies of the Kynurenine pathway

In our experiments NTG decreased the expression of the kynurenine pathway in the TNC and C1-C2 segments, which may indicate that fluctuation of these enzymes is involved in the NTG-triggered trigeminal activation. However, the question arises how NTG /NO can influence the kynurenine pathway. It is well-known that the nitrergic-system is able to alter the kynurenine system, e.g. NO inhibits IDO expression by reversible binding to the active site in macrophages (Thomas et al., 2007) and IDO activity is down-regulated by NO production in bone marrow cells (Hara et al., 2008). The other hand, it is also known that the expression of KMO influences NO production in human HEK293 cells (Wilson et al., 2016). Moreover, Backhaus and her colleagues showed in a mass spectrometry and NMR study that there is a direct interaction between kynurenine metabolites, e.g., 3-hydroxykynurenine and 3-hydroxyanthranilic acid and NO (Backhaus et al., 2008).

It is also important to note that kynurenines have a crucial role in immune regulation (Mandi and Vecsei, 2012). The transcriptional expression of IDO, KAT-II, KMO, KYNU is also under the control of interferons (Mandi and Vecsei, 2012), thus pro-inflammatory cytokines influence kynurenine pathway (Hassanain et al., 1993). Lögters and co-workers have shown that the kynurenine-tryptophan ratio was increased in the blood of patients with posttraumatic sepsis (Logters et al., 2009), proving that inflammation could modulate the kynurenine pathway.

In this context, it is pivotal that NO may cause neurogenic inflammation in the central nervous system. This is supported by observations, that NTG was able to increase the expression of NF-κB in the trigeminocervical complex of rats (Greco et al., 2005; Nagy-Grocz et al., 2016), which is a key player in the inflammation process controlled by cytokines.

In human studies, Tfelt-Hansen and his group demonstrated that infusion of NTG can trigger inflammatory response by inducing inflammatory mediators, which response was inhibited by the anti-inflammatory drug, prednisolone (Tfelt-Hansen et al., 2009).

On the other hand migraine can be characterized by an increase in glutamatergic function (Vecsei et al., 2013), yielding fully activated NMDA receptors by the high glutamate levels, which might be associated with low KYNA levels. Increased glutamate levels were found in the human cerebrospinal fluid, plasma and platelets of migraineurs (Cananzi et al., 1995; Peres et al., 2004).

Our findings are comparable with recent studies, which showed that chronic migraine and cluster headache are associated with altered levels of kynurenine metabolites, i.e., reduced levels of KYNA and L-KYN has been found in the serum of these patients (Curto et al., 2015a; Curto et al., 2015b). These findings are in accordance with the context of an increased release of glutamate might yielding to a hyperactivity of glutamate receptors.

To summarize the human and animal data, we can conclude that the kynurenine pathway is down-regulated under the different types of headaches and thus possibly providing less KYNA. These data are in line with the theory of hyperactive NMDA receptors having a role in the migraine pathophysiology since these receptors are key players in the mechanism of central sensitization (Sarchielli et al., 2007) and CSD (Pietrobon and Moskowitz, 2014).

Conclusion

The present data indicate that (i) NTG treatment activates the trigeminal system and the observed changes can be interpreted as a central sensitization phenomenon. Furthermore, (ii) NTG induced alterations are reversed by the administration of AEA suggesting the involvement of CBs in this process. Item, (iii) NTG is able to down-regulate the kynurenine pathway, with a potential influence on the glutamatergic system as well, contributing to the development of trigeminal activation and sensitization in animals.

These data suggest that the endocannabinoid system plays a significant role in the cellular mechanism of trigeminal sensitization and thus it may modulate the pathomechanism of migraine. Our present data strongly confirm that the kynurenine system has a relevant role in the pathomechanism of the trigeminal activation and sensitization, thus in the migraine pathology, as well. In summary, influencing the kynurenine pathway provides a possible new target in the future therapy of migraine.

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The modulatory effect of anandamide on nitroglycerin-induced sensitization in the trigeminal system of the rat

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Abstract

Background: One of the human and animal models of migraine is the systemic administration of the nitric oxide donor (NO) nitroglycerin (NTG). NO can provoke migraine-like attacks in migraineurs and initiates a self-amplifying process in the trigeminal system, probably leading to central sensitization. Recent studies suggest that the endocannabinoid system is involved in nociceptive signal processing and cannabinoid receptor (CB) agonists are able to attenuate nociception in animal models of pain.

Aim: The purpose of the present study was to investigate the modulatory effects of a CB agonist anandamide (AEA) on the NTG-induced expression of transient receptor potential vanilloid type 1 (TRPV1), neuronal nitric oxide synthase (nNOS), nuclear factor kappa B (NF-κB), cyclooxygenase-2 (COX-2) and kynurenine aminotransferase-II (KAT-II) in the upper cervical spinal cord (C1–C2) of the rat, where most of the trigeminal nociceptive afferents convey.

Methods: A half hour before and one hour after NTG (10 mg/kg) or placebo injection, adult male Sprague-Dawley rats ($n = 44$) were treated with AEA (2×5 mg/kg). Four hours after placebo/NTG injection, the animals were perfused and the cervical spinal cords were removed for immunohistochemistry and Western blotting.

Results and conclusion: Our results show that NTG is able to increase TRPV1, nNOS, NF-κB and COX-2 and decrease KAT-II expression in the C1–C2 segments. On the other hand, we have found that AEA modulates the NTG-induced changes, thus it influences the activation and central sensitization process in the trigeminal system, probably via CBs.

Keywords

Migraine, trigeminal system, nitroglycerin, anandamide, TRPV1, nNOS, NF-κB, COX-2, KAT-II

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Introduction

Migraine belongs to the group of primary headaches, and it is one of the most abundant neurological syndromes, which affects 15% of the European population (1). Despite intensive research, the exact pathomechanism of the disorder is not fully understood, but it is well known that activation and sensitization of the trigeminal system is essential during the attack (2). Continuous activation of peripheral trigeminal afferents leads firstly to peripheral (first-order) sensitization. Sustained inputs can lead to second-order, and ultimately to third-order central sensitization (3). Previous data have shown that after the onset of central sensitization during the migraine attack, the acute treatment becomes less effective (4), although this theory has been contested recently, stating that the severity of headache might be a better indicator than the symptoms of

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sensitization (5,6); it is generally accepted that the latter plays an essential role in the genesis of migraine (7).

One of the models of migraine is the systemic administration of nitroglycerin (NTG), generating a short-lasting headache in humans via the rapid vasodilatory effect of nitric oxide (NO) followed by a typical migraine without aura in migraine patients (8).

In rats, NTG is able to increase c-Fos-immunoreactivity in the caudal trigeminal nucleus (TNC) suggesting the activation of the second-order trigeminal neurons there (9). NO is also involved in the central sensitization process in the trigeminal system (10), probably acting via the activation of trigeminal Aδ and C fibers, since the destruction of the latter with capsaicin abolishes the effect of NTG (11).

Cannabinoid receptors (CBs) are G-protein-coupled receptors that have two subtypes: CB1 and CB2. CB1 is present in the central nervous system, liver and lung, and CB2 is expressed primarily in the immune system (12).

Animal models of pain have shown that fluctuations in the endocannabinoid levels in the nervous system are related to pain processing and antinociception (13). CB1 is present in the trigeminal ganglion (TG) and on the axon terminals of primary sensory neurons in the nociceptive areas in the spinal cord in rats (14). CB1 is able to inhibit the responses of trigeminal neurons with A- and C-fiber inputs from the dura mater (15), pointing to the importance of the endocannabinoid system in pain processing (16).

Although the psychoactive properties of cannabinoids (17) restrict their therapeutic application, the interactions between the endocannabinoid system and pain mediation is intensively studied in several laboratories.

N-Arachidonylethanolamide or anandamide (AEA) is the first discovered endocannabinoid, and is an agonist of CBs and transient receptor potential vanilloid type 1 (TRPV1), which is a nonselective cation channel activated by numerous stimuli, such as heat and vanilloids (18,19). AEA has vasodilatory actions (20) that are not mediated by CBs (21). It is well known that AEA is able to reduce NTG-induced hyperalgesia and c-Fos expression in the TNC in rats (22), which means that AEA is capable of modulating the activation of the trigeminal system.

TRPV1 is present in the spinal cord and is considered as a molecular integrator of chemical and physical stimuli that elicit pain (23). In addition, NO donors can activate TRPV1 resulting in an increase of intracellular calcium concentration in different cell types (24,25), which suggests that TRPV1 may be modulated by NO.

NO is synthesized from arginine by nitric oxide synthase, a neuronal isoform of which (nNOS) has an outmost importance in nociception and sensitization and is present in the trigeminal system (26). NO donors may trigger a self-amplifying process at the level of central projection site of the trigeminal system by increasing endogenous NO synthesis, which might be relevant in the central sensitization phenomenon (7).

Nuclear factor kappa B (NF-κB) has a crucial role in the inflammation process by controlling many genes including cytokines. Several studies have shown that proinflammatory cytokines contribute to the development of pain and hyperalgesia (27). Cyclooxygenase-2 (COX-2) is present in the dorsal horn of the spinal cord too and it has a substantial role in the processing of pain (28). Gao and Duan have found that COX-2 expression increased in the TNC after orofacial nociception (29).

Kynurenine aminotransferase-II (KAT-II) is a key enzyme in the kynurenine pathway that converts L-kynurenine (L-KYN) to kynurenic acid (KYNA), a known ionotropic glutamate receptor antagonist molecule (30) that can also block α-7-nicotinic acetylcholine receptors (31). In an NTG-induced animal model of migraine L-KYN, KYNA and KYNA analogs inhibited trigeminal activation and sensitization (32–34).

Thus modulatory effects of cannabinoids can be suggested on trigeminal activation both on peripheral and central levels. In the present paper we studied the effect of NTG injection on the TRPV1, nNOS, NF-κB, COX-2 and KAT-II expression levels in the superficial laminae of C1–C2 and its modulation by the CB agonist AEA.

Materials and methods

Animals

The procedures used in this study followed the guidelines for the Use of Animals in Research of the International Association for the Study of Pain and the directive of the European Economic Community (86/609/ECC). They were permitted 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). Forty-four adult male Sprague-Dawley rats weighing 200–250 g were used. The animals were raised and maintained under standard laboratory conditions with tap water and regular rat chow available ad libitium on a 12-hour dark-12-hour light cycle.

Drug administration

The animals were divided into four groups ($n = 6$ per group for immunohistochemistry, $n = 5$ per group for

Western blot analysis). The animals in the first group, called the placebo group, received only the vehicle solution (physiological saline) as treatment. In the second group, the rats were treated with an intraperitoneal injection of NTG (10 mg/kg bodyweight, Pohl Boskamp). In the third and fourth groups, the animals received intraperitoneal AEA (2 × 5 mg/kg bodyweight, Sigma Aldrich) a half hour before and one hour after the placebo or NTG treatment. Greco and colleagues have demonstrated that single 20 mg/kg doses of AEA before NTG administration is able to reduce NTG-induced c-Fos expression in the TNC (22). Since in our other running experiments (not reported here) we obtained results showing the efficacy of AEA at lower dosages, we used 2 × 5 mg/kg of AEA in this context using two injections because of the short half-life of the drug (35). AEA was dissolved in physiological saline. In the case of the first and second groups, animals were treated with physiological saline instead of AEA.

Immunohistochemistry

Four hours after the placebo/NTG injection, the rats were perfused transcardially with 100 ml phosphate-buffered saline (PBS, 0.1 M, pH 7.4), followed by 500 ml 4% paraformaldehyde in phosphate buffer under chloral hydrate (0.4 g/kg bodyweight) anesthesia. The C1–C2 segments of the cervical spinal cord between −5 and −11 mm from the obex were removed and postfixed overnight for immunohistochemistry in the same fixative. After cryoprotection, 30 μm cryostat sections were cut and serially collected in wells containing cold PBS. The free-floating sections were rinsed in PBS and immersed in 0.3% H_2O_2 in methanol or PBS for 30 minutes. After several rinses in PBS containing 1% Triton X-100, sections of C1–C2 were kept overnight at room temperature in anti-TRPV1 antibody (Santa Cruz, s.c.28759) at a dilution of 1:500, or for two nights at 4°C in anti-nNOS antibody (EuroProxima, 2263B220-1) at a dilution of 1:5000, or for two nights at 4°C in anti-NF-κB antibody (Abcam, ab97726) at a dilution of 1:100. The immunocytochemical reaction was visualized by the Vectastain Avidin-Biotin kit of Vector Laboratories (PK6101), and nickel ammonium sulfate-intensified 3,3'-diaminobenzidine. The specificity of the immune reaction was controlled by omitting the primary antisera.

Western blot analysis

Four hours after the placebo/NTG injection, the animals were perfused transcardially with 100 ml PBS and the dorsal horns of the C1–C2 segments were extracted. Until the measurements, the samples were stored at −80°C. The samples were sonicated in ice-cold lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% igepal, 0.1% cholic acid, 2 μg/ml leupeptin, 2 mM phenylmethylsulphonyl fluoride, 1 μg/ml pepstatin, 2 mM ethylenediaminetetraacetic acid (EDTA) and 0.1% sodium dodecyl sulfate (SDS). The lysates were centrifuged at 12,000 revolutions per minute (RPM) for 10 minutes at 4°C and supernatants were aliquoted and stored at −20°C until use. Protein concentration was defined with BCA Protein Assay Kit using bovine serum albumin as a standard. Prior to loading, each sample was mixed with sample buffer, and denaturated by boiling for 3 minutes. Equal amounts of protein samples (20 μg/lane) were separated by standard SDS polyacrylamide gel electrophoresis on 10% Tris-Glycine gel and electrotransferred onto Amersham Hybond-ECL nitrocellulose membrane (0.45 μm pore size). We used the Page Ruler Prestained Protein Ladder (10–170 kDa) to define approximate molecular weights. Following the transfer, membranes were blocked for one hour at room temperature in Tris-buffered saline containing Tween 20 (TBST) and 5% nonfat dry milk powder. Then, they were incubated in TBST containing 1% nonfat dry milk and nNOS antibody (BD Biosciences, 610308, dilution: 1:2000, incubation: overnight at room temperature), or COX-2 antibody (Proteintech, 12375-1-AP, dilution: 1:1000, incubation: overnight at room temperature), or KAT-II antibody (Santa Cruz, sc-67376, dilution: 1:10,000, incubation: overnight at room temperature) or β-actin antibody (Calbiochem, CP01, dilution: 1:100 000, incubation: overnight at room temperature). The next day, the membranes were incubated in TBST containing 1% nonfat dry milk and horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology, sc-2030, sc-2031) for two hours at room temperature. Protein bands were visualized after incubation of membranes with the SuperSignal West Pico Chemiluminescent Substrate using Carestream Kodak BioMax Light film.

Data evaluation

All evaluations were implemented by an observer blind to the experimental groups. The detailed methods were described previously (34,36,37).

Immunohistochemistry (TRPV1, nNOS, NF-κB). The photomicrographs of the stained sections of C1–C2 were taken using a Zeiss AxioImager microscope supplied with an AxioCam MRc Rev. 3 camera (Carl Zeiss Microscopy, Jena, Germany).

The area covered by TRPV1-immunoreactive fibers and nNOS-immunoreactive cells was determined by Image Pro Plus 6.2® image analysis software (Media Cybernetics). After image acquisition, the laminae I–II

in the dorsal horn were defined manually as areas of interest and a threshold gray level was validated with the image analysis software as described in an earlier study (32,36). The program calculated the area innervated by the immunoreactive fibers and cells as the number of pixels with densities above the threshold; the data were expressed as area fractions (%) of the corresponding immunolabelled structures.

We used the unbiased optical disector method to calculate the volume densities of the NF-κB-immunoreactive cells (37).

Western blot analysis (nNOS, COX-2, KAT-II). For densitometric analyses, films were scanned and quantified using Java ImageJ 1.47v analysis software (National Institutes of Health). The results were normalized to β-actin.

Statistical analysis

Statistical analysis of measurements were performed in SPSS Statistics software (version 20.0 for Windows, SPSS Inc) using one-way analysis of variance (ANOVA) followed by the Tukey or Tamhane post hoc test depending on variances of data, with $p < 0.05$ taken as statistically significant. Group values are reported as means ± SEM.

Results

NTG induced an increase in TRPV1 expression in the C1–C2, and AEA inhibited this phenomenon

On transverse sections of the C1–C2 segments, there were abundant TRPV1-positive fibers in the superficial

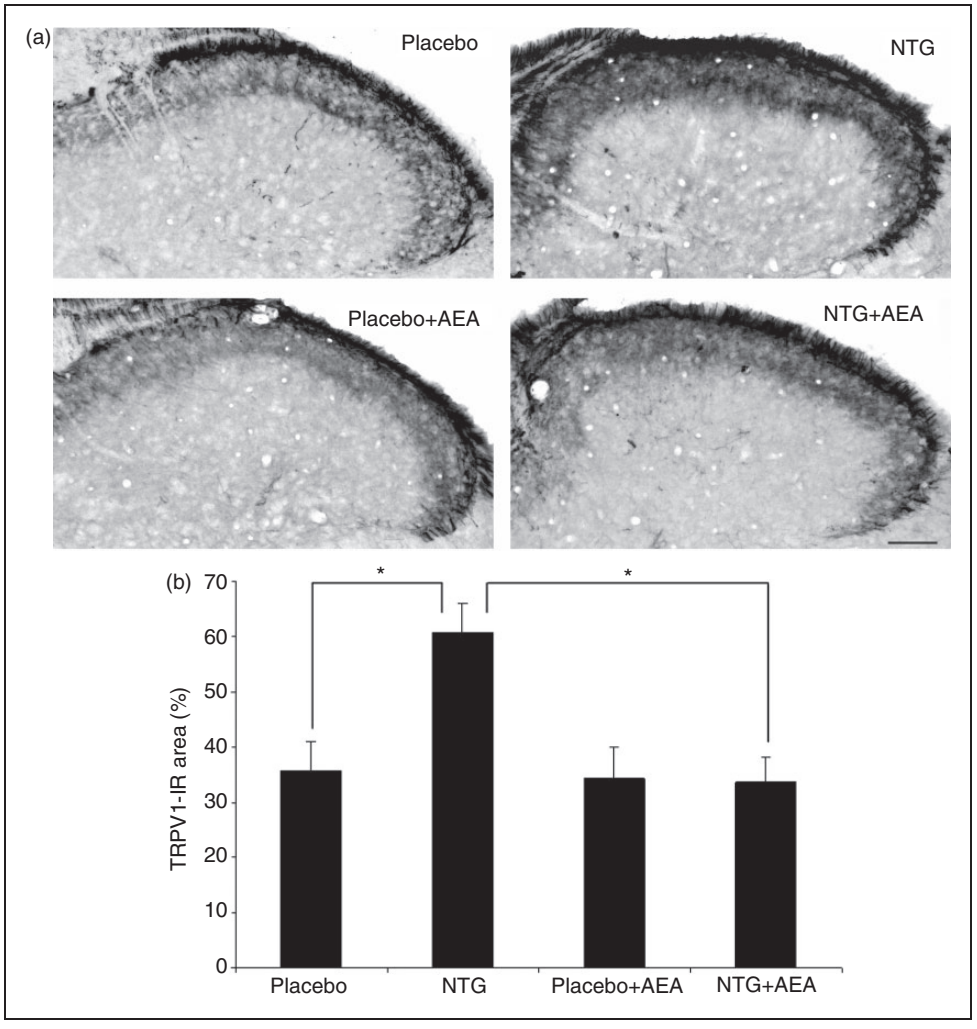

Figure 1. (a) Representative photomicrographs of the TRPV1 expression in the C1–C2 segments. (b) Changes in area fractions of TRPV1-immunoreactive fibers in superficial laminae I and II of the C1–C2 segments. In the NTG group, the area covered by TRPV1 was significantly higher than in the placebo group. AEA seemed to block this effect. Bar graph data presented here and in succeeding figures are means ± SEM. Scale bar: 100 μm, *$p < 0.05$. TRPV1: transient receptor potential vanilloid type 1; IR: immunoreactive; NTG: nitroglycerin; AEA: anandamide.

layers of the dorsal horn. The TRPV1-immunoreactive area in the NTG-treated group was significantly higher compared to the placebo-treated group ($p < 0.05$). The NTG-induced increase was attenuated by treatment with AEA ($p < 0.05$) (Figure 1).

NTG increased nNOS expression in the C1–C2, and AEA attenuated this effect

On transverse sections of the C1–C2 segments, nNOS-immunoreactive neurons and processes with cytoplasmic staining can be observed in the superficial layers of the dorsal horn. In the NTG group, the area fraction of nNOS-immunoreactive structures was significantly higher than in the placebo-treated group ($p < 0.01$). AEA treatment resulted in a decrease of nNOS-immunopositive structures ($p < 0.001$) (Figure 2).

Western blot analysis of the C1–C2 region confirmed the results obtained by nNOS immunohistochemistry. A band characteristic of the nNOS protein was identified at 155 kDa. Densitometric analyses confirmed that the nNOS bands were significantly enhanced ($p < 0.01$) in C1–C2 after NTG administration as compared with the placebo-treated animals. This effect was blocked by the treatment with AEA ($p < 0.05$) (Figure 3).

NTG treatment enhanced NF-κB expression, and AEA mitigated this phenomenon

In transverse sections of the C1–C2 region, a high number of NF-κB-positive cells can be seen in the superficial layers of the dorsal horn. In the NTG-treated animals the volume density of the NF-κB-positive

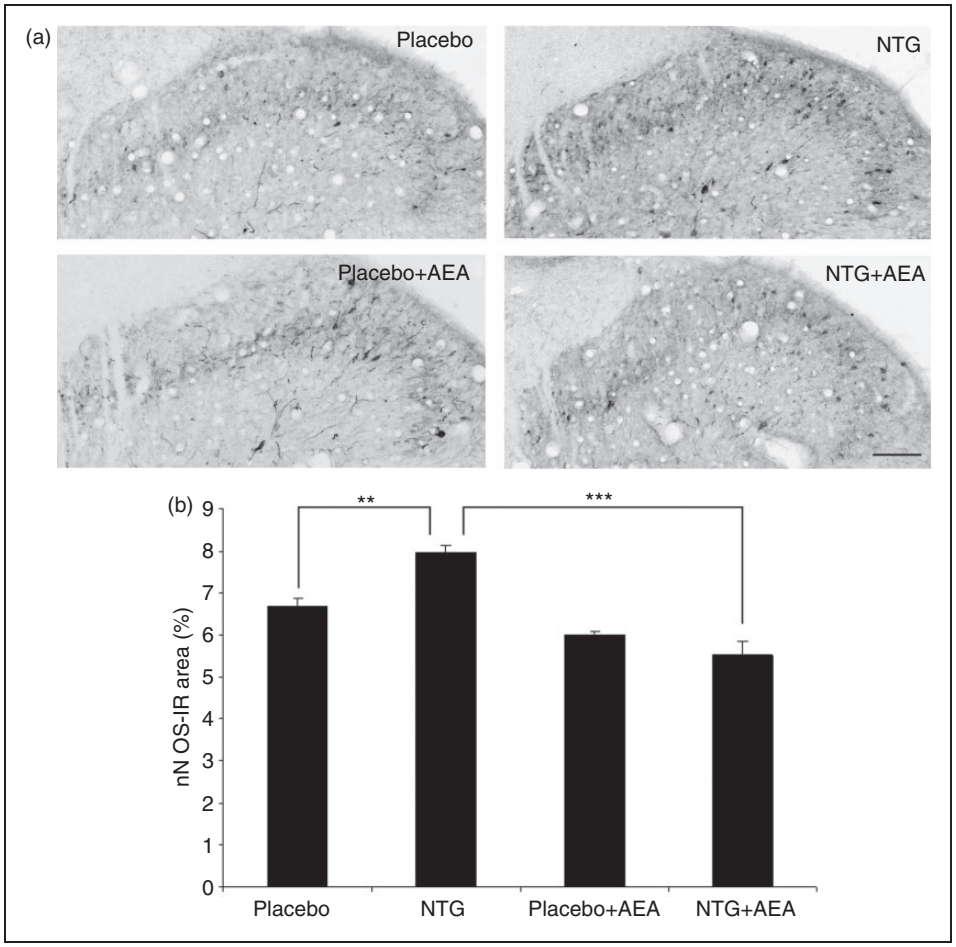

Figure 2. (a) Representative photomicrographs of nNOS expression in the C1–C2 segments. (b) Changes in the nNOS-immunoreactive area fractions. In the NTG group, the area fraction of nNOS-IR structures was increased compared to the placebo-treated group. AEA was able to attenuate this effect. Scale bar: 100 μm, $^{**}p < 0.01$; $^{***}p < 0.001$. nNOS: neuronal nitric oxide synthase; IR: immunoreactive; NTG: nitroglycerin; AEA: anandamide.

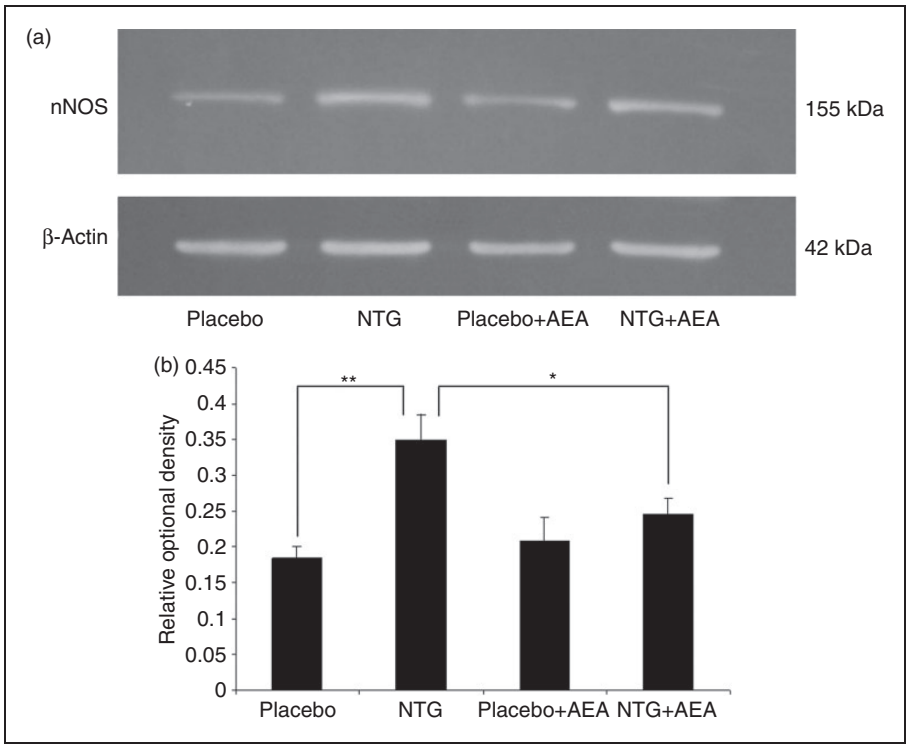

Figure 3. (a) Western blot of nNOS and β-actin expression in the C1–C2. (b) The quantitative analysis shows that in the NTG group, the relative optical density of the nNOS-specific band was significantly higher than in the placebo group. AEA treatment weakened this effect. *$p < 0.05$; **$p < 0.01$. nNOS: neuronal nitric oxide synthase; NTG: nitroglycerin; AEA: anandamide.

cells was significantly higher than in the placebo group ($p < 0.05$). This value decreased in the AEA-injected group ($p < 0.05$) (Figure 4).

NTG enhanced expression of COX-2 enzyme, and AEA inhibited this action

A band characteristic of the COX-2 protein was identified at 68 kDa in Western blot assay. Densitometric analyses showed that the COX-2 bands were significantly enhanced ($p < 0.01$) in segments C1–C2 after NTG administration as compared with the placebo-treated animals. The effect of NTG was decreased by the AEA treatment ($p < 0.01$) (Figure 5).

NTG decreased KAT-II expression, which was alleviated by AEA

A band characteristic of the KAT-II protein was referred at 60 kDa in Western blot assay. Densitometric analyses confirmed that the KAT-II bands were significantly weaker ($p < 0.05$) in segments C1–C2 after NTG administration as compared with the placebo-treated animals. This effect was reversed by treatment with AEA ($p < 0.05$) (Figure 6).

Discussion

The present data indicate that NTG treatment activates the trigeminal system, and the observed changes can be interpreted as a central sensitization phenomenon. The NTG-induced alterations are reversed by the administration of AEA suggesting the involvement of CBs in this process.

It is generally accepted that the trigeminal pain processing and sensitization is an extremely complex phenomenon involving numerous cellular and molecular components but the associated pathways are not fully known. Figure 7 shows a schematic and simplified flow diagram that indicates that the connections are sometimes indirect and the signaling molecules may modulate the complex response at different target points.

TRPV1

TRPV1 is present in the terminals of primary sensory neurons in the dorsal part of the spinal cord (38) and co-expressed with CB1 (39). Experimental data indicate that TRPV1 contributes to the peripheral sensitization, allodynia and hyperalgesia (40); central blockade of this receptor is able to attenuate central terminal sensitization

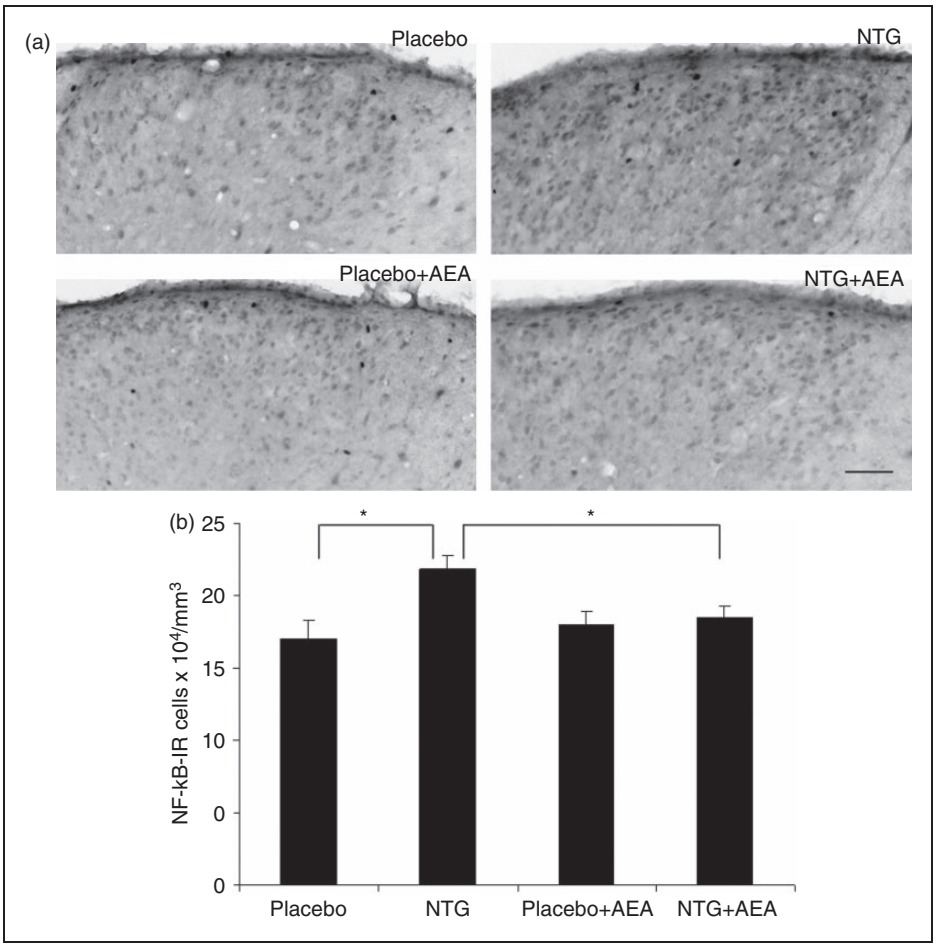

Figure 4. (a) Photomicrographs of the NF-κB expression in the C1–C2 segment. (b) The diagram shows that NTG treatment resulted in a significant increase in the volume density of NF-κB-immunoreactive cells. This effect was not observed in AEA-injected animals. Scale bar: 50 μm, $*p < 0.05$. NF-κB: nuclear factor kappa B; IR: immunoreactive; NTG: nitroglycerin; AEA: anandamide.

(41). Our results show that NTG significantly increases TRPV1 expression in the C1–C2 segments of the rat, thus it may be the indicator of sensitization phenomena in the trigeminal system in our experimental setting.

It is well evidenced that NO donors can activate TRPV1 in several cell types (24,42), and in inflammatory pain models increased receptor expression was also reported (43,44). This indicates that the NTG effect on TRPV1 expression is indirect—it may cause neurogenic inflammation (45), and the inflammatory mediators like serotonin (5-HT) and bradykinin (BK) can activate TRPV1 by stimulating trigeminal nociceptive neurons (46). Pro-inflammatory mediators, such as tumor necrosis factor α, interleukin 1 (IL-1), interleukin 6 (IL-6) and BK enhance TRPV1 (47). In the animal model of inflammation, Complete Freud's adjuvant increases TRPV1 messenger RNA (mRNA) expression in the dorsal root ganglion (48), this also supports the idea that inflammation upregulates TRPV1 expression. To summarize, we may assume that NTG is able to activate TRPV1 mainly via inflammatory mediators. On the other hand, there are research data indicating that systemic administration of calcitonin gene-related peptide (CGRP) increases the expression of TRPV1 in the TG of rats (49), which might also play a role in this process.

Our recent data also show that AEA, a CB receptor and TRPV1 agonist, attenuates the effect of NTG on TRPV1 changes. Activation of ionotropic CBs can result in inhibition of nociceptors and antihyperalgesia and antinociception in certain pain models (50). Intrathecal administration of AEA decreases thermal pain sensitivity and its effect can be altered with the TRPV1 antagonist capsazepine (51). AEA may also cause a desensitization of TRPV1 in skeletal muscle arterioles (52), suggesting that AEA is able to mitigate TRPV1 activity. On the other hand, AEA can inhibit neurogenic, CGRP- and NO-induced dural vasodilation, and this involves pre- and postsynaptic mechanisms (53). A recent report shows that the AEA level changes are able to modulate CGRP mRNA expression after NTG-treatment in human peripheral blood

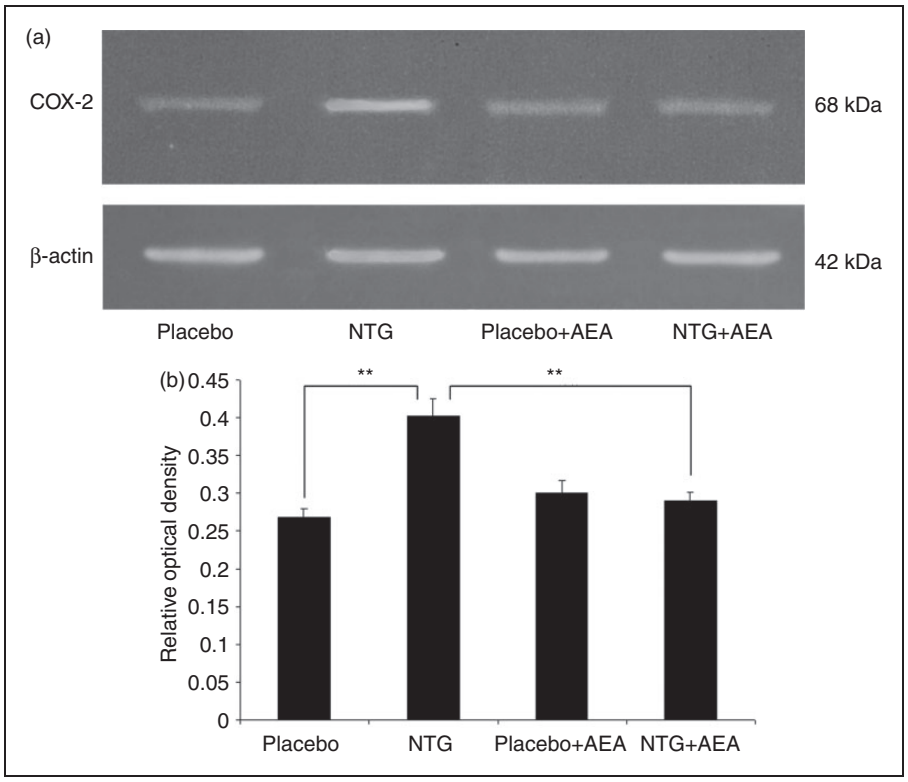

Figure 5. (a) Western blot of COX-2 and β-actin expression in the C1–C2. (b) Densitometry of the individual bands indicated that in the NTG-treated animals the expression of COX-2 was significantly higher than in the placebo group. No such effect was seen in the AEA-injected group. $^{**}p < 0.01$. COX-2: cyclooxygenase-2; NTG: nitroglycerin; AEA: anandamide.

mononuclear cells (54). We do not know exactly the role of TRPV1 in the AEA-modulated sensitization process, but based on the available literature data (15,55), we hypothesize that the role of CB1 is more pronounced than TRPV1 in this context.

nNOS

It is known that nNOS is a key player in nociception (56) and its role in the sensitization cascade is intensively studied. In our present experiment administration of NTG increased the expression of nNOS in the C1–C2, which is in line with earlier results (10). The most probable explanation for this phenomenon is that NO activates small-caliber fibers in the trigeminal system and the increase of nNOS expression in the second-order trigeminal neurons induces a self-amplifying mechanism (10,11). The present results indicate that AEA is able to inhibit this effect. Several studies have shown that there is an interaction between NO and the cannabinoid system, e.g. nNOS and CB1 are co-localized in neurons in lamina II of the spinal cord (57); NTG-induced hyperalgesia is associated with a fluctuation of the endocannabinoid system in various brain areas of rats (22). Our data are in line with the findings of Hillard and colleagues, who reported that CB1 agonists inhibit KCl-induced activation of nNOS in cultured cerebellar granule cells (58). In addition, Carney and colleagues have detected that cannabinoid agonist downregulated nNOS protein and mRNA in neuronal cells (59). These data suggest that NTG is able to generate a sensitization process and AEA inhibits this effect by blocking nNOS.

NF-κB

In our experiment NTG increased NF-κB expression in the superficial layers of the dorsal horn in the C1–C2 segments. A similar effect was reported by Reuter et al., who demonstrated that NTG infusion is able to trigger the activation of NF-κB in dura mater (60). It is not clear how NTG can activate the NF-κB-pathway; it might be related to a direct neuronal effect of NTG, or an indirect effect via dural inflammation (61). Concerning the cellular mechanisms it is important to note that both TRPV1 and nNOS might play a role in this effect. It was shown that Ca^{2+} influx through TRPV1 may modulate the nuclear translocation and increased the activity of NF-κB (62). On the other hand the increase in nNOS expression is accompanied by increased NF-κB expression and activation (63). Furthermore, Sancho and colleagues have noticed

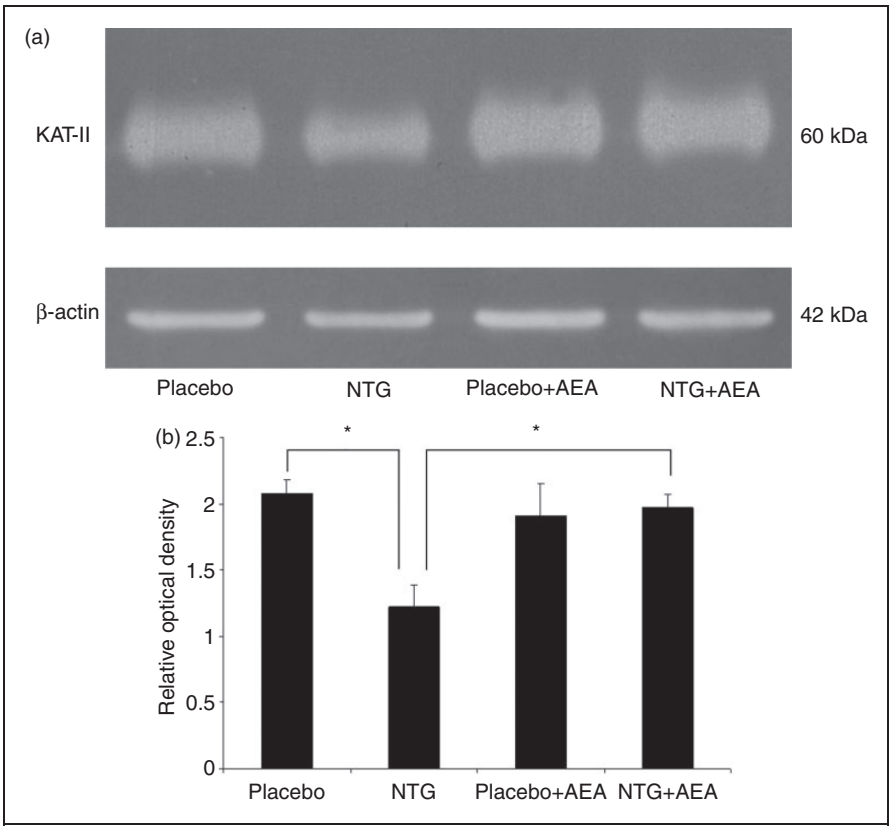

Figure 6. (a) Western blot of KAT-II and β-actin expression in the C1–C2. (b) Quantitative data demonstrate that in the NTG group, the relative optical density of KAT-II is significantly lower than in the placebo group. AEA reduced this effect. $^{*}p < 0.05$. KAT-II: kynurenine aminotransferase-II; NTG: nitroglycerin; AEA: anandamide.

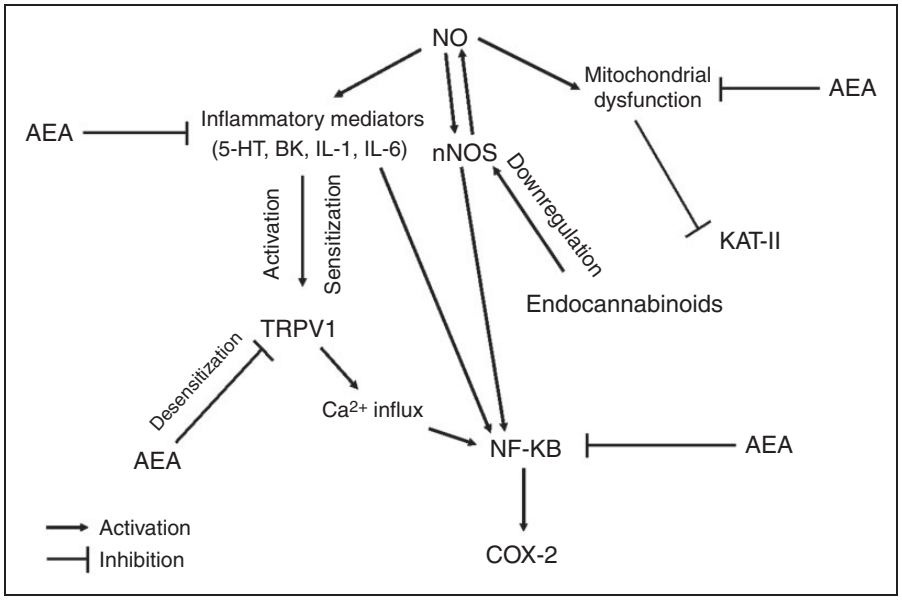

Figure 7. Schematic illustration of the interactions among the NO-influenced molecules involved in the trigeminal pain processing and sensitization showing the possible sites of action for cannabinoids.

that AEA inhibits tumor necrosis factor-α-induced activation by inhibition of a cytokine-induced cascade (64). In addition, Nakajima and colleagues have found that AEA also blocked lipopolysaccharide-induced activation, suggesting that AEA inhibits proinflammatory mediators by blocking NF-κB activation (65). Tassorelli and her group reported that parthenolide (inhibitor of NF-κB) attenuated NTG-induced c-Fos activation in TNC (66), which indicates that NF-κB may be important in the NTG-induced trigeminal activation and its inhibition is able to modulate the nociceptive process. Our data reconfirm that NTG is able to activate NF-κB, and thus can trigger neurogenic inflammation, which has a key role in sensitization phenomena. Furthermore, we detected that AEA is able to reduce this effect. Endocannabinoids might operate a negative feedback control over the proinflammatory process by suppressing the activation of transcription factors involved in the inflammatory action (67).

COX-2

NO may also cause neurogenic inflammation by increasing NF-κB levels, which may lead to the upregulation of COX-2 in inflammatory pain (68). It is well known that nonsteroidal anti-inflammatory drugs (NSAIDs), which exert their effects through the inhibition of COX-enzymes (69), are effective in the treatment of migraine and tension-type headache (70). In animal studies, it has been shown that COX-2 is involved in the NTG-induced activation and sensitization process of the trigeminal system. Pre-treatment with indomethacin (non-selective COX inhibitor) and NS398 (selective COX-2 inhibitor) reduced the NTG-induced c-Fos (11), nNOS and calmodulin-dependent protein kinase II alpha expression-changes in the TNC (71,72). Tassorelli and colleagues have demonstrated that COX-2 expression is increased in the hypothalamus and caudal brain stem after NTG injection (73), thus COX is one of the mediators of NTG-induced neuronal activation. Furthermore, NO is able to activate COX-enzymes in fibroblasts, probably by an interaction with the iron-hem center of the enzyme (74). It is important to note that AEA is one of the substrates of COX-2 producing prostaglandin and ethanolamids (75). In our study, we have found that AEA is able to inhibit the NTG-induced COX-2 increase. The cellular and molecular background of this effect remains to be determined. Our assumption is that it may be associated with a negative feedback mechanism, but it is possible that after the cleavage of AEA some metabolites may downregulate COX-2 expression. Since AEA is able to reduce cytokine-induced cascade and proinflammatory mediators, it is also possible that the reduction of the inflammatory process is able to downregulate the COX-2 expression.

KAT-II

In our experiments NTG decreased KAT-II expression in the C1–C2 segments, which may indicate that fluctuation of KAT is involved in the NTG-triggered trigeminal activation. Recent studies have shown that migraine is associated with mitochondrial dysfunction (76). It has also been demonstrated that an NO donor is able to downregulate the respiratory chain complex and cause release of the mitochondrial cytochrome C, thus causing a mitochondrial dysfunction (77). The decrease in KAT-II activity might be associated with mitochondrial damage as shown in the striatum of patients suffering in Huntington's disease (78). This is in line with our earlier data showing that administration of mitochondrial complex II inhibitor 3-nitropropionic acid is able to reduce KAT-II activity in rats (79). On the other hand, NTG may cause neurogenic inflammation and the released pro-inflammatory cytokines can modulate the kynurenine pathway (80), which may also contribute to the change in KAT-II expression. Nevertheless, in another model of trigeminal activation (electrical stimulation of the TG), decreased KAT-immunoreactivity in dural macrophages, Schwann cells and mastocytes was found in rats (81), suggesting a prominent role of this enzyme in the process of trigeminal activation.

In the present experiment, we have detected that AEA is able to mitigate NTG-induced KAT-II expression decrease. AEA and the other endocannabinoid molecule 2-arachidonoylglicerol cause reduction of calcium-induced cytochrome C release from mitochondria and protect mitochondria from cytochrome-mediated damage, which leads to DNA fragmentation and apoptosis (82,83). Taken together, it is possible that NTG may cause mitochondrial dysfunction, and AEA is able to inhibit this effect probably by decreasing cytochrome C release.

Conclusions

In conclusion, our results show that AEA has a modulating effect on central sensitization markers in our experimental setting. These data suggest that the endocannabinoid system plays a significant role in the cellular mechanism of trigeminal sensitization and thus it may modulate the pathomechanism of migraine.

Article highlights

- Systemic administration of nitroglycerin is able to activate and sensitize the trigeminal system in rats.
- This phenomenon is reflected by alteration of the expression of biological markers (transient receptor potential vanilloid type 1 (TRPV1), neuronal nitric oxide synthase (nNOS), nuclear factor kappa B (NF-κB), cyclooxygenase-2 (COX-2) and kynurenine aminotransferase-II (KAT-II)) of this process.
- Anandamide, a cannabinoid receptor agonist, is able to modulate these changes caused by nitroglycerin.
- Cannabinoid receptors are involved in the activation and sensitization of the trigeminal system.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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II.

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ORIGINAL RESEARCH
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The Effect of Systemic Nitroglycerin Administration on the Kynurenine Pathway in the Rat

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The primary headache disorders include migraine, which is one of the most frequent neurological disorders, which influences more than 14% of the whole population. Despite the research efforts, its exact pathomechanism is not fully revealed, but evidence points to the role of glutamate and its receptors. Kynurenic acid is an endogenous glutamate receptor antagonist produced by the kynurenine pathway (KP). Tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO) convert L-tryptophan to *N*-formyl-L-kynurenine, to be further transformed to L-kynurenine. Kynurenine aminotransferase-II (KAT-II), L-kynurenine hydrolase (KYNU), and L-kynurenine 3-monooxygenase (KMO) are key enzymes in the later steps of the KP. Nitroglycerin (NTG) administration serves as both human and animal model of migraine, causing the activation and sensitization in the trigeminal system. A previous study demonstrated a reduction of KAT-II expression following NTG administration in animals. The goal of current tests was to identify the potential modulatory effect of NTG on other metabolizing enzymes of the KP in the caudal trigeminal nucleus (TNC) of rats. Four hours following the intraperitoneal injection of NTG (10 mg/kg), the rats were perfused transcardially and the TNC was extracted for Western blotting. Western blot studies revealed that the expression of TDO2, IDO1, KYNU, and KMO decreased in the TNC. The results demonstrated that NTG is able to downregulate the KP, with a potential influence on the glutamatergic system as well, contributing to the development of trigeminal activation and sensitization in animals.

Keywords: migraine, nitroglycerin, kynurenic acid, L-kynurenine, kynurenine pathway

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INTRODUCTION

Migraine is a common primary headache characterized by severe head pain and numerous concomitant symptoms, e.g., vomiting, nausea, photophobia, and phonophobia. The disease affects about 14% of the total population (1). The exact pathophysiology of the disorder is not fully understood, but it is

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IDO, indoleamine 2,3-dioxygenase; KAT-II, kynurenine aminotransferase-II; KMO, L-kynurenine 3-monooxygenase; KYNA, kynurenic acid; KYNU, L-kynurenine hydrolase; KP, kynurenine pathway; L-KYN, L-kynurenine; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NTG, nitroglycerin; PBS, phosphate-buffered saline; QUIN, quinolinic acid; TBST, Tris-buffered saline containing Tween 20; TDO, tryptophan 2,3-dioxygenase; TNC, caudal trigeminal nucleus; Trp, tryptophan.

well known that the activation and sensitization of the trigeminal system is essential during the attack (2). Several lines of evidence have been put forth to support the hypothesis that glutamate receptors, principally *N*-methyl-D-aspartate (NMDA) receptors, have a pivotal aspect in these phenomena (3). Kynurenic acid (KYNA) is a neuroprotective metabolite that interacts with glutamate receptors, aryl hydrocarbon receptor, and G protein-coupled receptor 35 and elicits anti-glutamatergic actions. Series of data confirm that KYNA and its analogs have anti-nociceptive effects in several migraine models (4–6), probably by attenuating the trigeminal activation and sensitization. The initial process in the kynurenine pathway (KP) is the transformation of tryptophan (Trp) to *N*-formyl-L-kynurenine by tryptophan 2,3-dioxygenase (TDO1, 2) and indoleamine 2,3-dioxygenase (IDO1,2): the rate-limiting enzymes of Trp metabolism. *N*-formyl-L-kynurenine is further converted by formamidase to L-kynurenine (L-KYN), which is converted to KYNA by kynurenine aminotransferases (KATs), to 3-hydroxykynurenine by L-kynurenine 3-monooxygenase (KMO), and to anthranilic acid by L-kynurenine hydrolase (KYNU). The other metabolite of the KP is quinolinic acid (QUIN). In contrast to KYNA, QUIN is an agonist of the NMDA receptors and can provoke neuronal death and also causes lipid peroxidation and generates reactive oxygen species (7, 8) (**Figure 1**).

Systemic administration of nitroglycerin (NTG) can be utilized as a human and animal model of migraine. NTG is enzymatically converted to nitric oxide (NO) in the body, probably by a mitochondrial aldehyde dehydrogenase (9). The administration of NTG is able to activate and sensitize the trigeminal system in humans and animals (10–12). In our previous study, it was demonstrated that NTG decreased the expression of kynurenine aminotransferase-II (KAT-II) (13), which converts L-KYN to KYNA in the brain thus reducing KYNA levels, contributing to the hyperactivity of NMDA receptors.

The goal of this study was to explore the issue of NTG on the expression levels of TDO2, IDO1, KYNU, and KMO enzymes in the caudal trigeminal nucleus (TNC).

MATERIALS AND METHODS

Animals

We followed the directives for the Use of Animals in Research of the International Association for the Study of Pain and the policy of the European Economic Community (86/609/ECC). They were authorized by the local ethical committee of University of Szeged and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XXIV./352/2012). 44 adult male Sprague-Dawley rats of 200–250 g bodyweight were used. The rodents were raised and maintained under standard laboratory conditions with tap water and regular rat chow available *ad libitum* on a 12 h dark–12 h light cycle.

Drug Administration

The animals were separated into two groups ($n = 5$). The animals in the first group, called placebo group, received only the vehicle solution (physiological saline) as treatment. In the second group, the rats were treated with an intraperitoneal injection of NTG (10 mg/kg bodyweight, Pohl Boskamp).

Western Blot Analysis

Four hours after the placebo/NTG injection, the animals were perfused transcardially with 100 mL phosphate-buffered saline and the dorsal horns of TNC segments (+1 and −5 mm from the obex) were extracted. The samples were stored at −80°C and they were sonicated in ice cold lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 0.1% igepal, 0.1% cholic acid, 2 μg/mL leupeptin, 2 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin, 2 mM EDTA, and 0.1% sodium dodecyl sulfate. The homogenates were centrifuged for 10 min at 12,000 RPM at 4°C and supernatants were aliquoted and stored at −20°C until use. BCA Protein Assay Kit was used to measure protein concentration. Samples were mixed with sample buffer and were boiled for 3 min. Standard SDS polyacrylamide gel electrophoresis was performed with equal amounts of protein samples (20 μg/lane) loaded on 10% Tris–glycine gel and electrotransferred onto Amersham Hybond-ECL nitrocellulose membrane (0.45 μm pore size). Page Ruler Prestained Protein Ladder (10–170 kDa) was used to define approximate molecular weights. Non-specific binding was eliminated by blocking in Tris-buffered saline containing Tween 20 (TBST) and 5% non-fat dry milk for 1 h at room temperature. Then, membranes were incubated in TBST containing 1% non-fat dry milk and TDO antibody (LifeSpan BioSciences, LS-C111058, dilution: 1:500, incubation: overnight at room temperature), IDO antibody (Abcam, ab106134, dilution: 1:500, incubation: overnight at room temperature), KYNU antibody (Abcam, ab96365, dilution: 1:500, incubation: overnight at room temperature), KMO antibody (Abcam, ab83929, dilution: 1:4,000, incubation: overnight at room temperature), or glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Cell Signaling Technologies, 8884, dilution: 1:1,000, incubation: overnight at room temperature). On the following day, a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology, sc-2030) in TBST containing 1% non-fat dry milk was applied for 2 h at room temperature. SuperSignal West Pico Chemiluminescent Substrate was used to visualize bands on Carestream Kodak BioMax Light film. Western blot protocol was developed based on previous experiments (4, 14–16).

An observer blinded to the experimental groups carried out the measurements. The detailed methods were described previously (13).

Films were quantified by Java ImageJ 1.47v analysis software (National Institutes of Health). The data were standardized to GAPDH.

Statistical Analysis

Statistical analysis was carried out by SPSS Statistics software (Version 20.0 for Windows, SPSS Inc.). Normality was checked by Kolmogorov–Smirnov test, and group means were compared by independent *t*-test, with $p < 0.05$ taken as statistically significant. Group values are presented as means ± SEM.

RESULTS

NTG Induced a Decrease in TDO2 Expression in the TNC

TDO2 protein was identified at 50 kDa in Western blot assay. Densitometric analyses showed that the TDO2 bands were

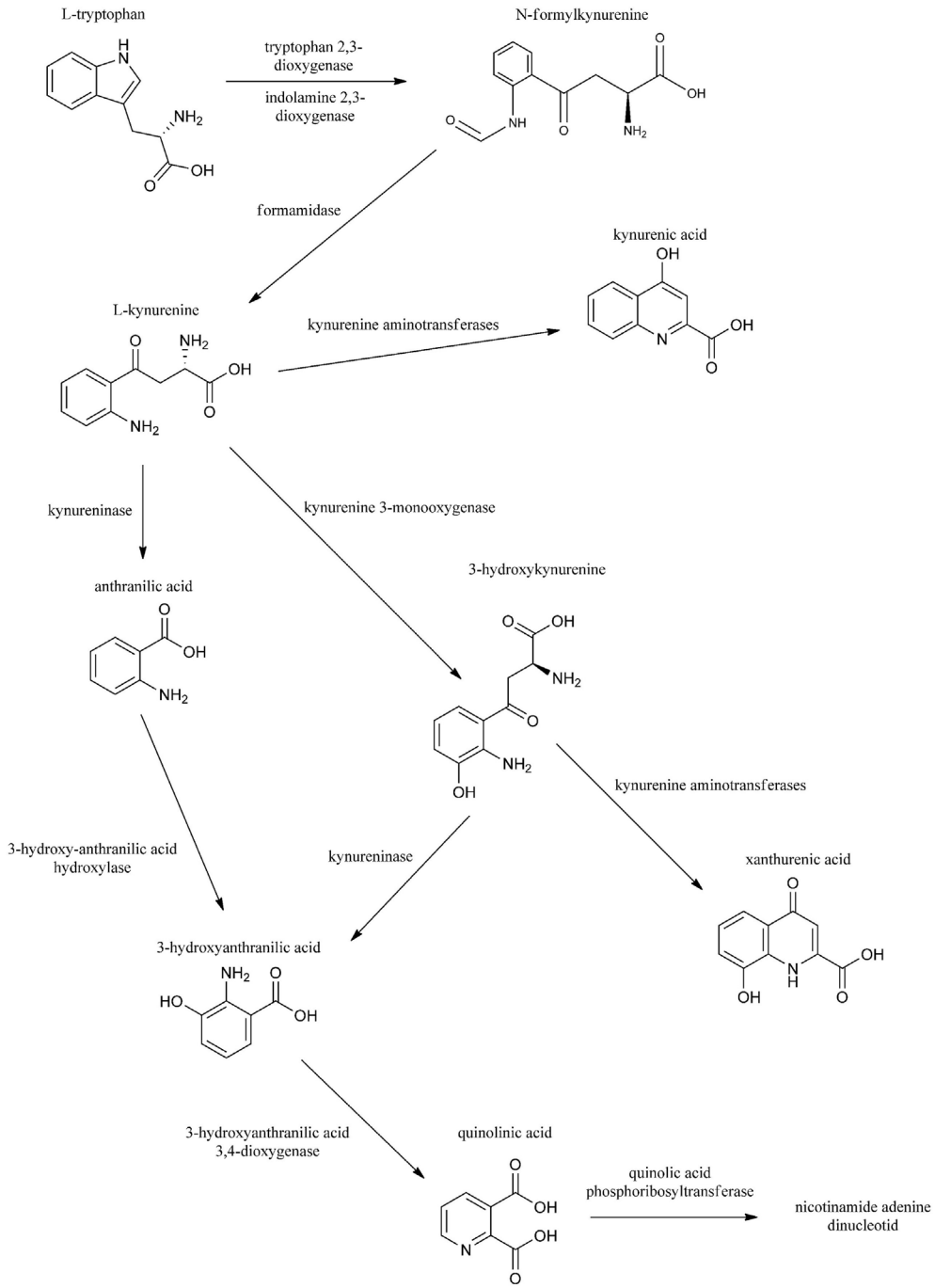

FIGURE 1 | The kynurenine pathway.

significantly decreased ($p < 0.05$) in the TNC after NTG administration as correlated with the placebo-treated animals (**Figure 2**).

NTG Treatment Resulted in a Diminished IDO1 Expression

A band characteristic of the IDO1 protein was referred at 45 kDa in Western blot assay. Densitometric analyses confirmed that the IDO1 bands were significantly weaker ($p < 0.05$) in the TNC after NTG administration as correlated with the placebo-treated animals (**Figure 3**).

NTG Was Able to Reduce the Expression of KYNU

We could determine a band at 35 kDa characteristic for the KYNU protein. In animals, which had received NTG, the density

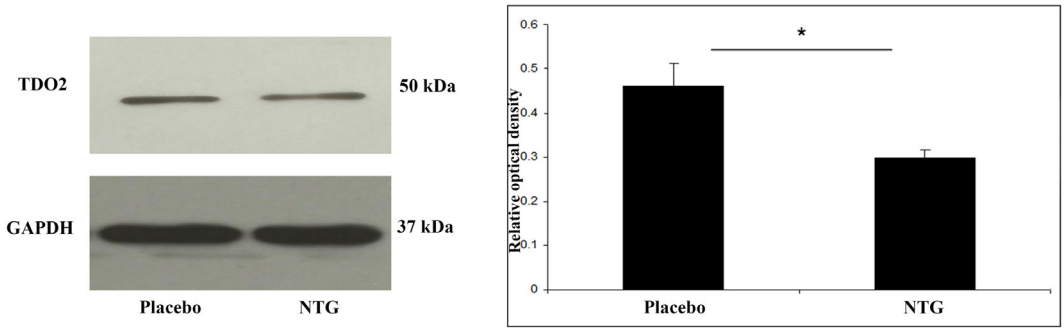

FIGURE 2 | Western blot analysis of TDO2 and GAPDH protein from the TNC. The quantitative analysis shows that in the NTG group the relative optical density of TDO2 specific bands were significantly less pronounced compared with the placebo group. *$p < 0.05$; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NTG, nitroglycerin; TDO2, tryptophan 2,3-dioxygenase 2; TNC, caudal trigeminal nucleus.

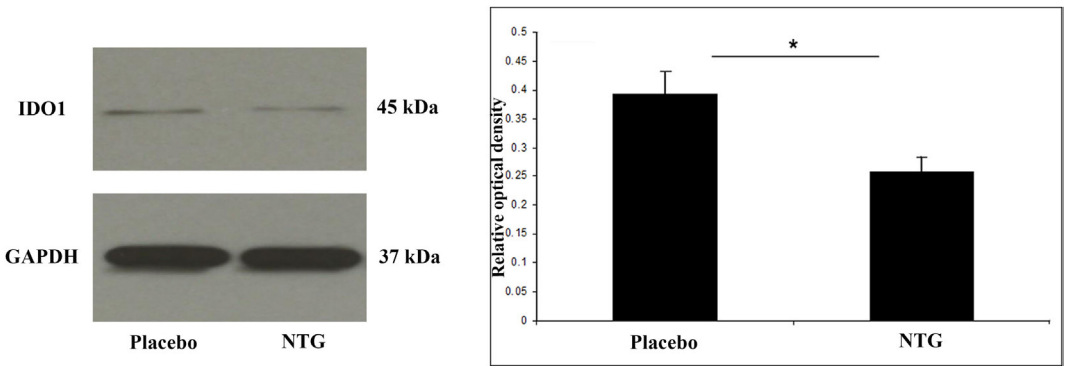

FIGURE 3 | Western blot of IDO1 and GAPDH expression in the TNC. The quantitative analysis shows that in the NTG group, the relative optical density of IDO1-specific bands was significantly decreased compared with the placebo group. *$p < 0.05$; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IDO1, indoleamine 2,3-dioxygenase; NTG, nitroglycerin; TNC, caudal trigeminal nucleus.

of KYNU protein bands was weaker in TNC segments ($p < 0.05$) as compared with the placebo-treated group (**Figure 4**).

KMO Expression Was Lower after NTG Administration

L-Kynurenine 3-monooxygenase protein was identified at 56 kDa in Western blot assay. Densitometric analyses showed that the KMO bands were significantly decreased ($p < 0.05$) in segments TNC after NTG administration as correlated with the placebo-treated animals (**Figure 5**).

DISCUSSION

The current data demonstrated that NTG is able to decrease the expression of the KP enzymes in rat TNC. However, the question arises how NTG/NO can influence the KP. It is well known that the nitrergic system is able to alter the KP, e.g., NO can inhibit IDO expression *via* reversible binding to the active site in macrophages (17) and IDO activity is downregulated by NO production in bone marrow cells (18). The other hand, it is also known that the expression of KMO influences NO production in human HEK293 cells (19). Moreover, Backhaus and her colleagues showed in a mass spectrometry and NMR study that there is a direct interaction between kynurenine metabolites (e.g., 3-hydroxykynurenine and 3-hydroxyanthranilic acid) and NO (20).

It is also important to note that kynurenines have a crucial role in immune regulation (21). The transcriptional expression of IDO, KMO, and KYNU is also under the control of interferons (21), thus pro-inflammatory cytokines influence KP (22). Lögters and co-workers have shown that the ratio of kynurenine–tryptophan was increased in the blood of patients with post-traumatic sepsis (23), which pointed out that inflammation could modulate KP.

In this context, it is crucial that NO may cause neurogenic inflammation in the central nervous system. This is supported by observations that NTG was able to increase the expression of nuclear factor κB in the trigeminocervical complex of rats (13, 24), which is a key player in the inflammation process controlled by cytokines. In human studies, Tfelt-Hansen and his group demonstrated that infusion of NTG can trigger inflammatory

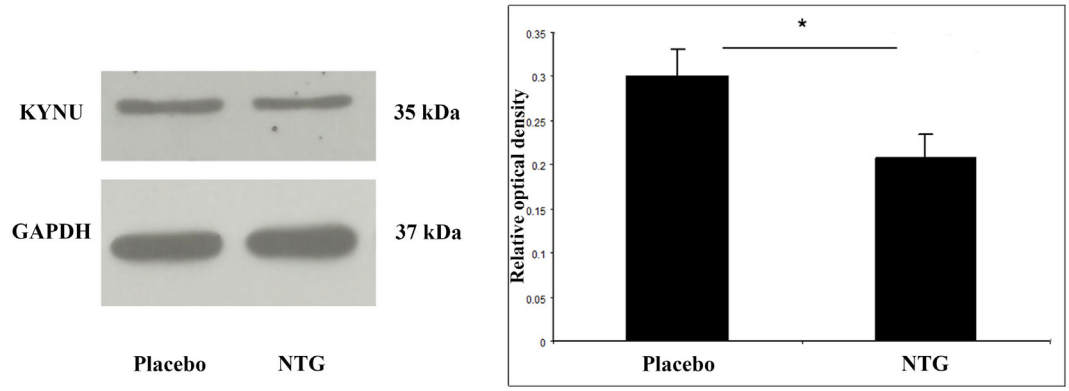

FIGURE 4 | Representative Western blot bands and diagram of KYNU and GAPDH in the TNC. The quantitative analysis shows that in the NTG group, the relative optical density of KYNU-specific bands were significantly smaller compared with the placebo group. *$p < 0.05$; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KYNU, kynurenine hydrolase; NTG, nitroglycerin; TNC, caudal trigeminal nucleus.

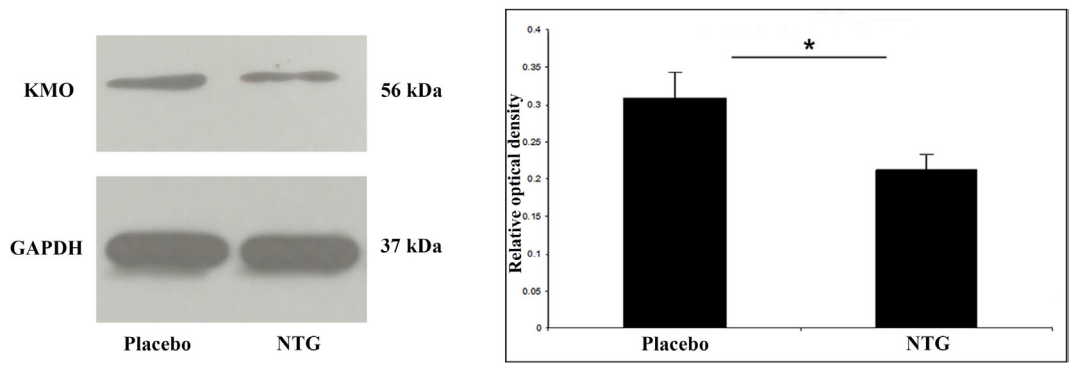

FIGURE 5 | Illustrative Western blot bands and diagram of KMO and GAPDH in the TNC. The quantitative analysis shows that in the NTG group, the relative optical density of KMO specific bands was significantly weaker compared with the placebo group. *$p < 0.05$; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KMO, L-kynurenine 3-monooxygenase; NTG, nitroglycerin; TNC, caudal trigeminal nucleus.

response by inducing inflammatory mediators, which response was inhibited by the anti-inflammatory drug, prednisolone (25).

In the previous study, we demonstrated that NTG was able to decrease the expression of KAT-II, which produces KYNA. Our present and earlier Western blot data showed that NTG could reduce the expression levels of the KP enzymes.

Our findings are comparable with recent studies, which showed that the chronic migraine and cluster headaches are associated with altered levels of kynurenine metabolites, i.e., reduced levels of KYNA and L-KYN have been found in the serum of these patients (26, 27). These findings are in accordance with the theory of an increased release of glutamate probably yielding to a hyperactivity of glutamate receptors.

Migraine can be characterized by an increase in glutamatergic function (28), yielding fully activated NMDA receptors by the high glutamate levels, which might combine with small KYNA levels. Increased glutamate levels were found in the human cerebrospinal fluid, platelets, and plasma of migraine sufferers (29, 30).

To summarize the human and animal data, we can conclude that the KP is downregulated under the different types of headaches and thus possibly providing less KYNA. These data are in line with the theory of hyperactive NMDA receptors having a crucial role in the migraine pathophysiology. These receptors are key players in the mechanism of central sensitization (31), which have a pivotal role in the pathophysiology of migraine. Our present data strongly confirm that the KP has a relevant role in the pathomechanism of the trigeminal activation and sensitization, thus in the migraine pathology as well. In summary, influencing the KP provides a possible new target in the future therapy of migraine.

ETHICS STATEMENT

We followed the directives for the Use of Animals in Research of the International Association for the Study of Pain and the policy of the European Economic Community (86/609/ECC). They were authorized by the local ethical committee of University of

Szeged and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XXIV./352/2012).

AUTHOR CONTRIBUTIONS

GN-G: participated in the design and implementation of experiments, statistical analysis, interpreted data, and wrote the manuscript. KL, GV, AB, ZB, DZ, AF-S, and ES: participated in the implementation of the experiments and statistical analysis. LV: participated in the design of the experiments and in the final approval of the version to be published. ÁP: participated in the conception and design of the experiments and in the interpretation of the data and writing. All authors: critical revision of the manuscript.

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