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

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Summary of Ph.D. Thesis

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Introduction

Migraine is a chronic neurological disorder characterized by recurrent headaches lasting for 4-72 hours and commonly accompanied by nausea, photophobia and phonophobia. This syndrome affects 16% of the total population 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 €.

It is well-known, that the activation and sensitization of the trigeminal system is essential during the attack. 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 manifested as allodynia when non painful stimuli are perceived as painful.

Despite the intensive research, the exact pathomechanisms of the migraine is not fully known, but the role of glutamate seems pivotal. One of the endogenous glutamate receptor antagonists is kynurenic acid (KYNA), which is produced by the kynurenine pathway.

KYNA is believed to be a neuroprotective metabolite of tryptophan that interacts with glutamate receptors, aryl hydrocarbon receptor, G protein-coupled receptor 35 and elicits anti-glutamatergic actions. Several lines of data confirm that KYNA and its analogues have anti-nociceptive effects in several migraine-models, 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. 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).

Animal models of pain have shown that fluctuations in the endocannabinoid levels in the nervous system are related to pain processing and antinociception. N-Arachidonylethanolamide or anandamide (AEA) is the first discovered endocannabinoid, and is an agonist of cannabinoid receptors (CBs) and transient receptor potential vanilloid type 1 (TRPV1).

An increasing amount of evidence suggests that nitroglycerin (NTG) is able to activate and sensitize the trigeminal system in humans. One of the side-effects of NTG is headache due to vasodilatation induced by nitric oxide (NO), which occurs immediately after its administration, but about four hours later it is followed by a typical migraine without aura in migraine patients, which can not be attributed to NO's prompt vasodilator effect. This observation and the results of a pilot study showing that treatment with nitric oxide sysnthase (NOS) inhibitor attenuates spontaneous migraine headaches in 67% of subjects, contributed to the implication of NO in migraine pathogenesis.

It is well known that AEA is able to reduce NTG-induced hyperalgesia and c-Fos expression in the caudal trigeminal nucleus (TNC) in rats, which means that AEA is capable of modulating the activation of the trigeminal system.

TRPV1 is a nonselective cation channel activated by numerous stimuli, such as heat and vanilloids. It is present in the spinal cord and is considered as a molecular integrator of chemical and physical stimuli that elicit pain. In addition, NO donors can activate TRPV1 resulting in an increase of intracellular calcium concentration in different cell types, which suggests that TRPV1 may be modulated by NO.

NO is synthesized from arginine by NOS, neuronal isoform of which (nNOS) has an outmost importance in nociception and sensitization and it is present in the trigeminal system. 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 central sensitization phenomenon.

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. 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. 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.

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

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). 54 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 hour dark-12 hour light cycle.

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 injection of NTG (10 mg/kg bodyweight, Pohl Boskamp). In the third and fourth group, animals received intraperitoneal AEA (2x5 mg/kg bodyweight, Sigma Aldrich) half hour before and one hour after the placebo or NTG treatment. 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 intraperitoneal injection of NTG (10 mg/kg bodyweight, Pohl Boskamp).

Immunohistochemistry

Four hours after the placebo/NTG injection, the rats were perfused transcardially and 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. After cryoprotection, 30 μm thick serial sections were cut from the C1-C2 and were processed for TRPV1, nNOS and NF-κB immunohistochemistry. 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. 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 disector method to calculate the volume densities of the NF-κB-immunoreactive cells.

Western blot analysis

Four hours after the placebo/NTG injection, the rats (n=5 per subgroup) were deeply anaesthetized with chloral hydrate, perfused transcardially and the C1-C2 segments of the cervical spinal cord between (-5) and (-11) mm from the obex and TNC between 1 and (-5) mm from the obex were removed and processed for nNOS, COX-2, β-actin, TDO2, IDO1, KAT-II, KYNU, KMO or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Western blotting.

Results

Immunohistochemistry

On transverse sections of the C1-C2 segments, there were abundant TRPV1-positive fibres, nNOS-immunoreactive neurons, NF-κB-positive cells in the superficial layers of the dorsal horn. The area covered by IR fibres and the number of IR cells did not differ significantly between sections located at the various levels along the rostrocaudal axis or between the right and left dorsal horns of the cervical segments.

The TRPV1-immunoreactive area in the NTG-treated group was significantly higher compared to the placebo-treated group (p<0.05). Furthermore, in the NTG group, the area fraction of nNOS-immunoreactive structures and the volume density of NF-κB-positive cells were significantly higher than in the placebo-treated group (p<0.01), (p<0.05).

AEA treatment resulted in a decrease of TRPV1-immunoreactice area (p<0.05) and nNOS-immunopositive structures (p<0.001), as well. NF-κB-positive cells were also decreased in the AEA injected group (p<0.05).

Western blotting

Western blotting analysis of the C1-C2 region confirmed the results obtained by nNOS immunohistochemistry. Densitometric analyses confirmed that the nNOS bands (155 kDa) were significantly enhanced (p<0.01) in dorsal horn of C1-C2 segments after NTG administration as compared with the placebo-treated animals. This effect of NTG on nNOS was attenuated by treatment with AEA (p<0.05).

A band characteristic of the COX-2 protein was identified at 68 kDa in Western blot assay. Statistical 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).

The TDO2 protein was identified at 50 kDa in Western blot assay. The TDO2 bands were significantly decreased (p < 0.05) in the TNC after NTG administration as compared with the placebo-treated animals.

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.

A band characteristic of the KAT-II protein was referred at 60 kDa in Western blot assay. Statistical 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.

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.

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.

Discussion

TRPV1 is present in the terminals of primary sensory neurons in the dorsal part of spinal cord and co-expressed with CB1. Experimental data indicate that TRPV1 contributes to peripheral sensitization, allodynia and hyperalgesia, central blockade of this receptor is able to attenuate central terminal sensitization. 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 and in inflammatory pain models increased receptor expression was also reported. This indicates that the NTG effect on TRPV1 expression is indirect - it may cause neurogenic inflammation, and the inflammatory mediators - like serotonin (5-HT), bradykinin (BK), etc. - can activate TRPV1 by stimulating trigeminal nociceptive neurons. Pro-inflammatory mediators, such as tumor necrosis factor α, interleukin 1 (IL-1), interleukin 6 (IL-6) and BK enhance TRPV1. In the animal model of inflammation, Complete Freud's adjuvant increases TRPV1 mRNA expression in the dorsal root ganglion, 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, 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.

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. Intrathecal administration of AEA decreases thermal pain sensitivity and its effect can be altered with the TRPV1 antagonist capsazepine. AEA may also cause a desensitization of TRPV1 in skeletal muscle arterioles, 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. Recent report shows that the AEA level changes are able to modulate CGRP mRNA-expression after NTG-treatment in human peripheral blood mononuclear cells. We do not know exactly the role of TRPV1 in the AEA modulated sensitization process, but based on the available

literature data, 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 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. 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. 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. NTG-induced hyperalgesia is associated with a fluctuation of the activity of endocannabinoid system in various brain areas of rats. 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. In addition, Carney and co-workers have detected that cannabinoid agonist downregulated nNOS protein and mRNA in neuronal cells. 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. 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. 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. On the other hand the increase in nNOS expression is accompanied by increased NF-κB expression and activation. Furthermore, Sancho and colleagues have noticed that AEA inhibits tumor necrosis factor-α-induced activation, by inhibition of a cytokine-induced cascade. 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. Tassorelli and her group reported that parthenolide (inhibitor of NF-κB) attenuated NTG-induced c-Fos activation in TNC, 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.

NO may also cause neurogenic inflammation by increasing NF-κB levels, which may lead to the upregulation of COX-2 in inflammatory pain. It is well-known, that NSAIDs, which exert their effects through the inhibition of COX-enzymes, are effective in the treatment of migraine and tension-type headache. 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, nNOS and calmodulin-dependent protein kinase II alpha expression-changes in the TNC. Tassorelli and colleagues have demonstrated that COX-2 expression is increased in the hypothalamus and caudal brain stem after NTG injection, 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. 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,

2006), it is also possible, that the reduction of the inflammatory process is able to downregulate the COX-2 expression.

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 and IDO activity is down-regulated by NO production in bone marrow cells. The other hand, it is also known that the expression of KMO influences NO production in human HEK293 cells. 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.

It is also important to note that kynurenines have a crucial role in immune regulation. The transcriptional expression of IDO, KAT-II, KMO, KYNU is also under the control of interferons, thus pro-inflammatory cytokines influence kynurenine pathway. Lögters and co-workers have shown that the kynurenine-tryptophan ratio was increased in the blood of patients with posttraumatic sepsis, 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, 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.

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.

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. 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, which is important in migraine pathophysiology.

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.

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

I. Nagy-Grócz G, Tar L, Bohár Z, Fejes-Szabó A, Laborc KF, Spekker E, Vécsei L, Párdutz Á.
The modulatory effect of anandamide on nitroglycerin-induced sensitization in the trigeminal system of the rat
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IF: 3.609

II. Nagy-Grócz G, Laborc KF, Veres G, Bajtai A, Bohár Z, Zádori D, Fejes-Szabó A, Spekker E, Vécsei L, Párdutz Á.
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IF: 3.552 (2016)

Cumulative impact factor of the publications directly related to the thesis: 7.161

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Nagy-Grócz G, Zádor F, Dvorácskó S, Bohár Z, Benyhe S, Tömböly C, Párdutz Á, Vécsei L.
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