The role of neurogenic inflammation and estradiol in migraine: animal experimental data

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Chronic 17β-estradiol pretreatment has pronociceptive effect on behavioral and morphological changes induced by orofacial formalin in ovariectomized rats.
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The effect of systemic nitroglycerin administration on the kynurenine pathway in the rat
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III. Nagy-Grócz G, Bohár Z, Fejes-Szabó A, Laborc KF, **Spekker E**, Tar L, Vécsei L, Párdutz Á.
Nitroglycerin increases serotonin transporter expression in rat spinal cord but anandamide modulated this effect.
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IV. Laborc KF, **Spekker E**, Bohár Z, Szűcs M, Nagy-Grócz G, Fejes-Szabó A, Vécsei L, Párdutz Á.
Trigeminal activation patterns evoked by chemical stimulation of the dura mater in rats. J Headache Pain. 2020 Aug 15;21(1):101. doi: 10.1186/s10194-020-01169-4. PMID: 32799798; PMCID: PMC7429748.
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List of abbreviations

AMPA – 2-amino-3-5-metil-3-oxo-1,2-oxazol-4-ilpropánsav

BDNF – brain-derived neurotrophic factor

CGRP – calcitonin gene-related peptide

GPR35 – G-protein coupled receptor 35

IR – immunoreactive

IS – inflammatory soup

KYNA – kynurenic acid

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NGF – nerve growth factor

nNOS – neuronal nitric oxide synthase

NO – nitric oxide

NTG – nitroglycerin

NMDA – N-methyl-D-aspartate

s.c. – subcutaneous

SIF – synthetic interstitial fluid

SP – substance P

TG – trigeminal ganglia

TNC – trigeminal nucleus caudalis

TS – trigeminal system

TRPV1 – transient receptor potential vanilloid-1 receptor

5-HT – 5-hydroxytryptamine

Introduction

Migraine a common neurological disorder, affecting up to 15% of the world's population is characterized by recurrent headaches lasting for 4-72 hours which are associated with allodynia, photo-and phonophobia, and decreased daily activity. Being three times more common in women than men, gonadal hormones, especially estrogen may play an important role in the appearance attacks. The pathomechanism of this disorder is not fully understood, but the activation and the sensitization of the trigeminal system is essential and animal studies where the trigeminovascular system is activated are widely accepted as models of these phenomena. It has been described that as a result of electrical or chemical stimulation the primary trigeminal neurons are activated and neurotransmitters are released from peripheral nerve endings, creating a sterile, neurogenic inflammation in the dura mater that can lead to both peripheral and central sensitization.

Calcitonin gene-related peptide (CGRP) is a multifunctional regulatory neuropeptide and a key player in migraine: serum concentrations of CGRP are elevated during the attack whereas intravenous infusion of CGRP can induce migraine-like headache in migraineurs. CGRP release from trigeminal nerve endings may mediate the inflammation and involved in the generation of mechanical allodynia and hyperalgesia.

Transient receptor potential vanilloid-1 receptor (TRPV1) is a nonselective cation channel, activated by numerous stimuli, such as heat and vanilloids. It is a molecular component of pain detection and modulation. TRPV1 activation leads to the release of neuropeptides, such as substance P and CGRP. These neuropeptides cause vasodilation and initiate neurogenic inflammation within the meninges under experimental conditions leading to activation and sensitization there.

Nitric oxide is synthesized from arginine by neuronal nitric oxide synthase (nNOS), which can be found in the superficial layers of the dorsal horn of the spinal cord underlining its importance in the trigeminal pain processing. It is involved in the inflammatory responses, cell communication, regulation of glutamate release in the spinal cord, and the development of hyperalgesia and allodynia.

There is evidence, that hormonal homeostasis, especially regarding ovarian hormones, plays a major role in the appearance of migraine, however, the underlying mechanisms are not yet known. Estrogen can elicit a pronociceptive effect by activating trigeminal afferents, enhancing glutamatergic tone and increasing the levels of certain neuropeptides such as brain-derived

neurotrophic factor (BDNF) or nerve growth factor (NGF); however, its antinociceptive proprieties have also been described which might be mediated by enhancing serotoninergic or opioidergic systems.

In migraine one of the most commonly used acute therapeutic agents are triptans, which act through 5-$HT_{1B/1D}$ receptors. They exert their effects by blocking vasoconstriction of the meningeal vessels, preventing the release of vasoactive neuropeptides, and blocking the depolarization of the trigeminal nerve inhibiting the neurotransmission in TNC neurons.

One promising molecule in the future migraine therapy is kynurenic acid (KYNA), a neuroprotective product of tryptophan metabolism, synthesized from L-kynurenine which can exert its effect through N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors. Furthermore, KYNA has an agonistic effect of on the G-protein coupled receptor 35 (GPR35), which can be found in the nervous system, with a relevant role in pain processing and neuroinflammation.

One possible way to model the neurogenic inflammation is to deliver inflammatory mediators to the surface of the dura mater. In animal experiments, topical application of IS locally causes vasodilation, and plasma extravasation promotes mast cell degranulation and results in CGRP release through activation of primary nociceptive afferents, similar to changes during a migraine attack.

Orofacial pain can appear during a migraine attack. It can be modeled in animal experiments by subcutaneous (s.c.) injection of diluted formalin into the whisker pad of rodents, which allows the study of both behavioral and neurophysiological aspects of this painful condition. The injection causes local tissue damage, inflammation occurs, and the activation of primary pain-sensing neurons. As a consequence, secondary neurons are activated and sensitization develops.

Aims

We planned to examine the effect of inflammatory soup induced dural inflammation on the selected makers of activation and sensitization (CGRP, TRPV1, nNOS) in the caudal trigeminal nucleus and to test whether pretreatment with sumatriptan or KYNA affects these changes.

The other aim of the study was to investigate trigeminal pain-related behavior and c-Fos immunoreactivity in rats with stable low and stable high estrogen levels in the orofacial

formalin model to acquire further data on the role of estradiol in the sex-related trigeminal nociception.

Materials and Methods

I. Animals

The procedures used in our study were approved by the Committee of the Animal Research of University of Szeged (I-74-49/2017) and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XI./1098/2018, XXIV/352/2012) and followed the guidelines the Use of Animals in Research of the International Association for the Study of Pain and the directive of the European Parliament (2010/63/EU).The animals 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. All efforts were made to minimize the number of animals used and their suffering.

II. Effect of dural application of inflammatory soup

Treatments

After craniotomy, the animals were divided into two groups of 6 rats (n = 6 per group for 2.5 hours and n = 6 per group for 4 hours). The rats in the first group called the placebo group, received synthetic interstitial fluid (SIF, 135 mM NaCl, 5 mM KCl, 1mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM glucose in 10 mM HEPES buffer, pH 7.3). In the second group, we applied inflammatory soup (IS, 1 mM bradykinin, 1 mM serotonin, 1 mM histamine, 0.1 mM prostaglandin in 10 mM HEPES buffer, pH 5.0) on the dural surface. In the third and fourth groups, the animals received s.c. sumatriptan (0.6 mg/kg) 10 minutes before the SIF or IS treatment, while in the fifth and sixth groups received intraperitoneal KYNA (189.17 mg/kg) pretreatment one hour before treatment. Both pretreatment protocols were based on the pharmacological properties of the substances. The dosage we used for both molecules was chosen based on previous reports. Two and a half hours or four hours after the SIF or IS administration, the animals were transcardially perfused.

Immunhistochemistry

Two and a half hours or hours after treatments, the trigemino-cervical complex was removed and processed for CGRP, TRPV1, and nNOS immunohistochemistry. In laminae I and II of

C1-C2, the area covered by CGRP and TRPV1 IR fibers was measured and number of nNOS IR cells were counted.

III. Effect of chronic 17β-estradiol treatment

The animals were ovariectomized, and divided into two groups. In the OVX group, the rats had two 15 mm long silastic capsules filled with cholesterol while in the OVX+E_2 group, the animals received capsules filled with a 1:1 mixture of 17β-estradiol and cholesterol.

Treatments

Both groups (OVX and OVX+E_2) of animals were divided further into two subgroups (n=10–12 per subgroup): In the OVX-Phys and OVX+E_2-Phys subgroups, the animals received a s.c. injection of 50 µl physiological saline solution while in the OVX-Form and OVX+E_2-Form subgroups, the rats were injected with s.c. 50 µl 1.5% formalin solution diluted in physiological saline via a 26-gauge needle into the right whisker pad.

Measurement of estradiol concentration

17β-estradiol concentration of serum was measured in the OVX and OVX+E_2 groups (n=5). The blood samples were taken weekly from the tail vein for 5 weeks and the concentrations were measured by using Estradiol EIA Kit.

Orofacial formalin test

After 10-minute habituation in the test box, the whisker pads of the rats were injected with formalin or physiological saline and the animals were replaced immediately back in the chamber for 45 minutes. The 45-minute recording period was divided into 15×3 minutes blocks and the total time (number of seconds) spent on rubbing directed to the injected area with the ipsilateral fore- or hindpaw was measured in each block and defined as the nociceptive score for that block. The grooming activity of physiological saline-injected animals was used as control based on an earlier study.

Immunohistochemistry

The medullary segment containing the TNC between +1 and –5 mm from the obex was removed and processed for c-Fos immunohistochemistry and the number of c-Fos IR cells were counted.

Results

I. Effect of dural application of inflammatory soup

In both time points IS treatment raised the amount of the area covered by fibers showing CGRP positivity compared to the control group. Pre-treatment with sumatriptan an KYNA was able to mitigate these changes. There was no relevant difference between the two time points in the area covered by fibers showing CGRP positivity.

After 4 hours we observed a significant increase in the amount of the TRPV1 IR fibers and in the number of nNOS IR cells in the IS-treated group compared to the control group. Sumatriptan and KYNA pretreatments were able to modulate the effect of the IS treatment. There was no difference between the results of the left and right side in any levels of the TNC either.

II. Effect of chronic 17β-estradiol treatment

After ovariectomy, the average serum concentration of estradiol in the OVX group was 25.93 pg/ml, while in the OVX+E_2 rats the level was 64.55 pg/ml, which was significantly higher.

Right after the formalin injection, the animals withdrew their heads, associated with vocalization and they rubbed their whisker pads continuously and vigorously with the ipsilateral forepaw accompanied often by the contralateral forepaw and occasionally scraped the perinasal area with the ipsilateral hind paw. As a result of the statistical analysis of the two phases, we found that the face rubbing activity in the OVX-Form and OVX+E_2-Form subgroups was significantly higher during both the first (*$P<0.01$; ***$P<0.001$) and the second phase (***$P<0.001$) than in the OVX-Phys and OVX+E_2-Phys subgroups. In both groups treated with formalin (OVX-Form and OVX + E_2-Form) the biphasic pain-related behavioral pattern can be observed, which was significantly stronger in the 17β-estradiol pretreated group.

In the OVX-Form and OVX+E_2-Form subgroups, the formalin treatment increased the number of c-Fos IR neurons in the dorsal, superficial area of the ipsilateral TNC when compared with the non-treated contralateral side. In the OVX+E_2-Form subgroup, the effect of formalin on the number of c-Fos IR neurons is more pronounced than that in the OVX-Form animals. After statistical analysis, it can be seen that the on the ipsilateral sides chronic 17β-estradiol-induced increase in the formalin-related c-Fos IR is significant (#$P<0.05$; ##$P<0.01$; ###$P<0.001$).

Discussion

I. Effect of dural application of inflammatory soup

In our rat model, IS is topically applied to activate and sensitize the trigeminovascular system, which causes hypersensitivity to mechanical and thermal stimulation and activates the primary and secondary trigeminal neurons followed by the release of neuropeptides (e.g. SP, CGRP) leading to a sterile neurogenic inflammation.

In our study, after two and a half hours, IS was able to increase the area covered by CGRP IR fibers in the dorsal horn of the cervical spinal cord. The cranial dura mater is densely innervated by CGRP IR fibers, thus the increased CGRP level might represent enhanced activation of the primary afferents, which may also be associated with increased CGRP release possibly causing a globally higher turnover e.g. intensive synthesis reflected by higher CGRP expression at the terminals.

Similar to CGRP, IS was able to significantly increase the amount of TRPV1 IR fibers in the dorsal horn after 4 hours. TRPV1 receptors are present in the human trigeminal ganglion (TG) and trigeminal afferents, which innervate the dura mater, and these nerve fibers also contain CGRP. Electrophysiological studies have shown, that TRPV1 is not just activated by capsaicin or painful heat stimulus, but it can be triggered by chemical factors, which are released during inflammation, thus the increased TRPV1 levels in our experimental setting can also be a part of the undergoing sensitization process in the TS.

Also, IS significantly enhanced the number of nNOS IR cells in the dorsal horn. The increase in NO production may contribute to an amplifying process in the meninges, which involves the release of CGRP and possibly prostaglandins and other mediators leading to rapid vasodilatation.

Interestingly, the increase of TRPV1 and nNOS levels are observed later compared to the changes of CGRP, which might reflect, that the changes of the latter are more likely related to the activation of the primary trigeminal nociceptors whereas TRPV1 and nNOS, which are more likely involved in the sensitization, show a delayed pattern of enhancement.

In our study, sumatriptan was able to modulate the effect of IS, successfully reduced the area covered by CGRP and TRPV1 IR fibers and the number of nNOS IR cells probably through 5-$HT_{1B/1D}$ receptors, which confirms the involvement of 5-$HT_{1B/1D}$ receptors in the sensitization process in the TS. These results suggest that 5-$HT_{1B/1D}$ agonism can inhibit IS-induced

activation and sensitization and these receptors are important in the dural inflammatory process.

KYNA also had a similar effect on the examined markers in our experimental setting. This phenomenon may be mediated through several different receptors: NMDA, AMPA, kainate, and metabotropic receptors are found in the TNC and it has been shown, that the antagonists of non-NMDA glutamate receptors also can inhibit the activation of secondary nociceptive neurons. It has been also reported that abnormalities of the kynurenine pathway are associated with headache disorders e.g. there is evidence that serum KYNA levels decrease during cluster headache and chronic migraine.

In the present study sumatriptan, which is a well-known antimigraine drug and KYNA were similarly effective mitigating the effects of the IS. They were likely to exert their effects through different receptors/pathways involved in the activation of the trigeminovascular system pointing to different sites of possible pharmacological modulation during this process with a similar efficacy at least in this animal model.

II. Effect of chronic 17β-estradiol treatment

In our model, the used chronic estradiol pretreatment resulted in an average serum 17β-estradiol level of 61.29 pg/ml in the OVX+E_2 group of rats, comparable with the value of serum concentration estradiol in cycling rats during the proestrus phase when the estrogen is at its peak level. Based on the results obtained in our experiment, the chronic 17β-estradiol treatment was pronociceptive in orofacial formalin test compared with the control, ovariectomized, female rats. In the behavioral test, the effect of estradiol was demonstrated in both phases of the orofacial formalin test, where the first phase is caused by direct chemical stimulation of nociceptors with the formalin solution, while the second phase can be the result of peripheral inflammation. In addition, this chronic estradiol treatment increased the number of c-Fos IR cells, one of the anatomical markers of pain-induced neuronal activity. This effect is probably related to the estrogen-dependent increase in TRPV1 and anoctamin 1 mRNA in the TNC, which play a key role in trigeminal pain sensation.

Conclusion

In our study, IS was able to activate and sensitize the TS - supported by changes in the levels of the selected markers - which might have relevance in migraine pathophysiology. The modulatory effect of sumatriptan supports the role of $5HT_{1B/1D}$ receptors during the neurogenic

inflammation of the dura and during the migraine attack as well. KYNA may have exerted its beneficial effects through its NMDA antagonism, which might be also relevant in these processes.

On the other hand, in a different model of trigeminal activation, the orofacial formalin test chronic estradiol treatment significantly increased the number of c-Fos IR cells and enhanced pain-related behavioral changes, thus having a pronociceptive effect on trigeminal pain and activation of TNC neurons. According to our hypothesis, estrogen can affect pain-induced neuronal processes in several ways: it might increase TRPV1 mRNA and anoctamin 1 in TNC or modulate the NF-κB pathway, but it is also possible, that its effects are due to the activation of extracellular signal-regulated kinase in the TG.

In summary, both acute and chronic modulation can be clearly demonstrated in various rat models of trigeminal activation which shows a multitude of separate pathways involved in this process enabling further different approaches in the future management of migraine patients.

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