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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Nitroglycerin increases serotonin transporter expression in rat spinal cord but anandamide modulated this effect.
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List of abbreviations

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

BDNF – brain-derived neurotrophic factor

CaMKIIα – calcium/calmodulin-dependent protein kinase type II subunit alpha

CFA – Complete Freund's adjuvant

CGRP – calcitonin gene-related peptide

CNS – central nervous system

DR – nucleus raphe dorsalis

DRG – dorsal root ganglion

ER – estrogen receptor

ERα – estrogen receptor alpha

ERß – estrogen receptor beta

ERK – extracellular signal-regulated kinase

FHM – familial hemiplegic migraine

GPR30 – G-protein coupled estrogen receptor 30

GPR35 – G-protein coupled receptor 35

H_2O_2 – hydrogen peroxide

IR – immunoreactive

IS – inflammatory soup

KYNA – kynurenic acid

LC – locus coeruleus

nAChR – α7-nicotinic acetylcholine receptor

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

NGF – nerve growth factor

nNOS – neuronal nitric oxide synthase

NO – nitric oxide

NRM – nuclei nucleus raphe magnus

NTG – nitroglycerin

NMDA – N-methyl-D-aspartate

PAG – periaqueductal grey matter

PBS – phosphate buffered saline

PBST – phosphate-buffered saline containing 1% Triton X-100

s.c. – subcutaneous

SEM - standard error of the mean

SIF – synthetic interstitial fluid

SP – substance P

TG – trigeminal ganglia

TMJ – temporomandibular joints

TNC – trigeminal nucleus caudalis

TS – trigeminal system

TRPV1 – transient receptor potential vanilloid-1 receptor

5-HT – 5-hydroxytryptamine

Summary

A migraine is a neurological condition that can cause various symptoms. Being three times more common in women than men, it has a clear sexual dimorphism, thus estrogen may play an important role in the appearance attacks. Despite continuous progress in migraine research, its exact pathomechanism is still unknown; however, the activation and sensitization of the trigeminal system (TS) play an essential role.

One of the animal models developed to mimic the events during migraine is the topical application of inflammatory soup (IS) on the dura mater. We used this method to evaluate various activation and sensitization markers in the trigeminal nucleus caudalis (TNC) and the possible modulatory effect of sumatriptan. a well-known antimigraine drug acting on $5HT_{1B/1D}$ receptors and kynurenic acid (KYNA), an endogenous molecule of tryptophan metabolism, an N-methyl-D-aspartate (NMDA) antagonist.

We also investigated the effect of chronic 17β-estradiol pretreatment on the trigeminal pain-related behavior and activation of trigeminal second-order neurons at the level of TNC in another model of headache, the orofacial formalin test.

Compared to placebo the topical IS application on the dura increased the expression of the activation and sensitization markers in the TNC, namely calcitonin gene-related peptide (CGRP), transient receptor potential vanilloid-1 receptor (TRPV1), and neuronal nitric oxide synthase (nNOS), which was attenuated by sumatriptan and KYNA, suggesting the involvement of $5\text{-}HT_{1B/1D}$ and NMDA receptors in the development of neurogenic inflammation and thus in migraine attacks.

Our results from the orofacial formalin test demonstrated, that chronic 17β-estradiol treatment had a strong pronociceptive effect on orofacial formalin-induced inflammatory pain, which was demonstrated by both by behavioral changes and increased c-Fos expression in the TNC. This suggests a modulatory action of estradiol on head pain through estrogen receptors, which are present throughout the TS.

Introduction

I. Migraine

Migraine is a common neurological condition that can cause a variety of symptoms besides recurrent headaches, such as allodynia, photo-and phonophobia, and decreased daily activity and can last from 4 to 72 hours without treatment *(IHS, 2013)*. Due to its own pathogenesis and the fact that no other cause can be associated with the disease's development, migraine belongs to the family of primary headache disorders.

The clinical appearance of migraine can be divided into different stages: the prodrome phase, a possible aura, followed by the headache and the recovery stage (postdrome). The prodrome phase typically occurs up to few days before the headache attack, changes in well-being and behavior are experienced, fatigue and impaired concentration occur as frequent complaints. In 25% of the migraineurs a temporary dysfunction of the central nervous system – the aura phenomenon – occurs. The most common symptoms are visual (e.g. visual field disturbances) but sensory or speech disturbances and rarely motor symptoms can be also observed. The typical aura is appearing before or at the beginning of the headache and lasts up to one hour. The headache in migraineurs is moderate or strong and throbbing, lasting 4-72 hours and associated with sensitivity to light/sound and nausea/vomiting. Physical activity worsens the symptoms, the patients seek rest.

Peripheral and central sensitization of the trigeminal nociceptive system is also observed during migraine headaches *(Malick and Burstein, 2000)*. The former means that under the influence of local mediators, the firing of primary sensory nociceptors increases even if their stimulation remain unchanged, which are reflected by the fact that migraine headaches are usually throbbing in nature and intensified by cough or physical activity. The latter is due to the continuous activity of the primary nociceptive neurons causing plastic changes in the nervous system leading to an increased firing of secondary nociceptors. an enhanced receptive field and an activation to non-painful stimuli.

During the attack two-thirds of migraineurs complain about allodynia, a condition when non painful stimuli are perceived as painful *(Selby and Lance,1960; Burstein et al., 2000)*. Allodynia is the clinical manifestation of a central sensitization process *(Burstein et al., 2000)* occurring at the level of TNC in second order trigeminal neurons and sometimes in the thalamus where the third order trigeminal nociceptors can be found. Since its presence

impairs the outcome of the acute treatment of the migraine attack *(Burstein et al., 2004),* the underlying mechanisms of central sensitization have outmost importance.

In the postdrome phase, the headache goes away, but patients usually still complain of weakness or impaired concentration which can last several days. In the post attack period, Bose et al. observed a significant blood flow decrease in most areas of the brain, which may explain the symptoms experienced by patients *(Bose et al., 2018).*

In the 1950s, Ray and Wolff developed the first theory about the pathomechanism of migraine. They believed that migraine pain was caused by extracranial vasodilation, while intracranial vasospasm was responsible for aura symptoms *(Ray and Wolff, 1940).* At that time this was in the line with the pharmacological observations, which showed that amyl nitrate a potent vasodilatator aborted the aura phase, meanwhile ergotamine with vasoconstricive proprieties decreased the headache *(Tunis and Wolff 1953).* Since vascular changes do not explain all the symptoms experienced during migraine attacks new theories emerged about the pathomechanism of the disease.

The most widely accepted theory is focusing on the so-called cortical spreading depression (CSD) first described by Leao *(Leao et al., 1945)*, which may be the equivalent of the aura phenomenon *(Ayata et al., 2010)* playing a role in the development of migraine attacks *(Lauritzen et al., 1994).* During CSD, depolarization following an excitatory wave across the cerebral cortex changes cerebral blood supply, increases tissue metabolism, releases amino acids and nitric oxide in the cortex, which activates nerves running in the dura, thus dilating the dural vessels, leading to sterile inflammation *(Read et al., 1997; Olesen et al., 2000).* Under experimental conditions, CSD can activate secondary trigeminal nociceptors *(Moskowitz et al., 2008)*, suggesting that susceptibility to CSD might be responsible for the appearance of the attack.

There are three known variants of a rare monogenic form of migraine, familial hemiplegic migraine (FHM). All three mutations cause changes in the central nervous system that facilitate the development of CSD *(Kors et al., 2003).* So, it can be assumed that a genetic factors not yet clarified facilitates the development of CSD in classic migraineurs which symptoms are comparable to FHM.

In this context or independently, Weiller and colleagues observed that the dorsolateral pons and the dorsal midbrain involving the nuclei nucleus raphe magnus (NRM), nucleus raphe

dorsalis (DR), locus coeruleus (LC), and the periaqueductal grey matter (PAG) are activated during a migraine attack, which persists even after triptan treatment *(Weiller et al., 1995).* These nuclei can influence TNC activity and they are involved in the transmission of pain. Brainstem serotonergic (NRM and PAG) and adrenergic (LC) nuclei contribute to the activation of the trigeminovascular system *(Edvinsson and Goadsby, 1995).* It is hypothesized that the altered function of these brainstem migraine generators also play a major role in attack development.

There are several theories concerning the mechanism of neurogenic inflammation appearance. Hormonal fluctuations or cortical spreading depression can initiate two types of processes: activating the TS to trigger the liberation of neuropeptides from the peripheral trigeminal afferents and/or degranulating the mast cells that can lead to the release of neuropeptides by activating and sensitizing the nociceptors *(Ramachandran et al., 2018).* In the rat Bolay and colleagues demonstrated that after local electrical stimulation of the cerebral cortex CSD is generated and it can trigger trigeminal activation, which causes meningeal inflammation occurring after the CSD disappearance *(Bolay et al., 2002).*

It is well-known, that the activation and sensitization of the TS plays a crucial role in the pathophysiology of migraine *(Goadsby et al., 2017).* The TS is responsible for processing painful stimuli from the head area; during its activation, neurotransmitters, such as CGRP, substance P (SP), and neurokinin A are released both at the peripheral and central arm of the primary sensory neurons *(Edvinsson et al., 2011).* The neuropeptide release can induce mast cell degranulation and plasma extravasation leading to neurogenic inflammation *(Fusco et al., 2003).* In the meantime activation of the second-order neurons occurs in the TNC *(Carpenter and Sutin, 1983)* and their axons ascend to terminate in the thalamus, and the nociceptive information is projected to the primary somatosensory cortex *(Cross, 1994).*

CGRP is a multifunctional regulatory neuropeptide *(Van Rossum et al., 1997)* and a key player in migraine: serum concentrations of CGRP are elevated during the attack *(Goadsby et al., 1993)* whereas intravenous infusion of CGRP can induce a migraine-like headache in migraineurs *(Lassen et al., 2008).* It has been reported that repetitive electrical stimulation of the dura mater causes an elevation in the expression level of CGRP in the trigeminal system *(Körtési et al., 2019).* CGRP release from trigeminal nerve endings may mediate the inflammation, contributing to the associated pain *(Brain et al., 1997).* Sun and colleagues described that CGRP was involved in the generation of mechanical allodynia and

hyperalgesia *(Sun et al., 2003)* and another study has shown that CGRP expression may be related to photophobia *(Noseda et al., 2011)*.

TRPV1, a nonselective cation channel, a molecular component of pain detection and modulation *(Caterina et al., 2000)*, is selectively expressed by small- to medium-diameter neurons within the dorsal root ganglion (DRG) and trigeminal ganglia (TG), co-localized with CGRP in the latter *(Caterina et al., 2000)*. 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 *(Lin et al., 2007)*. Amaya and colleagues described that after CFA-induced inflammation significant increase in TRPV1 mRNA expression was observed *(Amaya et al., 2003)*.

The synthesis of nitric oxide (NO) is catalyzed by 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 *(Saito et al., 1994)*. Furthermore, its presence is confirmed in dural mast cells, trigeminal nerve endings, and Gasserian ganglion cells *(Berger et al., 1994)*. Systemic administration of nitroglycerin (NTG), a nitric oxide donor can induce an immediate headache and in migraine patients, this is followed by a typical migraine attack without aura *(Sicuteri et al., 1987)*. The early headache can be explained by the vasodilatory effect of NO, which activates the dural nociceptors, while the delayed headache might be mainly due to an effect of NO on central nociceptors, causing a long-lasting endogenous synthesis of NO by enhancing nNOS resulting in a central sensitization process *(Pardutz et al., 2000)*. This can be paralleled with a human experiment, where Akerman et al. described that NTG can cause cranial allodynia in migraine patients *(Akerman et al., 2019)*.

Based on the above, in human studies NTG can activate and sensitize the trigeminal system, and this phenomenon can be seen in animal models as well: in the cervical section of the TNC, NTG reduces the area covered by CGRP-containing fibers, indicating neurotransmitter release and activation of primary trigeminal nociceptors *(Pardutz et al., 2002)*. NTG can increase the c-Fos expression, which reflects the activation of secondary trigeminal neurons and can enhance the nNOS and calmodulin-dependent protein kinase II (CaMKIIα) immunoreactivity, suggesting that central sensitization mechanisms are also present *(Pardutz et al., 2000; 2007)*.

Migraine is three times more common in women than men *(Stovner et al., 2013)*, but this difference only occurs after puberty. Besides this, menstrual cycle (with or without taking

hormonal contraceptives), pregnancy, and menopause may influence migraine incidence *(Stewart et al., 2008)*. MacGregor et al. examined the relationship between estrogen and migraine, and they found that perimenstrual estrogen withdrawal increases the number of migraine attacks *(MacGregor et al., 2006).* Wöber and colleagues found that menstruation can increase migraine prevalence *(Wöber et al., 2007)* and over half of the women with migraine reported a rise in the number of migraine attacks related to the menstrual cycle *(Karlı et al., 2012)*.

Several studies support that hormonal stability during pregnancy is associated with a reduced frequency of migraine attacks *(Sances et al., 2003)*; e.g. 80 percent of women had no migraine attack in the third trimester, almost all women had reported a return of attacks after childbirth *(Granella et al., 1993; Marcus et al., 1999)*. Migraine symptoms are often worsened during the menopausal transition, but they are usually abolished later when the hormonal levels become stable *(MacGregor et al., 2009)*.

This might indicate a strong relationship between primary headaches and hormone homeostasis, particularly related to ovarian hormones, but neither low or high estradiol level seems to be related to increased attack frequency, but rather changes in its level, therefore the exact modulatory mechanism remains to be discovered.

Previous studies have shown that estradiol acts on several neurotransmitters involved in the transmission or inhibition of pain *(Murphy et al., 1998; Foy et al., 1999; Aloisi et al., 2003)*. 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 effect has also been described which might be mediated by enhancing serotonergic or opioidergic systems *(Martin et al., 2008)*.

Numerous studies showed that the two genders perceive pain differently *(Peterlin et al., 2011; Bartley et al., 2013; Maurer et al., 2016)*. Several studies of temporomandibular joint pain demonstrated a higher prevalence in women than men *(Bagis et al., 2012; Shet et al., 2013; Witzel et al., 2015)*. Epidemiological studies find that pain is reported more frequently by women than by men *(Fillingim et al., 2009)* and women have longer lasting, and more severe headaches than men *(Celentano et al., 1990).*

Boes and Levy observed that in a neurogenic inflammation model, dural mast cell activation in intact female rats was consistently higher than in ovariectomized females and intact males, except the proestrus phase (when estradiol concentration is rising) *(Boes and Levy, 2012)*. Contrasting these results, in the NTG-induced neuronal activation model, Greco and colleagues found increased c-Fos activation in the hypothalamus and TNC in female rats in the proestrus phase compared to male rats *(Greco et al., 2013).*

The mechanism responsible for the difference is likely multifactorial and complex, but the gonadal hormones may play a role in nociceptive processing and the development of migraine attacks.

II. Triptans

Despite the recent emergence of several highly effective drugs to relieve migraine attacks, safe treatment has not yet been resolved in all cases. Triptans are selective 5-$HT_{1B/1D}$ receptor agonists and exert their effects by blocking vasoconstriction of the meningeal vessels, preventing the release of vasoactive neuropeptides, and blocking the depolarization of the trigeminal nerve. In addition, they block depolarization of the trigeminal nerve and inhibit neurotransmission in TNC interneurons *(Ferrari and Saxena, 1995)*.

In a human study, single oral doses of sumatriptan were able to reduce the severity of the headache after 30 minutes *(Nappi et al., 1994)*. Burstein et al. found that if sumatriptan is given early during the attack, it inhibits neurotransmission into second-order neurons in the TNC and prevents central sensitization *(Burstein et al., 2005)*. Wang and colleagues demonstrated that sumatriptan 20 mg nasal spray is an effective and well-tolerated treatment for patients with acute migraine attacks, and after 60 minutes, headache relief has occurred in more than half of the patients *(Wang et al., 2007)*.

In an animal model of migraine, after creating neurogenic inflammation in the dura mater, triptans were able to reduce the plasma protein extravasation, probably by inhibiting nociceptor activation and preventing neuropeptide release *(Goadsby et al., 1993)*. The increase of jugular vein CGRP concentration after the stimulation of the TG can be reduced by sumatriptan *(Goadsby et al., 1993, Goadsby et al., 1994)*. In the rat model of trigeminal neuropathic pain, triptans can selectively inhibit nociceptive *(Ottani et al., 2004)* and neuropathic pain behavior *(Kayser et al., 2002)* and evoked activity in trigeminal dorsal horn neurons *(Xiao et al., 2008)* in response to noxious stimulation of the trigeminal nerve area. In the TG, 5-$HT_{1B/1D}$ receptors and glutamate were co-localized in several neurons *(Ma et al., 2001)*, thus triptans may modulate glutamate release from trigeminal neurons through the 5-$HT_{1B/1D}$ receptors and possibly reduce pain *(Kayser et al., 2002)*.

III. Kynurenic acid

The kynurenine pathway is responsible for 95% of tryptophan metabolism *(Wolf, 1974)*, which occurs in the macrophages, microglial cells, astrocytes, and neurons *(Guillemin et al., 2003; 2007)* running parallel to 5-HT synthesis. 3-hydroxykynurenine and 3-hydroxyanthranilic acid may have neurotoxic effects by increasing the oxidative stress and the release of the free radicals *(Dykens et al. 1987; Eastman and Guilarte 1990)*. Another

intermediate product with excititoxic effect is the quinolinic acid, which administration into the striatum cause neuronal death probably via NMDA agonism *(de Carvalho et al. 1996)*.
On the other hand KYNA, another neuroactive product of the kynurenine pathway of tryptophan metabolism seems neuroprotective. KYNA can exert its effect through NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate receptors, and G-protein coupled receptor 35 (GPR35), and these receptors have a relevant role in pain processing and neuroinflammation *(Vécsei et al., 2013)*. A previous study suggests that KYNA has an anti-inflammatory effect on the TS *(Csáti et al., 2015)*. In rats, KYNA had an analgesic effect in the tail-flick test *(Heyliger et al., 1998)*. In an in vivo model of acetic acid-induced inflammatory pain, L-kynurenine, a precursor of KYNA, caused rise in the KYNA levels in the plasma and the central nervous system (CNS), thereby was able to elicit an anti-nociceptive effect *(Pineda-Farias et al., 2013)*.

IV. Animal models of neurogenic inflammation

The concept of neurogenic inflammation was introduced by the experiment of Goltz and Bayliss, in which skin vasodilation was observed during electrical stimulation of the dorsal horn, which could not be linked to the immune system *(Chiu et al., 2012)*. Dalessio was the first, who hypothesized a link between neurogenic inflammation and migraine and believed that headache is a result of vasodilation of cranial vessels associated with local inflammation *(Dalessio et al., 1974)*. This theory was later reworked by Moskovitz, who believed that upon activation, the neuropeptide release from trigeminal neurons has a role in increasing vascular permeability and vasodilation *(Moskowitz et al., 1984)*.

Chemical activation of meningeal trigeminal is possible in animal experiments. To activate the TS in the rat it is possible to place inflammatory mediators directly on the surface of the dura mater *(Phebus et al.. 2001; Andreou et al. 2010)* and it has been shown that trigeminal brainstem neurons have been sensitive to both subarachnoid superfusion and topical IS administration *(Burstein et al. 1998; Ebersberger et al. 1997)*.

One of the characteristic symptoms of migraine headaches is the development of allodynia, which can also be interpreted as a manifestation of central sensitization. This phenomenon can also be achieved by the chemical stimulation of the dura mater e.g. IS given between the skull and dura of free-moving, alert rats, repeated once two hours later, developed facial allodynia *(Wieseler et al. 2010)*.

V. Orofacial formalin model

The orofacial pain is appearing in primary headaches, such as migraine *(Nixdorf et al., 2008; Sokolov et al., 2012)* can be modeled by subcutaneous (s.c.) injection of diluted formalin in the whisker pad of the rodents, which allows the investigation of both behavioral and neurophysiological aspects of this pain condition. The injection causes actual tissue damage, inflammation occurs and the primary, then secondary pain-responsive neurons are activated and sensitization develops.

The typical time course of the response to a such formalin administration is biphasic, with an early and short-lasting first phase due to the direct chemical stimulation of the nociceptors usually appearing 3-5 minutes after the injection. After a short quiescent period, this is followed by a second prolonged phase which starts around the 15^{th} minute and lasts for approximately 40 minutes and is related to the formalin-induced inflammation *(Clavelou et al., 1995)*. Taken together the orofacial formalin model is also able to model two aspects of migraine, the activation and sensitization of the TS.

Aims

1. 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 TNC and to test whether pretreatment with sumatriptan or KYNA affects these changes.
2. The other aim of the study was to investigate trigeminal pain-related behavior and c-Fos IR – a marker for neuronal activity - in the rat TNC 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

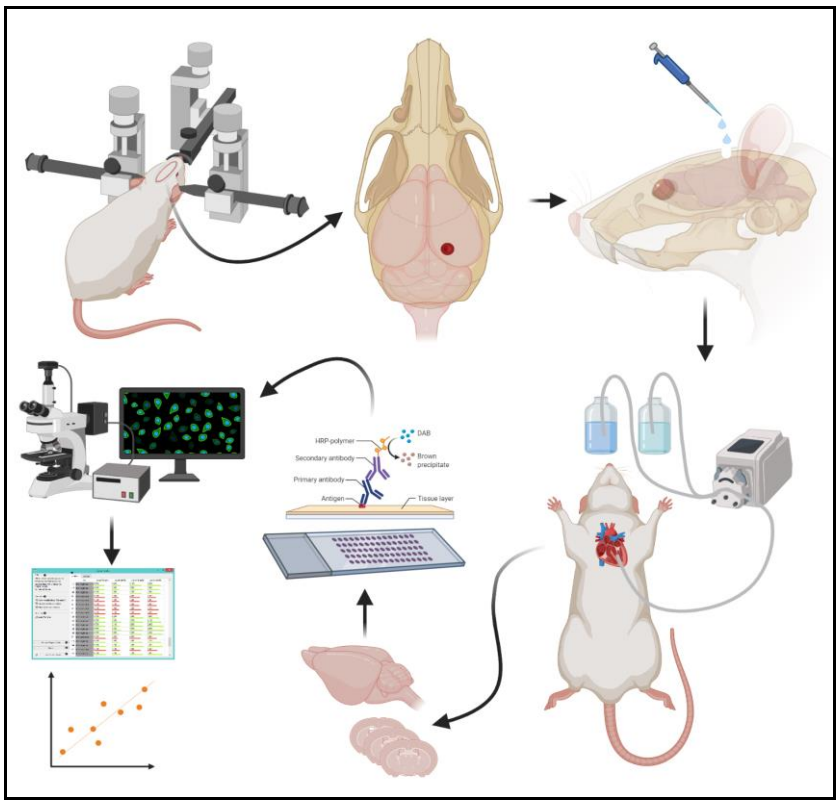
I. Animals

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) 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

Craniotomy

Seventy-two adult male Sprague-Dawley rats weighing 350-400 g were used. 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 animals were deeply anesthetized with an intraperitoneal injection of 4 % chloral hydrate (0.4 g/kg body weight, Sigma-Aldrich). The head of the animal was fixed in a stereotaxic frame and lidocaine (10 mg/ml, Egis) infiltration on the skull was performed before the interventions. A handheld drill was used to make a window on the skull. The hole was made posterolaterally (5 mm, 3 mm) to the bregma, on the right side without penetrating the dura mater.

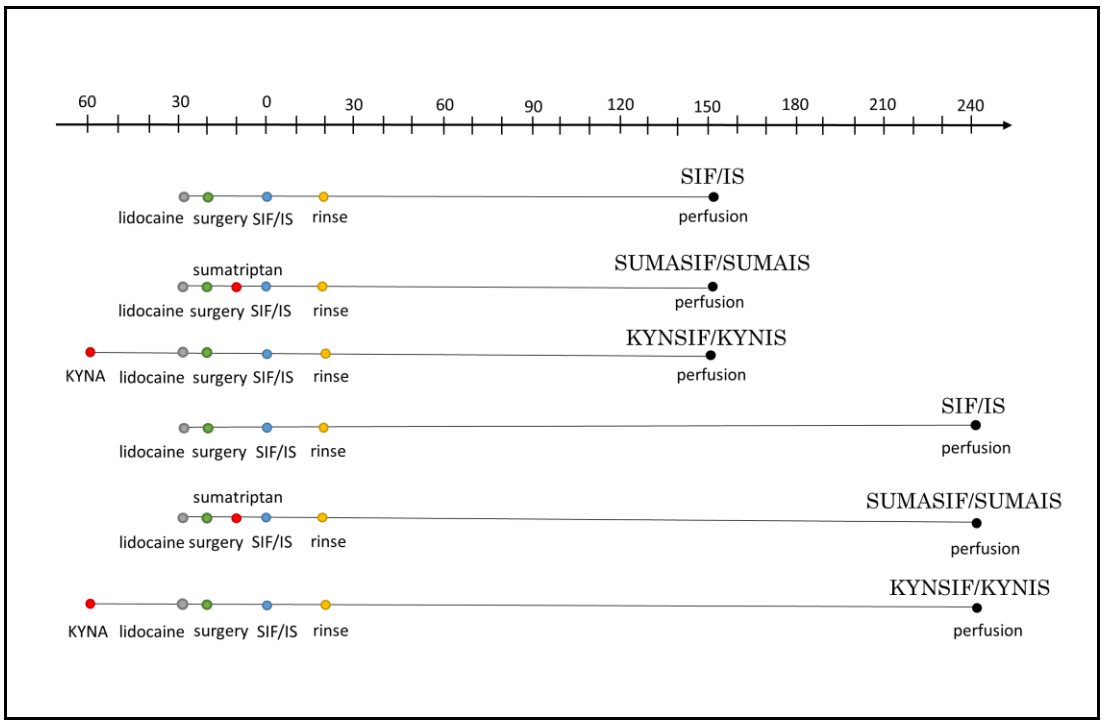


1. Figure Experimental setting

Following the craniotomy, the animals received SIF or IS treatment. After two and a half hours or four hours after the SIF or IS administration, the trigemino-cervical complex was processed for CGRP. TRPV1 and nNOS immunohistochemistry. We measured the covered area by the CGRP and TRPV1 IR fibers and counted the nNOS IR cells in the area of the dorsal horn, then statistical analyses were performed. (Created with BioRender.com)

Treatments

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 sc. 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 trigemino-cervical complex was processed for immunohistochemistry. Two survival times were used to examine changes over time.



2. Figure Schematic timeline of the experimental design.

The SIF or IS treatment was considered as "0" timepoint. The animals received lidocaine before the skin incision. 20 min after dural treatment, the surface of the dura was rinsed off with SIF. In the SUMASIF/SUMAIS groups, the animals got s.c. sumatriptan pretreatment 10 minutes before the SIF/IS treatment while In the KYNSIF/KYNIS groups, the animals received intraperitoneal KYNA pretreatment one hour before treatment. Two and a half hours or four hours after the dural treatment, the rats were thranscardially perfused.

Immunohistochemistry

Two and a half hours or four hours after SIF or IS administration, the animals were perfused transcardially with 50 ml phosphate-buffered saline (PBS, 0.1 M, pH 7.4), followed by 200 ml 4% paraformaldehyde in phosphate buffer under chloral hydrate anesthesia and the trigemino-cervical complex was 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 (CGRP staining) or PBS (nNOS and TRPV1 staining) for 30 minutes. After several rinses in PBS containing 1% Triton X-100, sections were kept overnight at room temperature in anti- CGRP antibody (Sigma, C8198) at a dilution of 1:20000, or TRPV1 antibody (Santa Cruz, s.c.28759) at a dilution of 1:1000, or for two nights at 4°C in anti-nNOS antibody (EuroProxima, 2263B220-1) at a dilution of 1:5000. The immunohistochemical reaction was visualized by the Vectastain Avidin-Biotin kit of Vector Laboratories (PK6101), and nickel ammonium sulfate-intensified 3,3'-diaminobenzidine. Control experiments included the omission of the primary antisera.

Data evaluation

All evaluations were performed by an observer blind to the experimental groups. The photomicrographs of the stained sections of trigemino-cervical complex were taken using a Zeiss AxioImager microscope supplied with an AxioCam MRc Rev. 3 camera (Carl Zeiss Microscopy). The area covered by TRPV1 immunoreactive (IR) and CGRP IR fibers 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. The program calculated the area innervated by the IR fibers as the number of pixels with densities above the threshold; the data were expressed as area fractions (%) of the corresponding immunolabelled structures. We measured the covered area by the CGRP and TRPV1 IR fibers and counted the nNOS IR cells in the area of the dorsal horn innervated by the ophthalmic nerve (V/1 area).

Statistical analysis

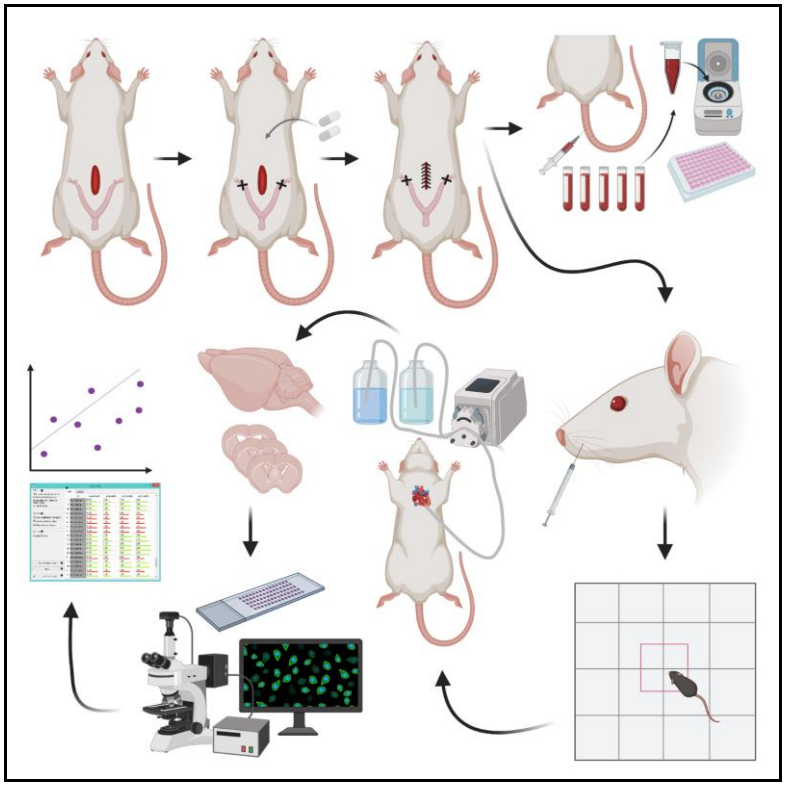
The Shapiro-Wilk test was used to determine the distribution of data. As our data followed a normal distribution in each case, the differences among the groups and sides were examined with a mixed ANOVA model. The pairwise comparisons were performed by paired and independent samples t-tests with Sidak corrections. All statistical analyses were performed using SPSS version 24.0 (IBM Corporation). Values $p<0.05$ were considered statistically significant. Our data are reported as means + standard error of the mean (SEM) for all parameters and groups.

III. Effect of chronic 17β-estradiol treatment

Ovariectomy

Fifty-five female Sprague Dawley rats weighing 150–250 g were used. The animals were ovariectomized under deep chloral hydrate (0.4 g/kg body weight, catalog ID: 23100; Sigma-Aldrich, St. Louis, MO, USA) anesthesia administered intraperitoneally. Prior to surgery, rats' back were shaved with electric clippers and furs were removed completely. Cutasept was applied to the shaved area to disinfect the skin. Ovariectomy was preceded by a midline dorsal skin incision, 3 cm long, approximately half way between the middle of the back and the base of the tail. 1.5 cm long peritoneal incisions were made bilaterally. After access into the peritoneal cavity, the ovary and associated fat were easily found, and exteriorized by

gentle retraction. Ligature of the blood vessels was also performed. The connection between the Fallopian tube and the uterine horn was cut and the ovaries were removed. Afterward, the animals were randomly divided into two groups: 1) In the OVX group, the rats had two 15 mm long silastic capsule (3.18 mm outer diameter and 1.57 mm inner diameter, catalog ID: 508–008; Dow Corning, Midland, Michigan, USA) filled with cholesterol (15 mg, catalog ID:C8667; Sigma-Aldrich) as control. 2) In the OVX+E_2 group, the animals received two 15 mm long silastic capsule filled with a 1:1 mixture of 17β-estradiol (7.5 mg, catalog ID: 75262; Fluka, Sigma-Aldrich) and cholesterol (7.5 mg), which provide a constant elevated serum estradiol level *(Smith et al., 1977)*. Capsules were inserted subcutaneously in the interscapular region. After implantation of capsules, peritoneal cavity and skin were closed with absorbable sutures. High degree of aseptic procedure was maintained throughout the operation. Surgical instruments were sterilized in 70% ethanol. During and after the surgery, animals were placed on heating plate and covered with paper in order to avoid hypothermia. The analgesia and attenuation of inflammation were provided by sc. administration of carprofen (5 mg/ kg body weight) three times: once before the operation and twice after the surgery (24 and 48 hours).



3. Figure Experimental setting

Following ovariectomy, animals received physiological saline or formalin treatment, then we examined the pain-related behavior. Four hours after the formalin or physiological saline injection, the rats were perfused transcardially, and the TNC was removed for immunohistochemistry, then we counted the c-Fos IR cells and statistical analysis was performed. (Created with BioRender.com)

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 sc injection of 50 µl physiological saline administered via a 26-gauge needle into the right whisker pad after 3 weeks of recovery following the ovariectomy. In the OVX-Form and OVX+E_2-Form subgroups, the rats were injected with s.c. 50 µl 1.5% formalin solution (containing 0.55% formaldehyde) diluted in physiological saline via a 26-gauge needle into the right whisker pad. According to Clavelou et al., this concentration is the most appropriate to detect changes in pain-related behavior of rats *(Clavelou et al.,1995)*.

Measurement of estradiol concentration

17β-estradiol concentration of serum was measured in both groups (n=5). The blood samples were taken weekly from the tail vein for 5 weeks. The serum was cleared from cellular components of the blood by centrifugation at 12,000 rpm for 10 minutes at 4°C and stored at –80°C until use. The concentrations were measured by using Estradiol EIA Kit (catalog ID: 582251; Cayman Chemical Company, Ann Arbor, MI USA) based on the guidelines of the manufacturer.

Behavior test

The testing procedures were performed during the light phase (between 8 a.m. and 2 p.m.) in a quiet room. The test box was a 30×30×30 cm glass terrarium with mirrored walls. For the off-line analysis of rubbing activity directed to the whisker pad, the behavior of the individually tested rats was recorded with a video camera (Logitech HD Webcam C615; Logitech Inc., Newark, NJ, USA) situated 1 m above the terrarium. After 10 minutes 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 rats did not receive any food or water during the observation period. The test box was cleaned and decontaminated after each animal. An observer blind to the experimental procedures analyzed the recorded videos. 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

Four hours after the formalin or physiological saline injection, the rats were perfused transcardially with 50 ml of phosphate-buffered saline, followed by 200 ml of 4% paraformaldehyde in phosphate buffer under deep chloral hydrate anesthesia. The medullary segment containing the TNC between +1 and –5 mm from the obex was removed, postfixed overnight for immunohistochemistry in the same fixative and cryoprotected (10% sucrose for 2 hours, 20% sucrose until the blocks sank and 30% sucrose overnight). Before sectioning, each segment was marked with a small incision on the ventral and left (contralateral) side of the tissue block, allowing side discrimination during the quantification process; 30 μm transverse cryostat sections were cut through the rostrocaudal axis from the beginning of the TNC and were serially collected in wells containing cold PBS. Each well contained every tenth section at 0.3 mm intervals along the rostrocaudal axis (15 levels sections/well). The free-floating sections were rinsed in PBS and immersed in 0.3% H_2O_2 in PBS for 30 minutes to suppress endogenous peroxidase activity. After several rinses in PBS containing 1% Triton X-100 (PBST), sections were incubated at room temperature overnight in PBST containing rabbit anti-rat c-Fos polyclonal antibody (catalog ID: sc-52, RRID: AB_2106783; Santa Cruz Biotechnology Inc., Dallas, TX, USA) at a dilution of 1:2000. The immunohistochemical reaction was visualized by using Vectastain Elite Avidin-Biotin Kits (catalog ID: PK6101; Vector Laboratories, Burlingame, CA, USA). Briefly, the sections were incubated at room temperature for 2 hours in PBST containing goat anti-rabbit biotinylated secondary antibody. After several rinses in PBST, and incubation at room temperature for 2 hours in PBST containing avidin and biotinylated horseradish peroxidase, the immunohistochemical labeling was visualized with 3,3′-diaminobenzidine intensified with nickel ammonium sulphate. The specificity of the immune reactions was checked by omitting the primary antiserum.

Cell counting

The counting of IR cells in the TNC was performed by an observer blinded to the experimental procedures under the 10× objective of a Nikon Optiphot-2 light microscope in every tenth transverse section in each animal. Before the counting, the location of each section along the rostrocaudal axis and the location of the TNC on each medullary section were determined by means of The Rat Brain in Stereotaxic Coordinates Atlas.23 The c-Fos neurons with obvious specific nuclear staining were counted in the TNC both ipsilaterally and contralaterally to the formalin or physiological saline injection.

Statistical analysis

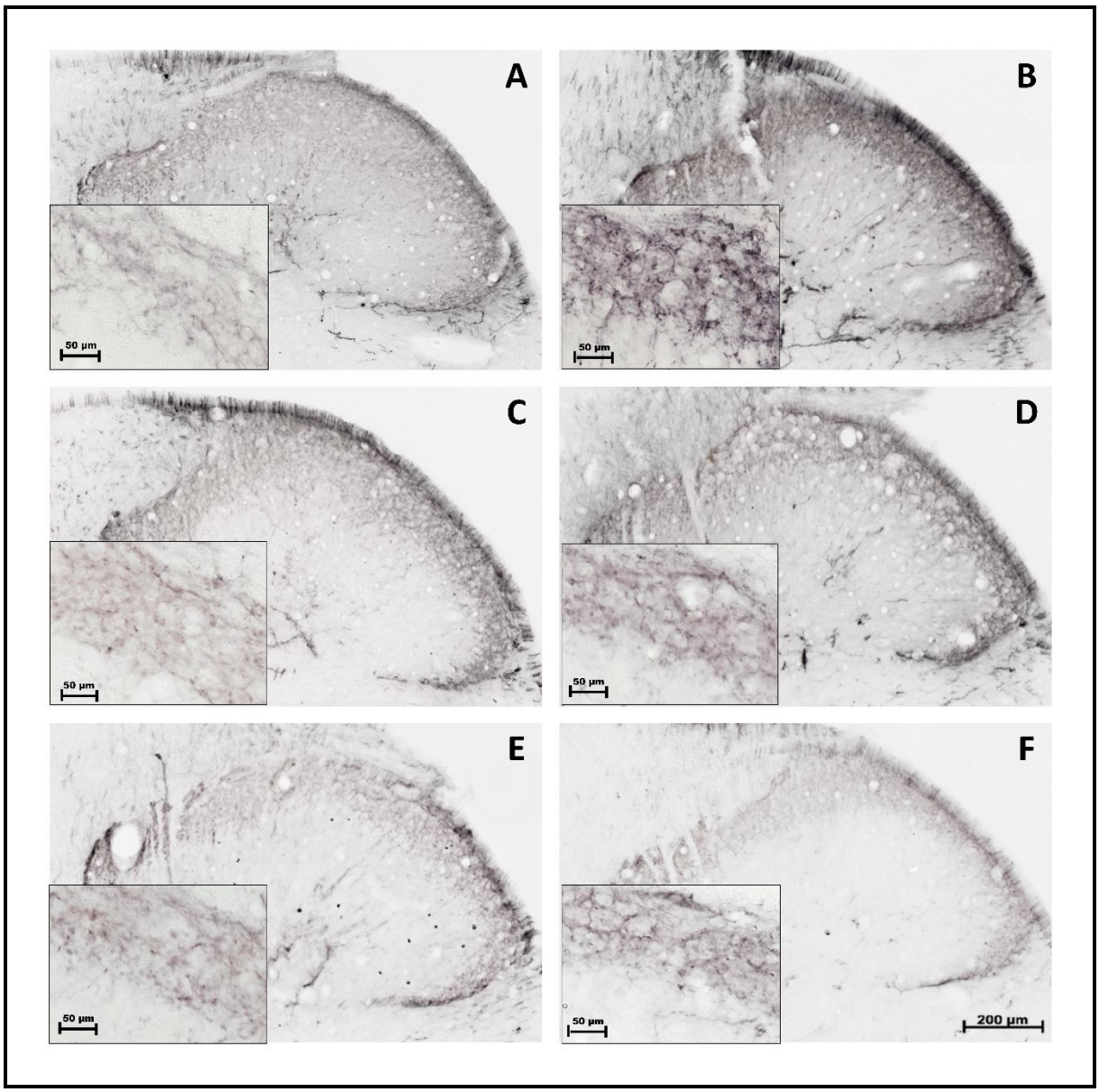
For statistical comparison of 17β-estradiol concentration of serum in the two groups (OVX and OVX+E_2), we used two- way repeated-measures ANOVA. Pairwise comparisons of group means were based on the estimated marginal means with Sidak adjustment for multiple comparisons. In the behavioral study, we compared the nociceptive responses in two time periods as described by Clavelou et al *(Clavelou et al.,1995)*. The first 3 minutes (block 1) are characterized by intensive rubbing activity and defined as the first phase. Following a relatively relaxed period, the rubbing intensifies again between about 12 and 45 minutes (second phase) and remains high for a longer period of time (blocks 5–15), which is defined as the second phase. For the comparison of the rubbing activities between subgroups (OVX-Phys, OVX-Form, OVX+E_2-Phys and OVX+E_2-Form) in the first and second phase, we used one-way ANOVA followed by the Tamhane post hoc test. The number of c-Fos IR neurons in the various subgroups (OVX-Phys, OVX-Form, OVX+E_2-Phys and OVX+E_2-Form) were compared at each level of 0.3 mm (15 levels) along the rostrocaudal axis by using two-way repeated-measures ANOVA. There was no significant difference in the number of c-Fos IR neurons between the contra- and ipsilateral sides in rats injected with s.c. physiological saline and the contralateral sides in animals injected with s.c. formalin (OVX-Form and OVX+E_2-Form); therefore, the data obtained from the contralateral sides of the subgroups injected with s.c. formalin (OVX-Form and OVX+E_2-Form) were used as controls in the statistical analysis. Pairwise comparisons of subgroup means were based on the estimated marginal means with Sidak adjustment for multiple comparisons. All tests were two-sided, and probability levels $P<0.05$ were considered to be statistically significant. Group values are reported as mean±SEM. Statistical analysis of measurements was carried out with IBM SPSS Statistics, version 20 software (IBM Corporation, Armonk, NY, USA).

Results

I. Effect of dural application of inflammatory soup

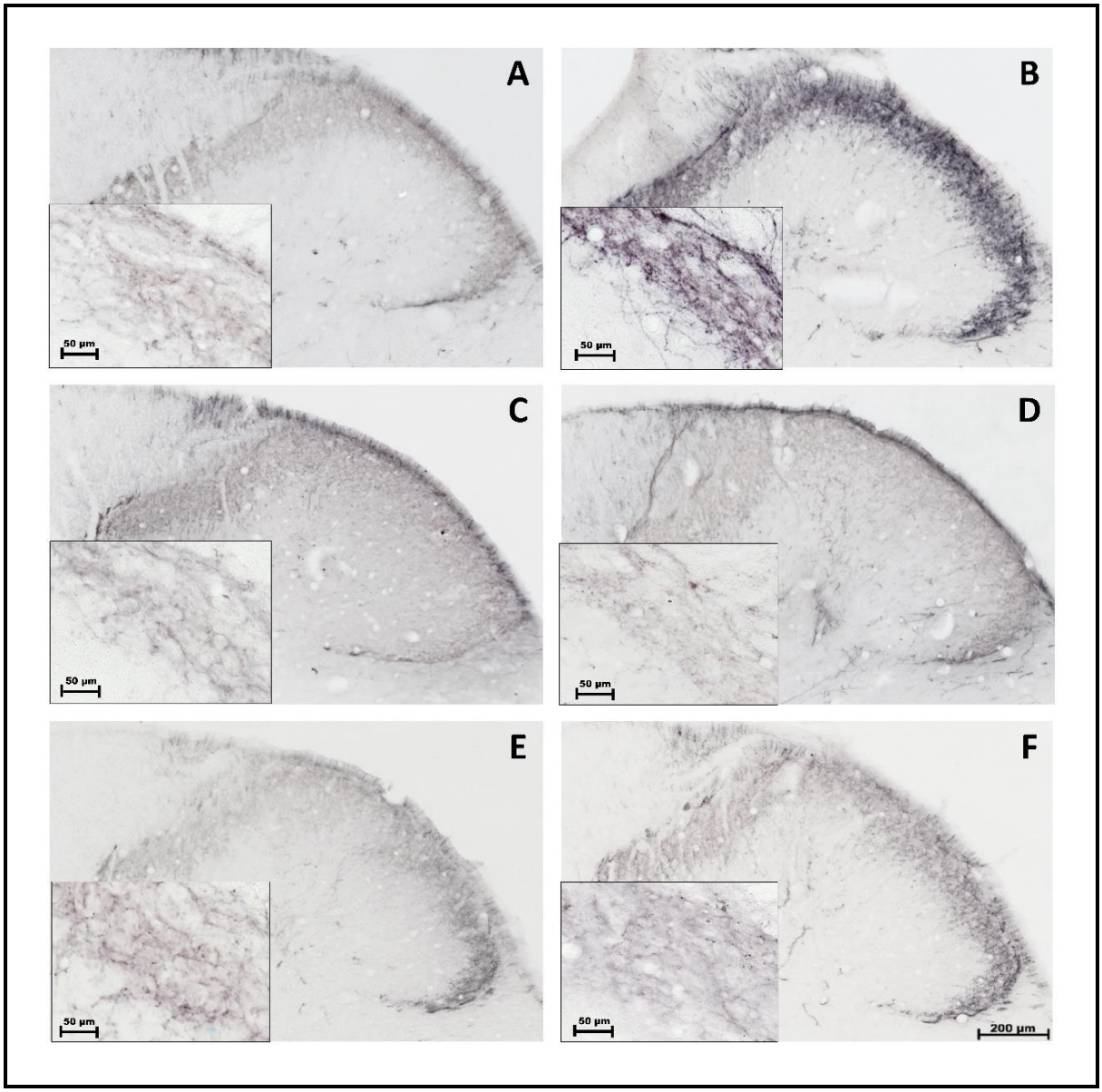
Changes in the CGRP expression after inflammatory soup treatment

In the dorsal horns CGRP IR axon fibers were distributed in the laminae I and II. The area covered by CGRP IR fibers did not differ significantly between sections located at the various levels along the rostrocaudal axis nor between the right and left dorsal horns of the cervical segments. 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 was able to mitigate these changes. Similar to sumatriptan, KYNA was also able to attenuate the effect of IS. There was no relevant difference between the two time points in the area covered by fibers showing CGRP positivity.



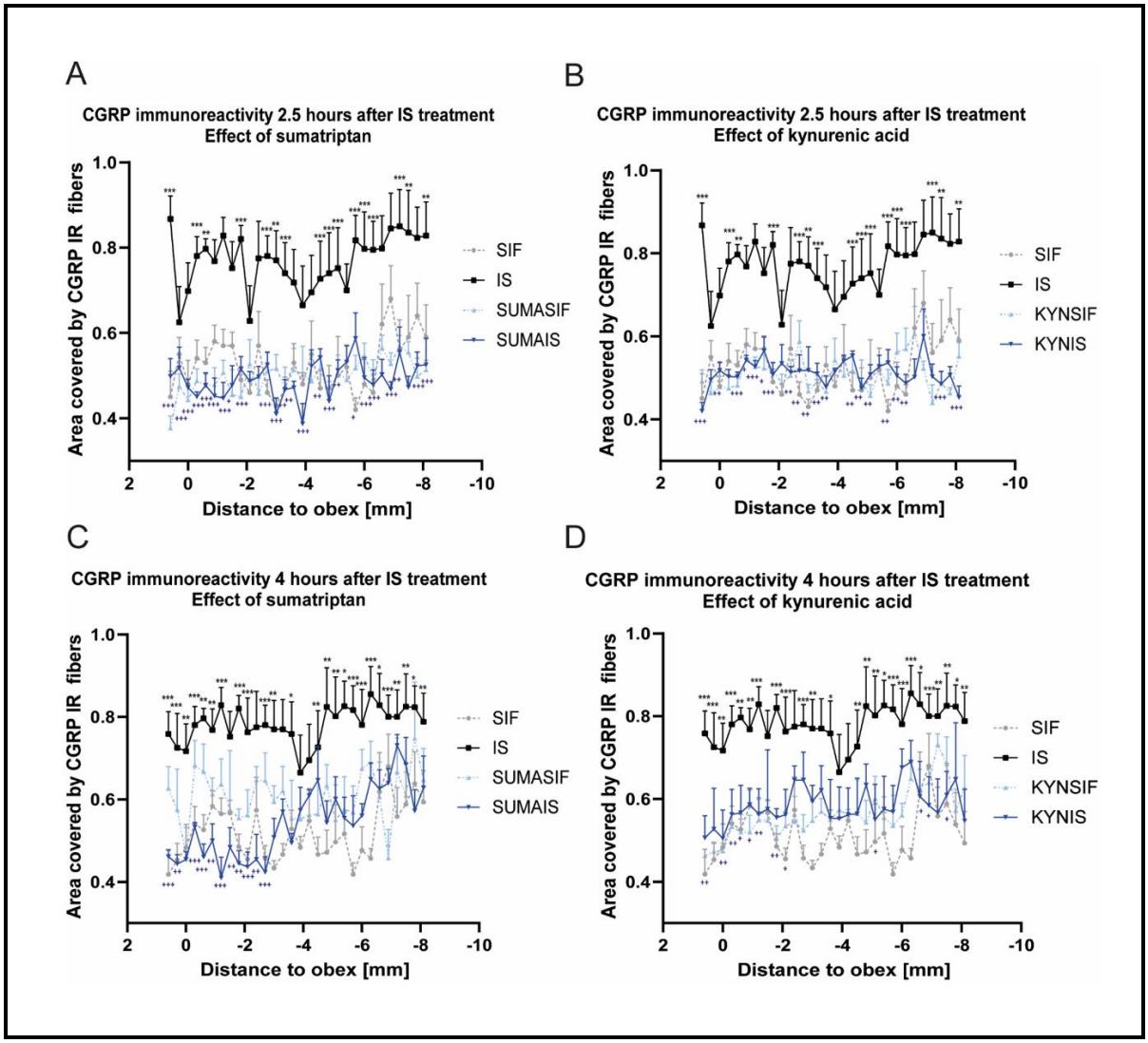
4. Figure CGRP immunoreactivity 2.5 hours after IS treatment

Representative photomicrographs of the CGRP expression in the trigemino-cervical segments after 2.5 hours. (A) – SIF, (B) – IS, (C) – SUMASIF, (D) – SUMAIS, € – KYNSIF, (F) – KYNIS. There is no difference between the groups. In the IS group, the CGRP staining was stronger than in the placebo group. Sumatriptan and kynurenic acid were able to attenuate this effect. SIF: synthetic interstitial fluid, IS: inflammatory soup, SUMASIF: sumatriptan + synthetic interstitial fluid, SUMAIS: sumatriptan + inflammatory soup, KYNSIF: kynurenic acid + synthetic interstitial fluid, KYNIS: kynurenic acid + inflammatory soup. Scale bars: 200 µm, 50 µm.



5. FigureCGRP immunoreactivity 4 hours after IS treatment

Representative photomicrographs of the CGRP expression in the trigemino-cervical segments after 4 hours. (A) – SIF, (B) – IS, (C) – SUMASIF, (D) – SUMAIS, € – KYNSIF, (F) – KYNIS. There is no difference between the groups. In the IS group, the CGRP staining was stronger than in the placebo group. Sumatriptan and kynurenic acid were able to attenuate this effect. SIF: synthetic interstitial fluid, IS: inflammatory soup, SUMASIF: sumatriptan + synthetic interstitial fluid, SUMAIS: sumatriptan + inflammatory soup, KYNSIF: kynurenic acid + synthetic interstitial fluid, KYNIS: kynurenic acid + inflammatory soup. Scale bars: 200 µm, 50 µm



6. Figure. Statistical analysis of CGRP staining 2.5 and 4 hours after IS treatment

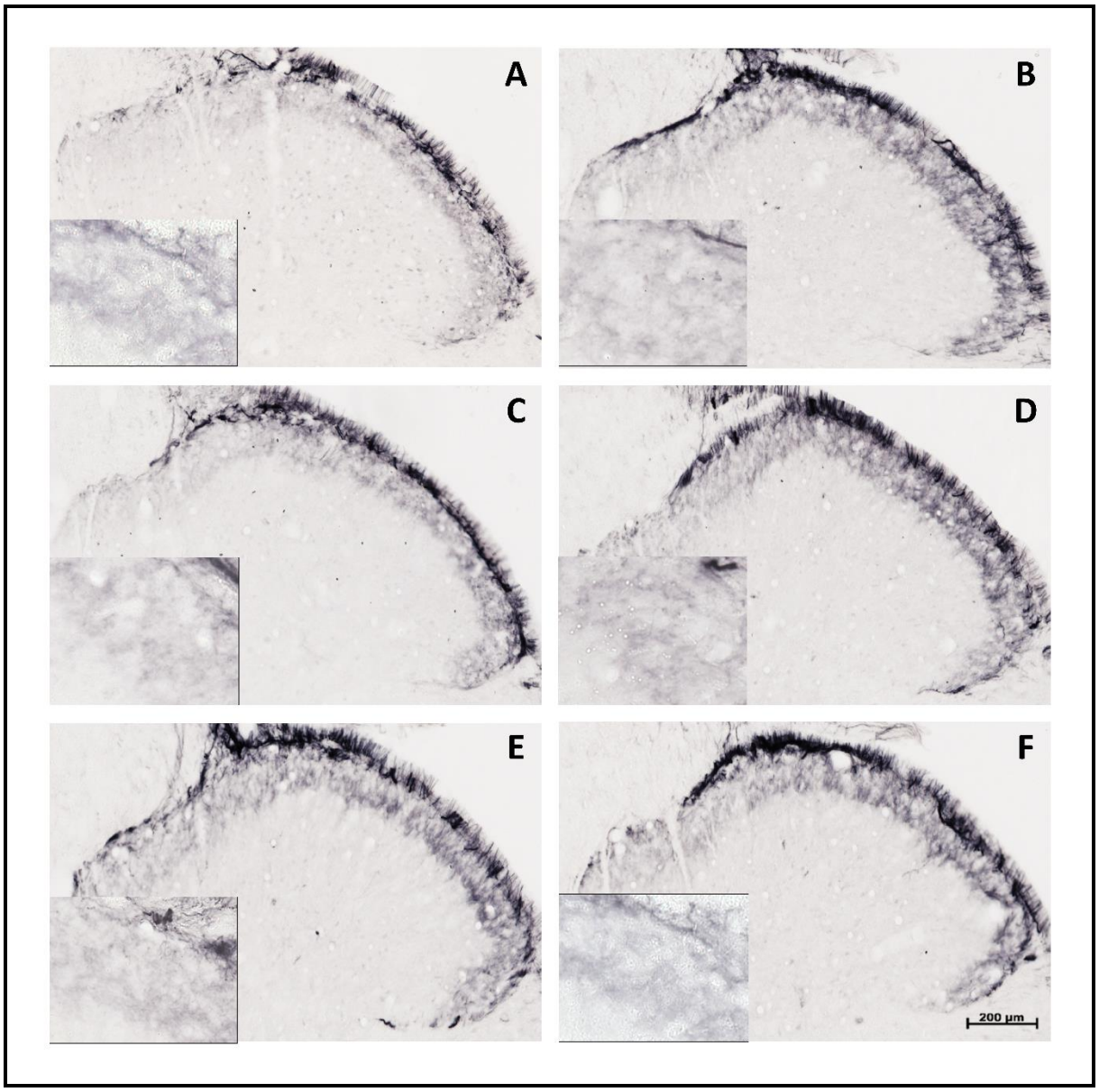
The quantitative analysis shows that in the IS group the area covered by fibers showing CGRP positivity is significantly higher than in the control group in both timepoints. (A) 2.5 hours after IS treatment, sumatriptan was able to attenuate this effect in the V/1 area. (B) Similar to sumatriptan kynurenic acid weakened the effect of IS in the V/1 area. (C) 4 hours after IS treatment, sumatriptan was able to mitigate this effect in the and V/1 area. (D) Kynurenic acid decreased the effect of IS in the V/1 area.

**p<0.05; **p<0.01, ***p<0.001 * means SIF-IS differences, + means IS-SUMA/KYNA. SIF: synthetic interstitial fluid, IS: inflammatory soup, SUMASIF: sumatriptan + synthetic interstitial fluid, SUMAIS: sumatriptan + inflammatory soup, KYNSIF: kynurenic acid + synthetic interstitial fluid, KYNIS: kynurenic acid + inflammatory soup*

The effect of inflammatory soup on the TRPV1 expression in the TNC

The transverse sections of the C1-C2 demonstrated TRPV1-positive fibers in the superficial layers (laminae I-II) of the dorsal horn. No significant difference was observed in staining along the rostrocaudal axis. After two and a half hours we have not seen considerable difference between the IS treated and placebo group, however after 4 hours we observed a significant increase in the amount of the TRPV1 IR fibers in the IS-treated group compared to the control group. Sumatriptan and KYNA pretreatments were able to modulate the effect

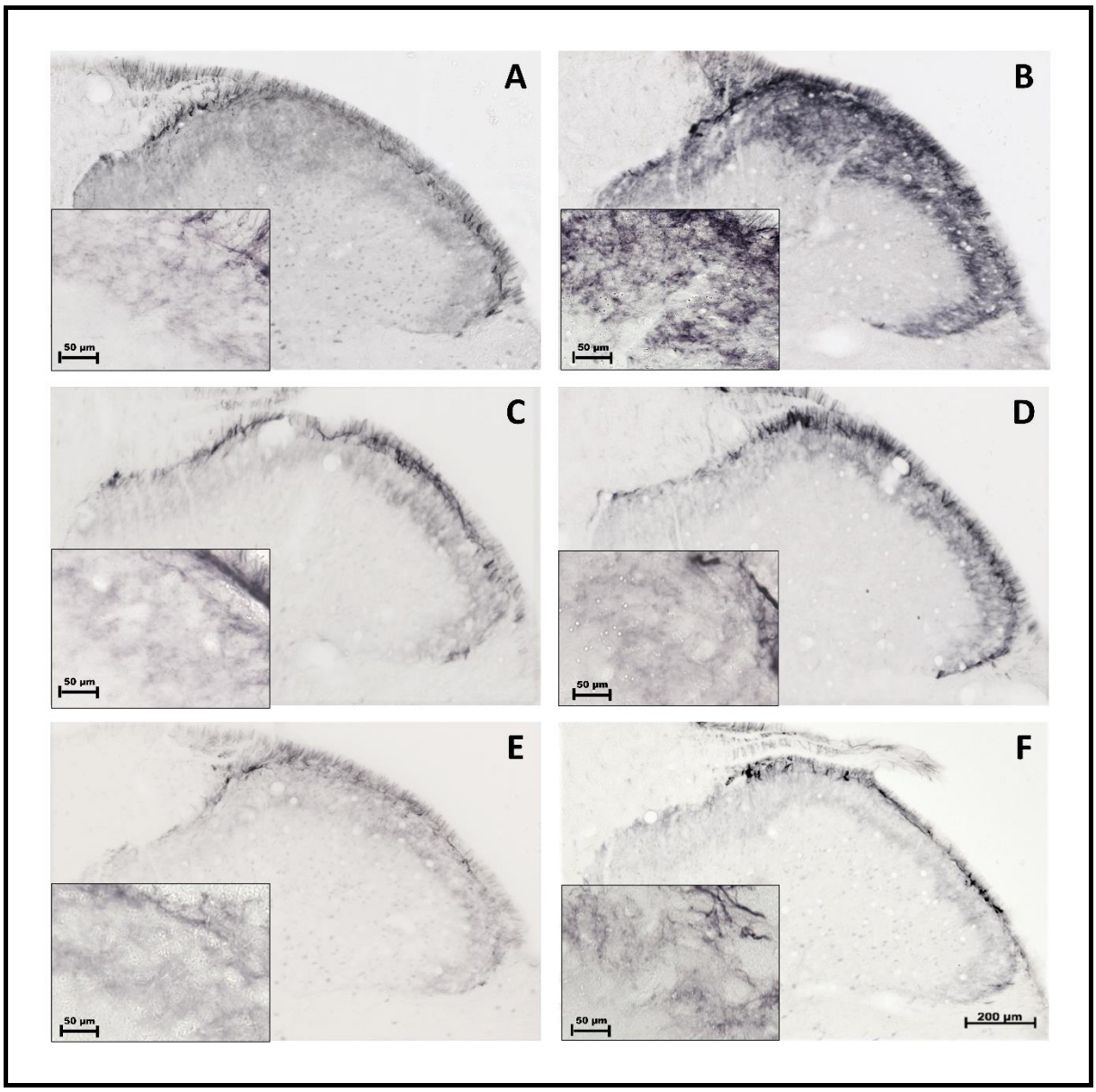
of the IS treatment. There was no difference between the results of the left and right side in any levels of the TNC either.



7. Figure TRPV1 immunoreactivity 2.5 hours after IS treatment

Representative photomicrographs of the TRPV1 expression in the trigemino-cervical segments after 4 hours. (A) – SIF, (B) – IS, (C) – SUMASIF, (D) – SUMAIS, € – KYNSIF, (F) – KYNIS. There is no difference between the groups.

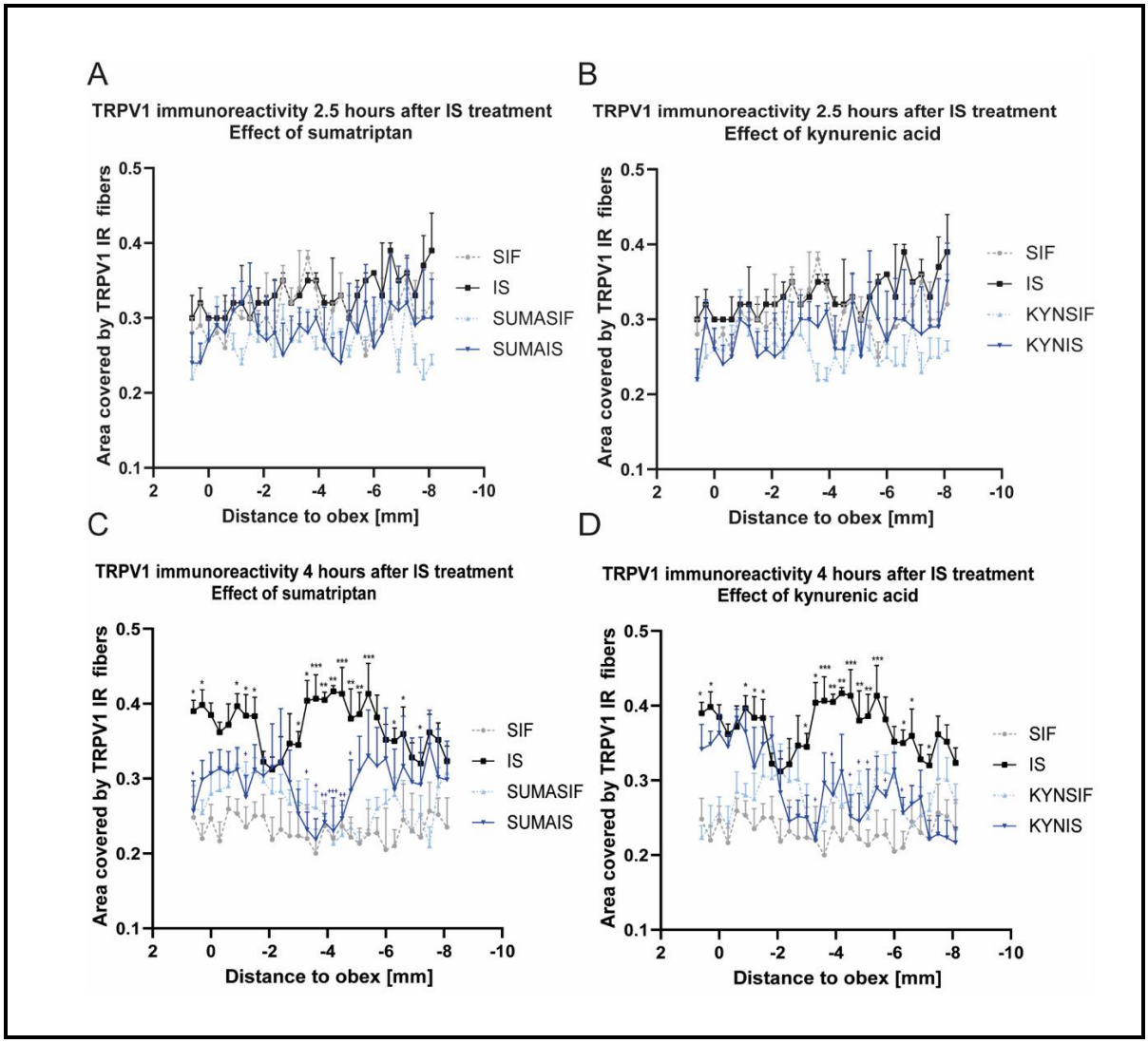
SIF: synthetic interstitial fluid, IS: inflammatory soup, SUMASIF: sumatriptan + synthetic interstitial fluid, SUMAIS: sumatriptan + inflammatory soup, KYNSIF: kynurenic acid + synthetic interstitial fluid, KYNIS: kynurenic acid + inflammatory soup. Scale bars: 200 µm, 50 µm



8. Figure TRPV1 immunoreactivity 4 hours after IS treatment

Representative photomicrographs of the TRPV1 expression in the trigemino-cervical segments after 4 hours. (A) – SIF, (B) – IS, (C) – SUMASIF, (D) – SUMAIS, € – KYNSIF, (F) – KYNIS. In the IS group, the area covered by TRPV1 was higher than in the placebo group. Sumatriptan and kynurenic acid were able to attenuate this effect.

SIF: synthetic interstitial fluid, IS: inflammatory soup, SUMASIF: sumatriptan + synthetic interstitial fluid, SUMAIS: sumatriptan + inflammatory soup, KYNSIF: kynurenic acid + synthetic interstitial fluid, KYNIS: kynurenic acid + inflammatory soup. Scale bars: 200 µm, 50 µm.



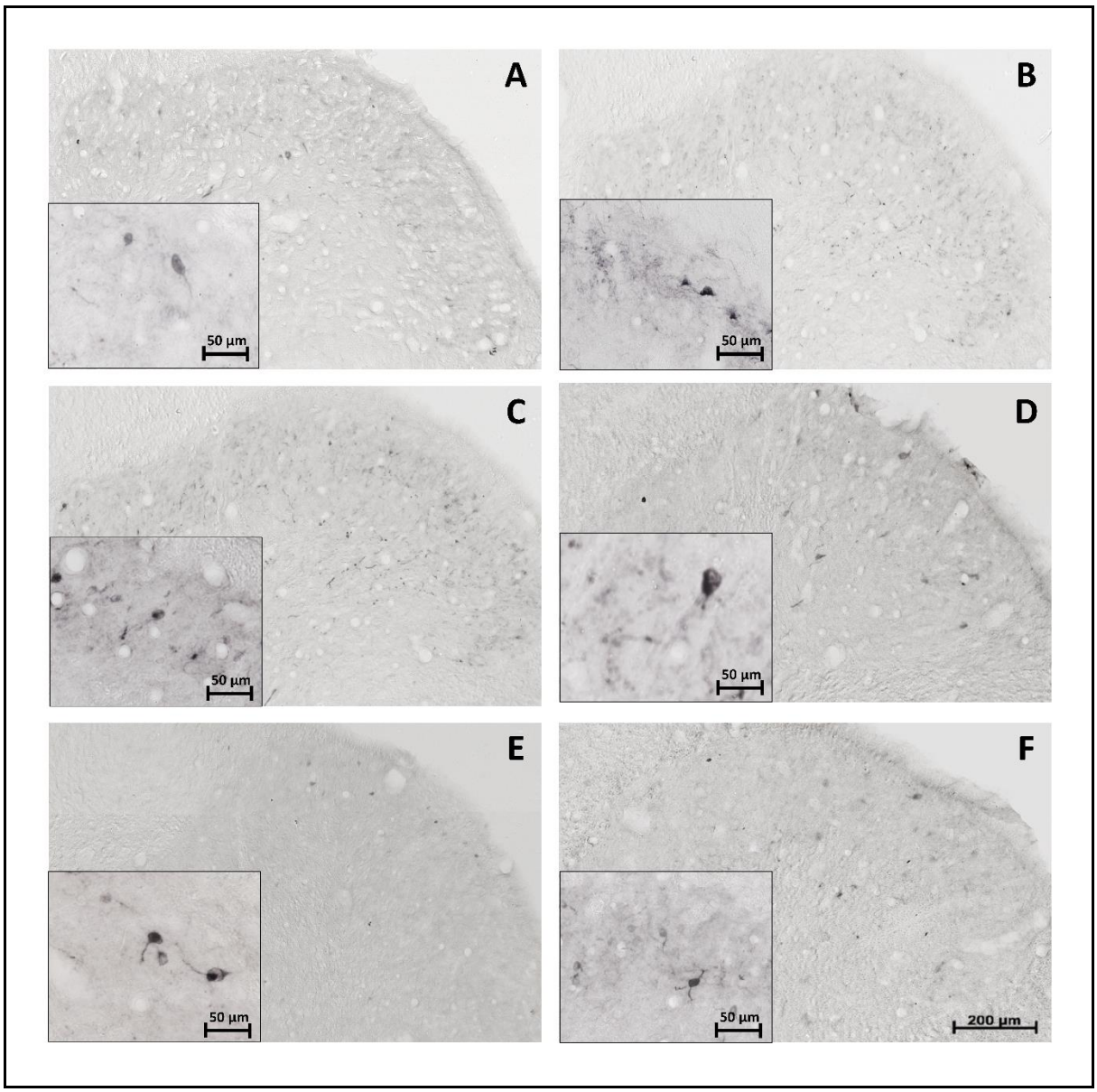
9. Figure Statistical analysis of TRPV1 staining 2.5 and 4 hours after IS treatment

The quantitative analysis shows that in the IS group the area covered by fibers showing TRPV1 positivity is significantly higher than in the control group after 4 hours. (A, B) 2.5 hours after IS treatment, no significant difference was observed in the V/1 area. (C) 4 hours after IS treatment, sumatriptan was able to mitigate this effect in the and V/1 area. (D) Similar to sumatriptan kynurenic acid also mitigated the effect of IS in the V/1 area after 4 hours.

**p<0.05; **p<0.01, ***p<0.001 * means SIF-IS differences, + means IS-SUMA/KYNA. SIF: synthetic interstitial fluid, IS: inflammatory soup, SUMASIF: sumatriptan + synthetic interstitial fluid, SUMAIS: sumatriptan + inflammatory soup, KYNSIF: kynurenic acid + synthetic interstitial fluid, KYNIS: kynurenic acid + inflammatory soup*

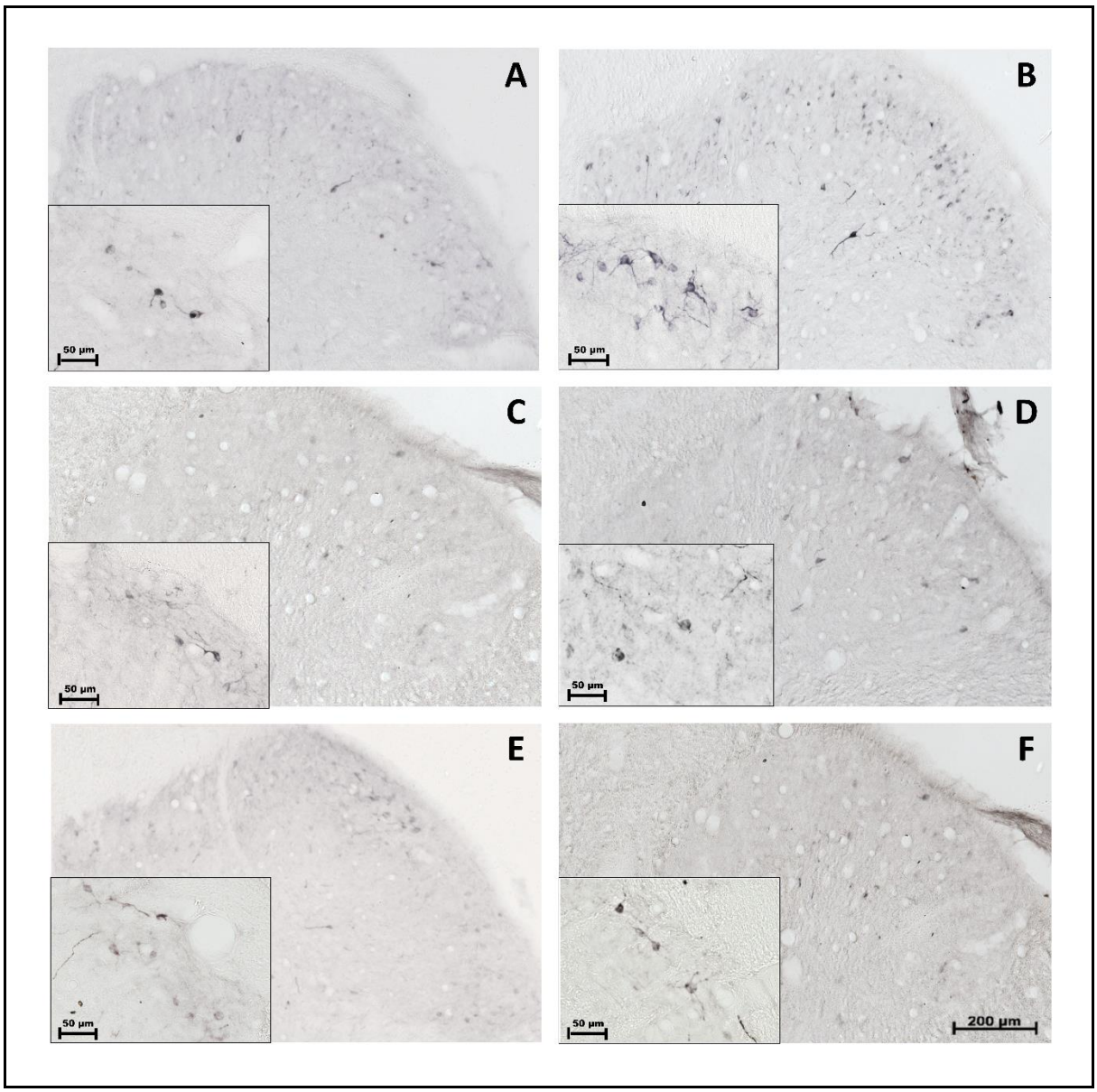
Increase in the nNOS levels after inflammatory soup administration

On transverse sections of the C1-C2 segments, nNOS IR neurons and processes with cytoplasmic staining can be observed in the superficial layers of the dorsal horns. There was a significant increase in the number of nNOS positive cells in the IS treated group compared to the placebo group in the V/1 area, but only after 4 hours. Both sumatriptan and KYNA pretreatments were able to modulate the changes caused by IS. Similar to the previous results no left-right difference was detected here.



10. Figure nNOS immunoreactivity 2.5 hours after IS treatment

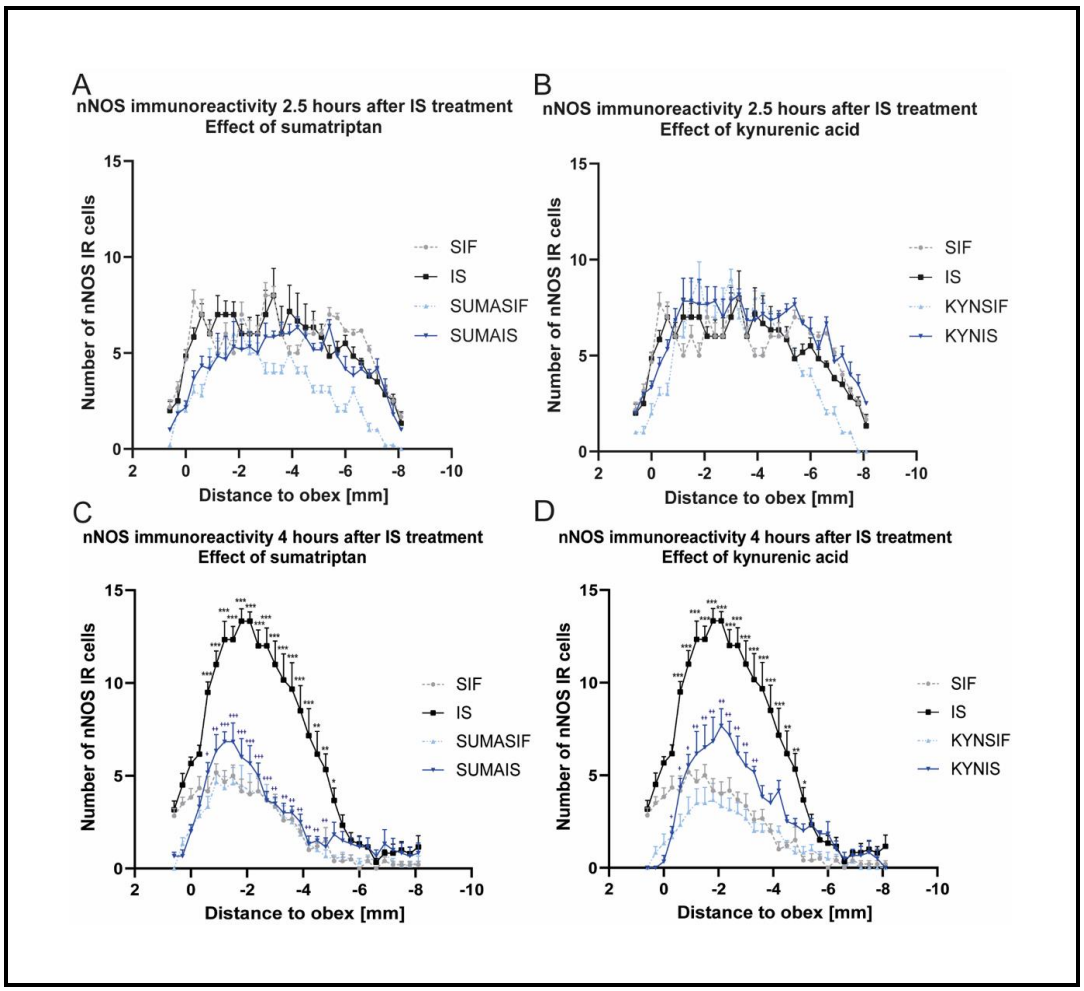
Representative photomicrographs of the nNOS expression in the trigemino-cervical segments after 2.5 hours. (A) – SIF, (B) – IS, (C) – SUMASIF, (D) – SUMAIS, (E) – KYNSIF, (F) – KYNIS. No difference was observed in the number of nNOS IR cells.
SIF: synthetic interstitial fluid, IS: inflammatory soup, SUMASIF: sumatriptan + synthetic interstitial fluid, SUMAIS: sumatriptan + inflammatory soup, KYNSIF: kynurenic acid + synthetic interstitial fluid, KYNIS: kynurenic acid + inflammatory soup. Scale bars: 200 µm, 50 µm.



11. Figure nNOS immunoreactivity 4 hours after IS treatment

Representative photomicrographs of the nNOS expression in the trigemino-cervical segments after 4 hours. (A) – SIF, (B) – IS, (C) – SUMASIF, (D) – SUMAIS, (E) – KYNSIF, (F) – KYNIS. In the IS group, the number of nNOS-IR cells was increased compared to the SIF- treated group. Sumatriptan and kynurenic acid were able to mitigate this effect.

SIF: synthetic interstitial fluid, IS: inflammatory soup, SUMASIF: sumatriptan + synthetic interstitial fluid, SUMAIS: sumatriptan + inflammatory soup, KYNSIF: kynurenic acid + synthetic interstitial fluid, KYNIS: kynurenic acid + inflammatory soup. Scale bars: 200 µm, 50 µm.



12. Figure Statistical analysis of nNOS staining 4 hours after IS treatment

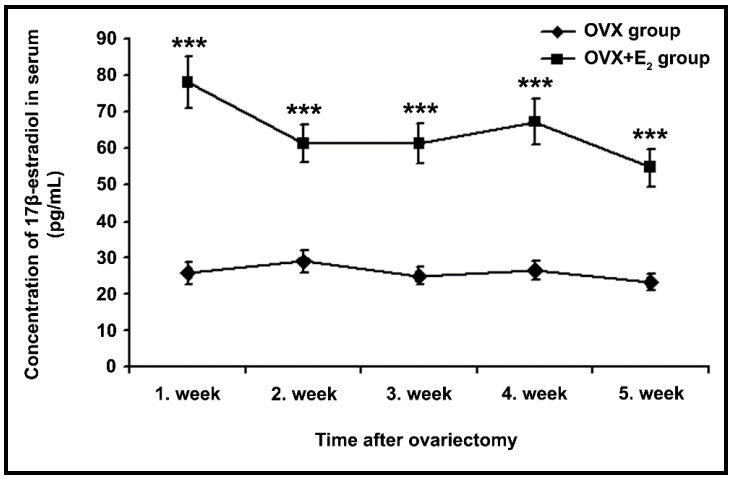
The quantitative analysis shows that in the IS group the number of nNOS IR cells is significantly higher than in the control group after 4 hours. (A,B) 2.5 hours no significant difference was observed.
(C) After 4 hours, in the V/1 area sumatriptan was able to abolish the increase in nNOS IR cells. (D) Kynurenic acid also weakened the effect of IS in the V/1 area after 4 hours
**p<0.05; **p<0.01, ***p<0.001. * means SIF-IS differences, + means IS-SUMA/KYNA differences. SIF: synthetic interstitial fluid, IS: inflammatory soup, SUMASIF: sumatriptan + synthetic interstitial fluid, SUMAIS: sumatriptan + inflammatory soup, KYNSIF: kynurenic acid + synthetic interstitial fluid, KYNIS: kynurenic acid + inflammatory soup*

II. Effect of chronic 17β-estradiol treatment

Estradiol concentrations

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 significantly higher at 64.55 pg/ml. In both animal groups estradiol levels seemed stable during the course of the experiment without any significant changes between the weekly serum concentrations. This is more or less in line with a previous observations, where the serum level of 17β-estradiol slightly began to decrease after the twenty-fourth day in similar conditions *(Mannino, 2005)*. For this reason, the potential modulating effect of chronic 17β-estradiol was examined on the third week following the ovariectomy and implantation of capsules, when the average estradiol

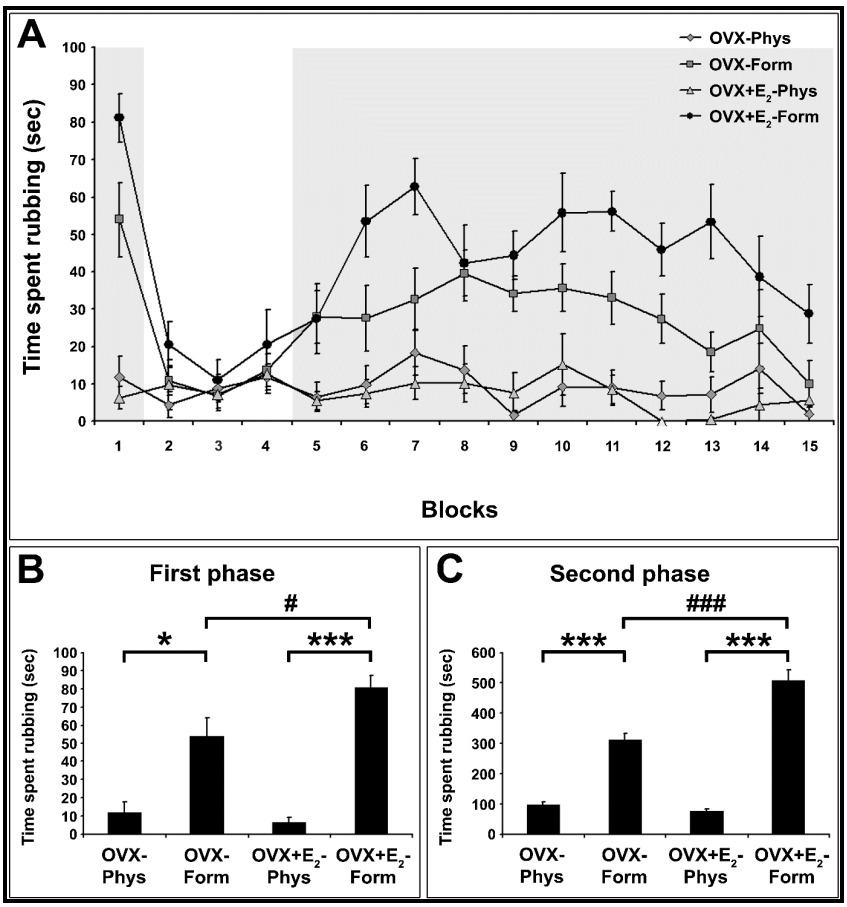
concentration in the serum was 25.01 pg/mL in the OVX group and 61.29 pg/ml in the OVX+E_2 group respectively.



*13. Figure The concentration of 17β-estradiol in serum (pg/mL) in the OVX and OVX+E_2 groups. The chronic 17β-estradiol treatment increases significantly the serum concentration compared to the OVX group ***p<0.001. OVX: ovariectomized rats + cholesterol, OVX +E_2: ovariectomized rats + 1:1 mixture of 17β-estradiol and cholesterol*

Behavioral changes

Right after the formalin injection, the animals withdrew their heads, associated with vocalization. After we put the animals back in the observation box, 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 after a quiet period of ~20 seconds. This is the period called the first phase, which lasted ~ 3-4 minutes, followed by a second phase preceded by a 9-10 minute rest period. During this phase, the rats rubbed their faces less intensely but continuously with both the ipsilateral and contralateral forepaw and lasted ~30–33 minutes. Similar behavior was not observed in the OVX-Phys and OVX + E_2-Phys subgroups, where facial rubbing was minimal. 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. Based on the pooled data from the two formalin treated groups, chronic 17β-estradiol pretreatment significantly increased the nociceptive behavior in both phases (#P<0.05; ###P<0.001).



14. Figure Diagrams show the time spent with pain-related behavior in OVX-Phys, OVX+E_2-Phys, OVX-Form and OVX+E_2-Form subgroups.

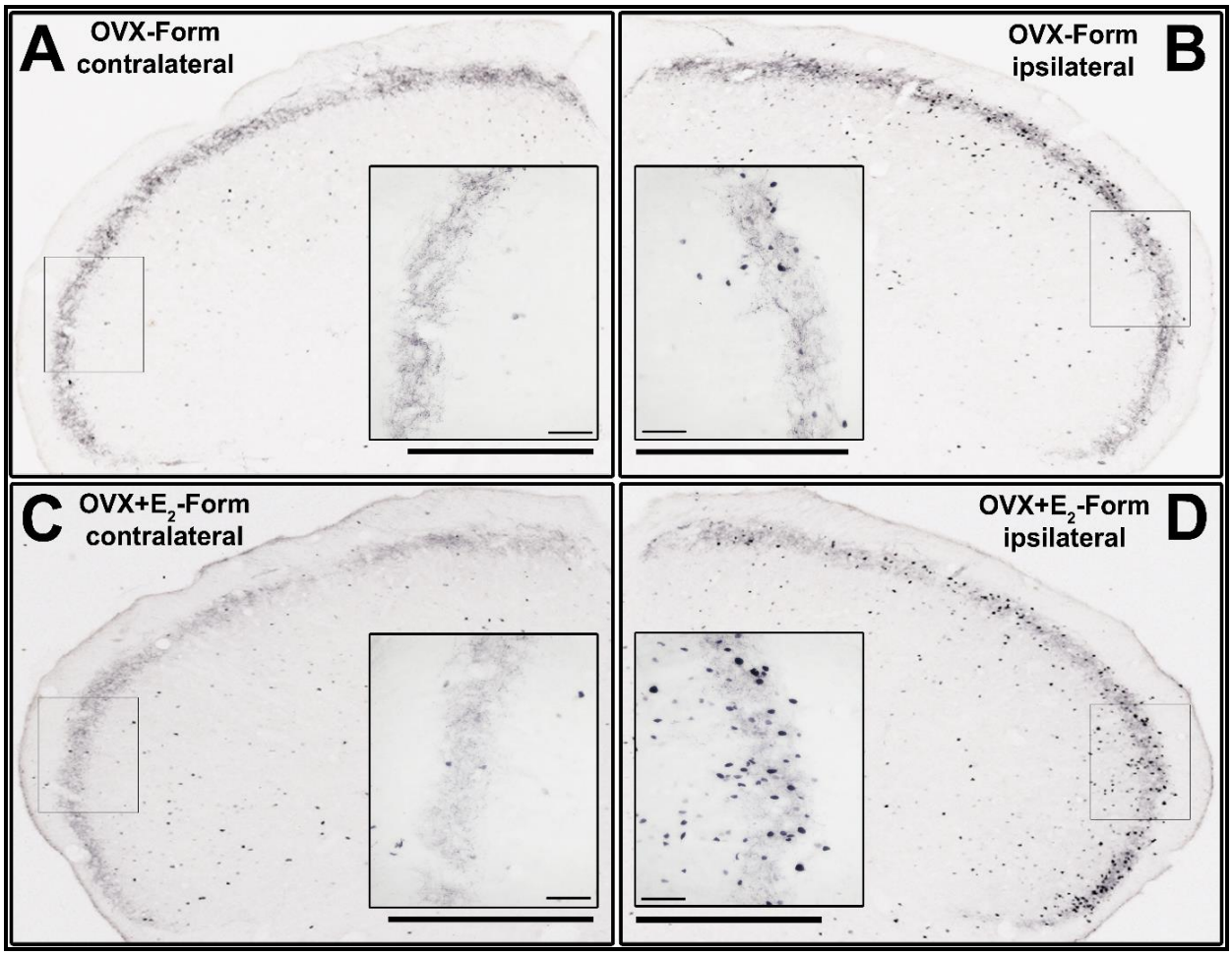
*(A) Diagram shows the time spent rubbing the injected area during 45 minutes of recording period in 3 minutes intervals (blocks 1–15) in all four subgroups. In the OVX-Form and OVX+E_2-Form subgroups, the two phases of formalin action are clearly distinguishable: the first phase (block 1, first grayed out area) lasted ~3–4 minutes and was followed by a quiescent period of 9–10 minutes (block 2–4); the second phase lasted ~30–33 minutes (block 5–15, second grayed out area). The rubbing activity in OVX+E_2-Form subgroup was more pronounced than that in the OVX-Form subgroup. The biphasic pain-related behavioral pattern does not appear in the OVX-Phys and OVX+E_2-Phys subgroups. (B, C) Diagrams show the rubbing activity in the two phases in all four subgroups. The subcutaneous formalin injection (OVX-Form andOVX+E_2-Form) induced a significant increase in rubbing activity in both the first and the second phase when compared with that in the saline-treated animals (OVX-Phys and OVX+E_2-Phys) (*P<0.01; ***P<0.001). In both phases, pretreatment with estradiol had a significant enhancing effect on the formalin-induced nociceptive behavior when compared with the OVX-Form subgroup (#P<0.05; ###P<0.001).*

OVX-Phys: ovariectomized rats + cholesterol + physiological saline solution, OVX- Form: ovariectomized rats + cholesterol + formalin, OVX-E_2-Phys: ovariectomized rats + 1:1 mixture of 17β-estradiol and cholesterol+ physiological saline solution, OVX-E_2-Form: ovariectomized rats + 1:1 mixture of 17β-estradiol and cholesterol+ formalin

C-Fos immunohistochemistry

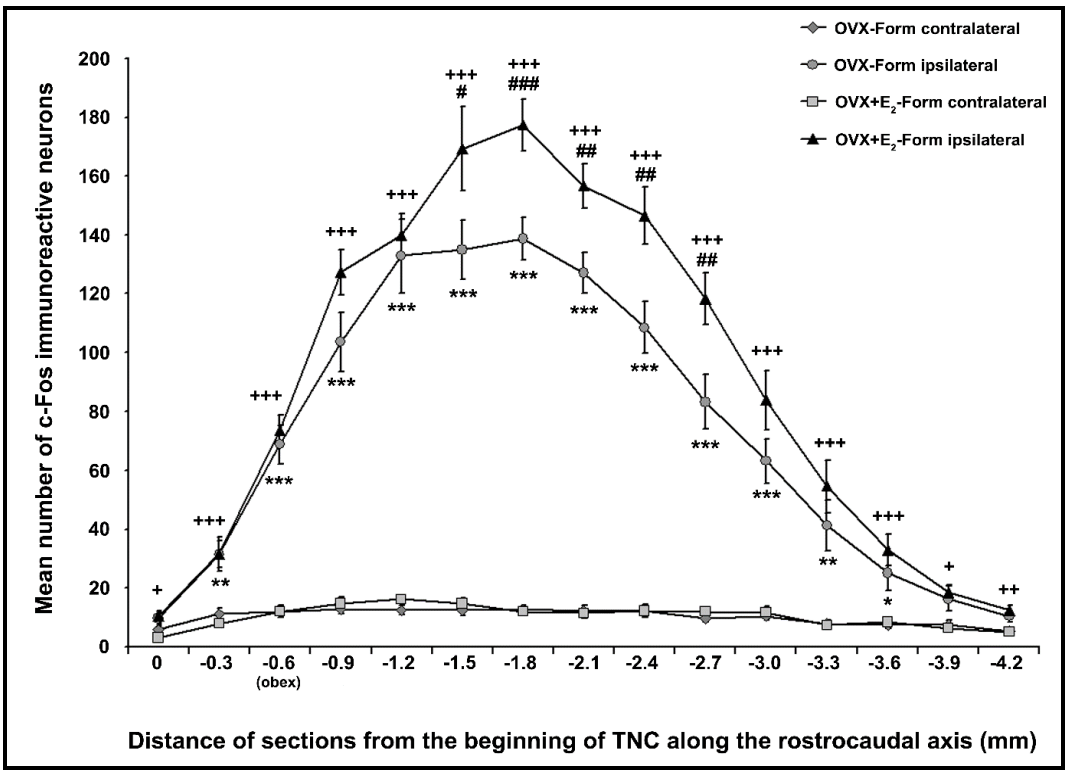
In the OVX-Form and OVX+E_2-Form subgroups, the formalin treatment increased the number of c-Fos IR neurons in the dorsal, superficial area of the ipsilateral TNC when compared with the non-treated contralateral side. This increase was significant at all levels along the rostrocaudal axis, in accordance with the somatotopic representation (*P<0.05; **P<0.01; ***P<0.001;+ P<0.05;++ P<0.01;+++ P<0.001). 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 at several levels of the TNC along the rostrocaudal axis (#P<0.05; ##P<0.01; ###P<0.001). On the contralateral sides of the TNCs, there were no significant differences between the subgroups nor between the different levels along the rostrocaudal axis.



15. Figure Photos of the c-Fos immunostained transverse sections of dorsolateral medulla containing the spinal TNC from the OVX-Form and OVX+E_2-Form subgroups.

Inserts show the framed regions in greater magnification. On the ipsilateral side (B, D), more c-Fos IR neurons can be observed than on the contralateral side (A, C) in both subgroups. After formalin injection, there is an increased number of c-Fos IR neurons on the ipsilateral side in the OVX+E_2-Form subgroup (D) compared to the OVX-Form subgroup (B). In these two subgroups, difference between the contralateral sides (A, C) cannot be observed. Scale bar: 500 and 50 µm. TNC, trigeminal nucleus pars caudalis. OVX-Phys: ovariectomized rats + cholesterol + physiological saline solution, OVX- Form: ovariectomized rats + cholesterol + formalin, OVX-E_2-Phys: ovariectomized rats + 1:1 mixture of 17β-estradiol and cholesterol+ physiological saline solution, OVX-E_2-Form: ovariectomized rats + 1:1 mixture of 17β-estradiol and cholesterol+ formalin



16. *FigureThe diagram shows the mean number of c-Fos IR cells in the superficial area of the TNC at different levels along the rostrocaudal axis.*

*Formalin produced a significant increase in the number of c-Fos IR neurons on the ipsilateral side of the TNC in the OVX-Form (*P<0.05; **P<0.01; ***P<0.001) and OVX+E_2-Form (+P<0.05; ++P<0.01; +++P<0.001) subgroups when compared with the contralateral side at different levels along the rostrocaudal axis.The effect of formalin was significantly increased by pretreatment with estradiol (#P<0.05; ##P<0.01; ###P<0.001). There was no significant difference between the contralateral sides.*

OVX-Phys: ovariectomized rats + cholesterol + physiological saline solution, OVX- Form: ovariectomized rats + cholesterol + formalin, OVX-E_2-Phys: ovariectomized rats + 1:1 mixture of 17β-estradiol and cholesterol+ physiological saline solution, OVX-E_2-Form: ovariectomized rats + 1:1 mixture of 17β-estradiol and cholesterol+ formalin

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 *(Strassman et al., 1996; Lukács et al., 2015).*

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 *(Messlinge et al.r, 1993)*, thus the increased CGRP level might represent enhanced activation of the primary afferents, which may also be associated with increased CGRP release *(Wattiez et al., 2019)* possibly causing a globally higher turnover e.g.

intensive synthesis reflected by higher CGRP expression at the terminals *(Greco et al., 2008)*. Likewise, stimulation of meningeal afferents by capsaicin can also result in CGRP release from dura mater *(Ebersberger et al., 1999)* and Miyamoto and colleagues observed higher CGRP levels in the DRG after they used mono-iodoacetate injection into the rat hip joints *(Miyamoto, 2017)*. In another study it has been shown, that intracisternal IS can raise the CGRP concentration in the jugular vein *(Hoffmann et al., 2012)*. Higher CGRP levels and its increased release might contribute to the activation and sensitization of primary and secondary nociceptors in the TS via the release of numerous pro-inflammatory agents (e.g. cytokines), which can stimulate the nociceptors *(Buckley et al., 1991)*.

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 TG *(Hou et al., 2003)* and trigeminal afferents, which innervate the dura mater *(Edvinsson et al., 2001)*, and these nerve fibers also contain CGRP *(Shimizu et al., 2007)*. 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 *(Jordt et al., 2000)*. After Complete Freund adjuvant-induced inflammation, the TRPV1 mRNA level is significantly increased in the DRG neurons and the excitation of TRPV1 receptors may directly activate mast cells, which are abundant in the dura mater, playing a crucial role in neurogenic inflammation *(Yu et al., 2008)*. TRPV1 expression is upregulated in painful inflammatory conditions in humans *(Matthews et al., 2004)*. In a clinical study, intranasal civamide, a TRPV1 agonist, decreased the intensity of headache two-third of patients *(Diamond et al., 2000)*. In chronic migraine patients, intranasal capsaicin was able to mitigate the migraine pain *(Fusco et al., 2003)* and TRPV1 agonists might effective in the acute treatment of migraine *(Wong et al., 2009)*, as well. Pharmacological studies have also shown that TRPV1 is an essential component of the cellular signaling mechanisms through which injury produces thermal hyperalgesia and pain hypersensitivity *(Davis et al., 2000)*. Altogether, the increased TRPV1 levels in our experimental setting can also be a part of the undergoing sensitization process in the TS.

In our experiment, IS significantly enhanced the number of nNOS IR cells in the dorsal horn after 4 hours due to the activation of primary trigeminal nociceptors conveyed to the second-order neurons *(Dubin et al., 2010)*. NO donors cause an increase and release of CGRP at the TG and TNC, and NO donors lead to a delayed enhancement of nNOS in the latter *(Dohrn et*

al., 1994). Also, NO is generated in abundance during human inflammatory reactions such as asthma and inflammatory mediators can also increase the amount of cyclic guanosine monophosphate the downstream effector of NO *(Sarker et al., 2002)*. Moreover, bradykinin and histamine trigger NO release from vascular endothelial cells in vitro, suggesting a strong interaction between NO and inflammation *(Palmer et al., 1987)*. An 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 *(Pardutz et al., 2000; Sarke et al.r, 2002)*. The latter can lead to the activation of primary afferent neurons and CGRP release, activating satellite glial cells that release NO, which can induce nNOS *(Capuano et al., 2009)*. In this context nNOS is can be considered as a significant marker of the sensitization process of the TS in animals. CGRP and nNOS also interact in the CNS and they can cause dilation of the meningeal blood vessels *(Majláth et al., 2016)*. Presumably, NO can induce CGRP release from the sensory fibers causing vasodilation *(Iyengar et al., 2017)*. Moreover, the activation of trigeminal nerves through inflammatory mediators can have similar effects on CGRP, which results in NO accumulation *(Ma et al., 2001)*.

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 and 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 suggesting an important involvement of 5-$HT_{1B/1D}$ receptors in the sensitization process in the TS. This is in line with previous results showing, that CGRP and TRPV1 are co-localized with 5-$HT_{1B/1D}$ receptors in trigeminal neurons *(Ma, 2001)* and sumatriptan presynaptically inhibits the release of nociceptive neuropeptides (e.g. CGRP) from primary afferents *(Arvieu et al., 1996)* and most of the effects of TRPV1 receptors are mediated through CGRP, which is released after TRPV1 activation *(Meng et al., 2009)* so 5-HT might have a role in modulation of TRPV1 function too. Similar to our results, after intracisternal injection of carrageenan, sumatriptan was able to inhibit the production of the nNOS enzyme in the brain stem of rats *(Demirpence et al., 2009)*. Taken together, 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 and this phenomenon may be mediated through several different receptors. Currently, the antagonist effect of KYNA on the α7-nicotinic acetylcholine receptor (nAChR) is contested *(Stone et al., 2020)*. Three hours after the local IS treatment of the dura, higher glutamate levels can be detected in the TNC *(Sarker et al., 2002)*. In addition to the NMDA receptors, both AMPA, kainate, and metabotropic receptors are found in the TNC *(Storer, 1999)* and it has been shown, that the antagonists of non-NMDA glutamate receptors also can inhibit the activation of secondary nociceptive neurons *(Dougherty et al., 1992)*. AMPA receptors can modulate c-fos expression and possibly the neurotransmission in the TS *(Mitsikostas et al., 1999)* and in a peripheral pain model, activation of the kainate receptors resulted in the appearance of mechanical, thermal hyperalgesia, and allodynia *(Dougherty et al., 1992)*. In the TNC, CGRP can stimulate glutamate expression and that can be inhibited by 5-$HT_{1B/1D}$ receptor agonists *(Choi et al., 2012)*. Hence, the relationship between the two systems can be assumed. KYNA pretreatment also modulated the IS induced nNOS expression in our animal model. This effect might be related to the anti-glutamatergic effect of KYNA, mainly on the NMDA receptors, which activation is associated with NO production in the spinal trigeminal nucleus *(Dohrn et al., 1994)*. Another possible explanation for the peripheral effects of KYNA is that it binds to GPR35, which receptor is present in the DRG *(Cosi et al., 2011)* and KYNA can inhibit adenylate cyclase activity there via G-protein-dependent mechanisms *(Ohshiro et al., 2008)* which might interact with nNOS *(Ohnishi et al., 2008; Boissel et al., 2004)*. 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 *(Heyes et al., 1992; Rejdak et al., 2002; Curto et al., 2015)*.

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 the present experiments, we examined the effect of chronic, stable high 17β-estradiol level in serum on trigeminal pain and trigeminal activation in orofacial formalin test of the rat. The source of estradiol we measured in OVX rats is probably extragonadal. 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 *(Edwards et al., 1999; Kaneko et al., 2004).* 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 *(Tjølsen et al., 1992)*. In addition, this chronic estradiol treatment increased the number of c-Fos IR cells, one of the anatomical markers of pain-induced neuronal activity.

The molecular basis for estrogen to directly regulate the pain transmission at the level of the TS is mediated by estrogen receptors (ER), which have three known types: estrogen receptor alpha (ERα), ERβ, and G-protein-coupled estrogen receptor (GPR30). These receptors are present in the TS. ERα and GPR30 are present in the TG of female rodents. Expression of ERα or GPR30 by most nociceptors in the TG suggests a basis for the high sensitivity of the TS to estrogen *(Liverman et al., 2009)*. In superficial laminae of rat TNC, ERα and ERβ proteins are co-expressed by neurons *(Bereiter et al., 2005)*, and in this area, ERα was shown to be present in nociceptive-responsive neurons *(Amandusson et al., 2010)*. Experimental data show that the modulation of these receptors results in well-defined changes of trigeminal pain processing *(Kou et al., 2011)*. ERs have also been reported in various parts of the TS *(Liverman et al., 2009)*, which may mediate estrogen-induced pronoiceptive responses. Liverman and colleagues also showed that specific agonists of the ER enhanced mechanical allodynia *(Liverman et al., 2009)*. Nociceptive input activates the extracellular signal-regulated kinase (ERK) that mediates hyperalgesia *(Ji et al.,1999; Karim et al.,2006)*. ERK is activated by peripheral inflammation in the dorsal root ganglion and the dorsal horn of spinal cord *(Doya et al., 2005)*. In the TS, ERK is activated in the nucleus caudalis by passive jaw movement in TMJ inflammation *(Suzuk et al.i, 2007)* and by capsaicin injection of the tooth

pulp *(Shimizu et al., 2006)*. Estrogen can exert its effects in two ways: a slow genomic *(Heldring et al., 2007)* and a rapid non-genomic *(Srivastava et al., 2013)* mechanism and these pathways are both involved in the regulation of trigeminal pain processing. In an in vitro experiment, it was found that estrogen treatment can affect the expression of several chemokines, cytokines and immune genes in trigeminal neurons that play an important role in migraine *(Puri et al., 2011)*. Furthermore, estrogen increases the activation of ERK, which is involved in changes in sensory neurons during inflammatory and neuropathic pain *(Puri et al., 2011)*. Estrogen can activate intracellular signaling pathways via non-genomic, membrane-mediated mechanisms also *(Tashiro et al., 2009; Liverman et al., 2009)*. These cellular mechanisms affect numerous processes, which are essential in trigeminal pain perception, including the function of the endogenous antinociceptive system, the modulation of the excitability of TNC neurons.

Earlier studies support our present data on the pronociceptive effect of estradiol *(Bereiter et al., 2001; Bereiter et al., 2005; Allen et al., 2005)*. After CFA- induced masseter muscle inflammation, single s.c estradiol-valerate administration enhanced the primary and secondary facial allodynia in ovariectomized rats *(Liverman et al., 2009)*. In a model of temporomandibular joint inflammation, the ovariectomized rats received s.c. 17β-estradiol treatment for 10 days, which increased the inflammatory response and decreased the food intake of the animals *(Kou et al., 2011)*. In another study, s.c. carrageenan injection caused orofacial inflammation and thermal hyperalgesia, which was worsened by single s.c.17β-estradiol treatment *(Nag et al., 2016)*. In capsaicin-induced eye wipe test, high estradiol treatment for 2 days intensified the pain-related behavior and increased the c-Fos immunoreactivity in the TNC, which is a marker of the activation of trigeminal neurons *(Yamagata et al., 2016)*. 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.

However, numerous studies support that estrogen has antinociceptive effect *(Sanoja et al., 2005; Kramer et al., 2009; Hunter et al., 2011)*. Flores et al. described that intracisternal administration of N-methyl-d-aspartic acid could cause behavioral changes associated with facial pain and 18-24 hours before a single s.c. estradiol treatment was able to moderate this effect *(Flores et al., 2001)*. Furthermore, in the animal model of temporomandibular joint inflammation, peripheral estradiol had an antinociceptive effect by activating the nitric

oxide/cyclic guanosine monophosphate signaling pathway *(Fávaro-Moreira et al., 2009)*. There may be several reasons for these opposite results, such as the various mechanisms mediated by ERs, changes in ERs expression during the menstrual cycle and the proportion of ERs in different tissues. Nevertheless, in our model stable high estradiol levels proved to be pronociceptive.

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. KYNA may have exerted its beneficial effects through NMDA receptors, so it may play an important role 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 effect are due to the activation of ERK 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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I.

Effect of dural inflammatory soup application on activation and sensitization markers in the caudal trigeminal nucleus of the rat and the modulatory effects of sumatriptan and kynurenic acid

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Abstract

Background The topical inflammatory soup can model the inflammation of the dura mater causing hypersensitivity and activation of the trigeminal system, a phenomenon present in migraineurs. Calcitonin gene-related peptide, transient receptor potential vanilloid-1 receptor, and neuronal nitric oxide synthase are important in the sensitization process there.

5-$HT_{1B/1D}$ receptor agonists, triptans are used as a treatment of migraine. Kynurenic acid an NMDA antagonist can act on structures involved in trigeminal activation.

Aim We investigated the effect of inflammatory soup induced dural inflammation on the calcitonin gene-related peptide, transient receptor potential vanilloid-1 receptor, and neuronal nitric oxide synthase levels in the caudal trigeminal nucleus. We also tested whether pretreatment with a well-known antimigraine drug, such as sumatriptan and kynurenic acid, a compound with a different mechanism of action, can affect these changes and if their modulatory effects are comparable.

Material and methods After subcutaneous sumatriptan or intraperitoneal kynurenic acid the dura mater of adult male Sprague-Dawley rats (n=72) was treated with inflammatory soup or its vehicle (synthetic interstitial fluid). Two and a half or four hours later perfusion was performed and the caudal trigeminal nucleus was removed for immunohistochemistry.

Results and conclusion Inflammatory soup increased calcitonin gene-related peptide, transient receptor potential vanilloid-1 receptor, and neuronal nitric oxide synthase in the caudal trigeminal nucleus compared to placebo, which was attenuated by sumatriptan and kynurenic acid. This suggests the involvement of 5-$HT_{1B/1D}$ and NMDA receptors in neurogenic inflammation development of the dura and thus in migraine attacks.

Keywords: migraine, trigeminal system, inflammatory soup, sumatriptan, kynurenic acid, CGRP, TRPV1, nNOS

Introduction

Migraine is a common condition affecting up to 15% of the world's population (1). The pathomechanism of this disorder is not fully understood, but the sterile neurogenic inflammation of the dura mater and the activation and sensitization of the trigeminal system (TS) play a crucial role in the attack (2).

In animal models, stimulation of trigeminal nerves causes a release of neuropeptides, resulting in meningeal blood vessel dilation, plasma extravasation, platelet activation, and mast cell degranulation characteristic of neurogenic inflammation (3). Calcitonin gene-related peptide (CGRP) is a multifunctional regulatory neuropeptide (4) and a key player in migraine: Serum concentrations of CGRP are elevated during the attack (5) whereas intravenous infusion of CGRP can induce a migraine-like headache in migraineurs (6). In a rat model of migraine, electrical stimulation of the trigeminal ganglion was able to increase blood flow on the same side of the facial skin which was reduced by intravenous administration of the CGRP antagonist, CGRP 8-37 (7). Transcranial electrical stimulation in rats was able to cause CGRP release and vasodilation, which was prevented by olcegepant, a CGRP receptor blocker (8). These results are in line with the clinical data showing that CGRP antagonists are effective in the acute treatment of this disease (9, 10, 11). On the other hand, there are other pathways involved in neurogenic inflammation which are not directly related to CGRP e.g. the appearance of cortical spreading depression might also contribute to this phenomenon (12).

Transient receptor potential vanilloid-1 receptor (TRPV1), a nonselective cation channel, a molecular component of pain detection and modulation (13), is selectively expressed by small- to medium-diameter neurons within the dorsal root ganglion (DRG) and trigeminal ganglia (TG), co-localized with CGRP in the latter (14). TRPV1 activation leads to the release of

66 neuropeptides, such as substance P and CGRP. These neuropeptides cause vasodilation and
67 initiate neurogenic inflammation within the meninges under experimental conditions (15).

68 The synthesis of nitric oxide (NO) is catalyzed by neuronal nitric oxide synthase (nNOS),
69 which can be found in the superficial layers of the dorsal horn of the spinal cord underlining
70 its importance in the trigeminal pain processing (16). Furthermore, its presence is confirmed in
71 dural mast cells, trigeminal nerve endings, and Gasserian ganglion cells (17). Systemic
72 administration of nitroglycerin (NTG), a nitric oxide donor can induce an immediate headache
73 and in migraine patients, this is followed by a typical migraine attack without aura (18). The
74 immediate headache can be explained by the vasodilatory effect of NO, which activates the
75 dural nociceptors, while the delayed headache might be mainly due to an effect of NO on
76 central nociceptors, causing a long-lasting endogenous synthesis of NO by enhancing nNOS
77 resulting in central sensitization process (19).

78 Triptans are used to relieve migraine attacks, being an agonist on 5-hydroxytryptamine
79 receptors (5-$HT_{1B/1D}$), they can cause the constriction of dilated cranial arteries and the
80 inhibition of CGRP release (20). They block the depolarization of the trigeminal nerves and
81 inhibit the neurotransmission at the level of interneurons of TNC (21). In an animal model of
82 migraine, after creating neurogenic inflammation in the dura mater, triptans were able to reduce
83 the plasma protein extravasation, probably by inhibiting nociceptor activation and preventing
84 neuropeptide release (5). The increase of jugular vein CGRP concentration after the stimulation
85 of the TG can be reduced by sumatriptan (5, 22). In the rat model of trigeminal neuropathic
86 pain, triptans can selectively inhibit nociceptive (23) and neuropathic pain behavior (24) and
87 evoked activity in trigeminal dorsal horn neurons (21) in response to noxious stimulation of
88 the trigeminal nerve area. In the TG, 5-$HT_{1B/1D}$ receptors and glutamate were co-localized in
89 several neurons (25), thus triptans may modulate glutamate release from trigeminal neurons
90 through the 5-$HT_{1B/1D}$ receptors and possibly reduce pain (24).

Kynurenic acid (KYNA) is a neuroactive product of the kynurenine pathway of tryptophan metabolism, which can exert its effect through N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate receptors, and G-protein coupled receptor 35 (GPR35), and these receptors have a relevant role in pain processing and neuroinflammation (26). A previous study suggests that KYNA has an anti-inflammatory effect on the TS (27). In rats, KYNA had an analgesic effect in tail-flick test (28). In an in vivo model of acetic acid-induced inflammatory pain, L-kynurenine, which is a precursor of KYNA, caused rise in the KYNA levels in the plasma and the central nervous system (CNS), thereby, was able to elicit anti-nociceptive effect (29).

Based on the results of clinical trials and animal experiments, trigeminal activation and sensitization occur during the migraine attack. Local administration of inflammatory soup (IS) on the dura mimics this process (30), which might be characterized by the alteration of selected molecular markers. On the other hand, since sumatriptan is effective in the acute treatment it may prevent these alterations and we wanted to compare its possible modulatory effects with a compound with a different pharmacological mechanism of action.

Thus, in our present study, we investigated the effect of IS induced dural inflammation on markers of the sensitization process in the trigemino-cervical complex, namely: CGRP, TRPV1, and nNOS. We also tested whether pretreatment with sumatriptan or KYNA has an effect on the IS induced changes.

Materials and Methods

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) 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).

72 adult male Sprague-Dawley rats weighing 350-400 g were used. 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.

Drug administration

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 animals were deeply anesthetized with an intraperitoneal injection of 4 % chloral hydrate (0.4 g/kg body weight, Sigma-Aldrich). The head of the animal was fixed in a stereotaxic frame and lidocaine (10 mg/ml, Egis) infiltration on the skull was used before the interventions. A handheld drill was used to make a window on the skull. The hole was made posterolaterally (5 mm, 3 mm) to the bregma, on the right side without penetrating the dura mater (31).

The animals 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 subcutaneous 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. Sumatriptan has a short half-life, its receptor binding is reversible and the onset of action is 10-15 minutes after administration. The half-life time of KYNA is about an hour. The dosage we used for both molecules was chosen based on previous reports (32, 33, 34). Two and a half

hours or four hours after the SIF or IS administration, the trigemino-cervical complex was processed for immunohistochemistry. Two survival times were used to examine changes over time.

Immunohistochemistry

Two and a half hours or four hours after the SIF or IS administration, the rats were perfused transcardially with 50 ml phosphate-buffered saline (PBS, 0.1 M, pH 7.4), followed by 200 ml 4% paraformaldehyde in phosphate buffer under chloral hydrate anesthesia, and the trigemino-cervical complex was 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 (CGRP staining) or PBS (nNOS and TRPV1 staining) for 30 minutes. After several rinses in PBS containing 1% Triton X-100, sections were kept overnight at room temperature in anti- CGRP antibody (Sigma, C8198) at a dilution of 1:20000, or TRPV1 antibody (Santa Cruz, s.c.28759) at a dilution of 1:1000, or for two nights at 4°C in anti-nNOS antibody (EuroProxima, 2263B220-1) at a dilution of 1:5000. The immunohistochemical reaction was visualized by the Vectastain Avidin-Biotin kit of Vector Laboratories (PK6101), and nickel ammonium sulfate-intensified 3,3'-diaminobenzidine. Control experiments included the omission of the primary antisera.

Data evaluation

All evaluations were performed by an observer blind to the experimental groups. The photomicrographs of the stained sections of trigemino-cervical complex were taken using a Zeiss AxioImager microscope supplied with an AxioCam MRc Rev. 3 camera (Carl Zeiss Microscopy). The area covered by TRPV1-immunoreactive and CGRP-immunoreactive fibers 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. The program calculated the area innervated by the immunoreactive fibers as the number of pixels with densities above the threshold; the data were expressed as area fractions (%) of the corresponding immunolabelled structures. We measured the covered area by the CGRP and TRPV1 immunoreactive fibers and counted the nNOS immunoreactive cells in the area of the dorsal horn innervated by the ophthalmic nerve (V/1 area).

Statistical analysis

The Shapiro-Wilk test was used to determine the distribution of data. As our data followed a normal distribution in each case, the differences among the groups and sides were examined with a mixed ANOVA model. The pairwise comparisons were performed by paired and independent samples t-tests with Sidak corrections. All statistical analyses were performed using SPSS version 24.0 (IBM Corporation). Values $p<0.05$ were considered statistically significant. Our data are reported as means+SEM for all parameters and groups.

Results

Inflammatory soup and CGRP

In the dorsal horn, CGRP immunoreactive (IR) axon fibers were distributed in the laminae I and II. IS treatment was able to increase the amount of the area covered by fibers showing CGRP positivity in both time points (Fig. 1., Fig. 3.). Sumatriptan and KYNA pretreatments were able to attenuate this effect (Fig. 2., Fig. 3.). There was no relevant difference between the two time points in the area covered by fibers showing CGRP positivity.

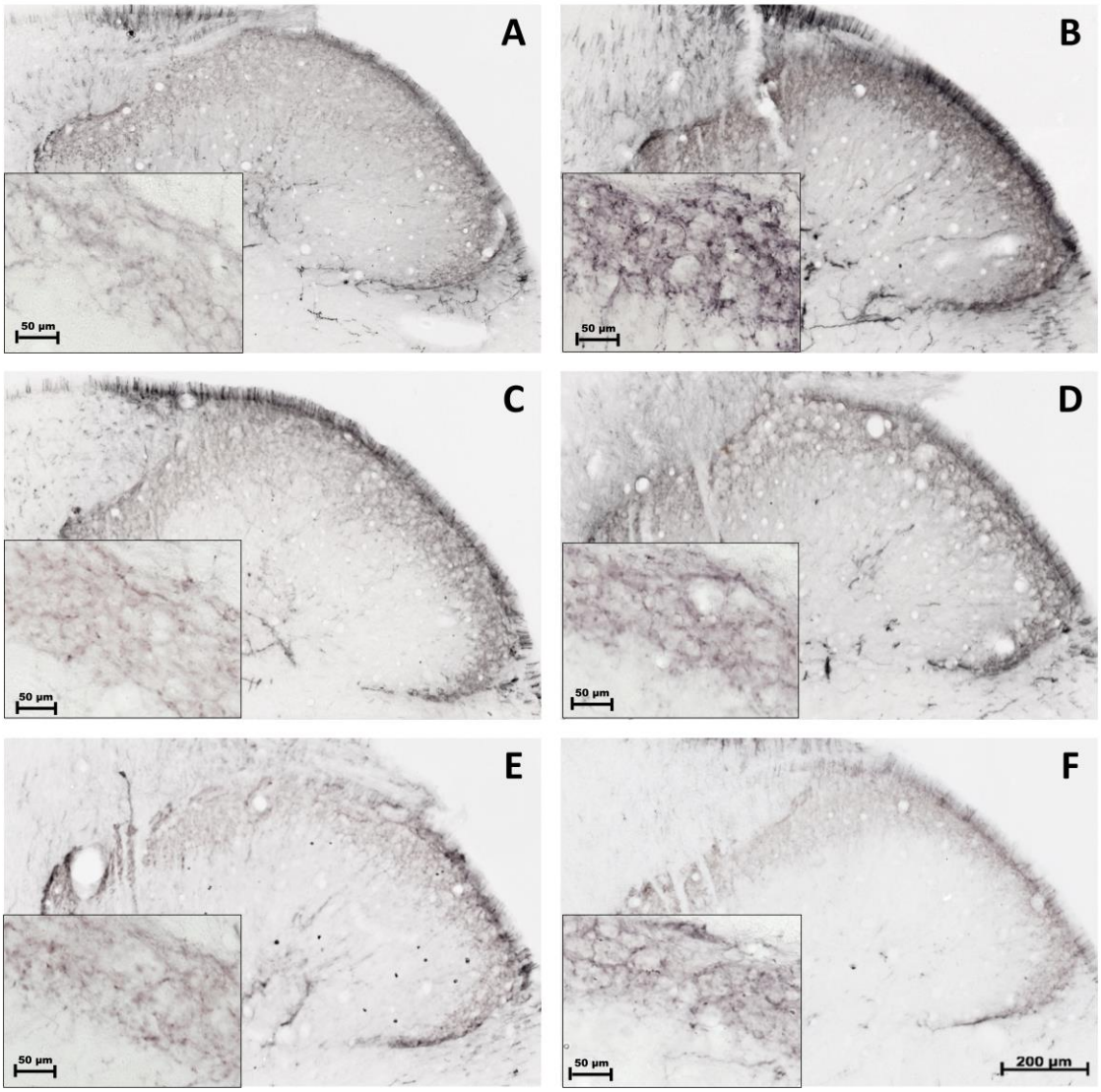
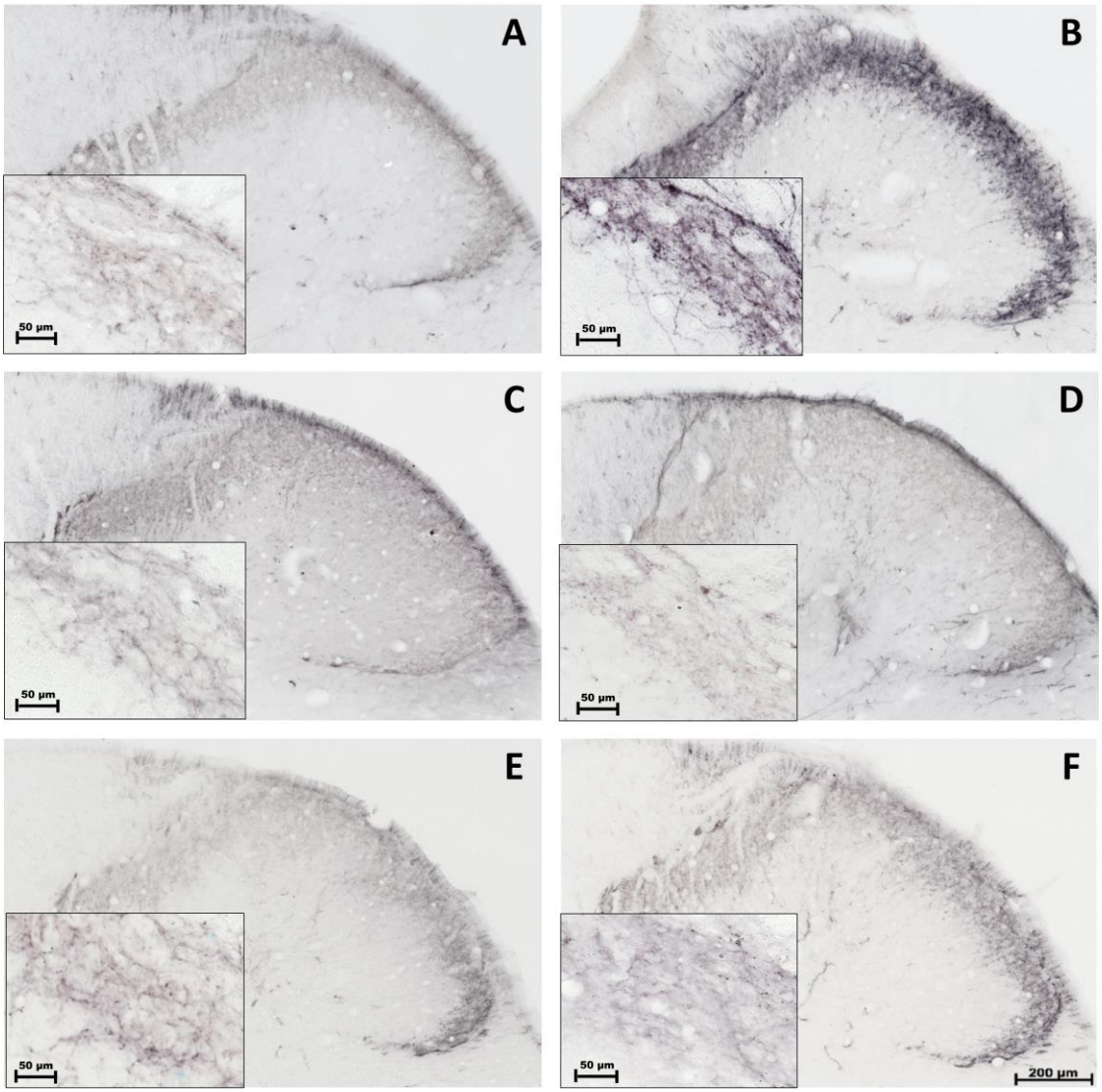


Figure 1. CGRP immunoreactivity 2.5 hours after IS treatment Representative photomicrographs of the CGRP expression in the trigemino-cervical segments after 2.5 hours. **A** – SIF, **B** – IS, **C** – SUMASIF, **D** – SUMAIS, **E** – KYNSIF, **F** – KYNIS. In the IS group, the CGRP staining was stronger than in the placebo group. Sumatriptan and kynurenic acid were able to attenuate this effect. SIF: synthetic interstitial fluid, IS: inflammatory soup, SUMASIF: sumatriptan + synthetic interstitial fluid, SUMAIS: sumatriptan + inflammatory soup, KYNSIF: kynurenic acid + synthetic interstitial fluid, KYNIS: kynurenic acid + inflammatory soup. Scale bars: 200 µm, 50 µm.



192

193 **Figure 2**. CGRP immunoreactivity 4 hours after IS treatment Representative photomicrographs of the CGRP
194 expression in the trigemino-cervical segments after 4 hours. **A** – SIF, **B** – IS, **C** – SUMASIF, **D** – SUMAIS, **E** –
195 KYNSIF, **F** – KYNIS. In the IS group, the CGRP staining was stronger than in the placebo group. Sumatriptan
196 and kynurenic acid were able to attenuate this effect. SIF: synthetic interstitial fluid, IS: inflammatory soup,
197 SUMASIF: sumatriptan + synthetic interstitial fluid, SUMAIS: sumatriptan + inflammatory soup, KYNSIF:
198 kynurenic acid + synthetic interstitial fluid, KYNIS: kynurenic acid + inflammatory soup. Scale bars: 200 µm, 50
199 µm.

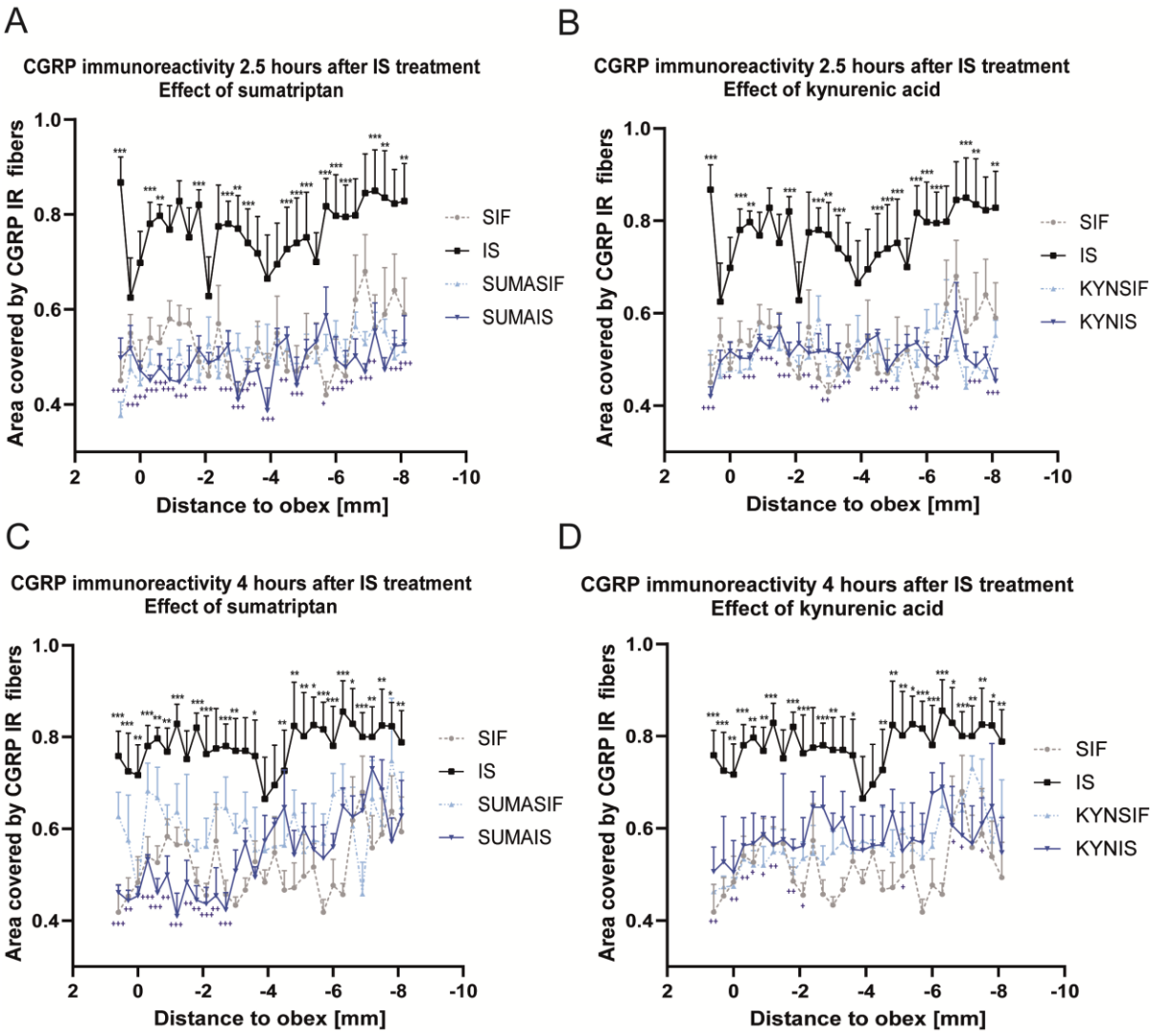


Figure 3. Statistical analysis of CGRP staining 2.5 and 4 hours after IS treatment The quantitative analysis shows that in the IS group the area covered by fibers showing CGRP positivity is significantly higher than in the control group in both timepoints. **A** 2.5 hours after IS treatment, sumatriptan was able to attenuate this effect in the V/1 area. **B** Similar to sumatriptan kynurenic acid weakened the effect of IS in the V/1 area. **C** 4 hours after IS treatment, sumatriptan was able to mitigate this effect in the and V/1 area. **D** Kynurenic acid decreased the effect of IS in the V/1 area. *p<0.05; **p<0.01, ***p<0.001 * means SIF-IS differences, + means IS-SUMA/KYNA. SIF: synthetic interstitial fluid, IS: inflammatory soup, SUMASIF: sumatriptan + synthetic interstitial fluid, SUMAIS: sumatriptan + inflammatory soup, KYNSIF: kynurenic acid + synthetic interstitial fluid, KYNIS: kynurenic acid + inflammatory soup

Inflammatory soup and TRPV1

After 2.5 hours there was no significant difference between the IS treated and placebo group (data not shown), but after 4 hours we observed a significant increase in the amount of the TRPV1 IR fibers in the IS-treated group, compared to the placebo. Sumatriptan and KYNA pretreatments were able to mitigate the effect of the IS treatment (Fig. 4., Fig. 6.).

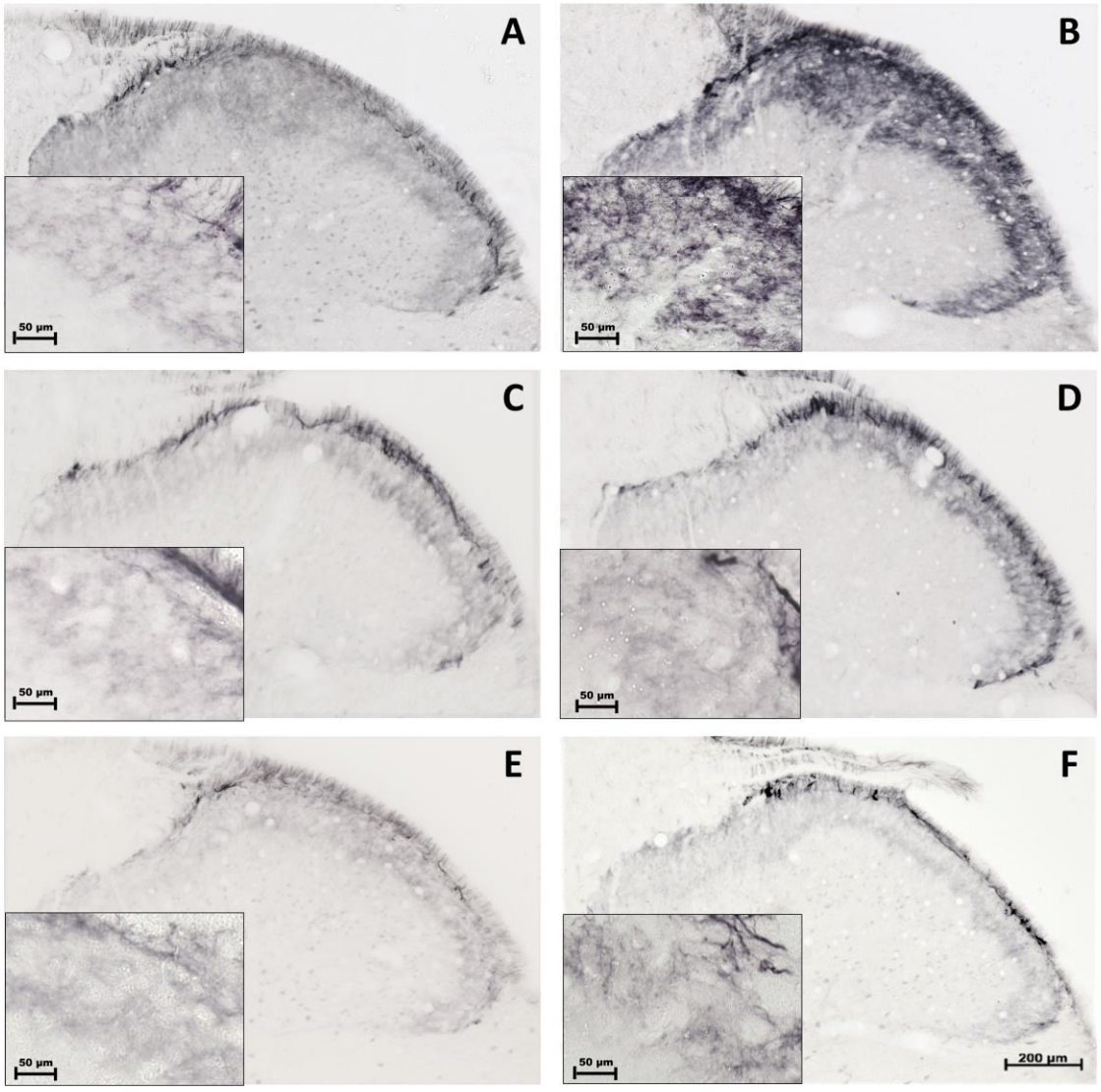


Figure 4. TRPV1 immunoreactivity 4 hours after IS treatment Representative photomicrographs of the TRPV1 expression in the trigemino-cervical segments after 4 hours. **A** – SIF, **B** – IS, **C** – SUMASIF, **D** – SUMAIS, **E** – KYNSIF, **F** – KYNIS. In the IS group, the area covered by TRPV1 was higher than in the placebo group. Sumatriptan and kynurenic acid were able to attenuate this effect. SIF: synthetic interstitial fluid, IS: inflammatory soup, SUMASIF: sumatriptan + synthetic interstitial fluid, SUMAIS: sumatriptan + inflammatory soup, KYNSIF:

kynurenic acid + synthetic interstitial fluid, KYNIS: kynurenic acid + inflammatory soup. Scale bars: 200 µm, 50 µm.

Inflammatory soup and nNOS

A significant increase of nNOS was observed only after 4 hours in the IS treated group compared to the placebo group in the V/1 area. Sumatriptan and KYNA pretreatments were able to modulate the effect of IS administration (Fig. 5., Fig. 6.).

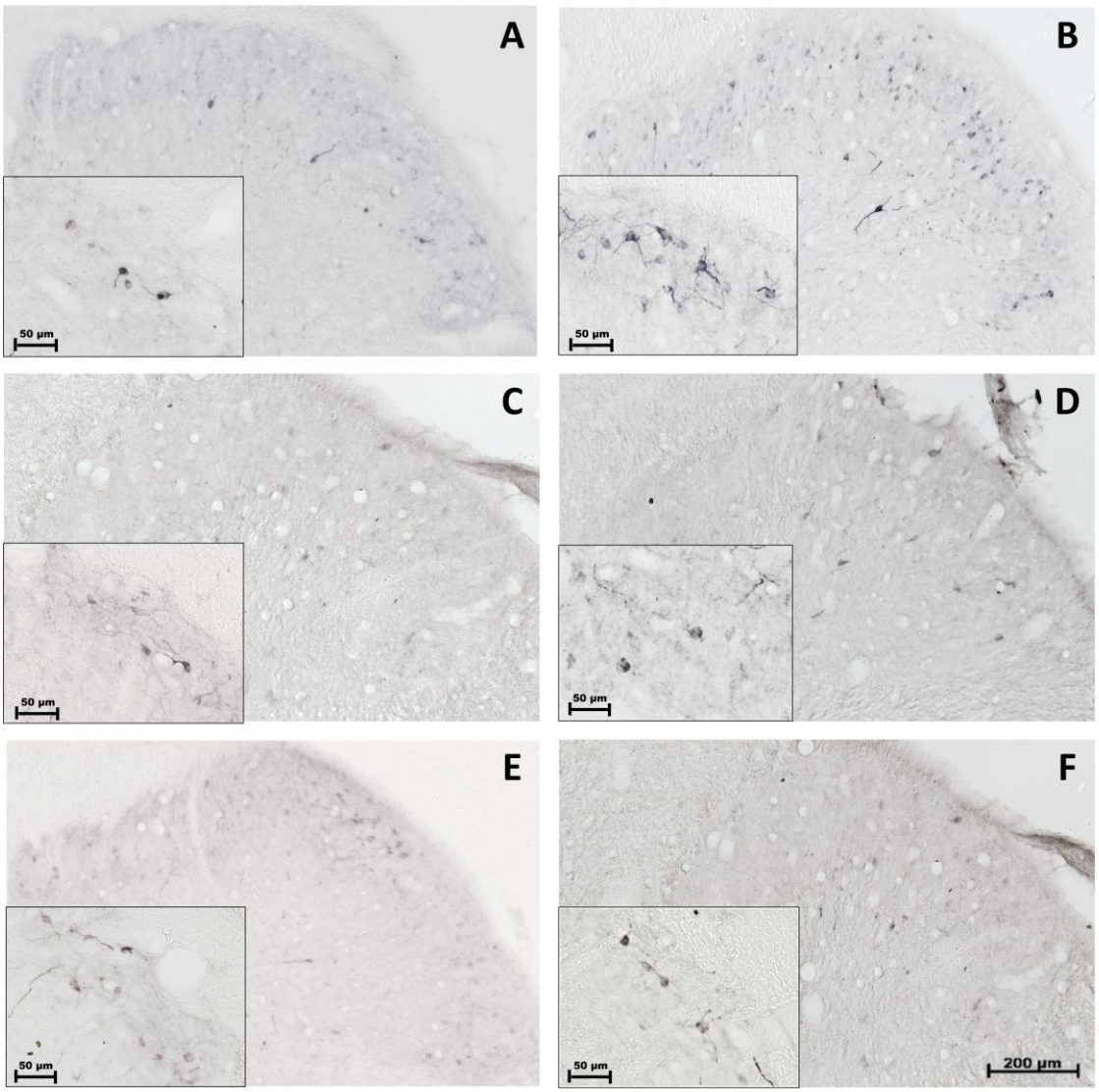


Figure 5. nNOS immunoreactivity 4 hours after IS treatment Representative photomicrographs of the nNOS expression in the trigemino-cervical segments after 4 hours. **A** – SIF, **B** – IS, **C** – SUMASIF, **D** – SUMAIS, **E** –

KYNSIF, **F** – KYNIS. In the IS group, the number of nNOS-IR cells was increased compared to the SIF- treated group. Sumatriptan and kynurenic acid were able to mitigate this effect. SIF: synthetic interstitial fluid, IS: inflammatory soup, SUMASIF: sumatriptan + synthetic interstitial fluid, SUMAIS: sumatriptan + inflammatory soup, KYNSIF: kynurenic acid + synthetic interstitial fluid, KYNIS: kynurenic acid + inflammatory soup. Scale bars: 200 µm, 50 µm.

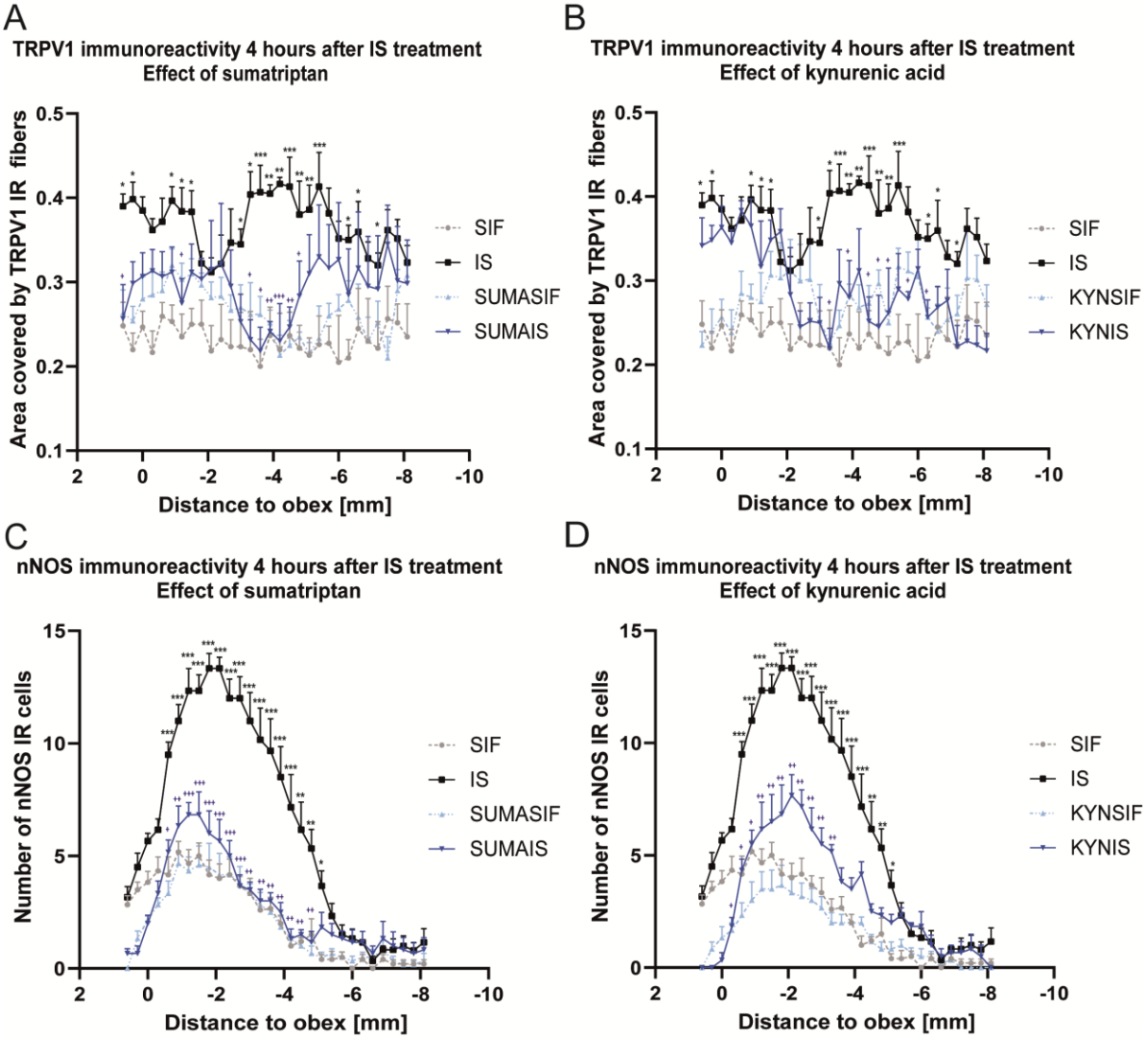


Figure 6. Statistical analysis of TRPV1 and nNOS staining 4 hours after IS treatment The quantitative analysis shows that in the IS group the area covered by TRPV1 IR fibers and the number of nNOS IR cells is significantly higher than in the control group after 4 hours. **A** Sumatriptan was able to attenuate the increase in TRPV1 IR fibers in the V/1 area. **B** Similar to sumatriptan kynurenic acid also mitigated the effect of IS in the V/1 area. **C** In the V/1 area sumatriptan was able to abolish the increase in nNOS IR cells. **D** Kynurenic acid also weakened the effect of IS in the V/1 area *p<0.05; **p<0.01, ***p<0.001. * means SIF-IS differences, + means IS-SUMA/KYNA differences. SIF: synthetic interstitial fluid, IS: inflammatory soup, SUMASIF: sumatriptan +

synthetic interstitial fluid, SUMAIS: sumatriptan + inflammatory soup, KYNSIF: kynurenic acid + synthetic interstitial fluid, KYNIS: kynurenic acid + inflammatory soup

Discussion

The activation and sensitization of the TS are essential hallmarks of migraine pathomechanism. In our rat model, the topically applied IS activated the trigeminovascular system (35, 36), and raised the levels of all the three selected markers in the TNC area. Although other pathways, neurotransmitters (e.g. glutamate, 5HT, prostaglandins) and mechanisms might be involved, the release of neuropeptides from the activated peripheral nociceptive terminals may contribute to the development of neurogenic inflammation in this case (3). These peptides lead to a cascade of inflammatory tissue responses including vasodilation, plasma protein extravasation, and degranulation of mast cells (3), at least in rats.

In our study, as early as two and a half hours after administration, the IS was able to increase the area covered by fibers showing CGRP positivity in the dorsal horn of the cervical spinal cord. The cranial dura mater is densely innervated by CGRP IR fibers (37) thus the increased CGRP level might represent enhanced activation of the primary afferents, which may also be associated with increased CGRP release from the terminals (9) possibly causing a globally higher turnover e.g. intensive synthesis reflected by higher CGRP expression at the terminals (38). It has been also shown, that intracisternal IS can raise the CGRP concentration in the jugular vein which also reflects release from the nerve endings (39). This phenomenon might contribute to the activation and sensitization of primary and secondary nociceptors in the TS via the release of numerous pro-inflammatory agents (e.g. cytokines), which can stimulate the nociceptors (40).

In our experiment, IS was able to significantly increase the amount of TRPV1 IR fibers in the dorsal horn after 4 hours. Amaya and colleagues described that TRPV1 expression is showing

up-regulation in DRG neurons after local inflammation in rats (41). TRPV1 activation by high temperature or capsaicin allows the entry of Ca^{2+}, leading to the release of neuropeptides (42, 43), which are able to influence the development of edema and neurogenic inflammation (44, 45). In another study, after CFA-induced inflammation, increased TRPV1 expression was observed in the digital nerves of the inflamed hindpaw (46). Pharmacologic studies have also shown that TRPV1 is an essential component of the cellular signaling mechanisms through which injury produces thermal hyperalgesia and pain hypersensitivity (47).

TRPV1 is present in the human TG (48) and trigeminal afferents, which innervate the dura mater (49), and these nerve fibers also contain CGRP (50). TRPV1 expression is also upregulated in painful inflammatory conditions in humans (51). In chronic migraine patients, intranasal capsaicin was able to mitigate the migraine pain (52) and TRPV1 agonists might be effective most likely due to desensitization in the acute treatment of migraine (53) as well. Taken together these data point to the fact, that TRPV1 is involved in the trigeminovascular activation and sensitization both in animal models and humans.

In our study, IS significantly enhanced the number of nNOS IR cells in the dorsal horn after 4 hours due to the activation of primary trigeminal nociceptors conveyed to the second-order neurons (54). NO donors cause an increase and release of CGRP at the TG and TNC, and NO donors lead to a delayed enhancement of nNOS in the latter (55). Moreover, bradykinin and histamine trigger NO release from vascular endothelial cells in vitro, suggesting a strong interaction between NO and inflammation (56). An 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 (57, 58). The latter can lead to the activation of primary afferent neurons and CGRP release, activating satellite glial cells that release NO, which can induce nNOS (59). In this context nNOS is can be considered as a significant marker of the sensitization process of the TS in animals.

Interestingly, the increase of TRPV1 and nNOS levels are observed later compared to the changes of CGRP reflecting, 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 increase of CGRP levels and the TRPV1 activity probably through 5-$HT_{1B/1D}$ receptors. This is in line with previous results showing, that CGRP and TRPV1 are co-localized with 5-$HT_{1B/1D}$ receptors in trigeminal neurons (60) and sumatriptan presynaptically inhibits the release of nociceptive neuropeptides (e.g. CGRP) from primary afferents (61) and most of the effects of TRPV1 receptors are mediated through CGRP, which is released after TRPV1 activation (62) so 5-HT might have a role in modulation of TRPV1 function too. This is paralleled with the observations, where sumatriptan mitigated the TRPV1 activity after the intracisternal application of IS (63).

In our experiment, sumatriptan also prevented the increase in the number of nNOS IR cells in the rat TNC after 4 hours suggesting an important involvement of 5-$HT_{1B/1D}$ receptors in the sensitization process in the TS. On the periphery, sumatriptan inhibits presynaptically the release of vasoactive peptides from primary afferents and impairs the development of neurogenic inflammation (25). Sumatriptan prevented the increased NOS production in the brainstem after intracisternal carrageenan injection (64). In humans, NTG-induced headache has been reported to respond to sumatriptan as well (65). Taken together, these results suggest that 5-$HT_{1B/1D}$ agonism can inhibit IS-induced activation and sensitization present in dural inflammatory process.

Compared to sumatriptan, KYNA had a similar effect on the examined markers in our experimental setting and this phenomenon may be mediated through several different receptors. First KYNA is an endogenous, non-selective ionotropic glutamate receptor

316 antagonist, which acts on the non-competitive glycine site of NMDA receptors and it is also a
317 GPR35 ligand (26). Currently, the antagonist effect of KYNA on the α7-nicotinic acetylcholine
318 receptor (nAChR) is contested (66).

319 Three hours after the local IS treatment of the dura, higher glutamate levels can be detected in
320 the TNC (57). In addition to the NMDA receptors, both AMPA, kainate, and metabotropic
321 receptors are found in the TNC (67) and it has been shown, that the antagonists of non-NMDA
322 glutamate receptors also can inhibit the activation of secondary nociceptive neurons (68).
323 AMPA receptors can modulate c-fos expression and possibly the neurotransmission in the TS
324 (69) and in a peripheral pain model, activation of the kainate receptors resulted in the
325 appearance of mechanical, thermal hyperalgesia, and allodynia (68). In the TNC, CGRP can
326 stimulate glutamate expression and that can be inhibited by 5-$HT_{1B/1D}$ receptor agonists (70).
327 Hence, the relationship between the two systems can be assumed.

328 In humans, painful stimulation leads to an increase in glutamate concentration in the trigemino-
329 cervical complex (71) and glutamate levels are elevated both ictally and interictally in migraine
330 sufferers (72). Based on observations in animals and humans, we believe that among others the
331 modulation of glutamatergic neurotransmission is the key event here mitigating CGRP
332 changes.

333 TRPV1 and NMDAR are co-expressed in the TG (73). In a mechanical hyperalgesia test of
334 rats, it was found, that NMDAR and TRPV1 functionally interact probably via the
335 calcium/calmodulin-dependent protein kinase type II (CaMKII) and protein kinase C signaling
336 cascades in rat trigeminal sensory neurons and this interaction has a role in the development of
337 mechanical hyperalgesia (73). GPR35 and TRPV1 co-localize in small- and medium diameter
338 DRG neurons. GPR35 may regulate TRPV1 channel activity by modulating cyclic adenosine
339 monophosphate /protein kinase A pathway (74).

KYNA pretreatment also modulated the IS induced nNOS expression in our animal model. This effect might be related to the anti-glutamatergic effect of KYNA, mainly on the NMDA receptors, which activation is associated with NO production in the spinal trigeminal nucleus (75). Furthermore, Cosi and colleagues described that elevation of KYNA concentration in the brain could decrease the extracellular glutamate levels in the nervous tissue and reduce inflammatory pain (76). Another possible explanation for the peripheral effects of KYNA is that it binds to GPR35, which receptor is present in the DRG (77) and KYNA can inhibit adenylate cyclase activity there via G-protein-dependent mechanisms (78) which might interact with nNOS (79, 80). It has been also reported that abnormalities of the kynurenine pathway are associated with headache disorders e.g. there is evidence that serum kynurenic acid levels decrease during cluster headache and chronic migraine (81, 82, 83).

In the present study, sumatriptan and KYNA were similarly effective mitigating the effects of the IS model. 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.

Conclusion

In our experiment, IS was able to cause sterile neurogenic inflammation in the dura mater. As a consequence of inflammation, changes occur in CGRP, TRPV1, and nNOS levels, which indicates activation and sensitization. Sumatriptan probably acted through the 5-$HT_{1B/1D}$ receptors to reduce the expression of the activation and sensitization markers in the TNC by direct (presynaptic) and indirect (lowered dural inflammation) effects on the periphery. In our present study, KYNA possibly acted primarily on peripheral trigeminal nociceptors and secondary sensory neurons and was able to mitigate the activation of these markers in the TNC predominantly through the inhibition of the glutamate system and thereby blocking the

364 sensitization processes, which is important in primary headaches. These findings can help us
365 to understand the pathomechanism processes in migraine.

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Ethics or Institutional Review Board Approval

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) 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).

Declaration of conflicting interests

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

Author contributions

ES and KFL carried out the experiments and analyzed the data. ES wrote the manuscript with support from ZSB, GNG, AFSz, KFL, and ÁP. MSz helped to select and perform statistical analysis. ÁP supervised the project. LV participated in the conception and design of the experiments and writing. All authors provided critical feedback and helped shape the research and manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon request.

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Consent for Publication

Not applicable.

List of abbreviations:

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

CaMKII - calcium/calmodulin-dependent protein kinase type II

CGRP - calcitonin-gene related peptide

CNS - central nervous system

IR - immunoreactive

IS - inflammatory soup

DRG - dorsal root ganglion

KYNA - kynurenic acid

nAChR - α7-nicotinic acetylcholine receptor

nNOS - neuronal nitric oxide synthase

NMDA - N-methyl-D-aspartate

NO - nitric-oxide

NTG - nitroglycerin

PBS - phosphate-buffered saline

PBS-T - PBS containing 1% Triton-X-100

PFA - paraformaldehyde

SIF - synthetic interstitial fluid

TG - trigeminal ganglion

TNC - caudal trigeminal nucleus

TRPV1 - transient receptor potential vanilloid-1 receptor

TS - trigeminal system

V/1 - ophthalmic nerve

5-HT - 5-hydroxytryptamine

Article Highlights or Key Findings

Inflammatory soup can cause an increase in the CGRP, TRPV1, and nNOS levels in the TNC.

Sumatriptan was able to mitigate the effect of inflammatory soup.

Kynurenic acid could modulate the effect of inflammatory soup.

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II.

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ORIGINAL RESEARCH

Chronic 17β-estradiol pretreatment has pronociceptive effect on behavioral and morphological changes induced by orofacial formalin in ovariectomized rats

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Background: The prevalence of craniofacial pain disorders show sexual dimorphism with generally more common appearance in women suggesting the influence of estradiol, but the exact cause remains unknown. The common point in the pathogenesis of these disorders is the activation of trigeminal system. One of the animal experimental models of trigeminal activation is the orofacial formalin test, in which we investigated the effect of chronic 17β-estradiol pretreatment on the trigeminal pain-related behavior and activation of trigeminal second-order neurons at the level of spinal trigeminal nucleus pars caudalis (TNC).

Methods: Female Sprague Dawley rats were ovariectomized and silicone capsules were implanted subcutaneously containing cholesterol in the OVX group and 17β-estradiol and cholesterol in 1:1 ratio in the OVX+E_2 group. We determined 17β-estradiol levels in serum after the implantation of capsules. Three weeks after operation, 50 µL of physiological saline or 1.5% of formalin solution was injected subcutaneously into the right whisker pad of rats. The time spent on rubbing directed to the injected area and c-Fos immunoreactivity in TNC was measured as the formalin-induced pain-related behavior, and as the marker of pain-related neuronal activation, respectively.

Results: The chronic 17β-estradiol pretreatment mimics the plasma levels of estrogen occurring in the proestrus phase and significantly increased the formalin-induced pain-related behavior and neuronal activation in TNC.

Conclusion: Our results demonstrate that the chronic 17β-estradiol treatment has strong pronociceptive effect on orofacial formalin-induced inflammatory pain suggesting modulatory action of estradiol on head pain through estrogen receptors, which are present in the trigeminal system.

Keywords: c-Fos, headache, pain, sexual dimorphism, trigeminal system

Introduction

The perception of intensity of pain or the responses to painkillers show differences between the sexes.[1,2] Lower tolerance for pain, greater ability to discriminate painful sensations, lower pain thresholds and higher pain ratings can be observed in women.[1–3] Moreover, the localization of pain according to body parts can amplify this sex-related difference, because this discrepancy is more pronounced in the case of craniofacial pain, which is usually more common in women. For example, temporomandibular disorders, mainly manifested in paroxysmal pain in the masticatory muscles and temporomandibular joints,[4] are three times more prevalent in women.[5] Also, more women than men

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suffer from trigeminal neuralgia,[6,7] which is characterized by recurrent unilateral brief electric shock-like pains, abrupt in onset and termination, limited to the distribution of one or more divisions of the trigeminal nerve and triggered by innocuous stimuli.[8] Significant sex-related differences can be observed among the primary headache disorders as well. Women also have higher prevalence of tension-type headache than men,[9] and migraine is three times more common in females compared to males.[10] In contrast, cluster headache is five times more frequent in men than women.[11]

These data indicate that sex hormones may influence the development of trigeminal pain conditions, which is underlined by the observations, that migraine without aura usually starts after menarche, tends to be related to menstrual cycle and ameliorates during pregnancy and after menopause in women.[12] In addition, the appearance of migraine without aura is thought to be related to the fall in estrogen concentrations prior to menstruation,[12,13] while in migraine with aura an increase in attack frequency can be observed related to high estrogen levels, eg, pregnancy.[14] Similarly, the marked female predominance appears only after puberty in the case of tension-type headache as well.[15]

Animal research data also show the presence of the sexual dimorphism and the modulatory effect of sex hormones on the orofacial pain;[16] moreover, site-specific effect of sex hormones on nociception was detected in rats as well.[17] These experimental data might give useful information concerning the molecular mechanisms underlying the sex differences in pain conditions, but they are rather controversial.

Concerning the possible mechanisms, these sex-related differences in craniofacial pain disorders suggest that trigeminal neurons are sensitive to sex hormones, which can modulate their function. Hormonal receptors are present in both the trigeminal ganglion and the spinal trigeminal nucleus pars caudalis (TNC) providing the molecular basis for direct modulatory action on the peripheral and central sensitization in the trigeminal system.[18–21] At present, however, the exact mechanisms underlying sex-related differences in the prevalence of these craniofacial pain conditions remain obscure.

To get further data on the role of estradiol in the sex-related trigeminal nociception, we investigated trigeminal pain-related behavior and c-Fos immunoreactivity – a morphological marker of trigeminal activation – in rats with stable low and stable high estrogen levels in the orofacial formalin test.

Materials and methods

Animals

Fifty-five female Sprague Dawley rats weighing 150–250 g were used. The animals were raised and housed under standard laboratory conditions (in an air-conditioned, humidity-controlled and ventilated room), with drinking water and regular rat chow available ad libitum on a 12 h-12 h dark–light cycle. The procedures used in this study followed the guidelines of the International Association for the Study of Pain and the directive of the European Economic Community (86/609/ECC). They were approved by the Committee of Animal Research at the University of Szeged (I-74-12/2012) and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XXIV/352/2012). All efforts were made to minimize the number of animals used and their suffering.

Ovariectomy

The animals were ovariectomized under deep chloral hydrate (0.4 g/kg body weight, catalog ID: 23100; Sigma-Aldrich, St. Louis, MO, USA) anesthesia administered intraperitoneally. Prior to surgery, rats' back were shaved with electric clippers and furs were removed completely. Cutasept was applied to the shaved area to disinfect the skin. Ovariectomy was preceded by a midline dorsal skin incision, 3 cm long, approximately half way between the middle of the back and the base of the tail. 1.5 cm long peritoneal incisions were made bilaterally. After access into the peritoneal cavity, the ovary and associated fat were easily found, and exteriorized by gentle retraction. Ligature of the blood vessels was also performed. The connection between the Fallopian tube and the uterine horn was cut and the ovaries were removed. Afterward, the animals were randomly divided into two groups: 1) In the OVX group, the rats had two 15 mm long silastic capsule (3.18 mm outer diameter and 1.57 mm inner diameter, catalog ID: 508–008; Dow Corning, Midland, Michigan, USA) filled with cholesterol (15 mg, catalog ID: C8667; Sigma-Aldrich) as control. 2) In the OVX+E_2 group, the animals received two 15 mm long silastic capsule filled with an 1:1 mixture of 17β-estradiol (7.5 mg, catalog ID: 75262; Fluka, Sigma-Aldrich) and cholesterol (7.5 mg). Capsules were inserted subcutaneously in the interscapular region. After implantation of capsules, peritoneal cavity and skin were closed with absorbable sutures. High degree of aseptic procedure was maintained throughout the operation. Surgical instruments were sterilized in 70% ethanol. During and after the surgery, animals were placed on heating plate and covered with paper in order to avoid hypothermia. The analgesia and attenuation of inflammation were provided by subcutaneous (sc) administration of carprofen (5 mg/kg body weight) three times: once before the operation and twice after the surgery (24 and 48 hours).

Measurement of estradiol concentration

17β-estradiol concentration of serum was measured in both groups (n=5). The blood samples were taken weekly from the tail vein for 5 weeks. The serum was cleared from cellular components of the blood by centrifugation at 12,000 rpm for 10 minutes at 4°C and stored at –80°C until use. The concentrations were measured by using Estradiol EIA Kit (catalog ID: 582251; Cayman Chemical Company, Ann Arbor, MI USA) based on the guidelines of the manufacturer.

Behavioral test

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 sc injection of 50 µL physiological saline administered via a 26-gauge needle into the right whisker pad after 3 weeks of recovery following the ovariectomy. In the OVX-Form and OVX+E_2-Form subgroups, the rats were injected with sc 50 µL 1.5% formalin solution (containing 0.55% formaldehyde) diluted in physiological saline via a 26-gauge needle into the right whisker pad. According to Clavelou et al, this concentration is the most appropriate to detect changes in pain-related behavior of rats.[22]

The testing procedures were performed during the light phase (between 8 a.m. and 2 p.m.) in a quiet room. The test box was a 30×30×30 cm glass terrarium with mirrored walls. For the off-line analysis of rubbing activity directed to the whisker pad, the behavior of the individually tested rats was recorded with a video camera (Logitech HD Webcam C615; Logitech Inc., Newark, NJ, USA) situated 1 m above the terrarium. After 10 minutes 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 rats did not receive any food or water during the observation period. The test box was cleaned and decontaminated after each animal. An observer blind to the experimental procedures analyzed the recorded videos. 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.[22]

c-Fos immunohistochemistry

Four hours after the formalin or physiological saline injection, the rats were perfused transcardially with 100 mL of phosphate-buffered saline, followed by 500 mL of 4% paraformaldehyde in phosphate buffer under deep chloral hydrate anesthesia. The medullary segment containing the TNC between +1 and –5 mm from the obex was removed, postfixed overnight for immunohistochemistry in the same fixative and cryoprotected (10% sucrose for 2 hours, 20% sucrose until the blocks sank and 30% sucrose overnight). Before sectioning, each segment was marked with a small incision on the ventral and left (contralateral) side of the tissue block, allowing side discrimination during the quantification process; 30 µm transverse cryostat sections were cut through the rostrocaudal axis from the beginning of the TNC and were serially collected in wells containing cold PBS. Each well contained every tenth section at 0.3 mm intervals along the rostrocaudal axis (15 levels [sections]/well). The free-floating sections were rinsed in PBS and immersed in 0.3% H_2O_2 in PBS for 30 minutes to suppress endogenous peroxidase activity. After several rinses in PBS containing 1% Triton X-100 (PBST), sections were incubated at room temperature overnight in PBST containing rabbit anti-rat c-Fos polyclonal antibody (catalog ID: sc-52, RRID: AB_2106783; Santa Cruz Biotechnology Inc., Dallas, TX, USA) at a dilution of 1:2000. The immunohistochemical reaction was visualized by using Vectastain Elite Avidin-Biotin Kits (catalog ID: PK6101; Vector Laboratories, Burlingame, CA, USA). Briefly, the sections were incubated at room temperature for 2 hours in PBST containing goat anti-rabbit biotinylated secondary antibody. After several rinses in PBST, and incubation at room temperature for 2 hours in PBST containing avidin and biotinylated horseradish peroxidase, the immunohistochemical labeling was visualized with 3,3′-diaminobenzidine intensified with nickel ammonium sulphate. The specificity of the immune reactions was checked by omitting the primary antiserum.

The counting of immunoreactive (IR) cells in the TNC was performed by an observer blinded to the experimental procedures under the 10× objective of a Nikon Optiphot-2 light microscope in every tenth transverse section in each animal. Before the counting, the location of each section along the rostrocaudal axis and the location of the TNC on each medullary section were determined by means of The Rat Brain in Stereotaxic Coordinates Atlas.[23] The c-Fos neurons with obvious specific nuclear staining were counted in the TNC both ipsilaterally and contralaterally to the formalin or physiological saline injection.

Statistical analysis

For statistical comparison of 17β-estradiol concentration of serum in the two groups (OVX and OVX+E_2), we used

two-way repeated-measures ANOVA. Pairwise comparisons of group means were based on the estimated marginal means with Sidak adjustment for multiple comparisons.

In the behavioral study, we compared the nociceptive responses in two time periods as described by Clavelou et al.[22] The first 3 minutes (block 1) are characterized by intensive rubbing activity and defined as the first phase. Following a relatively relaxed period, the rubbing intensifies again between about 12 and 45 minutes (second phase) and remains high for a longer period of time (blocks 5–15), which is defined as the second phase. For the comparison of the rubbing activities between subgroups (OVX-Phys, OVX-Form, OVX+E_2-Phys and OVX+E_2-Form) in the first and second phase, we used one-way ANOVA followed by the Tamhane post hoc test.

The number of c-Fos-IR neurons in the various subgroups (OVX-Phys, OVX-Form, OVX+E_2-Phys and OVX+E_2-Form) were compared at each level of 0.3 mm (15 levels) along the rostrocaudal axis by using two-way repeated-measures ANOVA. There was no significant difference in the number of c-Fos-IR neurons between the contra- and ipsilateral sides in rats injected with sc physiological saline and the contralateral sides in animals injected with sc formalin (OVX-Form and OVX+E_2-Form); therefore, the data obtained from the contralateral sides of the subgroups injected with sc formalin (OVX-Form and OVX+E_2-Form) were used as controls in the statistical analysis. Pairwise comparisons of subgroup means were based on the estimated marginal means with Sidak adjustment for multiple comparisons.

All tests were two-sided, and probability levels $P<0.05$ were considered to be statistically significant. Group values are reported as mean ± standard error of the mean (SEM).

Statistical analysis of measurements was carried out with IBM SPSS Statistics, version 20 software (IBM Corporation, Armonk, NY, USA).

Results

Estradiol concentration

Following the ovariectomy, in both groups, an approximate steady state status in serum concentration of 17β-estradiol was maintained for 5 weeks with an average value of 25.93 pg/mL in the OVX group and 64.55 pg/mL in the OVX+E_2 group (Figure 1). In OVX+E_2 group, the ovariectomized female rats received two silastic capsules containing in all 15 mg 17β-estradiol, which chronic pretreatment keeps the serum concentration of 17β-estradiol at significantly higher level compared with the OVX group (***$P<0.001$; Figure 1). Although we did not find significant difference between the serum levels measured weekly in OVX+E_2 group, a tendency of lower level in the fifth week can be observed (Figure 1). This result suggests that serum concentration of 17β-estradiol began to decrease in the fifth week similarly to data published in an earlier work.[24] Since a stable serum concentration was found from second to fourth week (Figure 1), we examined the potential modulating effect of chronic 17β-estradiol pretreatment in the third week following the ovariectomy and implantation of capsules. At this time point, the average estradiol concentration in the serum was 25.01 pg/mL in OVX group and 61.29 pg/mL in OVX+E_2 group (Figure 1).

Nociceptive response

The behavioral pattern following orofacial injection of formalin observed in the rats is in accordance with

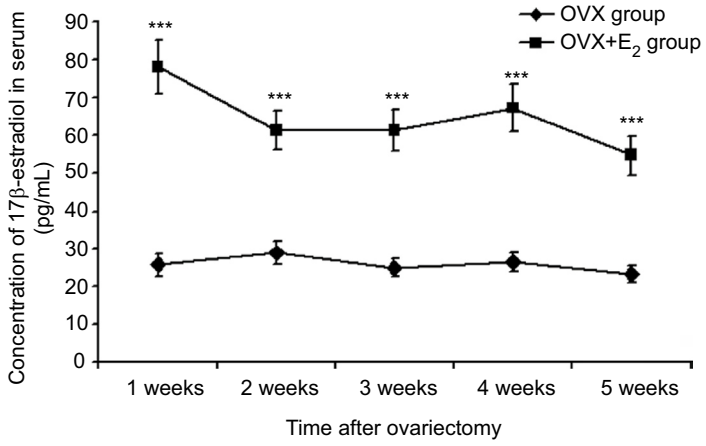

Figure 1 The concentration of 17β-estradiol in serum (pg/mL) in the OVX and OVX + E2 groups.
Notes: The chronic 17β-estradiol treatment significantly increases the serum concentration compared with the OVX group (***$P<0.001$).

previous findings.[22,25,26] After the formalin injection, the rats immediately withdrew their heads, often accompanied by vocalization. Following their return to the observation box, the rats started to rub their whisker pad continuously and intensely with the ipsilateral forepaw accompanied often by the contralateral forepaw, and occasionally scraped the perinasal area with the ipsilateral hindpaw after a quiet period of ~20 seconds. This period, referred to as the first phase, lasted ~3–4 minutes, and was followed by a quiescent period of 9–10 minutes, separating the first phase from the second phase (Figure 2). The second phase was characterized by less intense, but continuous rubbing of the face, predominantly with the ipsilateral forepaw consorted often by the contralateral forepaw as well. This tonic phase lasted ~30–33 minutes (Figure 2). In both subgroups injected with formalin (OVX-Form and OVX+E_2-Form), the biphasic pain-related behavioral pattern can be observed (Figure 2). In the OVX+E_2-Form subgroup, this pattern was clearly more pronounced than that in the OVX-Form subgroup (Figure 2), and such behavior was not detected at all in the OVX-Phys and OVX+E_2-Phys subgroups, where the animals displayed very little rubbing activity (Figure 2).

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 (Figure 2).

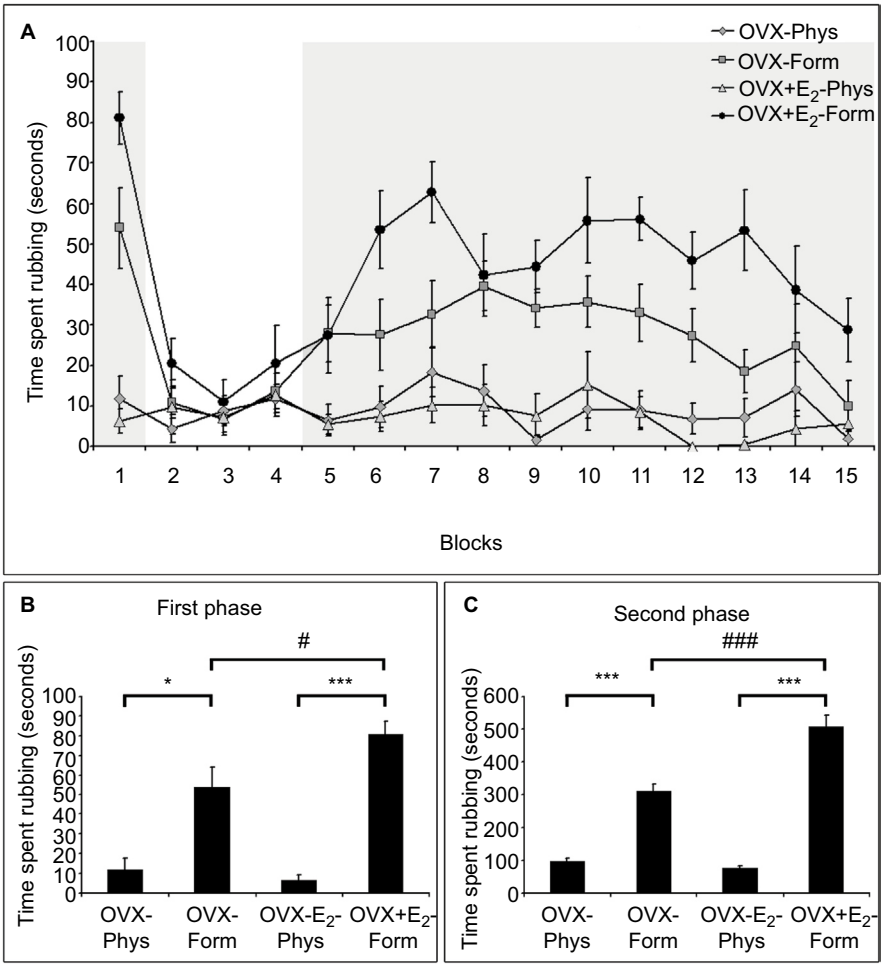

Figure 2 Diagrams show the time spent with pain-related behavior in OVX-Phys, OVX+E2-Phys, OVX-Form and OVX+E2-Form subgroups.

Notes: (**A**) Diagram shows the time spent rubbing the injected area during 45 minutes of recording period in 3 minutes intervals (blocks 1–15) in all four subgroups. In the OVX-Form and OVX+E_2-Form subgroups, the two phases of formalin action are clearly distinguishable: the first phase (block 1, first grayed out area) lasted ~3–4 minutes and was followed by a quiescent period of 9–10 minutes (block 2–4); the second phase lasted ~30–33 minutes (block 5–15, second grayed out area). The rubbing activity in OVX+E_2-Form subgroup was more pronounced than that in the OVX-Form subgroup. The biphasic pain-related behavioral pattern does not appear in the OVX-Phys and OVX+E_2-Phys subgroups. (**B**, **C**) Diagrams show the rubbing activity in the two phases in all four subgroups. The subcutaneous formalin injection (OVX-Form and OVX+E_2-Form) induced a significant increase in rubbing activity in both the first and the second phase when compared with that in the saline-treated animals (OVX-Phys and OVX+E_2-Phys) (*P<0.01; ***P<0.001). In both phases, pretreatment with estradiol had a significant enhancing effect on the formalin-induced nociceptive behavior when compared with the OVX-Form subgroup ($^{\#}P$<0.05; $^{\#\#\#}P$<0.001).

Data obtained from OVX-Form and OVX+E_2-Form subgroups show that chronic 17β-estradiol pretreatment significantly increased the nociceptive behavior in both phases (#P<0.05; ###P<0.001; Figure 2).

c-Fos in the TNC

Microscopic examination of the immunostained transverse sections of medulla containing the TNC revealed c-Fos immunoreactivity in the nuclei of the neurons. In the OVX-Form and OVX+E_2-Form subgroups, unilateral sc formalin injection produced an increase in the number of c-Fos-IR neurons in the dorsal, superficial area of the ipsilateral TNC when compared with the non-treated contralateral side (Figure 3). This increase was significant at all levels along the rostrocaudal axis, in accordance with the somatotopic representation (*P<0.05; **P<0.01; ***P<0.001;+ P<0.05;++ P<0.01;+++ P<0.001; Figure 4).

In the OVX+E_2-Form subgroup, the effect of formalin on the number of c-Fos-IR neurons seems to be similar, but more pronounced than that in the OVX-Form subgroup (Figure 3). The results of statistical analysis show that this chronic 17β-estradiol-induced increase in the formalin-related activation of the second-order trigeminal neurons is significant at several levels of the TNC along the rostrocaudal axis (#P<0.05; ##P<0.01; ###P<0.001; Figure 4). On the contralateral sides of the TNCs, there were no significant differences either between the subgroups or between the different levels along the rostrocaudal axis (Figure 4).

Discussion

Although, there is a clear female predominance in craniofacial pain disorders suggesting the modulatory role of sex hormones, there are only relatively few and conflicting studies, which investigated their influence on the trigeminal nociception. In the present experiments, we examined the effect of chronic, stable high 17β-estradiol level in serum on trigeminal pain and trigeminal activation in orofacial formalin test of rat.

The source of estradiol found in OVX rats is probably extragonadal.[27] On the other hand, the used chronic estradiol

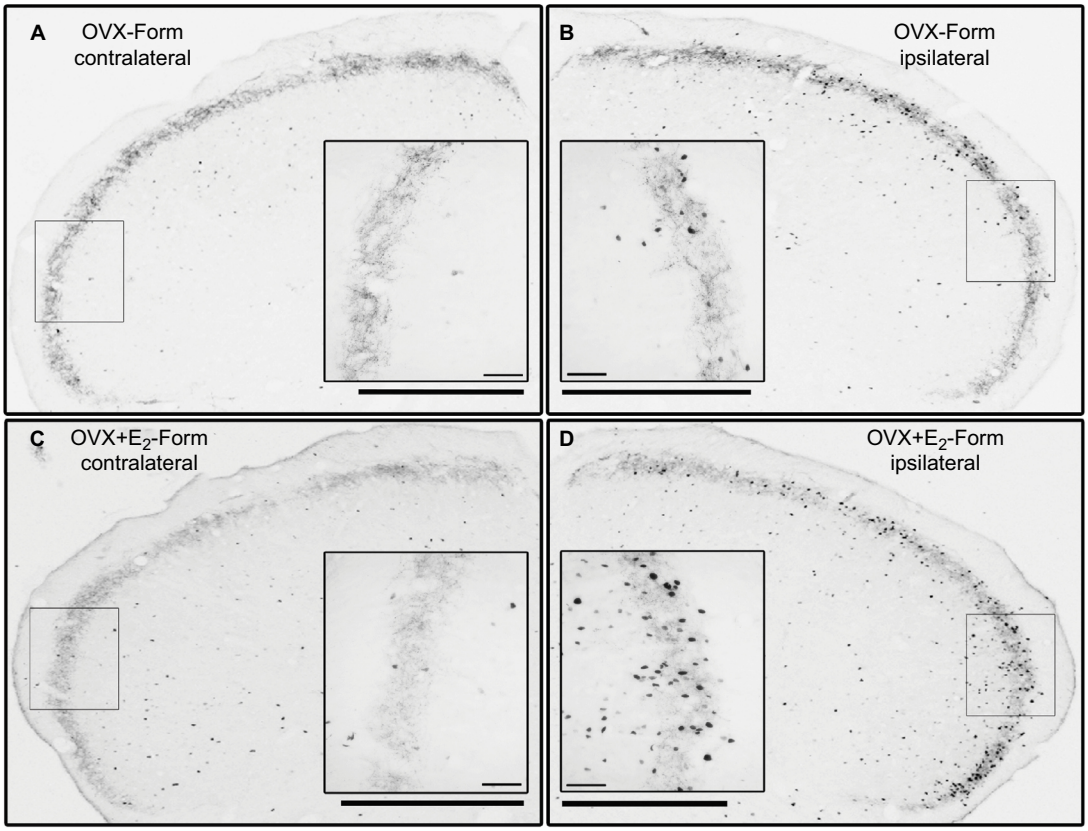

Figure 3 Photos of the c-Fos immunostained transverse sections of dorsolateral medulla containing the spinal TNC from the OVX-Form and OVX+E_2-Form subgroups. Inserts show the framed regions in greater magnification.

Notes: On the ipsilateral side (**B, D**), more c-Fos-immunoreactive neurons can be observed than on the contralateral side (**A, C**) in both subgroups. After formalin injection, there is an increased number of c-Fos-immunoreactive neurons on the ipsilateral side in the OVX+E_2-Form subgroup (**D**) compared to the OVX-Form subgroup (**B**). In these two subgroups, difference between the contralateral sides (**A, C**) cannot be observed. Scale bar: 500 and 50 μm.

Abbreviation: TNC, trigeminal nucleus pars caudalis.

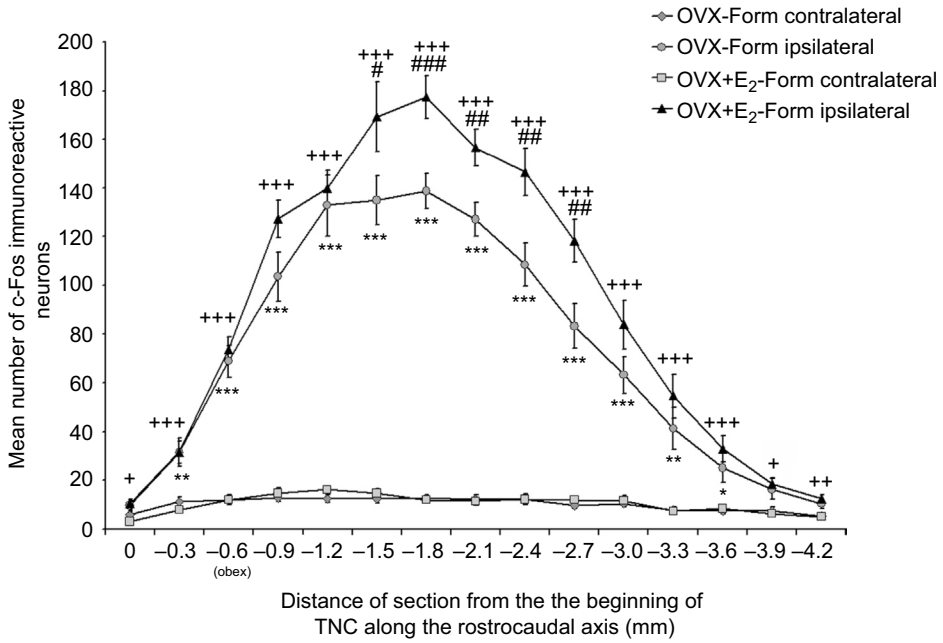

Figure 4 Diagram shows the mean number of c-Fos-immunoreactive cells in the superficial area of the spinal trigeminal nucleus pars caudalis (TNC) at different levels along the rostrocaudal axis.

Notes: Formalin produced a significant increase in number of c-Fos-immunoreactive neurons on the ipsilateral side of the TNC in the OVX-Form (**P*<0.05; ***P*<0.01; ****P*<0.001) and OVX+E_2-Form (^{+}P<0.05; ^{++}P<0.01; ^{+++}P<0.001) subgroups when compared with the contralateral side at different levels along the rostrocaudal axis. The effect of formalin was significantly increased by pretreatment with estradiol ($^{\#}P$<0.05; $^{\#\#}P$<0.01; $^{\#\#\#}P$<0.001). There was no significant difference between the contralateral sides.

pretreatment resulted in an average serum 17β-estradiol level of 61.29 pg/mL in OVX+E_2 group of rats, which is comparable with the value of serum concentration of estradiol in cycling rats during the proestrus phase, when the estrogen concentration is at its peak level.[28–31]

Our data show that the chronic 17β-estradiol treatment was pronociceptive in orofacial formalin test compared with the control, ovariectomized, female rats. The effect of estradiol was shown in both the first and the second phase of orofacial formalin test, where the first phase is caused by the direct chemical stimulation of the nociceptors by the formalin solution, while the second phase is the result of peripheral inflammation.[32] Furthermore, this chronic estradiol treatment enhanced the formalin-induced trigeminal activation at the level of second-order trigeminal neurons located in the TNC, as reflected by the increased c-Fos immunoreactivity, which is one of the anatomical markers of the pain-induced neuronal activity.[33]

The molecular basis for estrogen to directly regulate the pain transmission at the level of trigeminal system is mediated by estrogen receptors, which has three known types: estrogen receptor alpha (ERα), ERβ and G-protein-coupled estrogen receptor (or G-protein-coupled receptor-30 [GPR30]). These receptors are present in the trigeminal system. ERα can be observed in 22% of primary trigeminal neurons of rat, where it is found mainly in nuclei of cells with larger diameter and in cytoplasm of smaller neurons.[34] Satellite glia in the trigeminal ganglion of rat also express ERα.[35] ERβ is also present in both small to large neurons in the Gasserian ganglion of rat, but not in the satellite glia.[35] GPR30 receptor can be observed in 35% of neurons in trigeminal ganglion of rat and shows cytoplasmatic localization mainly in small diameter neurons with unmyelinated axons, but it is present in neurons with myelinated axons with a broad range of cell sizes, too.[34] ERα and GPR30 are colocalized in 10% of primary trigeminal neurons of rat.[34] In superficial laminae of rat TNC, ERα and ERβ proteins are co-expressed by neurons,[18] and in this area, ERα was shown to be present in nociceptive-responsive neurons.[36] GPR30 IR cells were also localized in the mouse TNC.[37] In human TNC, ERα immunostaining was found in the nucleus and cytoplasm of neurons and glial cells and in the nerve fibers; ERβ was detected in the cytoplasm of neuronal cells.[38] Experimental data show that the modulation of these receptors results in well-defined changes of trigeminal pain processing. In temporomandibular joint inflammation induced by Complete Freund's Adjuvant (CFA), estradiol potentiated the effect in dose-dependent manner and the blocking of estrogen receptors by an antagonist was able to

evolve anti-inflammatory action.[39] It was also shown that estrogen receptors located in different parts of the trigeminal system might mediate pronociceptive responses to estrogen. In the same animal model, estrogen receptor stimulation by specific agonists enhanced the secondary mechanical allodynia and the authors found in the trigeminal ganglion an increased immunoreactivity of the activated extracellular signal-regulated kinase, which is a specific marker of pain-induced activation of nociceptor.[34] The involvement of trigeminal ganglion in this process was shown in primary trigeminal cultures: microarray and protein activity assays also demonstrated the estrogen-induced activation of ERK.[40]

Effects of estrogen may manifest through two different pathways: a slow genomic and a rapid non-genomic mechanism[41,42] and both pathways play important role in regulation of trigeminal pain processing. By influencing the transcription of certain genes (*mitogen-activated protein kinase-1*, *interleukin-1 receptor type I*, *bradykinin B2 receptor*, *GABA transporter protein*, *GABA A receptor subunit α6, opioid receptor-like 1 receptor*, *purinoreceptor P2X3*, *transient receptor potential vanilloid 1* and *neuropeptide Y*) with potential relevance to craniofacial pain, long-lasting changes were reported in different cells.[35,40,43–46] Estrogen can activate intracellular signaling pathways via non-genomic, membrane-mediated mechanisms also, which may occur within seconds or minutes.[40,47–50] These cellular mechanisms affect numerous processes, which are essential in trigeminal pain perception including the function of endogenous antinociceptive system,[51,52] the modulation of the excitability of TNC neurons.[53] In other studies, alteration in the activation mechanisms,[54] in the neuronal firing activity[48] and in the glutamatergic neurotransmission[55,56] was demonstrated. On the other hand, estrogen-dependent changes were reported in the expression of several factors such as calcium/calmodulin-dependent protein kinase II α, calcitonin gene-related peptide or serotonin, as well. These molecules are playing important role during pain processing.[57–59]

Our present data on the pronociceptive effect of estradiol are supported by earlier studies. In ovariectomized rats, estradiol valerate replacement, administered as a single, sc injection, increased primary (in masseter muscle) and secondary (in whisker pad) facial allodynia after CFA-induced inflammation of masseter muscle mediated by ERK activation in the trigeminal ganglion.[49] Kou et al[39] reported that in ovariectomized rats 17β-estradiol, subcutaneously administered for 10 days, potentiated the inflammation and exacerbated the pain-induced decrease in the food intake in temporomandibular joint inflammation model. The inflammation was induced by intraarticular injection of CFA and the effect was dose-dependent. The authors discuss the possibility of estrogen effect through the nuclear factor-κB (NF-κB) pathway inducing the enhancement of the DNA-binding activity of NF-κB and the increased transcription of its target genes in the synovial membrane. In another experiment, a single sc injection of 17β-estradiol, administered 48 hours before the testing, worsened the thermal hyperalgesia in orofacial inflammation caused by sc injected carrageenan in ovariectomized rats, which may be caused by the decreased α_2-adrenoceptor-mediated inhibition of nociception and hyperalgesia.[60] High 17β-estradiol for 2 days, mimicking the plasma levels of estrogen in proestrus, significantly increased the duration of pain-related behavior (eye wipe test induced by capsaicin) and the activation of trigeminal neurons in TNC indicated by c-Fos immunoreactivity. The authors conclude that the effect may be in part due to the estrogen-dependent increases in mRNA of transient receptor potential vanilloid 1 and anoctamin 1 in TNC, which have important role in trigeminal pain.[46]

The complexity of the possible mechanisms involved may explain the conflicting data on the effect of estrogen on trigeminal pain and trigeminal system. Antinociceptive effect of single sc estradiol replacement administered 18–24 hours prior to the nociceptive testing was shown by Flores et al[61] on facial pain-related behavior induced by intracisternally injected *N*-methyl-D-aspartic acid. In other experiments, pronociceptive effect of low serum level of estrogen was reported in rats in the temporomandibular joint inflammation,[50,62,63] in the orofacial formalin model,[17] in the basic facial mechanical pain threshold[45] or after ligature of the masseter tendon.[64] There are also results, however, which show that serum level of estrogen do not have any effect on trigeminal pain for example in the eye wipe test after ten-day long pretreatment with daily sc 17β-estradiol-3-benzoate[54] and in the masseter inflammation in normally cycling female and male rats.[51] Similarly, estradiol benzoate, given subcutaneously 48 hours prior to the nociceptive testing, had no effect in rats on facial pain-related behavior induced by intracisternally injected NMDA in males, in ovariectomized females and in normally cycling females tested at proestrus or diestrus stages.[65]

Conclusion

We can conclude that chronic 17β-estradiol pretreatment was able to significantly influence both the formalin-induced nociceptive behavior and the c-Fos expression at the level of trigeminal system suggesting its pronociceptive effect on the trigeminal pain and the potentiation of activation of TNC

neurons. For the first time, we maintained continuously high 17β-estradiol serum level for a relatively longer period (21 days) in ovariectomized female rats, excluding the effect of other sexual hormones in the orofacial formalin test. The estrogen receptors are present in all key areas of trigeminal nociception, including the trigeminal ganglion and the TNC, and we hypothesize that the hormone may influence the neuronal processes induced by pain through the increase of mRNA of transient receptor potential vanilloid 1 and anoctamin 1 in TNC, or modulation of the NF-κB pathway or activation of ERK in the trigeminal ganglion.

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Author contributions

The specific contributions of the authors to this research were as follows: AFSz participated in the design and implementation of experiments, collected data for statistical analysis, interpreted the data and wrote the manuscript. ES, LT and GNG participated in the design and in the implementation of the experiments and in the critical revision of the manuscript. ZB participated in the design of the experiments and in the critical revision of the manuscript. KFL participated in the critical revision of the manuscript. LV participated in the design of the experiments, in the critical revision of the manuscript and in the final approval of the version to be published. ÁP participated in the design of the experiments, the interpretation of the data and the writing and critical revision of the manuscript. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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