Activation of the trigeminal system in the animal models of migraine

Klaudia Flóra Laborc M.D.

Ph.D. thesis summary

Supervisor: Árpád Párdutz M.D., Ph.D.

Doctoral School of Clinical Medicine
Experimental and Clinical Neuroscience Program
Department of Neurology
Albert Szent-Györgyi Medical School
University of Szeged
Szeged, 2023

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

I.

Klaudia Flóra Laborc, Eleonóra Spekker, Zsuzsanna Bohár, Mónika Szűcs, Gábor Nagy-Grócz, Annamária Fejes-Szabó, László Vécsei, Árpád Párdutz
"Trigeminal activation patterns evoked by chemical stimulation of the dura mater in rats"
The Journal of Headache and Pain 2020 Aug 15;21(1):101
IF: **7.277**

II.

Gábor Nagy-Grócz, Zsuzsanna Bohár, Annamária Fejes-Szabó, **Klaudia Flóra Laborc**, Eleonóra Spekker, Lilla Tar, László Vécsei, Árpád Párdutz
Nitroglycerin increases serotonin transporter expression in rat spinal cord but anandamide modulated this effect
Journal of Chemical Neuroanatomy 2017 Nov;85:13-20
IF: **2.162**

Cumulative impact factor of the publications directly related to the thesis: 9.439

Publications not directly related to the Ph.D. thesis:

I.
Gábor Nagy-Grócz, Lilla Tar, Zsuzsanna Bohár, Annamária Fejes-Szabó, **Klaudia Flóra Laborc**, Eleonóra Spekker, László Vécsei, Árpád Párdutz
The modulatory effect of anandamide on nitroglycerin-induced sensitization in the trigeminal system of the rat
Cephalalgia 2016 Aug;36(9):849-61
IF: **3.609**

II.
Gábor Veres, Annamária Fejes-Szabó, Dénes Zádori, Gábor Nagy-Grócz, Anna M László, Attila Bajtai, István Mándity, Márton Szentirmai, Zsuzsanna Bohár, **Klaudia Laborc**, István Szatmári, Ferenc Fülöp, László Vécsei, Árpád Párdutz
A comparative assessment of two kynurenic acid analogs in the formalin model of trigeminal activation: a behavioral, immunohistochemical and pharmacokinetic study
Journal of Neural Transmission 2017 Jan;124(1):99-112
IF: **3.886**

III.
Gábor Nagy-Grócz**, Klaudia Flóra Laborc**, Gábor Veres, Attila Bajtai, Zsuzsanna Bohár, Dénes Zádori, Annamária Fejes-Szabó, Eleonóra Spekker, László Vécsei, Árpád Párdutz
The effect of systemic nitroglycerin administration on the kynurenine pathway in the rat
Frontiers in Neurology 2017 Jun 14;8:278
IF: **3.508**

IV.
Scott J Denstaedt, Joanna L Spencer-Segal, Michael W Newstead, **Klaudia Laborc**, Anne P Zhao, Alexander Hjelmaas, Xianying Zeng, Huda Akil, Theodore J Standiford, Benjamin H Singer
S100A8/A9 Drives Neuroinflammatory Priming and Protects against Anxiety-like Behavior after Sepsis.
Journal of Immunology 2018 May 1;200(9):3188-3200
IF: **4.718**

V.
Annamária Fejes-Szabó, Eleonóra Spekker, Lilla Tar, Gábor Nagy-Grócz, Zsuzsanna Bohár, **Klaudia Flóra Laborc**, László Vécsei, Árpád Párdutz
Chronic 17β-estradiol pretreatment has pronociceptive effect on behavioral and morphological changes induced by orofacial formalin in ovariectomized rats.
Journal of Pain Research 2018 Sep 25;11:2011-2021
IF: **2.236**

VI.
Scott J Denstaedt, Joanna L Spencer-Segal, Michael Newstead, **Klaudia Laborc**, Xianying Zeng, Theodore J Standiford, Benjamin H Singer
Persistent Neuroinflammation and Brain Specific Immune Priming in A Novel Survival Model of Murine Pneumosepsis.
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VII.
Joanna L. Spencer-Segal, Benjamin H. Singer, **Klaudia Laborc**, Khyati Somayaji, Stanley J. Watson, Theodore J. Standiford, Huda Akil
"Sepsis survivor mice exhibit a behavioral endocrine syndrome with ventral hippocampal dysfunction"
Psychoneuroendocrinology 2020 Jul;117:104679
IF: **4.732**

VIII.
Eleonóra Spekker, **Klaudia Flóra Laborc**, Zsuzsanna Bohár, Gábor Nagy-Grócz, Annamária Fejes-Szabó, Mónika Szűcs, László Vécsei, Árpád Párdutz
"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"
The Journal of Headache and Pain 2021 Mar 31;22(1):17.
IF: **8.592**

IX.
Swapnill Gavade, Qiang Wie, Colin Johnson, Savannah Kounelis-Wuillaume, **Klaudia Laborc**, Salisha Baranwal, Huda Akil, Joanna L. Spencer-Segal
Forebrain glucocorticoid receptor overexpression alters behavioral encoding of hippocampal CA1 pyramidal cells in mice
eNeuro 2022 Dec 9;9(6):ENEURO.0126-22.2022
IF: **4.363** (2021)

Cumulative impact factor of publications not directly related to the thesis: 39.098

Total impact factor: 48.537

List of abbreviations

5-HT – serotonin
5-HTergic – serotonergic
AEA – anandamide
CB – cannabinoid receptor
CFA – Complete Freund's Adjuvant
CGRP – calcitonin gene-related peptide
ECs – endocannabinoid system
IR – immunoreactive
IS – inflammatory soup
nNOS – neuronal nitric oxide synthase
NTG – nitroglycerin
PET – positron emission tomography
PHYS – physiologic saline
SERT – serotonin transporter
SIF – synthetic interstitial fluid
TNC – caudal trigeminal nucleus
TRPV1 – transient receptor potential receptor 1
V/1 – ophthalmic nerve
V/2 – maxillary nerve
V/3 – mandibular nerve

Introduction

Migraine is a common and often debilitating disease, affecting one billion people worldwide. The International Headache Society defines migraine as a recurrent throbbing headache that usually lasts 4-72 hours and can be accompanied by other symptoms such as photophobia, phonophobia, nausea, vomiting, and allodynia.

Migraine is a substantial public health problem due to its significant impact on the affected patients and society. The headache attacks can cause severe disability, leading to decreased productivity and reduced quality of life. The total cost of migraine affects the economy as well due to absenteeism and healthcare costs, which was estimated at €50 billion to €111 billion per year in Europe (2010).

Despite the increasing availability of migraine-specific drugs, the effectiveness of these therapies remains relatively low, with an efficacy rate of around 60%. There is high demand for more effective treatments, although the current lack of knowledge of the underlying causes hinders the development of potential new drugs.

The exact pathomechanism of migraine is still not fully understood, but it is known that the activation and sensitization of the trigeminal system have a crucial role in headaches. All three branches of the trigeminal nerve provide sensory fibers to the dura mater and the face, forming a plexus of nerve fibers in the dural - and pial vessel walls. The vasodilation of these vessels can activate the first-order neurons in the trigeminal ganglion, and as a result, various vasoactive mediators like calcitonin gene-related peptide (CGRP) and substance-P are released. These neurotransmitters increase the sensitivity of these ganglion cells to stimuli; this process is called peripheral sensitization and contributes to the throbbing nature of the headache. The ongoing overactivation of first-order neurons and the release of mediators also affect the second-order sensory neurons, leading to central sensitization in the caudal trigeminal nucleus (TNC), which can cause allodynia, a condition when non-painful stimuli become painful.

Serotonin (5-HT) is a monoamine neurotransmitter involved in various physiological and pathological processes, including migraine and pain perception. 5-HT has been shown to have both pronociceptive and antinociceptive effects, depending on the site of action, receptor subtype, and cell type. 5-HT acts as a pronociceptive mediator in the periphery after nerve

injury or inflammation, and it can activate and sensitize nociceptors, while in the descending pain modulatory system, it can either reduce or enhance spinal pain transmission.

It has been known for a long time that 5-HT plays a key role in migraine pathophysiology. It was first suggested after increased hydroxy-indoleacetic acid (the breakdown product of 5-HT) level was observed in the urine of migraineurs experiencing headaches. There is a growing amount of evidence suggesting that decreased levels of 5-HT in the brain may cause migraine. For instance, the use of the drug reserpine, which reduces 5-HT levels, has been shown to trigger attacks. Another study using rats found that lower levels of 5-HT were linked to increased number of c-Fos immunoreactive (IR) cells in the TNC after the dura was stimulated with inflammatory soup (IS).

Certain brainstem areas are known to play a role in the onset of migraines, including the periaqueductal gray, rostral ventromedial medulla, and nucleus raphe magnus. Activation of these structures has been shown to reduce pain and increase the spinal release of 5-HT, this effect can be prevented with nonselective 5-HT receptor antagonists, indicating that pain alleviation is mediated by 5-HT. These findings suggest a complex regulating role of 5-HT in pain and migraine headache, but the details still need to be understood.

The serotonin transporter (SERT) is implicated in the regulation of 5-HT levels in the extracellular space and synaptic cleft through its reuptake function. A positron emission tomography (PET) imaging study revealed that migraine patients exhibit higher levels of SERT in the mesopontine brainstem compared to healthy controls, probably due to either inherent elevated levels of the transporter or increased expression in response to abnormal 5-HT neurotransmission. To summarize, 5-HT has a multifaced role in regulating of inflammation and pain transmission, and an increasing amount of evidence suggests that variations in 5-HT levels and its regulation might be involved in the pathophysiology of migraine.

Fos is a protein expressed in various cell types, including neurons and glial cells, and is commonly utilized as a marker to investigate functional pain pathways. c-Fos is classified as an immediate early gene, its expression is initiated shortly after a stimulus and has a half-life of two hours. Although c-Fos is believed to be somewhat non-specific, measurement of its expression is a widely accepted method for studying activation patterns and identifying specific

pathways. Careful control of experimental conditions can provide a reliable means of comparing the effect of different stimuli and testing potential drugs in animal models.

Nitroglycerin (NTG), also known as glyceryl trinitrate, is a medication that has been used in medical settings for decades. It has the ability to pass through membranes easily due to its lipophilic properties, allowing it to reach the central nervous system through the blood-brain barrier. Despite having a short half-life of 1-4 minutes in the bloodstream, NTG accumulates in lipophilic tissues, including the brain. NTG is a potent vasodilator that increases the cGMP levels, which leads to the relaxation of smooth muscle in the blood vessels. However, NTG can cause headaches in healthy individuals, and particularly in migraine patients. In about 80% of these patients, NTG has a biphasic effect, causing an immediate headache followed by a delayed migraine-like one 4-6 hours later, which meets the criteria of the migraine headache. The immediate headaches are believed to be the result of its vasoactive properties, and symptoms resembling the prodrome phase, as well as activation of the hypothalamus and periaqueductal gray, have been observed after NTG administration. The origin of the delayed headache that can occur after NTG use is not fully understood, but a PET scan study has shown brainstem activation during these headaches, similar to migraine. Additionally, increased levels of CGRP and substance P have been detected in the external jugular vein during NTG-induced headaches, suggesting the involvement of proinflammatory pathways, which has also been confirmed in animal studies. Lysine-acetyl-salicylate pretreatment was able to prevent an increase in levels of neuronal nitric oxide synthase (nNOS) in the TNC of rats, which suggests that the cyclo-oxygenase pathway may be involved after the administration of NTG. In addition to nNOS, NTG administration has been shown to increase the synthesis of inducible nitric oxide synthase. Due to its ability to cause headaches, NTG is often used as a model for migraine. Several animal studies support the idea that NTG can also activate and sensitize the trigeminal system, resulting in an increase in c-Fos protein expression reaching its peak four hours after NTG injection, similar to the timing of the onset of the delayed headache in migraine patients. NTG was also found to cause hyperalgesia and allodynia in animals in multiple behavior studies.

In various experiments, chemical substances such as capsaicin, Complete Freund's Adjuvant (CFA), and IS were used to stimulate the meninges. The IS contains four different inflammatory mediators, including 5-HT, bradykinin, prostaglandin E2, and histamine. It was demonstrated that IS not only activates secondary neurons, but also sensitizes them to mechanical stimulation.

This was confirmed through behavioral studies in rats, which showed that the IS induced cutaneous allodynia and decreased the withdrawal threshold to von Frey filament stimulation. CFA, a suspension of dried, killed Mycobacterium tuberculosis, is commonly used to induce inflammation in joints and skin. When applied to the dura, both CFA and IS increased the presence of mediators, such as interleukin-1β, in the trigeminal ganglion, suggesting the development of neurogenic inflammation after chemical stimulation of the meninges. Both migraine-specific drugs (e.g., sumatriptan) and nonsteroid anti-inflammatory drug (such as naproxen and ketorolac) were effective in both clinical trials and after chemical stimulation of the dura in rodents, indicating the translational potential of the model.

In summary, using NTG and chemically stimulating the dura in rodents have been shown to activate and sensitize the trigeminal system, resulting in pain and allodynia-related behavior resembling symptoms of migraine. These experimental methods have accurately predicted the effectiveness of migraine medications, showing the prospective use of the models in future studies.

The endocannabinoid system (ECs) is involved in various physiologic processes, such as appetite, energy metabolism, immune function, memory, sleep, thermoregulation, and pain modulation. The ECs performs these functions through the activation of cannabinoid receptors (CB), specifically CB1 and CB2. However, research studies have shown that in certain cell types within the same brain region, agonist binding can lead to the action of different signaling pathways. The ECs plays a crucial role in pain modulation, and the administration of cannabinoids, either peripherally or intrathecally, has been shown to reduce hyperalgesia and allodynia, alleviate local edema and decrease nociceptor firing. In addition, studies examining brain structures that are involved in pain processing found that endocannabinoids are abundant in the spinal cord dorsal horn and TNC. The pain-relieving effects of endocannabinoids, such as anandamide (AEA), are related to CB1 and CB2 receptors, although AEA may also exert its neuromodulatory effects through the transient receptor potential receptor 1 (TRPV1).

The ECs influences the serotonergic (5-HTergic) system in the periphery and central nervous system, which may also contribute to their ability to reduce pain. A specific CB1 agonist, arachidonoyl-2'-chloroethylamide, decreased 5-HT levels in the peripheral blood, and this effect was blocked with pretreatment with a CB1 antagonist, indicating that cannabinoids can

alter 5-HT levels in the periphery. Furthermore, selective inhibition of CB1 decreased the firing rate of 5-HTergic cells in the dorsal raphe in vitro, suggesting that endocannabinoids may regulate the tonic activity of 5-HTergic cells in the dorsal raphe.

It has been proposed that deficiencies in the ECs may be present in various pain-related conditions, like migraine, fibromyalgia, and irritable bowel syndrome. This hypothesis was based on the observation that female migraineurs have higher levels of fatty acid amide hydrolase activity in platelets, which suggests increased breakdown of AEA in female migraine patients. The deficiency of the ECs was further supported by a study that detected lower levels of AEA in the cerebrospinal fluid of chronic migraineurs compared to healthy controls. In addition, a negative correlation was found between cerebrospinal fluid levels of AEA and CGRP as well as AEA and nitrate in these patients. In a migraine animal model, intraperitoneal administration of AEA also decreased the number of Fos positive cells in the TNC following NTG injection, suggesting that AEA may modulate nociceptive transmission in the trigeminal system.

Aims

1. To examine the expression of SERT in the dorsal horn of TNC after systemic administration of NTG.

2. To investigate whether the AEA is affecting the expression of SERT in the NTG model.

3. To map the activation pattern in the dorsal horn of the TNC after chemical stimulation of the dura mater.

Materials and methods:

The procedures used in our study followed the guidelines outlined by the Use of Animals in Research of the International Association for the Study of Pain and the European Parliament (86/609/ECC; 2010/63/EU), as well as the directive of the Committee of the Animal Research of University of Szeged (I-74-12/2012; I-74-49/2017) and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XI./352/2012; XI./1098/2018).

I. The effect of NTG and AEA on the expression of SERT

Animals, experimental groups:

Forthy-four Sprague-Dawley rats (200-250 g) were utilized in our study. The animals were housed and raised in a standard laboratory setting and provided *ad libitum* access to standard rat chow and water under a 12-h light/dark cycle. The rats were randomly allocated into four groups, with six and five animals per group selected for immunohistochemistry and Western blot. Group 1 received physiological saline (PHYS) as a pretreatment and vehicle as a treatment. Group 2 was injected with both PHYS and NTG. Groups 3 and 4 were given AEA as pretreatment and NTG or vehicle after.

Immunohistochemistry

Four hours post NTG or vehicle injection, all animals were perfused. The segments corresponding to the TNC (obex -5mm to -11 mm) were removed and post-fixed overnight in formaldehyde. After cryoprotection, 30-µm transverse sections were cut using a cryostat and stained for SERT.

To take photomicrographs of the stained sections, a Zeiss AxioImager microscope with an AxioCam MRc Rev.3 camera (Carl Zeiss Microscopy, Jena, Germany) was used with a 20× objective. The laminae I, II, and III of the dorsal horn were chosen as the area of interest, and the density of IR fibers was quantified using Image-Pro Plus 6.21 software. This density was calculated as the percentage of the designated region covered by pixels with densities above the threshold. To assess the size of the IR varicosities, additional images were taken at a higher magnification of 40×. The borders of laminae I, II, and III were manually defined as the region of interest. The relative area covered by immunopositive fibers was calculated for each lamina and presented as a percentage of the corresponding structures. To examine the size of the IR varicosities, 40× magnification photomicrographs were taken using the same digital system. Individual immunolabeled processes in focus were defined as single objects, and their areas were determined using the same method described above.

Western blot

The expression levels of the SERT in the TNC were quantified using Western blot. Dorsal horns of the TNC were removed and separated and subsequently stored in cold lysis buffer containing

50 mM Tris-HCl and 150 mM NaCl until use. Care-Stream Kodak Biomax Light film was utilized for the visualization of the protein bands. Films were scanned then densitometric analysis was conducted with ImageJ 1.47v analysis software (National Institute of Health). Results were normalized to the reference protein glyceraldehyde-3-phosphate as an internal control to ensure equal protein loading across samples.

II. Activation patterns in the TNC following chemical stimulation of the dura

Forty-eight adult male Sprague Dawley rats with a body weight of 240-430 grams were assigned to twelve groups. The animals were maintained under standard laboratory conditions with a light-dark cycle of 12-12 hours and were provided access to standard chow and water *ad libitum*. Two groups did not undergo craniotomy and were only exposed to anesthesia (FRE) or anesthetized and fixed in a stereotaxic instrument (NAT) for a two-hour duration and perfused after. The animals of 2PHYS/2CFA and 4PHYS/4CFA groups were deeply anesthetized, and craniotomy was drilled carefully using PHYS for cooling, and 10 µL CFA or PHYS was applied on the dural surface. After the twenty-minute treatment, the dura was rinsed with PHYS, and rats were perfused after two or four hours. In the LPHYS/LCFA groups, animals had the same procedure as the 2CFA/2PHYS groups, with the additional administration of local lidocaine to the scalp prior to the incision. 2SIF/2IS and 4SIF/IS groups underwent the same surgery as LPHYS/LCFA, but synthetic interstitial fluid (SIF) or IS was put on the dura mater, and after 2.5- and 4-hour, the animals were perfused.

Rats were anesthetized with i.p. chloral hydrate (4%; 400 mg/kg) prior the surgery. We examined the effect of two different inflammatory substances in our study: CFA is dried and inactivated Mycobacterium tuberculosis in mineral oil (Sigma-Aldrich, St. Louis, MO, USA), while IS contained 1 mM bradykinin, 100 µM PGE_2, 1 mM 5-HT, 1 mM histamine, (pH 5.0) in 10 mM HEPES buffer. 0.9% PHYS or SIF (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM glucose, in 10 mM HEPES buffer, pH 7.3) was used in the control groups. We infiltrated the skin with lidocaine (1%, 10 mg/mL; dosage of 4.5 mg/kg; Egis, Budapest, Hungary) for local anesthesia.

After perfusion, the brain with the cervical spinal cord was extracted for the c-Fos immunohistochemistry and left overnight in the fixative. Following cryoprotection, 30 µm transverse sections were made with cryostat and stained for c-Fos.

The IR cells in laminae I-II of the dorsal horn and also according to the somatotopic representation of the ophthalmic nerve (V/1), maxillary nerve (V/2), and mandibular nerve (V/3) (based on Strassman and Vos, 1993) were counted by an observer blind to the procedures. Nikon Optiphot-2 light microscope (Nikon, Tokyo, Japan) equipped with 10× objective was used for the cell counting, while the representative photographs were taken by an AxioImager M2 microscope (Carl Zeiss, Germany) with AxioCam MRc rev.3 camera under 20x objective.

Results:

I. The effect of NTG and AEA on the expression of SERT

We found abundant SERT-positive fibers in the dorsal horn's superficial layers (laminae I-III). The relative area covered by SERT immunolabelled fibers was significantly higher in NTG-treated animals compared to animals in the placebo group ($p < 0.01$), and AEA had the same effect on the SERT fibers ($p < 0.01$; $p < 0.001$). Surprisingly, the combined NTG+AEA treated group had significantly decreased SERT area fractions ($p < 0.01$) compared to either NTG or AEA groups.

The average size of the fiber varicosity was significantly increased in the NTG or AEA group compared to the placebo group ($p < 0.05$). Combined NTG+AEA treatment reduced the average size of varicose fibers in comparison to NTG or AEA treatment.

Western blot analysis showed the same tendency as we obtained in SERT immunohistochemistry. The densitometric analysis confirmed that NTG and AEA treatment caused significantly enhanced SERT protein band density ($p < 0.01$) compared to the placebo group. Compared to NTG- or AEA-treated groups, the protein band of the NTG+AEA group was significantly less dense ($p < 0.05$; $p < 0.01$) in comparison to either NTG- or the AEA-treated groups.

II. Activation patterns in the TNC following chemical stimulation of the dura

Placement of the ear bars or snout fixation in a stereotaxis frame caused a bilateral dense, localized increase in the number of IR cells in the somatotopic area of the V/2 nerve. Anesthetic drug (chloral hydrate) and perfusion (FRE group)

The effect of chloral hydrate and the perfusion in the TNC was negligible.

Dural application of CFA did not cause a notable increase in the number of IR cells compared to PHYS, neither two hours nor four hours after administration. We did not find a difference in the number of cells between the right and left dorsal horns, neither in 2CFA nor in 2PHYS groups.

No difference was found between the 4CFA and 4PHYS groups four hours after CFA application on the dura. The number of c-Fos IR cells was less after four hours compared to the two hours group, and we found the same tendency in cell distribution as in 2CFA and 2PHYS groups. S.c. applied lidocaine decreased the number of c-Fos immunopositive cells compared to the 2CFA group, particularly in the V/1 branch area.

After applying IS on the dura, animals showed significantly increased number of c-Fos immunolabelled cells in the TNC. These changes were more prominent in the 2IS group, especially in the somatotopic area of the V/1 nerve. In the 2IS group, we found a significant difference between the right (ipsilateral) and left (contralateral) sides at the same rostrocaudal levels. The cell distribution in the 4IS group is comparable to the peak seen in the 2IS group in the V/1 area; this suggests sustained stimulation of the nerve. Four hours after the IS application, we found a significant difference between the 4SIF and 4IS groups, both in the dorsal horn and V/1 area.

Discussion

I. SERT expression following systemic administration of NTG and AEA

SERT is a monoamine transporter protein that regulates the concentrations of 5-HT in both synaptic and extracellular spaces. Previous research demonstrated that inhibiting 5-HT synthesis can exacerbate headache attacks in migraine patients, suggesting that SERT may play a role in the pathomechanism of the condition. In our experiment, treatment with NTG led to an increase in the amount of SERT IR fibers and expression of SERT in the segments corresponding to TNC. It is known that systemic administration of NTG can also enhance the number of 5-HT IR fibers in the TNC, which might be indicative of reduced 5-HT release from the terminals or increased neurotransmitter turnover. Prior research has identified a potential connection between NO and the 5-HTergic system, as the physical interaction between nNOS and SERT was observed, which suggests the possibility of reciprocal regulation between these systems. Similarly, pretreatment with AEA resulted in an increase of SERT-immunopositive

fibers and transporter expression within the dorsal horn of TNC. A link between the cannabinoid and 5-HTergic system was suggested earlier after the treatment with selective CB1 receptor agonist was able to decrease 5-HT levels in the periphery, an effect that could be prevented by pretreatment with CB1 receptor antagonist. While cannabinoids were shown to decrease peripheral 5-HT levels, they also were able to increase central 5-HT release via the descending pain control system, potentially contributing to their analgesic effects. This was supported by the finding that selective lesions of the spinal cord 5-HTergic pathways could attenuate the antinociceptive effects of cannabinoids.

It is also possible that AEA indirectly increases the expression of SERT, and this effect was mediated through the modulation of NO levels. Our previous study showed that AEA could modulate the expression of nNOS, and our results demonstrated that the pretreatment with AEA before systemic NTG did not alter the SERT expression and amount of SERT IR fibers in the TNC compared to controls. However, as both NTG and AEA alone were shown to increase the expression of SERT, it is possible that negative feedback mechanisms were induced in response to the combined treatment, leading to no differences between the two groups. Our previous study demonstrated that NTG enhanced the levels of sensitization markers in the TNC, and this effect was prevented by pretreatment with AEA. This suggests that additional mechanisms might be involved in the regulation of SERT expression that could be the subject of further research.

II. Activation pattern in the trigeminal system following chemical stimulation of the dura

The expression of c-Fos was measured to assess activation patterns in response to chemical stimulation of the dura in the TNC. Our experiment aimed to learn the effect of multiple experimental variables in the animal model of migraine. Urethane is a commonly used anesthetic known to elicit activation of the trigeminal system; therefore, first, we examined the effect of chloral hydrate. However, similar to pentobarbital, perfusion in chloral hydrate anesthesia did not induce a significant change in the number of c-Fos IR cells in the TNC. The heads of the animals were fixed in the apparatus after the animals were deeply anesthetized. This caused pronounced and variable c-Fos activation in the TNC, primarily in the V/2 area, while only a small number of immunopositive cells were detected in the V/3 area. The head of

the rats was fixed at three points, and the use of the stereotaxic frame was inevitable for performing the craniotomy. The observed increase in c-Fos IR cells in the V/2 area may be due to the fixation of the upper incisors, which receive sensory innervation from the V/2. In contrast, the V/3, cervical spinal nerves, and Arnold's nerve are primarily responsible for innervating the external ear. These results are showing the baseline activation patterns in the TNC after craniotomy and enables specific examination of the inflammation mediated c-Fos expression.

In this study, we also aimed to examine the impact of CFA on the secondary trigeminal neurons within the TNC. CFA, a solution of inactivated Mycobacterium suspended in mineral oil, is often used to elicit inflammation in various tissues. We compared the number of c-Fos IR cells in the TNC after two and four hours following the application of CFA or PHYS. We found that CFA did not significantly alter the number of immunopositive cells in the TNC in comparison to the control PHYS, even when the scalp was anesthetized with lidocaine prior to the surgical incision. Earlier studies showed that CFA applied on the dura mater can lead to an increase in the levels of phosphorylated extracellular kinase, CGRP, and interleukin-1β in the primary trigeminal neurons after four hours. However, the maximal effect of CFA may require more time to act, potentially due to the presence of substances in the solution that prolong its degradation and extend its duration of action. CFA was suggested to trigger a type IV or late-type hypersensitivity reaction, a T-cell-dependent response that takes longer to develop. This property may make it suitable for modeling headache chronicity. When injected into the temporomandibular joint, CFA is able to induce prolonged inflammation, which is associated with the release of various mediators, such as CGRP, resulting in a self-amplifying process. This phenomenon, referred to as neurogenic neuroinflammation, can be comparable to repeated migraine attacks. While these findings suggest a longer duration of action for the CFA, its prolonged use was also associated with severe adverse effects, including the development of skin ulcers, local tissue necrosis, and granuloma formation, leading us to not extend our experiments to longer survival times.

The pathomechanism of migraine may involve neuroinflammatory processes, which can be modeled by the application of proinflammatory mediators on the surface of the dura mater. A few of these substances, such as prostaglandin E2, bradykinin, and 5-HT, are the components of the IS that can directly irritate tissues, including meninges. In our experimental model, the administration of IS caused significant increase in the c-Fos IR cells in the TNC after two and

four hours, with the greatest effect in the V/1 area. It was previously demonstrated that using IS on the dura was able to cause trigeminal sensitization characterized by decrease in action potential threshold and increase in the receptive field. Behavioral manifestations of sensitization, such as allodynia in both facial and limb regions, were also described and tend to peak at approximately three hours and resolve after six hours. The prolonged activation observed in our experiments may result from trigeminal sensitization induced by IS.

Conclusion

To summarize, in the present study, we examined the effects of NTG and AEA on the expression of SERT in rodents. Our findings revealed that both NTG and AEA individually increased SERT expression, however, this effect was prevented when the substances were administered together. These results suggest that NTG may modulate the 5-HTergic system in addition to activating and sensitizing the trigeminal system. The endocannabinoid AEA might cause this change in SERT expression by activating the descending pain control system or increasing nNOS activity. The combination of NTG+AEA may counteract these effects through negative feedback mechanisms. Our data support the hypothesis that the 5-HTergic and endocannabinoid systems might be connected and that the 5-HTergic pathways may play a role in the analgesic effects of cannabinoids. Our results also showed that IS, when applied to the dura mater of rats, activated cells in the TNC, which were most prominent in the somatotopic area of the V/1. It is important to note that surgical procedures may affect and mask the activity changes induced by chemical stimulation of the dura, we demonstrated the necessity of rigorous experimental controls. In conclusion, the results of this study suggest a potential relationship between the 5-HTergic and endocannabinoid systems and provide a reliable method for migraine modeling in rodents, thereby contributing to a better understanding of the underlying pathomechanisms in migraine.

Acknowledgment

First of all, I would like to express my gratitude to my advisor, Dr. Árpád Párdutz, for his mentorship and giving me the opportunity to work in his lab. I sincerely thank him for his patience, support, and guidance throughout my time in the lab and beyond; I will be forever grateful for it.

I would like to thank Professor László Vécsei and Professor Péter Klivényi for providing me with the opportunity to work in the Department of Neurology.

I wish to thank all of my coworkers in the Department of Neurology. Dr. Zsuzsanna Bohár, Dr. Annamária Fejes-Szabó, and Dr. Gábor Nagy-Grócz introduced the world of neuroscience to me when I was only an undergraduate student, they taught me the nitty-gritty of performing experiments, and kept me enthusiastic about scientific research throughout the years. I am also thankful for Dr. Eleonóra Spekker's help. I want to thank Valéria Vékonyné Széll and Erzsébet Lukács for their assistance and guidance in the lab.

I am grateful to all the people who shaped my view about neuroscience and provided me more learning opportunities, especially Dr. Joanna Spencer-Segal and Dr. Kathryn Hilde.

Finally, I am truly indebted to my family and friends, especially my mom and my brother, for believing in me and encouraging me over the years. Most of all, I am deeply grateful to my husband, without his constant support and love, none of this would have been possible.