NEUROINFLAMMATORY MECHANISMS IN THE DEVELOPMENT OF STRESS-INDUCED PAIN IN A MOUSE MODEL

Doctoral (PhD) Thesis

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1. 1. GENERAL INTRODUCTION, LITERATURE REVIEW, RESEARCH CONCEPT

1.1 Chronic primary pain and nociplastic pain

According to the definition published by the World Health Organization, chronic primary pain disorders are pain conditions that persist for more than three months and cannot be explained by tissue or nerve damage (1–3). Diseases belonging to this group include, for example, fibromyalgia (FM) and complex regional pain syndrome (CRPS).

Chronic primary pain can be classified into the group of so-called nociplastic pain, in which disturbances of the central nervous system processing underlie the pain. This third pain entity is distinct from the classical nociceptive (caused by tissue injury) and neuropathic (associated with nerve injury) pain types (4).

1.2. The relationship between chronic stress and pain

Persistent psychosocial stress and chronic primary pain are closely intertwined, mutually aggravating conditions (5). Plasticity changes in the limbic system (hippocampus, amygdala, and prefrontal cortex) in response to stress may contribute to the development of central sensitization, where pain becomes independent of the initial stimulus and may become chronic (6–8). In parallel, dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis also plays a significant role in the maintenance and aggravation of both pain and frequent psychiatric comorbidities (anxiety, depression, sleep disturbances) (9).

In my dissertation, I present the results of our preclinical research, obtained from a chronic stress-induced pain mouse model with FM-like mechanisms.

1.3. Clinical characteristics and treatment of fibromyalgia (FM)

FM is a disorder characterized by spontaneous musculoskeletal pain occurring throughout the body and persisting for at least three months, often accompanied by sleep disturbances, cognitive decline, fatigue, and anxiety. Patients typically exhibit increased pain sensitivity (allodynia, hyperalgesia) and usually experience cold intolerance as well (10). The diagnosis is currently based on the diagnostic criteria issued by the American College of Rheumatology, which considers the extent of symptoms by mapping painful areas as well as the severity of complaints (11).

The treatment strategy requires a multidisciplinary approach, with physiotherapy and psychological management also playing essential roles in the disease therapy (12,13). Classical analgesic drugs, nonsteroidal anti-inflammatory drugs, and opioids are not effective in relieving

pain in FM patients. First-line adjuvant analgesics – primarily antidepressants (duloxetine, amitriptyline) and antiepileptics (pregabalin, gabapentin) – have "A" level, strong evidence for the treatment of FM (14,15). However, the development of the therapeutic effect of these drugs often requires long-term administration, which is limited by the numerous side effects that restrict their applicability (16).

1.4. Etiology of FM

In FM, no local organ or tissue alterations can be detected at the site of pain; however, recent studies suggest disturbances in central pain modulation and the importance of neuroinflammatory processes. Imaging techniques (PET CT, fMRI) have revealed increased glial cell activation in brain regions responsible for pain processing in FM patients (17,18).

1.5. Neuroinflammation

Neuroinflammation refers to the immune response of the central nervous system to various harmful stimuli (e.g., infection, trauma, neurodegeneration, or psychosocial stress). The key players of this process are primarily microglia and astrocyte cells, which, upon activation, release inflammatory mediators, cytokines, chemokines, and reactive oxygen and nitrogen species. These substances initially play a protective role, for instance, by promoting the elimination of damaged cells or supporting regeneration processes. However, excessive activation or prolonged effects may become neurotoxic and contribute to neuronal dysfunction or even cell death. Neuroinflammation is therefore a dual-natured process in which the protective and damaging mechanisms of the immune response operate in a delicate balance (19,20).

1.6. Fractalkine receptor (CX3CR1)

Among the numerous signaling pathways involved in neuroinflammation, the protein fractalkine (FKN), produced by cells in response to neuronal injury, plays a vital role by acting as a chemoattractant for immune cells at the site of injury. FKN binds to its specific receptor, the fractalkine receptor (C-X3-C chemokine receptor 1 – CX3CR1), which is found on T cells and monocytes in the periphery and primarily on microglial cells in the central nervous system, thereby inducing cell activation (21). The "danger" signal (e.g., cell damage, oxidative stress) is transmitted by microglia, for example, via the NOD-like receptor protein 3 (NLRP3) inflammasome, partly through the release of the proinflammatory cytokine interleukin-1 (IL-1) to surrounding cells (22,23).

Literature data indicate that activation of this receptor contributes to mood disorders, the formation of stress response, as well as the development of neuropathic and inflammatory pain, and it also plays a role in central pain sensitization (24–26). Glia–neuron interactions influenced by this receptor are important in the development of psychosomatic disorders. Although higher FKN concentrations have been measured in the blood of FM patients (27), limited data are available on the role of the CX3CR1 receptor in fibromyalgia.

1.7. NLRP3 inflammasome

Inflammasomes are multiprotein complexes that, as part of the innate immune system, play a key role in regulating inflammatory processes. These intracellular sensor proteins are activated by various pathogens or cell damage and mediate the maturation and release of inflammatory cytokines (28).

The NLRP3 inflammasome is responsible for the production of IL-1β and IL-18. IL-1β is one of the key inflammatory mediators, closely associated with neuroinflammatory processes (29).

1.8. Interleukin-1

Cytokines are low-molecular-weight (10–40 kDa) glycoproteins that mediate intercellular communication. Their diverse effects on cells and immune processes are exerted by binding to cytokine receptors located on the cell membrane, initiating intracellular signaling cascades.

Our research focuses on IL-1α and IL-1β (IL-1) within the IL-1 superfamily, the most extensively studied proinflammatory cytokines of the group, as they are key players of the inflammatory response and primary mediators of numerous pathological processes (30,31). In the central nervous system, IL-1 is mainly produced by microglia, and through the IL-1 surface receptor (IL-1R), it activates multiple cell types (NK, T and B cells, astrocytes), and by binding to receptors on neurons, it can transmit the proinflammatory signal to several sites. Moreover, in an autocrine manner, it promotes the activation of additional microglia and astrocytes and is indispensable in the development of neuroinflammation (30,31).

Both acute and chronic stress reactions have been shown to elevate IL-1 levels (32). IL-1 has also been associated with the development of depression; however, changes in IL-1 levels have not yet been clearly demonstrated in the plasma of FM patients (33).

1.9. Hemokinin-1

In the development of neuroinflammation, not only glial and immune cells, but also neuropeptides released by neurons play a crucial role. The tachykinins are one of the most extensively studied neuropeptide families, including Substance P (SP), neurokinin A and B, and

the most recently described hemokinin-1 (HK-1), which primarily acts through the neurokinin-1 (NK1) receptor (34). The role of the SP–NK1 system in pain processing is well established, and although NK1 antagonists have proven effective in animal models for alleviating neuropathic pain, they have not been successful in clinical trials (35,36). In the case of HK-1, the mediated effects are not exclusively linked to the NK1 receptor, suggesting that other, yet unidentified structures may also be involved. HK-1 expression can be detected in several tissues (brain, airways, liver, kidney, uterus), and it plays a role in inflammatory and immune responses (37,38). During microglial activation, its levels increase, in HK-1-deficient mice, a traumatic mononeuropathy model showed reduced pain and lack of trauma-induced microglial activation (39). Higher HK-1 levels have been reported in fibromyalgia patients, indicating its possible pathogenic significance (40). Overall, HK-1 is a vital regulator of inflammation, pain, and immunomodulation; however, its role in fibromyalgia remains unclear.

1.10. FM models in mice

Numerous mouse models have been described in the literature for modeling human FM, among which the chronic restraint stress (CRS) model accurately reflects the symptom characteristics of FM. During CRS, prolonged non-invasive stress induces mechanical and cold hyperalgesia, as well as neuroinflammatory changes.

In our institute, ongoing experiments aimed at characterizing the CRS model have shown, during pharmacological validation, that classical analgesics ineffective in FM patients (e.g., diclofenac, tramadol) also failed to significantly alleviate stress-induced pain in this model. However, “A” level evidence-based adjuvant drugs (duloxetine, gabapentin) were effective. Our results further strengthen the translational value of the CRS model, and since our institute has several years of experience with this well-reproducible model, we chose this protocol.

2. AIMS

During my PhD work, I investigated the central nervous system neuroinflammatory mechanisms of chronic stress-induced pain, involving the CX3CR1 fractalkine receptor located on the surface of microglia, the NLRP3 inflammasome found in glial cells, and the proinflammatory cytokine IL-1 produced within it, as well as HK-1 encoded by the Tac4 gene. Our series of experiments aimed to examine the role of these key elements in a chronic restraint stress-induced pain mouse model, using knockout mice and pharmacological tools. Since FM occurs more frequently in women, particular attention was paid in our experiments to the possible role of sex differences.

3. EXPERIMENTAL MODELS AND METHODS

3.1. Animals

For our studies, we used 12–16-week-old male and female C57Bl/6J (wild-type, WT), as well as IL-1αβ knockout (IL-1 KO), CX3CR1 receptor knockout (CX3CR1 KO), and Tac4 knockout (Tac4 KO) mice. The animals were bred and housed in the Animal Facility of the Department of Pharmacology and Pharmacotherapy at the University of Pécs. For environmental enrichment, polycarbonate shelters were placed in the cages to provide the animals with safety and hiding places.

3.2. Chronic restraint stress (CRS)

To establish the model of chronic stress-induced pain, the animals of the stressed group were placed daily for 6 hours into well-ventilated tubes that greatly restricted their movements, for two or four weeks (41).

3.3. Experimental design

Baseline pain threshold measurements were conducted one week before the application of stress. To determine the baseline nociceptive threshold of each animal, the tests performed (mechanical and cold tolerance – see section 3.5) were repeated three times on non-consecutive days. The results were averaged and recorded as "baseline" values. During the two- or four-week CRS, nociceptive tests were performed once weekly. In contrast, behavioral tests were conducted once during the last week of the stress protocol (either the second or fourth week).

All tests began in the afternoon, at least two hours after immobilization. The experimenter was blinded to group allocation and, in the case of pharmacological protocols, to the administered treatment. At the end of the stress protocol, animals were perfused, followed by tissue sample processing.

3.4. Compounds testing

The role of CX3CR1, NLRP3, and IL-1 in chronic stress-induced pain was also investigated using pharmacological blockade with antagonist compounds. Along with to the CRS experimental protocol described above, we administered the CX3CR1 antagonist AZD8797 (1 mg/kg, twice daily (42)), the NLRP3 inflammasome blocker MCC950 (10 mg/kg, once daily (43)), and the clinically available IL-1 receptor antagonist anakinra (10 mg/kg, once daily (44)). These treatments were given intraperitoneally once daily during the two-week CRS period.

3.5. Nociception testing

3.5.1. Measurement of mechanonociceptive threshold

The mechanical pain threshold of mice was determined using a dynamic plantar aesthesiometer (DPA). The animals were placed in plastic compartments, and after 10 minutes of conditioning, a gradual increase in pressure was applied to the mid-plantar surface with a blunt needle (maximum 10 g, within 4 seconds). Upon a pain response (paw withdrawal, shaking, licking), the device stopped, and the value was recorded in grams. Three measurements were taken on each paw, and the average was considered the pain threshold. Values measured during CRS were analyzed as a percentage change from baseline thresholds (45).

3.5.2. Cold tolerance test

The cold pain threshold was determined by measuring paw withdrawal latency. One hind paw of the mouse was immersed in water near 0 °C containing crushed ice, ensuring no direct contact with the ice. The cut-off point was 180 seconds. Upon pain response (paw withdrawal), the measurement was stopped, and the reaction time was recorded in seconds. Changes were expressed relative to baseline values (46).

3.6. Behavioral tests

3.6.1. Forced Swim Test (FST)

The FST was used to assess depression-like behavior. Mice were placed into a transparent plastic cylinder filled with tap water at 22–24 °C to a height of 17 cm. The test lasted 6 minutes, during which immobility time was recorded in the last 4 minutes. Increased immobility is directly proportional to the lack of coping and is interpreted as depression-like behavior (47).

3.6.2. Light–Dark Box (LDB)

This test was used to measure anxiety levels. In a box divided into a light and a dark compartment, mice could freely move between the two areas. During the 5-minute test, the time spent in the light compartment was inversely proportional to the level of anxiety. Parameters were analyzed with EthoVision® XT software from video recordings (48).

3.6.3. Open Field Test (OFT)

The spontaneous locomotor activity and anxiety-like behavior of mice were measured in a 48×48×55 cm illuminated arena. The 5-minute test was recorded with a camera system and analyzed using EthoVision® XT software (49).

3.7. Perfusion and sample processing

After 2 or 4 weeks of the study, and following the behavioral tests, mice were euthanized with a combination of ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.m.). The thoracic cavity was opened, and transcardial perfusion was performed: first with 0.1 M phosphate-buffered saline (PBS, pH 7.4), then with 4% paraformaldehyde (PFA) solution in Millonig buffer (pH 7.4).

3.8. Processing of brain tissue

After one day of postfixation in 4% PFA, brains from six randomly selected mice per group were cut into 30 μm-thick coronal sections using a Vibratome (Leica VT1000 S, Leica Biosystems, Richmond, IL, USA).

3.9. Conventional avidin–biotin immunoperoxidase immunohistochemistry

3.9.1. Ionized calcium-binding adaptor molecule-1 (IBA1)

Immunoreactivity of the microglial marker IBA1 (n = 6/group) was examined using a conventional avidin–biotin immunoperoxidase protocol. After preparatory and blocking steps, sections were incubated with rabbit anti-IBA1 primary antibody (019-19741, Wako Chemicals GmbH, Neuss, Germany, dilution 1:10,000) diluted in PBS with 2% normal goat serum. This was followed by incubation with 1.5% biotinylated goat anti-rabbit IgG (Vector Labs). After incubation with the avidin–biotin complex, the reaction was visualized using 3,3-diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) with 0.003% H_2O_2 (50).

3.9.2 Glial fibrillary acidic protein (GFAP)

Immunoreactivity of the astrocyte marker GFAP (n = 5–7/group) was examined on sections stained with the same conventional avidin–biotin immunoperoxidase protocol. The detailed steps were identical to the IBA1 immunolabeling, with the following differences: in the GFAP immunostaining, nonspecific binding sites were blocked with 2% normal horse serum (Vector Laboratories, Burlingame, CA, USA) in PBS, and mouse monoclonal anti-GFAP primary antibody (NCL-LGFAP-GA5, Novocastra, dilution 1:1000) diluted in PBS with 2% normal horse serum was used. As a secondary antibody, 1.5% biotinylated anti-mouse IgG (Vector Labs) was applied. The signal was visualized with DAB, similar to the IBA1 staining (51).

3.9.3 Immunohistochemical data analysis – IBA1, GFAP

Images were taken from the sections using an Olympus IX81 microscope and an Olympus DP74 digital camera with a 10X objective. Quantitative analysis of integrated density (using the built-in plugin of ImageJ), cell count, and activation measurements (assessing relative protein

expression levels and morphological changes of cells on a scale from 1 [resting state] to 5 [fully activated]) was performed with ImageJ 1.48 software in brain regions involved in stress and pain processing (51). Four brain areas associated with pain and stress processing were examined:

- Primary somatosensory cortex – hindlimb representation region (S1HL, approx. -0.46 to -0.7 mm posterior to bregma)
- Periaqueductal gray matter (PAG, approx. -7.3 to -8.3 mm posterior to bregma)
- Hippocampal cornu ammonis 3 region (CA3, approx. -2.18 to -2.46 mm posterior to bregma)
- Central amygdala (CeA, approx. -1.82 to -2.06 mm posterior to bregma)

3.10. Immunofluorescent staining

In sections prepared from the S1HL region, immunofluorescent staining was also performed to investigate microglia–cortical neuron interactions (n=5/group) in male animals. After preparatory and blocking steps, sections were incubated in PBS containing 3% normal goat serum, rabbit anti-IBA1 primary antibody (019-19741, Wako Chemicals GmbH, Neuss, Germany, 1:10,000 dilution), and Kv2.1 primary antibody (75-014, NeuroMab, Antibodies Incorporated, Davis, CA, USA, 1:1000 dilution). Subsequently, Alexa 647-conjugated goat anti-rabbit IgG (A32733, Invitrogen Antibodies, 1:500 dilution), Alexa 568-conjugated goat anti-mouse IgG (A11031, Invitrogen Antibodies, 1:500 dilution), and 4′,6-diamidino-2-phenylindole (DAPI) for nuclear labeling were applied (52).

3.10.1 Immunohistochemical data analysis – IBA, Kv2.1, and DAPI

Confocal images were captured using a Nikon Eclipse Ti2-E confocal microscope with a 60x objective. Z-stacks (1024 × 1024 pixel resolution) were acquired. Somatic microglia contacts, i.e., direct contact surfaces between microglial processes and cortical pyramidal neuron perikarya, were examined based on previous descriptions (52). The ratio and surface area of microglia-neuron contacts were analyzed. The studied neurons were classified into three groups:

- Satellite microglia (with somata in direct contact with the neuronal perikaryon),
- Microglial processes contacting neurons,
- Microglia not in contact with neurons.

3.11. Ethical approvals

All experimental methods fully comply with the provisions of Act XXVIII of 1998 on the Protection and Humane Treatment of Animals, as well as with the European Parliament's Directive (2010/63). The Animal Welfare Committee of the University of Pécs approved the experimental protocols (Permit numbers: BA02/2000-40/2016 and BA02/2000-25/2023).

3.12. Statistical analysis

Baseline data were compared using independent samples t-tests. For repeated-measures experiments, data with normal distribution were evaluated using two-way repeated measures ANOVA (Two-way RM ANOVA) followed by Sidak's post hoc test. The effects of stress and genotype on single-timepoint, normally distributed data were analyzed using two-way ANOVA followed by Tukey's post hoc test.

For all comparisons between groups, $*p<0.05$ values were considered statistically significant. Statistical analyses were performed with GraphPad Prism 8.

4. RESULTS

4.1. Stress-induced mechanical hyperalgesia did not develop in the absence of IL-1

The average baseline mechanonociceptive thresholds were significantly higher in both WT and IL-1 KO male mice compared to females of the same genotype. Furthermore, the baseline nociceptive threshold of KO females was significantly higher than that of WT females. In males, such genotype-related differences were not observed.

Following CRS, a significant reduction in mechanonociceptive thresholds appeared as early as the first week in males. In contrast, by the second week, both sexes exhibited an approximate 15–20% decrease, indicative of mechanical hyperalgesia. In males, this hyperalgesia persisted throughout the 4-week experiment, whereas in females it disappeared after 3 weeks.

In male mice, IL-1 knockout only tended to (but not significantly) attenuate stress-induced mechanical hyperalgesia, whereas in females, it prevented the significant reduction in pain threshold observed after the second week of stress.

4.2. IL-1 deficiency did not affect the development of stress-induced cold hyperalgesia

Baseline cold withdrawal latencies from icy water test were significantly lower in both WT and IL-1 KO males compared to females of the same genotype. No genotype differences were observed within either sex at baseline.

Upon CRS, both sexes exhibited a 60–70% reduction in cold tolerance from the first week onwards. This cold hyperalgesia remained unchanged throughout the entire experimental period in all groups. IL-1 deficiency did not influence stress-induced cold hyperalgesia in either sex.

4.4. Stress-induced microgliosis did not develop in IL-1 KO females in pain-related brain regions

In females, baseline IBA1 integrated density values were similar in WT and IL-1 KO non-stressed animals. After 2 weeks of CRS, stressed WT mice exhibited significantly increased IBA1 integrated density, while no stress-induced changes were detected in KO animals. Microglial cell activation was not significantly influenced by either genotype or stress exposure.

4.5. Stress-induced astrogliosis did not develop in IL-1 KO females in the CA3 region

In females, GFAP+ cell integrated density values showed no differences between groups. CRS did not significantly affect astrocytic integrated density values in PAG or CA3 regions.

In contrast, CRS induced significant astrocyte activation in the hippocampal CA3 region in WT mice, which was not observed in IL-1 KO animals. No such changes were found in the PAG region in either genotype. GFAP+ astrocytes were not detectable in the CeA and S1HL regions.

4.6. Behavioral studies in WT and IL-1 KO animals

In females, after 2 weeks of CRS, a significant reduction in immobility time during the FST was observed in IL-1 KO mice, but not in WT animals. After the 4-week protocol, the group differences disappeared.

In the LDB test, time spent in the light compartment was unaffected by CRS at either 2 or 4 weeks. However, at the end of week 2, a significant difference was found between non-stressed groups: IL-1 KO mice spent more time in the light compartment than WT mice.

In the OFT, after 2 weeks of CRS, IL-1 KO mice exhibited significantly increased locomotor activity, while WT mice showed a similar trend, but it did not reach statistical significance.

4.7. The IL-1 receptor antagonist anakinra prevented stress-induced mechanical hyperalgesia and reduced cold hyperalgesia

In stressed male mice, the mechanical threshold of the vehicle-treated group significantly decreased already during the first week compared to the non-stressed, vehicle-treated controls. By the second week, vehicle-treated animals showed an approximately 30% reduction in mechanonociceptive thresholds due to chronic stress. However, this stress-induced reduction was not observed in the group treated with the IL-1 receptor antagonist anakinra.

Similarly, CRS-induced cold hyperalgesia was significantly less pronounced in anakinra-treated animals compared to vehicle-treated controls during the second week.

4.8 Stress-induced mechanical hyperalgesia did not develop in the absence of CX3CR1

The average baseline mechanonociceptive thresholds were significantly higher in both WT and CX3CR1 KO male mice compared to females of the same genotype. Furthermore, female KO mice exhibited significantly lower baseline nociceptive thresholds than WT females. In males, such genotype-related differences were not observed.

Upon CRS, both male and female mice exhibited a 15–20% reduction in mechanonociceptive thresholds by the second week, indicative of mechanical hyperalgesia. However, in both sexes, the absence of the CX3CR1 gene prevented the development of stress-induced mechanical hyperalgesia.

4.9. The absence of CX3CR1 reduced the development of stress-induced cold hyperalgesia

Baseline cold sensitivity values were significantly lower in males compared to females of the same genotype, in both WT and CX3CR1 KO mice. No genotype-related differences were detected within either sex at baseline.

Following CRS, both sexes showed a 60–70% reduction in cold tolerance already from the first week. However, the extent of cold hyperalgesia was significantly lower in CX3CR1 KO mice of both sexes compared to WT controls.

4.11. Stress-induced microgliosis did not develop in CX3CR1 KO males in pain- and stress-related brain regions

In males, IBA1 integrated density values were significantly higher in non-stressed CX3CR1 KO mice compared to WT animals in the S1HL and PAG regions. After 2 weeks of CRS, stressed WT animals exhibited significantly elevated IBA1 integrated density in the S1HL and PAG, whereas no such changes were observed in KO mice. No differences were detected in the CA3 and CeA regions across groups.

Microglial activation was significantly increased by stress in the S1HL and CA3 regions in WT animals, but this effect was absent in KO mice. In the PAG and CeA regions, neither genotype nor stress had a significant influence on the proportion of activated microglia.

4.12. Higher microglia–neuron contact ratio in the primary somatosensory cortex in CX3CR1 KO males

In male mice, immunofluorescent labeling with IBA1 and Kv2.1 revealed that microglial cells exhibited larger contact surface areas with neurons in CX3CR1 KO animals compared to WT controls, independent of stress exposure. The distribution of microglia types—non-contacting,

somatic-contact, or satellite microglia—did not differ across groups, regardless of genotype or stress exposure.

4.13. Stress-induced astrogliosis did not develop in CX3CR1 KO males in the periaqueductal gray

In male WT animals, CRS significantly increased the integrated density of GFAP+ cells in the PAG region. This increase was absent in CX3CR1 KO mice. No differences were observed among groups in the CA3 region. Neither genotype nor CRS significantly affected GFAP+ astrocyte activation in the PAG or CA3 regions. GFAP+ astrocytes were not detectable in the CeA or S1HL regions.

4.14. Stress-induced microgliosis did not develop in CX3CR1 KO females in the hippocampal CA3 region

In females, integrated density values in the S1HL, PAG, CA3, and CeA regions were not significantly influenced by either genotype or stress. However, CRS significantly increased the proportion of activated IBA1+ microglia in the hippocampal CA3 region of WT mice, an effect that was absent in KO animals. No significant group differences were observed in the other brain regions.

4.15. Stress-induced astrogliosis did not develop in CX3CR1 KO females in pain-related brain regions

In non-stressed CX3CR1 KO female mice, integrated GFAP density values were significantly higher in the PAG region compared to WT controls. Following CRS, integrated GFAP density was significantly increased in WT females in both the PAG and CA3 regions, whereas no such changes were detected in KO mice.

Similarly, the proportion of activated GFAP+ cells was significantly increased by CRS in the PAG and CA3 regions of WT females, but this effect was absent in CX3CR1 KO animals. No GFAP+ astrocytes were detectable in the CeA or S1HL regions.

4.16. Behavioral tests in WT and CX3CR1 KO animals

In the FST, neither genotype nor the applied stress caused significant differences between the groups in either sex.

In the LDB, the time spent in the light compartment did not change under CRS in WT animals; however, in female CX3CR1 KO animals, we observed a significant increase in this parameter following 2 weeks of CRS. At the same time, in non-stressed male animals, a significant difference was detectable: CX3CR1 knockout mice spent more time in the light compartment than their WT counterparts.

In the OFT test, significantly lower spontaneous locomotor activity was shown in stressed males and in non-stressed females in the CX3CR1 knockout groups.

4.17. The CX3CR1 antagonist AZD8797 prevented stress-induced mechanical, but not cold hyperalgesia

In vehicle-treated male animals, chronic stress caused a significant decrease in the mechanonociceptive threshold by the time of the second-week measurement; however, this decrease was not observed in the group receiving the CX3CR1 antagonist compound AZD8797. The extent of CRS protocol-induced cold hyperalgesia was identical in both the vehicle- and AZD8797-treated groups.

4.18. The NRLP3 blocker MCC950 prevented stress-induced mechanical but not cold hyperalgesia

In vehicle-treated male animals, chronic stress caused a significant decrease in the mechanonociceptive threshold by the second week; however, this decrease did not form in the group receiving the NRLP3 inflammasome antagonist compound, MCC950. CRS-induced cold hyperalgesia developed to the same extent in both the vehicle- and MCC950-treated groups.

4.19. In the absence of HK-1, stress-induced mechanical hyperalgesia did not develop

The baseline mechanonociceptive thresholds in both WT and Tac4 KO male mice were significantly higher than those in females of the same genotype. No genotype differences were observed in the baseline values in either male or female animals. As a result of CRS, the mechanonociceptive threshold significantly decreased in wild-type animals; however, in the absence of HK-1, the stress-induced mechanical hyperalgesia did not develop.

4.20. The absence of HK-1 did not affect the development of stress-induced cold hyperalgesia

The baseline paw withdrawal latency values in the cold sensitivity test were significantly lower in both male WT and Tac4 KO animals compared to females of the same genotype. No genotype differences were observed in the baseline values in either male or female animals.

As a result of CRS, we observed a 50–70% decrease in cold tolerance in both male and female mice starting from the first week, which was similar in wild-type and Tac4 knockout mice. In the non-stressed groups, a significant decrease in cold tolerance was also measurable; however, during the first two weeks of the experiment, this decrease was significantly smaller in male animals in the absence of HK-1.

5. DISCUSSION

Our studies provide the first data indicating that microglial CX3CR1, as well as the NLRP3 inflammasome and the IL-1 it produces, and HK-1 play a crucial role in the development of chronic stress-induced pain, presumably through influencing glia-neuron interactions in brain regions essential for pain processing, such as the PAG and the S1HL. The observed morphological changes in microglia and astrocytes indicate the presence of neuroinflammatory processes in both male and female mice. Furthermore, pharmacological inhibition of the CX3CR1–NRLP3–IL-1 axis may offer a new therapeutic option for the treatment of stress-induced pain disorders.

In the CRS model, well-defined pain parameters, brain neuroinflammatory changes, and sex differences can be observed

In baseline threshold measurements, female mice exhibited lower cold sensitivity and higher mechanical sensitivity compared to males, regardless of genotype, a phenomenon previously described in the literature (53–55).

As a result of the CRS model, significant mechanical and cold hyperalgesia developed in both male and female wild-type mice. The significant decrease in the mechanical pain threshold occurred at a slower rate (starting from the 2nd week), whereas the increased pain response to cold stimuli developed during the first week. While no habituation was observed in cold tolerance, we did observe habituation in mechanical sensitization. Furthermore, the results of behavioral tests, in which no differences were observed between the stressed and non-stressed groups, also indicate that pain and behavioral parameters differ in the time course and extent of habituation, a finding supported by literature data (56–58).

By using cell number, activation, and integral density measurements suitable for objective quantification (59,60), we demonstrated that CRS induces significant microgliosis and astrogliosis in several brain regions involved in pain and stress regulation in both male and female animals. Our results are supported by findings that several stress-based FM models have demonstrated neuroinflammation, morphological changes in microglial cells, or increased levels of molecules involved in neuroinflammatory signaling pathways in various brain regions, as also discussed in a recent review published by our research group (61). Clinical studies have shown that increased glial cell activation is present in brain regions involved in pain processing in FM patients, which correlates well with their subjective pain levels (17). In our experiments, along with microglia, we also observed increased astrocyte activation under stress. Under

physiological conditions, astrocytes play a protective and supportive role, participating in neuronal communication (62). However, under pathological conditions, they can become reactive, a phenomenon linked to several neurological diseases. Activated microglial cells release proinflammatory cytokines, such as TNF-α and IL-1, which can trigger a toxic, reactive astrocyte state (63). Based on literature data and our own results, it is conceivable that, under stress, enhanced microglial activation further stimulates astrocytes in wild-type animals (62). In the future, we plan to investigate this further using astrocyte-microglia double labeling.

HK-1 is indispensable in the development of stress-induced pain

HK-1 is a widely expressed inflammatory modulator produced by immune and epithelial cells, and it plays a role in airway, skin, and intestinal inflammation as well (64–66). The peptide encoded by the Tac4 gene shows stable distribution in the central nervous system in addition to peripheral tissues (37). In our experiments, stress-induced mechanical hyperalgesia was absent in the lack of HK-1, while cold hyperalgesia remained unchanged, suggesting that HK-1 mainly contributes to pain sensitization through central mechanisms. In animal models, HK-1 enhanced pain responses, which can be partly explained by the modulation of neuron–glia interactions and neurotransmitter systems (67). Microglial inhibition decreased HK-1 levels and neuropathic pain, while anti-HK-1 antibodies effectively reduced diabetic pain (68). In clinical samples, HK-1 expression correlated with the severity of lumbosacral back pain and was also found to be elevated in fibromyalgia (69, 70). Based on these data, HK-1 plays a central role in the development of pain and may represent a potential therapeutic target.

The CX3CR1–IL-1 cascade plays a decisive role in pain perception

The baseline mechanical pain threshold of CX3CR1 KO female mice was lower compared to that of wild-type animals. However, previous data suggest that this knockout does not alter baseline pain thresholds (71,72). The receptor is mainly found on microglia in the central nervous system, but is also present on several immune cells in the periphery. In preclinical models, the loss of CX3CR1 abolished mechanical and thermal sensitivity in a neuropathy mouse model (73), whereas administration of FKN increased pain (26); in contrast, FKN-neutralizing antibodies reduced traumatic mononeuropathic pain in rats (74). Elevated FKN levels have been described in the cerebrospinal fluid of FM patients (27). In our experiments, stress-induced mechanical hyperalgesia did not develop in CX3CR1 KO mice in either sex. Furthermore, cold hypersensitivity was also reduced in KO animals, especially in females,

which may be explained by altered peripheral processes or disrupted interactions between immune cells and sensory neurons in the absence of the gene.

In our experiments, the baseline mechanical threshold of female IL-1 KO mice was higher than that of WT animals, a finding also supported by literature data (75–78). The absence of IL-1 prevented the development of stress-induced mechanical hyperalgesia in females. IL-1β can directly activate nociceptive C-fibers and also enhances the synthesis of sensitizing mediators (79). Its receptor is primarily expressed on capsaicin-sensitive neurons, and its activation triggers multiple intracellular signaling pathways (80–82).

Clinical studies also support the role of IL-1 in pain: elevated IL-1 levels have been demonstrated in chronic pain and neuropathic patients (83). Although this is less clear in fibromyalgia, IL-1 signaling may be particularly important in stress-induced pain in females. In contrast, in male animals, IL-1 gene deficiency had only a minor effect on CRS-induced pain development.

Regarding stress-induced cold hyperalgesia, we found no sex differences: IL-1 knockout did not affect the stress-induced decrease in cold tolerance in either male or female animals. This suggests that IL-1 primarily influences mechanical pain through central sensitization (84), while cold sensitivity of peripheral origin (85) is less dependent on this mediator.

Overall, our results indicate that both IL-1 and CX3CR1 play important roles in the development of stress-induced central pain sensitivity, whereas cold hypersensitivity appears to depend on CX3CR1 but not on IL-1. These molecules may represent promising targets in the study of female-dominated chronic pain syndromes, contributing to a better understanding of sex differences.

The CX3CR1–IL-1 pathway plays a role in mood regulation

In CX3CR1 KO animals, mice spent more time in the light compartment during the LDB test, moved less in the OFT, and proved more resilient to stress in anxiety- and depression-like behavioral tests. Previous data have also confirmed that the absence of CX3CR1 increases stress resilience and reduces depression-like behavior (86,87). In our experiments, IL-1 KO mice were more active in the FST and spent more time in the light compartment. In a neuropathic model, IL-1 gene knockout alleviated both pain and depression-like symptoms in mice (88). Clinical studies have shown elevated IL-1 levels in the peripheral blood of depressed patients, supporting the role of inflammatory cytokines in psychiatric symptoms (89,90).

The CX3CR1–IL-1 signaling axis plays a central role in CRS-induced neuroinflammatory processes

The CX3CR1 receptor and the IL-1 cytokines are key mediators of microglia–neuron communication, playing a crucial role in regulating neuroinflammation and homeostatic responses. Although the exact mechanism of stress-induced microglial activation is not fully understood, the FKN–CX3CR1 pathway appears to be a promising target (91).

In mouse models, the absence of CX3CR1 prevented chronic stress-induced microglial and neuronal changes, such as the reduced arborization and plasticity observed in the CA1 region (92). In cortical regions and the striatum, microglia of CX3CR1 KO mice displayed reduced arborization, associated with lower inflammatory gene expression (93). However, in the hippocampus, CX3CR1 KO mice exhibited shorter microglial processes and aberrant activation (94), which is consistent with our own measurements. In non-stressed KO animals, we found higher IBA1 integrated density in the S1HL and PAG regions.

In our experiments, the absence of CX3CR1 or IL-1 prevented CRS-induced IBA1+ microglial and GFAP+ astrocyte activation, as well as the increase in integrated density. Since the PAG region plays a central role in pain regulation, the absence of stress-induced cellular activation observed in KO animals supports the importance of the CX3CR1 and IL-1 genes in the development of stress-induced pain and neuroinflammation.

Pharmacological inhibition of the neuroinflammatory cascade effectively reduces pain.

To complement the knockout models, we also investigated the potential analgesic role of pharmacological inhibitors (AZD8797 – CX3CR1 antagonist, MCC950 – NLRP3 inhibitor, anakinra – IL-1R antagonist) in the stress-induced sensitization of these structures. With these agents, compensatory distortions of knockout models can be avoided, thus better modeling the potential for clinical application.

The CX3CR1 antagonist AZD8797, which is under development (95), also reduced the decrease in mechanical pain threshold but did not affect cold hyperalgesia, suggesting inhibition of central neuroinflammation rather than the attenuation of peripheral sensitization.

The NLRP3 inflammasome inhibitor MCC950 prevented the development of stress-induced mechanical hyperalgesia but did not affect cold sensitivity. This also confirms that NLRP3 plays a role in central pain mechanisms (84).

Anakinra, an already approved IL-1R blocker, significantly reduced chronic stress-induced mechanical and cold hyperalgesia, indicating a central and peripheral role of IL-1 signaling (96).

In summary, all three inhibitors – targeting the CX3CR1, NLRP3, and IL-1R pathways – effectively reduced chronic stress-induced pain. Based on our results, these molecules may be promising targets for the treatment of chronic pain syndromes such as fibromyalgia.

6. SUMMARY OF NEW RESULTS, CONCLUSIONS

1. In our studies, we characterized the chronic immobilization stress model, the pain parameters induced by it, and the neuroinflammatory changes.
2. HK-1 plays a key role in the development of stress-induced mechanical hyperalgesia.
3. Using KO animals, our experiments demonstrated that the microglial CX3CR1 receptor is indispensable for the development of CRS-induced mechanical and cold hyperalgesia in both sexes.
4. Our studies revealed that IL-1 plays an important role in the development of chronic stress-induced mechanical pain, mainly in female animals.
5. In our experiments, pharmacological inhibition of the CX3CR1–NLRP3–IL-1 signaling pathway prevented the development of stress-induced pain.

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8. PUBLICATIONS

8.1. Publications forming the basis of the dissertation

1. Fülöp, Barbara; Hunyady, Ágnes; Bencze, Noémi; Kormos, Viktória; Szentes, Nikolett; Dénes, Ádám; Lénárt, Nikolett; Borbély, Éva ✉ ; Helyes, Zsuzsanna
IL-1 Mediates Chronic Stress-Induced Hyperalgesia Accompanied by Microglia and Astroglia Morphological Changes in Pain-Related Brain Regions in Mice
INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES 24 : 6 Paper: 5479 , 25 p. (2023) (IF: 4,9)

2. Borbély, Éva ✉; Kecskés, Angéla*; Kun, József; Kepe, Eszter; Fülöp, Barbara; Kovács-Rozmer, Katalin; Scheich, Bálint; Renner, Éva; Palkovits, Miklós; Helyes, Zsuzsanna
Hemokinin-1 is a mediator of chronic restraint stress-induced pain
SCIENTIFIC REPORTS 13 : 1 Paper: 20030 , 15 p. (2023) (IF: 3,8)

3. Barbara Fülöp, Ágnes Király, Rebeka Petrák, Júlia Müller, Tünde Biró-Sütő, Viktória Kormos, Valéria Tékus, Katalin Rozmer, Ádám Dénes, Éva Borbély, Zsuzsanna Helyes
Microglial CX3CR1 signaling mediates stress-induced pain behavior in mice.
Submitted for publication to *Brain, Behavior, and Immunity*.

Cumulative impact factor of the publications forming the basis of the dissertation: 8,7
Total independent citations of the publications forming the basis of the dissertation: 13

8.2. Additional publications

1. Fülöp, B. ✉ ; Borbély, É. ; Helyes, Z.
How does chronic psychosocial distress induce pain? Focus on neuroinflammation and neuroplasticity changes
BRAIN BEHAVIOR IMMUNITY - HEALTH 44 Paper: 100964 , 8 p. (2025) (IF: 3,5)

Cumulative impact factor of other publications: 3,5
Total independent citations of other publications: 1

10. LIST OF CONGRESS PRESENTATIONS

Somatostatin 4 receptor agonists in mouse models of neuropathic pain, anxiety and depression-like behaviour
Barbara Fülöp, Éva Borbély, Ágnes Hunyady, Boglárka Kántás, János Szolcsányi, Erika Pintér, Zsuzsanna Helyes
Frigyes Korányi Science Forum – RECOOP Student Conference, 2018, Budapest, Magyarország

Effect of orally administered somatostatin 4 receptor agonists in neuropathic pain, anxiety and depression-like behaviour in mouse models
Barbara Fülöp, Boglárka Kántás, Ágnes Hunyady, Éva Borbély, Erika Pintér, János Szolcsányi, Zsuzsanna Helyes
YES Meeting (Young European Scientist Meeting), 2019, Porto, Portugália

Interleukin-1 mediates chronic restraint stress-induced neuropathic hyperalgesia in a mouse model
Barbara Fülöp, Ágnes Hunyady, Éva Borbély, Nikolett Szentes, Ádám Dénes, Nikolett Lénárt, Zsuzsanna Helyes
HMAA Conference, 2019, Sarasota, FL, USA

Interleukin-1 mediates chronic restraint stress-induced neuropathic hyperalgesia in a mouse model
Barbara Fülöp, Ágnes Hunyady, Éva Borbély, Nikolett Szentes, Ádám Dénes, Nikolett Lénárt, Zsuzsanna Helyes
11th ISCTICO – HUPHAR – IUPHAR Conference, 2021, Pécs, Magyarország

Microglia-surface Fractalkine receptor mediates chronic stress-induced pain in mice
Barbara Fülöp, Ágnes Hunyady, Nikolett Szentes, Éva Borbély, Zsuzsanna Helyes
Białystok International Medical Congress, 2021, Białystok, Lengyelország *(runner up in the section)*

Interleukin-1 mediates chronic restraint stress-induced hyperalgesia in a mouse model
Barbara Fülöp, Ágnes Hunyady, Nikolett Szentes, Viktória Kormos, Ádám Dénes, Nikolett Lénárt, Éva Borbély, Zsuzsanna Helyes
HMAA Hungary Chapter Conference, 2022, Balatonfüred, Magyarország *(Best poster award)*

Az Interleukin-1 szerepe a krónikus immobilizációs stressz-okozta fájdalom kialakulásában egérmodellben
Barbara Fülöp, Ágnes Hunyady, Nikolett Szentes, Viktória Kormos, Ádám Dénes, Nikolett Lénárt, Éva Borbély, Zsuzsanna Helyes
Magyar Élettani Társaság Konferencia, 2022, Budapest, Magyarország *(Unicam special award)*

IL-1 mediates chronic stress-induced hyperalgesia accompanied by microglia and astroglia morphological changes in pain-related brain regions in mice
Barbara Fülöp, Á. Hunyady, N. Bencze, V. Kormos, N. Szentes, Á. Dénes, N. Lénárt, É. Borbély, Z. Helyes
Euoropen Pain School, 2023, Siena, Olaszország

Fractalkine receptor (CX3CR1) mediates chronic restraint stress-induced pain behavior in a mouse model
Fülöp, Barbara; Dénes, Ádám ; Lénárt, Nikolett ; Kormos, Viktória ; Borbély, Éva ; Helyes,Zsuzsanna
European Psychoneuroimmunology Network Autumn School, 2023, Giessen, Németország

Stress-induced pain is mediated by central nervous system sensitization
Barbrara, Fülöp; Rebeka, Petrák; Júlia, Müller; Viktória, Kormos; Katalin, Rozmer; Ágnes, Király, Ádám Dénes; Éva, Borbély; Zsuzsanna, Helyes
MEDPécs konferencia, 2024, Pécs

Potential analgesic effect of fractalkine receptor (CX3CR1) antagonist in mouse model of chronic stress-induced pain
Barbrara, Fülöp; Viktória, Kormos; Katalin, Rozmer; Ádám Dénes; Nikolett, Lénárt; Éva, Borbély; Zsuzsanna, Helyes
Annual Meeting of the Hungarian Neuroscience Society *(best poster award)* és 8th Hungarian Neuroscience Doctoral Conference 2024, Pécs

Stress-induced mechanical and thermal pain sensitisation mediated through central nervous system cell signalling
Barbara Fülöp, Viktória Kormos, Katalin Rozmer, Ágnes Király, Ádám Dénes, Nikolett Lénárt, Éva Borbély, Zsuzsanna Helyes
9th Conference of the Federation of European Pharmacological Societies (EPHAR), 2024, Athén, Görögország

Stress-induced mechanical and thermal pain sensitisation mediated through NLRP3 inflammasome activation
Barbrara, Fülöp; Viktória, Kormos; Katalin, Rozmer; Ágnes, Király, Ádám Dénes; Nikolett, Lénárt; Éva, Borbély; Zsuzsanna, Helyes
Annual Meeting of the Hungarian Neuroscience Society, és 8th Hungarian Neuroscience Doctoral Conference 2025, Debrecen

Molecular Mechanisms of stress-induced Pain and new types of Analgesic Drug Candidates in rodents
Barbara Fülöp, Ágnes Király, Rebeka Petrák, Júlia Müller, Viktória Kormos, Katalin Rozmer, Ádám Dénes, Éva Borbély, Zsuzsanna Helyes
HUPHAR Conference, 2025, Mátraháza, Magyarország *(best poster award)*

A stressz által kiváltott fájdalom molekuláris mechanizmusainak feltérképezése, és új típusú fájdalomcsillapító gyógyszerjelöltek vizsgálata rágcsálókban
Fülöp Barbara, Király Ágnes, Petrák Rebeka, Müller Júlia, Kormos Viktória, Rozmer Katalin, Dénes Ádám, Borbély Éva, Helyes Zsuzsanna
37. Országos Tudományos Diákköri Konferencia (OTDK) Orvos- és Egészségtudományi Szekciója: „Roska Tamás Tudományos Előadás”, 2025, Pécs

Stress-induced mechanical and thermal pain sensitisation mediated through central nervous system cell signalling
Barbara Fülöp, Ágnes Király, Viktória Kormos, Rebeka Petrák, Júlia Müller, Katalin Rozmer, Ádám Dénes, Éva Borbély, Zsuzsanna Helyes
European Pain Federation Congress (EFIC 2025 – Pain in Europe XIII), 2025, Lyon, Franciaország

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