Master's Thesis of Natural science

Sexual dimorphic mechanism of GT1b-induced pain central sensitization

GT1b에 의한 통증 중추 감작의 성적 이형성 기전

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Abstract

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Previous study has shown that GT1b, one of the four major gangliosides in the central nervous system, can trigger activation of spinal microglia that mediates pain central sensitization as an endogenous agonist of Toll-like receptor 2. However, GT1b administration induced pain central sensitization exclusive in male. In this study, we investigated the sexual dimorphism of GT1b-induced pain central sensitization and the underlying mechanisms. Spinal tissue transcriptomic comparison between male and female mice after GT1b injection suggested the putative involvement of estrogen (E2)-mediated signaling in the sexual dimorphism of GT1b-induced pain sensitization. Upon ovariectomy-reducing systemic E2, female mice became susceptible to GT1b-induced pain central sensitization, which was completely reversed by systemic E2 supplementation. Meanwhile, orchiectomy of male mice did not affect pain sensitization. As an underlying mechanism, we present evidence that E2 inhibits GT1b-induced inflammasome activation and subsequent IL-1β production. Our findings demonstrate that E2 is responsible for sexual dimorphism in GT1b-induced pain central sensitization.

Keyword : GT1b, pain central sensitization, sexual dimorphism, estrogen, IL-1β

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1. Introduction

1.1. Background study

Neuronal diseases affect more than 1 billion individuals, and extensive research has revealed significant sex differences in their occurrence, severity, and progression [1]. These differences have been observed not only in clinical study but also in preclinical and basic studies on neuronal diseases. Notably, women are more likely to suffer from Alzheimer’s disease, depression and anxiety disorders, whereas men are more susceptible to Parkinson’s disease, attention deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) [2]. According to a large meta-analysis, the prevalence of Parkinson’s disease is more than twice as high in men compared to women [3], while the proportion of women affected by Alzheimer’s disease is three times higher than men [4]. Moreover, women with Alzheimer's disease tend to exhibit a faster rate of cognitive decline [3; 5].

Sexual dimorphism has also been observed in chronic pain [6]. In 2007, the Sex, Gender and Pain special interest group of the International Association for the Study of Pain brought attention to the fundamental question, “Do I really need to study females?” and raised the topic to the surface [7]. Since then, numerous studies have emerged the mechanisms underlying sex differences in pain and analgesia while offering new perspectives on the subject.

Aligned with this trend, our aim was to address this notable subject by comparing distinct pain responses and mechanisms, ultimately proposing a more comprehensive approach to understanding sexual dimorphism in pain central sensitization.

1.1.1 Pain central sensitization

Pain central sensitization is a physiological symptom stimulated by painful stimulus in chronic pain and refers to a state in which reactivity by nociceptors increased and the patient's nervous system is continuously activated [8]. The pain types of central sensitization include allodynia, in which pain occurs in response to stimuli that would not normally cause pain, and hyperalgesia that has greater pain than usual sensitivity to stimuli, and secondary hyperalgesia, which is characterized by a widespread injury site [8]. These symptoms tend to be persistent, become chronic, and are less sensitive to treatments.

Central sensitization arises from an increase in synaptic efficacy and membrane excitability or a decrease in inhibitory currents in the nociceptive pathway, leading to an enhanced functional status of the neuron and circuits. Central sensitization is induced through the activation of postsynaptic glutamate NMDA receptors and the insertion of AMPA receptors into the neuronal membrane surface, which is induced by glutamate, substance P, CGRP secreted from primary afferents, and microglial brain-derived neurotrophic factor (BDNF), interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) [9; 10]. These mechanisms are activated in response to pain by immune cells.

Among the immune cells, microglia are the primary innate immune cells and are involved in various developmental and physiological processes in the central nervous system (CNS) [11]. They play a critical role in the initiation of central sensitization, making them an essential target for pain intervention. In addition, microglia have a neuroprotective function, change cell function, and secrete various bio-active diffusible factors such as inflammatory cytokines, chemokines, and induce phagocytosis when external stimuli comes [12; 13]. When activated, microglia undergo morphological changes characterized by increased soma volume, decreased process length and complexity [14; 15].

These microglial-mediated processes are able to induce pain central sensitization, which can be classified into neuropathic pain and inflammatory pain [16]. Although neuropathic pain and inflammatory nociceptive pain share a similar mechanism, neuropathic pain is caused by nerve damage while inflammatory pain is due to tissue damage. Furthermore, there are differences in the progression of the time course or the relative contribution of each mechanism.

1.1.1.1 Neuropathic pain

Neuropathic pain is a widespread and notorious disease of the sensory nervous system that occurs continuous and intermittent pain and reduces quality of life due to sleep disturbances, anxiety, and depression. Generally, neuropathic pain is caused by dysfunction of the somatosensory nervous system, postherpetic neuralgia and stroke, or neuronal damage after spinal cord surgery or itself, leading to the disturbance and alteration of sensory signal transmission [17].

In peripheral nerve injury (PNI)-induced pain, hypersensitivity is mediated by crucial signaling changes within microglia: upregulation of the purinergic receptor P2X4 (P2X4R) [18], activation of p38 mitogen-activated protein kinase (p38MAPK) and release of BDNF [19; 20; 21]. These P2X4R-p38MAPK-BDNF pathway trafficked to the surface of the microglia, where they are activated by adenosine triphosphate (ATP), which has been shown to be released in the dorsal horn [22].

Not all peripheral neuropathy or central nervous injury lead to neuropathic pain, but up to 60% of patients with severe clinical neuropathy experience neuropathic pain [23]. Furthermore, according to a neuropathic pain scale analysis of United Kingdom cities, females (6%) experience neuropathic pain lasting longer than 3 months more often than males (3%) [24]. Despite of prevalence of sex differences in pain experience, pain research has largely overlooked this aspect, evidence suggests that during

the 1990s, approximately 45% of articles published in 100 reputable neuroscience journals did not consider sex at all and only utilized male subjects [25]. However, in recent years, numerous studies have emphasized the importance and necessity of considering sex in pain research, leading to a significant increase in active research in this area. In 2019, more than half of the papers about neuropathic pain published in the Journal of Pain focused on sexual dimorphism [26].

Taken together, these studies indicate that proportion of patients with neuropathic pain shows sex differences, and immune response has a sexual dimorphic pathway [27]. As mechanistic research through male-biased studies is inaccurate for females, female-based mechanistic studies are needed to develop appropriate neuropathic pain medications and treatment strategies.

1.1.1.2 Inflammatory nociceptive pain

Inflammatory nociceptive pain is a spontaneous, stimulus-dependent pain that occurs when nociceptors detect noxious or potentially harmful stimuli. Inflammation is an essential process in the body that recruits lymphocytes such as neutrophils and macrophages in response to tissue injury and induces tissue repair and healing. However, as a side effect, it activates nerves and induces inflammatory nociceptive pain [28]. These injuries can occur in bone, skin, muscles, and can also occur in physical injuries such as cancer, cut, bruise or broken bone.

The nociceptive pathway first begins with the conversion of noxious stimuli into electrical signals and transmits the signals to pain projection neurons in the spinal cord via afferent nerves. During this process, biological mediators or signaling molecules (such as glutamate, BDNF, CGRP, substance P, chemokines, and cytokines) are released, leading to the sensitization of neuronal circuits and the induction of pain by interacting with surface receptors [29; 30; 31].

Previous report showed that male sensory neurons are more sensitive to mitochondrial stress, which accelerates neuronal damage in autoimmune disease, whereas female sensory neurons are relatively resistant to mitochondrial stress, leading to greater nociceptor hyperexcitability or pain-mediated inflammatory phenotypes. Collectively, sex differences in pain are readily observable, and sex is a spotlighted topic to conquering central sensitization and developing treatments from preclinical to clinical studies [32].

1.1.1.3 Limitation of treatment

Current pain treatments have presented several limitations. First, different treatment effects between male and female are frequently observed in chronic pain as I mentioned above. There have been numerous reports of functional and morphological differences in the central nervous system between sexes, ranging from basic research to clinical settings in both human and animal studies. Also, pain treatments only provide short term pain relief effects instead of addressing the underlying pain itself. Since the effectiveness of treatment for neuropathic pain varies among patients and side effects are frequently observed, “personalized treatment” that considers the optimal drug type and administration regimen for each patient is being emphasized [33].

Correctively, sex should be taken into consideration when developing more effective personalized treatments because it is affected by a variety of factors, including genes, sex hormones, sociocultural factors, and environmental factors [34; 35; 36].

1.1.2 Sexual dimorphism in pain

1.1.2.1 Clinical study

Chronic pain is not only common in women, but also more frequent and severe than men [27]. Analysis of pain responses to multiple stimuli in

both sexes has yielded remarkably consistent findings. Specifically, by examining eight stimuli, including pressure pain threshold, cold pain threshold, and heat pain threshold, women have consistently demonstrated greater sensitivity to pain than men [37]. In a survey conducted by Nadia, 1000 participants were recruited through Kuwait's hospital medical record system to analyze the proportion of patients with neuropathic pain. In the results, neuropathic pain was found in 37.2% of women and 23.6% of men, and the degree and presence of symptoms of neuropathic pain such as burning (women; 27.6%, men; 14.6%), pins and needles (women; 40.4%, men; 26.8%), numbness (women; 53.5%, men; 42.3%) were higher in women.

In addition, studies by Ruau et al. compared sex differences in pain severity in 11,000 patients using electronic medical records (EMRs), a large database of electronic medical records. EMR data were provided from Stanford Hospital and Clinics, and average pain scores of males and females were analyzed for 47 diagnosis sections. As a result, women's pain scores were higher in 72% of the sections, which was equally observed in 95% of the pain diagnosis results [38].

Most of the research on sexual dimorphism in pain has been advanced in the adult literature. However, recent studies have examined children's reactions to experimental pain and reported that there is a significant difference between children and adolescents. According to studies conducted on children, 85.7% showed no difference in pain sensitivity between sexes, but when differences observed, girls were more sensitive to stimulation than boys. These characters were remarkable in children over 12 years of age, during pubertal development, where 14.3% of girls were more affected by pain than boys [39]. These findings suggest that pubertal development and sex hormones play a role in the differences in pain response between adolescent males and females [40].

1.1.2.2 Animal pain model

To gain a better understanding of the underlying mechanisms of chronic pain, preclinical studies typically use various animal pain models that focus on discrete peripheral nerve damage or injury resulting in neuropathic pain. Such animal models offer an effective platform for investigating the pathophysiology of chronic pain, elucidating the molecular mechanisms of central sensitization, and identifying potential therapeutic targets for treating neuropathic pain in humans.

Spared nerve injury (SNI), one of the commonly used neuropathic pain models, exhibits sex differences even after being treated. Metformin, a medication used to treat type 2 diabetes, is also used as a pain reducer in the SNI neuropathic pain model. It regulates MAPK by activating AMP activated protein kinase (AMPK) and reduces chronic pain by controlling microglia activation. However, these positive neuropathic pain-modifying effects were only observed in males. In females, metformin does not inhibit microglial activation or has any analgesic effects on neuropathic pain [41]. Mechanical allodynia between males and females in these models was also different. Similarly, the administration of minocycline (a microglia inhibitor), Mac-1-saporin (used to deplete microglia), and TNP-ATP (a P2X inhibitor and regulator of microglial activation) reverse allodynia only in male mice although both SNI-injured male and female mice manifested mechanical allodynia [42].

The above data show that microglia play a more important role in males than females when promoting chronic neuropathic pain, and microglia play different roles in each sex. It can be one possibility of sex differences in peripheral immune system or central nervous system processes of neuropathic pain [43].

Another neuropathic pain model which highlights the sexual dimorphic pain is chronic constriction injury (CCI). Stephens and colleagues analyzed similarities and differences in gene regulation between

males and females using RNA seq in DRG after CCI. First of all, there are 210 genes which are differentially expressed between naïve male mice and naïve female mice DRG, and 130 of these genes related to 'positive regulation of nervous system development' encoding cytokines (e.g., Il6, Il1r1), chemokines (e.g., Cxcl13, Cxcl9), and enzymes (e.g., Doux2, Gch1, Tnik). Among these genes, 30 genes (14.2%) are downregulated, and 4 genes (1.9%) are upregulated in CCI females [44]. In addition, the researchers confirmed that estrogen plays an antinociceptive effect in CCI injury. They found that structural and functional changes induced by CCI are completely different in male and female mice. Male mice showed a gradual decrease in allodynia after CCI and complete recovery after 61 weeks, while female mice still showed allodynia and gliosis 4 months after allodynia induction. Interestingly, administration of estrogen can significantly attenuate the difference between the sexes and reduce allodynia in females and completely recover after 13 weeks [45; 46]. Overall, the researchers confirmed that there are several differences in the CCI situation because neurochemical mediators are differentially expressed by sex, and estrogen can regulate CCI [44].

1.1.2.3 Inflammatory factors

Lipopolysaccharide (LPS), a type of endotoxin found in the outer membrane of gram-negative bacteria, is considered an acute inflammatory stress when it enters the body. Once LPS enters the body, the LPS activates TLR4, inducing an immune response and mechanical allodynia [47]. However, a different response is observed between the male and female groups in the spinal cord. Upon LPS administration in the rat spinal cord, inflammatory cytokines, including IL-1β, were increased in the male rat group, and male astrocyte show a significant upregulation of TNF-α compared to female astrocyte [48]. In contrast to the immune response activation and allodynia observed in the male group, the female group

showed no allodynia. Furthermore, orchiectomized male or testosterone deficient male mice exhibited reduced allodynia after LPS administration, whereas testosterone replacement reinstated the pain. In the meanwhile, when LPS is administered to the brain or hind paw, hypoxia and allodynia developed in both males and females. These findings demonstrate that spinal cord is the only organ that exhibits a sexual dimorphic and hormone-dependent response to LPS stimulus [49; 50].

Recent studies have shown that colony stimulating factor 1 (CSF1) also triggers the sexual dimorphic response. CSF1, which is derived from injured sensory neurons, is transported to the spinal cord and induces microglial proliferation and leading to pain hypersensitivity [21]. However, CSF1-induced pain was only apparent in males. Although intrathecal administration of CSF1 proliferates spinal microglia in both sexes, microglia activation and robust transcriptional changes associated with inflammatory activation were observed in males, not in females. This reveals that other immune cells could be suppressing the microglial response to CSF1 in female. Indeed, several studies have observed that regulatory T cells (T reg) expansion induced by CSF-1 inhibits microglia activation and suppresses pain hypersensitivity in females. These findings indicated distinct factors between male and female in microglial activation and pain hypersensitivity [51].

1.1.3 GT1b induced sexual dimorphic pain

GT1b, a type of ganglioside molecule, has been found to exhibit a sexual dimorphic pain response similar to LPS and CSF1. This molecule is abnormally upregulated in injured sensory neurons and functions as an endogenous agonist of Toll-like receptor 2 (TLR2), leading to pain sensitization [52]. Upon peripheral nerve injury, St3gal2 is upregulated in the injured sensory neurons, which in turn produces GT1b. Then, GT1b is transported to and released in the spinal cord, which activates spinal cord

microglia and upregulated inflammatory cytokines such as IL-1β, TNF-α, and NADPH oxidase 1 (Nox2) which led to pain central sensitization (Figure 1).

However, our investigation revealed a sexually dimorphic response to the injection of GT1b in mice, as females did not exhibit mechanical hypersensitivity induced by these molecules. Intriguingly, despite this difference in pain response, both male and female mice demonstrated proliferation of spinal cord microglia following GT1b injection [53]. Nevertheless, the precise molecular mechanisms underlying this sexual dimorphism remain unclear, prompting us to conduct further studies aimed at unraveling these specific mechanisms.

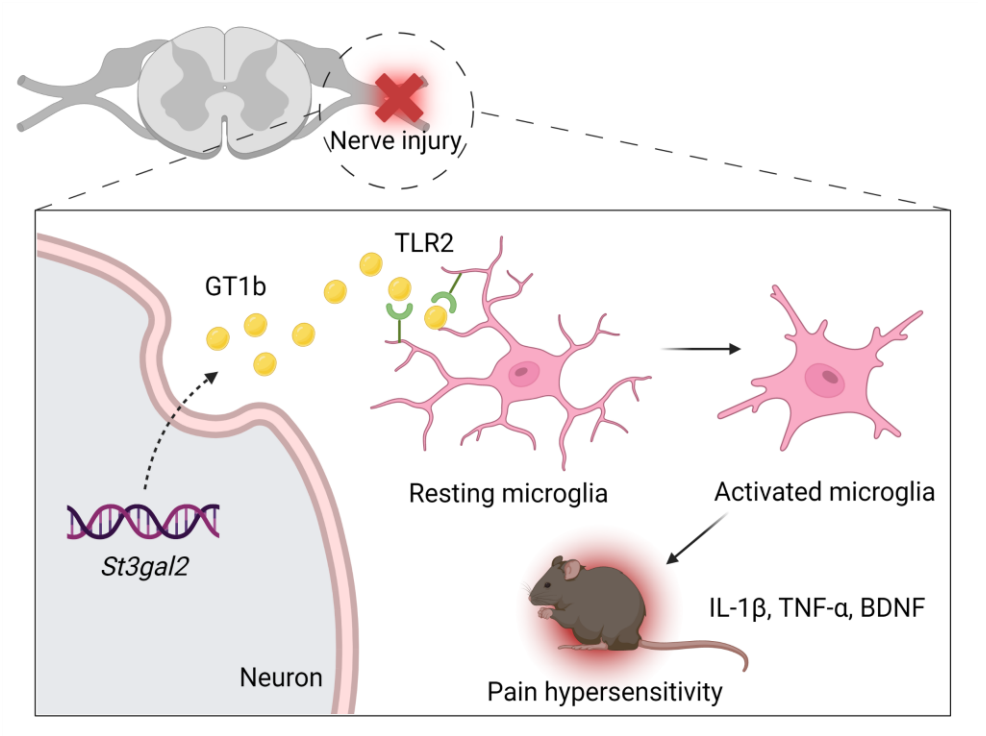


Figure 1. Pain inducing mechanism by GT1b after nerve injury or stimulation.

1.2. Purpose of Research

Despite the growing evidence on sexual dimorphism in pain research, there is still a dearth of studies investigating this phenomenon, particularly the underlying mechanisms. To address this gap in knowledge, we conducted a study based on our previous research that examined pain behavior induced by GT1b.

Our primary aim was investigating sexual dimorphic features of microglia activity and confirming effects of sex hormones on GT1b-induced pain. Additionally, we examined RNA levels of inflammatory cytokines to gain mechanistic insight into the inflammatory mechanisms underlying GT1b-induced pain. By conducting these experiments, we aimed to expand our understanding on the differences in pain central sensitization between males and females.

Our findings are particularly noteworthy, as they shed new light on the sexual dimorphism in pain central sensitization and suggest potential avenues for developing more effective treatments.

2. Materials and Methods

2.1 Animals

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University. Male and female C57BL/6J mice (8~10 weeks of age) were purchased from Daehan Biolink (DBL, Eumsung, Korea), and all animals were housed and maintained in a controlled environment at 22°C–24°C and 55% humidity with a 12-h light/dark cycle in a specific pathogen-free (SPF) environment. They were given access to food and water ad libitum. All protocols were performed in accordance with the guidelines from the International Association for the Study of Pain.

2.2 Intrathecal injection

The mice were anesthetized with isoflurane in an O2 carrier (induction at 2% and maintenance at 1.5%), and the GT1b (25 μg/5 μL; Matreya LLC, Cat # 1548, State College, PA, USA) in saline solution was administered using a 10-μL Hamilton syringe (Hamilton Company, Cat # 701LT, Reno, NV, USA) with a 30-gauge needle into the subarachnoid space as previously described [54]. The success of intrathecal injection was assessed by monitoring a slight tail-flick when the needle penetrated the subarachnoid space.

2.3 Immunohistochemistry

Mice were transcardially perfused with 0.1 M phosphate buffer (pH 7.4) followed by 4% paraformaldehyde, and the L5 spinal cord was removed and post-fixed in the same solution at 4°C overnight. Spinal cord samples were transferred to 30% sucrose for at least 48 h and coronally cut into 16-μm-thick sections using a cryostat (Leica, Cat # CM1860, Wetzlar, Germany). The spinal cord sections were blocked in a solution containing

5% normal goat serum, 2.5% bovine serum albumin (BSA), and 0.2% Triton X-100 for 1.5 h at room temperature. Then, spinal cord sections were incubated with rabbit anti-Iba-1 antibody (1:1,000; Wako, Cat # 019-19741, Osaka, Japan). After rinsing 5 times with 0.1 M PBS, samples were incubated with CY3-conjugated secondary antibodies (1:200; Jackson ImmunoResearch Laboratories, Cat # 111-165-003, West Grove, PA, USA) for 1.5 h at room temperature. The samples were mounted on glass slides with Vectashield mounting medium (Vector Laboratories, Cat # H-1000-10, Burlingame, CA, USA).

2.4 Confocal Microscopy and 3D IMARIS Analysis

Images were captured using an LSM 800 confocal microscope (Carl Zeiss, Oberkochen, Germany). For the 3D reconstruction of microglia, we took Z-stack images (6 µm depth, 460 µm steps) of spinal dorsal horns using an LSM 800 (1024×1024 pixels, 16-bit depth, 0.624 mm pixel size). Raw image files (.czi) were converted and analyzed using IMARIS (Version 9.8.0, Oxford Instruments, Abingdon, UK). The morphology of the single microglia (from 4 to 6 mice/group) was analyzed using the Filament Tracer Tool with the following settings: Autopath algorithm; Dendrite starting point diameter, 16.3 µm; Dendrite seed point diameter, 1 µm.

2.5 Behavioral Tests

All animal experimental procedures were reviewed and approved by the IACUC, Seoul National University. Mechanical allodynia tests were performed as previously reported [52]. All the behavior tests occurred between 10:00 a.m. and 3:00 p.m., and the experimenter was blind to group assignments throughout the experiment. The mechanical sensitivity of the right hind paw was assessed using a calibrated series of von Frey hairs (0.02–6 g; Stoelting, Wood Dale, IL, USA), following an up-down method [55]. Thermal sensitivity was determined by measuring

paw withdrawal latencies in response to radiant heat [56]. Rapid paw withdrawal, licking, and flinching were interpreted as pain responses. Tests were performed after at least three habituations at 24-h intervals. Assessments were made 1 day before surgery for baseline and 1, 3, and 7 days after surgery or injection. All behavioral tests were performed blinded.

2.6 RNA extraction and Transcriptome Analysis

Total RNA was extracted from mouse spinal cords using TRIzol reagent (Thermo Fisher Scientific, Cat # 15596026, Waltham, MS, USA). For transcriptome analysis, five spinal cords were pooled to synthesize the cDNA library for each group after 24 h of intrathecal injection of GT1b (25 μg/5 μL). RNA sequencing was conducted by E-Biogen (Seoul, Korea). Briefly, RNA quality and quantity were evaluated with the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and NanoDrop 2000 (Thermo Fisher Scientific). Library construction was achieved following the QuantSeq 3′ mRNA-seq library prep kit FWD (LEXOGEN, Cat # 015.96 Vienna, Austria) manufacturer's protocol. The cDNA libraries were sequenced on the Illumina NextSeq500 platform (Illumina, San Diego, CA, USA). Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) was performed using DAVID 2021 (https://david.ncifcrf.gov/, accessed on 21 December 2021) [57]. The heatmap of RNA-seq transcriptome analysis was generated for 23,281 genes after 24 h of i.t. injection of GT1b, and hierarchical clustering analysis (HCL) was conducted using TM4/MeV software (version 4.9.0) to analyze RNA sequencing data and compare GT1b-induced transcriptional changes between male and female [58]. Analyzed DAVID GO terms were visualized with a GOcircle plot in MATLAB dis-playing the fold change of each gene.

2.7 Ovariectomy

Eight-week-old female C57BL/6J mice received bilateral ovariectomy (OVX). Mice were anesthetized with isoflurane in an O2 carrier (induction at 2% and maintenance at 1.5%) and subsequently subjected to OVX or sham operation via a bilateral back incision. For the OVX group, we excised the anterior uterine horns to remove the ovaries and mitigated bleeding using the High Temp Cautery Kit (FST, Cat # 18010-00, Foster City, CA, USA). Incisions were closed with sterile sutures.

2.8 Orchiectomy

Eight-week-old male C57BL/6J mice received bilateral orchiectomy (ORX). Mice were anesthetized with isoflurane in an O2 carrier (induction at 2% and maintenance at 1.5%). A midline scrotal incision was made, and bilateral spermatic cords were ligated. Testes along with epididymal adipose were excised distal to the ligature, and bleeding was mitigated using the High Temp Cautery Kit. Incisions were closed with sterile sutures.

2.9 ELISA Assay

Mouse blood was collected via cardiac puncture in microtainer K2E (BD, Cat # 365974, Franklin Lakes, NJ, USA) and centrifuged at 1,600 × g for 15 min at 4°C to measure plasma 17β-estradiol levels. The supernatant plasma was collected, and the levels of 17β-estradiol were assessed using the 17 beta Estradiol ELISA kit (Abcam, Cat # ab108667, Cambridge, UK) following the manufacturer’s protocol. To measure IL-1β release from the GT1b-stimulated primary mixed glia, we treated ATP 30 min prior to supernatants collection to induce secretion of IL-1β, and collected supernatants of mixed glia centrifuged at 500 × g for 5 min to discard cell debris. IL-1β levels were measured using the mouse IL-1 beta Quantikine ELISA kit (R&D Systems Inc., Cat # MLB00C, Minneapolis, MN, USA) following the manufacturer’s protocol.

2.10 *In vitro* Primary Mixed Glia Culture

Primary mixed glia cultures were prepared from 1–2-day-old mice, as previously described [59]. In brief, brain glial cells were cultured in DMEM, high-glucose formula supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 1x penicillin/streptomycin, and 1x nonessential amino acid mixture at 37°C in a 5% CO2 incubator. Media was renewed every 5 days. After 15 days, primary mixed glia were harvested with 0.25% Trypsin-EDTA and plated in 6-well plates at a density of 5 x 10^5 cells per well for real-time RT-PCR and ELISA.

2.11 Real-Time RT-PCR

Real-time RT-PCR experiments were performed using the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) following the 2−ΔΔCt method [60]. Total RNA from the L5 spinal cord tissue in deeply anesthetized mice and primary mixed glia was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using TOPscript RT DryMIX (Enzynomics, Cat # RT200, Daejeon, Korea). All the ΔCt values were normalized to the corresponding GAPDH values and were represented as the fold induction. The following PCR primer sequences were used: Gapdh forward, 5′-AGT ATG ACT CCA CTC ACG GCA A-3′; Gapdh reverse, 5′-TCT CGC TCC TGG AAG ATG GT-5′; Il-1β forward, 5′-GTG CTG TCG GAC CCA TAT GA-3′; Il-1β reverse, 5′-TTG TCG TTG CTT GGT TCT CC-3′; Casp1 forward, 5′-CTG ACA AGA TCC TGA GGG CA-3′; Casp1 reverse, 5′-AAA GAT TTG GCT TGC CTG GG-3′; Nlrp3 forward 5′-CCA TCA ATG CTG CTT CGA CA-3′; Nlrp3 reverse 5′-GAG CTC AGA ACC AAT GCG AG-3′; Tlr2 forward, 5′-CTC CCA CTT CAG GCT CTT TG -3′; and Tlr2 reverse, 5′-ACC CAA AAC ACT TCC TGC TG-3′.

2.12 Statistical Analysis

Data were analyzed using Student's t-test for comparisons between two groups. One- and two-way analysis of variance (ANOVA) with Bonferroni's post hoc test were used for the statistical analysis of multiple comparisons. All data are presented as mean ± standard error of the mean (SEM), and differences were considered statistically significant when the p-value was < 0.05

3. Results

3.1 GT1b-induced pain central sensitization and spinal cord microglia activation are sexually dimorphic

To test if GT1b-induced pain central sensitization is sexually dimorphic, we administered GT1b into the spinal cord of male and female mice through intrathecal injection and compared the mechanical threshold to the von Frey stimuli (Figure 2A). As previously reported, GT1b administration induced mechanical allodynia 1 and 3 days post-injection (dpi) in the male mice (Figure 2B). However, the female mice were completely resistant to GT1b-induced pain sensitization (Figure 2B), thus indicating sexual dimorphism in GT1b-induced pain central sensitization. We have reported that GT1b activates spinal cord microglia via TLR2, which leads to pain central sensitization [52]. Therefore, we tested TLR2 transcript expression in the spinal cords of female mice, and similar levels of TLR2 transcript were detected in the spinal cords of the female mice compared to the male mice (Figure 2C).

To test sex difference in spinal cord microglia activation upon GT1b administration, we assessed spinal cord microglia activation using Iba-1 immunohistochemistry. The GT1b injection induced spinal cord microglia activation in female mice at 3 dpi, although the activation was not as significant as in male microglia at 1 dpi (Figure 3A, B).

We then characterized the morphological features of the GT1b-activated microglia in the female mice by analyzing the soma size, process length, and the branch point of each activated microglia (Figure 4A). In the female mice, GT1b injection increased the microglia soma size (1 dpi) and reduced the process length and branch point number (Figure 4B), which are typical morphological features of the activated microglia [61]. Intriguingly, GT1b injection did not reduce the process length or branch number of spinal cord microglia in male mice; rather, it increased the process length at 1 dpi

(Figure 4B). Regarded together, our data indicate that i.t. GT1b administration induces spinal cord microglia activation both in male and female mice, but with distinct kinetics and morphological activation features.

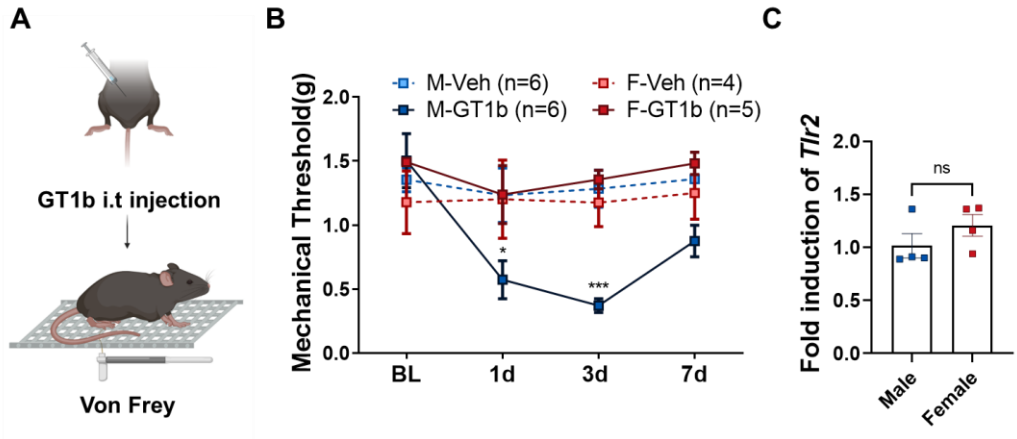


Figure 2. Pain central sensitization by GT1b is sexually dimorphic.

(**A**) Schematic diagram of i.t administration of GT1b and von Frey allodynia behavior test. (**B**) Mechanical threshold of von Frey tests on hind paws after GT1b administration (n = 4 to 6/group, two-way ANOVA with Tukey's multiple comparison test post hoc, * vs. M-Veh). (**C**) Relative TLR2 transcript levels in the spinal cords of female mice compared to male mice. (n = 4, Unpaired t-test, ns: not significant).

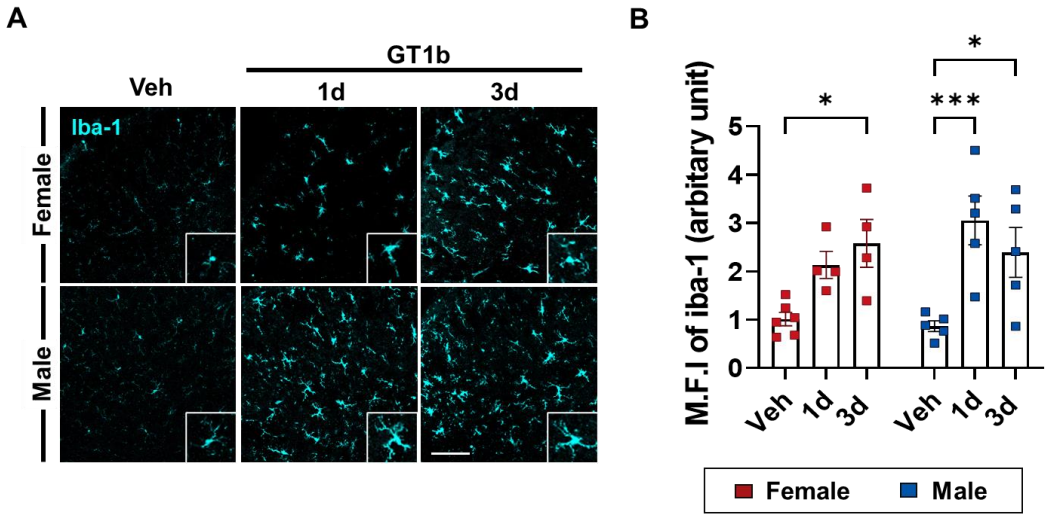


Figure 3. Comparison of microglial activation and intensity induced by GT1b administration.

(**A**) Representative image of a spinal cord immunostained with Iba-1 (Scale bar, 100 μm). Below right panels show magnified images of a typical microglia. (**B**) Mean fluorescence intensity of Iba-1 (n = 4 to 6/group, two-way ANOVA with Bonferroni's multiple comparison test post hoc). Data are presented as mean ± SEM. *$P < 0.05$, ***$P < 0.001$. 1, 3d: 1, 3 days after injection. Veh: vehicle control group. M.F.I: mean fluorescence intensity.

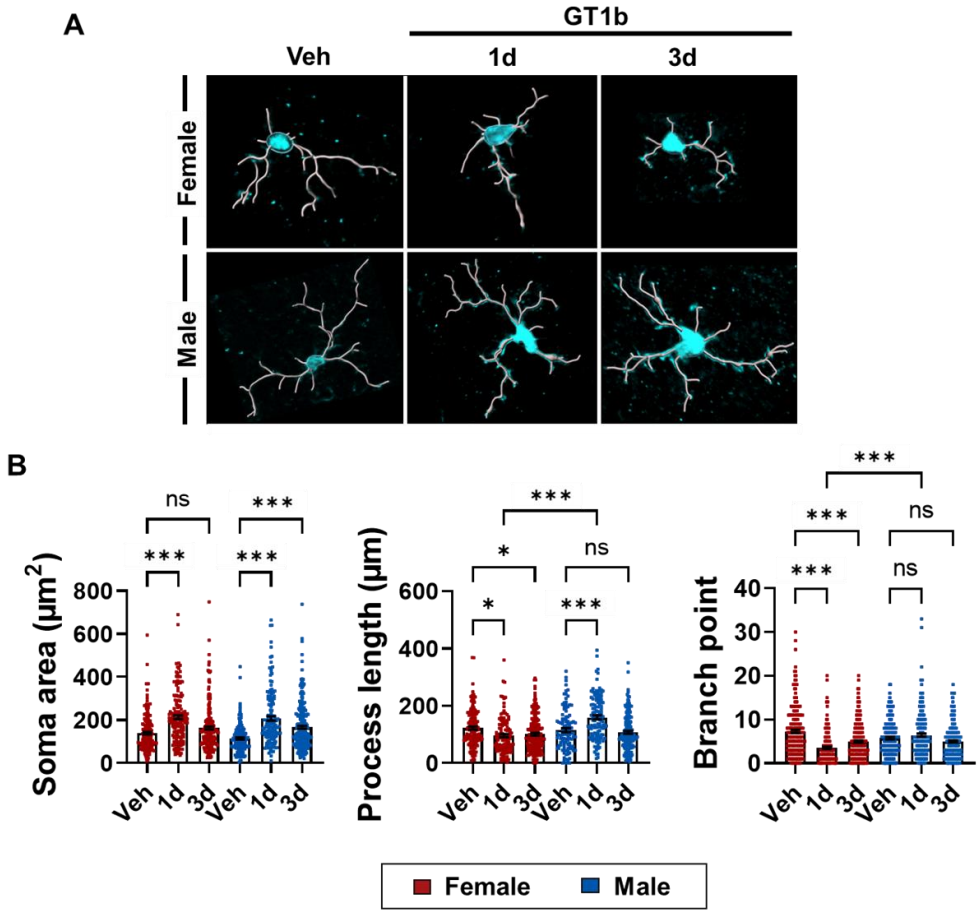


Figure 4. Morphological and dynamics characterization of microglia between male and female during GT1b administration.

(**A**) Representative image of microglial morphology analyzed using IMARIS. (**B**) Soma area, process, and branch points of microglia (n = from 139 to 235 microglia/group, two-way ANOVA with post hoc Tukey's multiple comparison test). Data are presented as mean ± SEM. ns: not significant, *$P < 0.05$, ***$P < 0.001$. 1, 3d: 1, 3 days after injection. Veh: vehicle control group. M.F.I: mean fluorescence intensity.

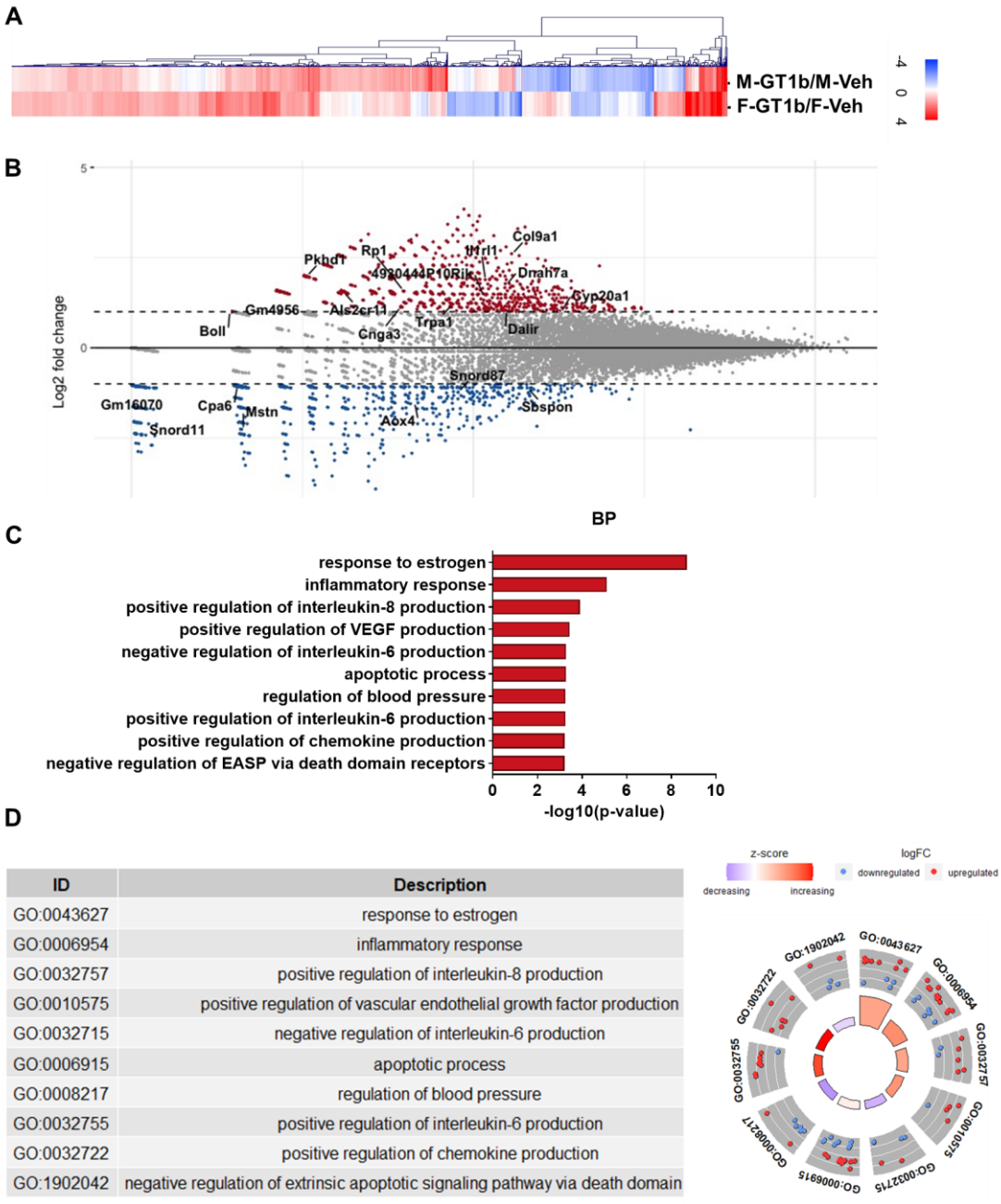
3.2 Sexually dimorphic transcriptome profiles of GT1b-stimulated mouse spinal cords

To further compare the activation features between male and female mouse spinal cord microglia, we analyzed and compared the gene expression profiles in the spinal cord dorsal horn after GT1b administration in the male and female mice, respectively, using RNA-seq and hierarchical clustering analysis (Figure 5A). Spinal cord samples were taken one day after ganglioside administration. In search of genes associated with the sexual dimorphism, we screened for differentially expressed genes (DEGs) with expression levels more than 2-fold different in the females compared to the males (Figure 5B).

To account for differences in basal gene expression profiles between male and female mice, we conducted additional transcriptome comparisons and annotations using DAVID Gene Ontology (GO) analysis [58; 62]. Considering male and female have different basal level gene expression profile, we compared the transcriptome profile between male and female (M-GT1b/M-Veh vs. F-GT1b/F-Veh); however, we did not find any GO terms related to pain. Although we did not find GO terms in between Vehicle M/GT1b M vs. Vehicle F/GT1b F, we assumed that GO term between GT1b-male vs. GT1b-female gives information regarding on pain sensitivity.

Then we compared and annotated the transcriptome of GT1b-administered males exhibiting pain behavior with that of GT1b-administered females not showing pain behavior. We identified the top 10 enriched categories of biological processes (BP) (Figure 5C). Based on DAVID gene ontology analysis, the genes involved in estrogen-related signaling pathways were found to be the most significantly different between the male and female mice (Figure 5D). Meanwhile, the other genes putatively involved in pain sensitization at the spinal cord level (e.g., proinflammatory cytokines, chemokines, etc.) were comparably regulated

by GT1b administration (Figure 6). Based on this context, we hypothesized that estrogen might contribute to the sexual dimorphism in GT1b-induced pain central sensitization.



ID	Description
GO:0043627	response to estrogen
GO:0006954	inflammatory response
GO:0032757	positive regulation of interleukin-8 production
GO:0010575	positive regulation of vascular endothelial growth factor production
GO:0032715	negative regulation of interleukin-6 production
GO:0006915	apoptotic process
GO:0008217	regulation of blood pressure
GO:0032755	positive regulation of interleukin-6 production
GO:0032722	positive regulation of chemokine production
GO:1902042	negative regulation of extrinsic apoptotic signaling pathway via death domain

Figure 5. Transcriptomic analysis of GT1b-injected mouse spinal cords. **(A)** Heat map of RNA-seq transcriptome analysis for 23281 genes after 24h of i.t. injection of GT1b **(B)** MA plot for DEGs of GT1b-stimulated female compared to GT1b-stimulated male mice. Greater than two-fold upregulated or downregulated genes are plotted. Y-axis shows the fold induction of

DEGs, and the X-axis shows the basal expression level of each gene. **(C)** The top 10 enriched GOs were analyzed by DAVID functional gene ontology analysis of DEGs (BP: Biological process). **(D)** GOcircle plot displaying the fold change (logFC) of each gene in the top 10 enriched BP GO terms. The chart displays the annotation categories of each GO. Z-scores are displayed in the inner circles. M-GT1b: GT1b administered male. M-Veh: vehicle administered male. F-GT1b: GT1b administered female. F-Veh: vehicle administered female.

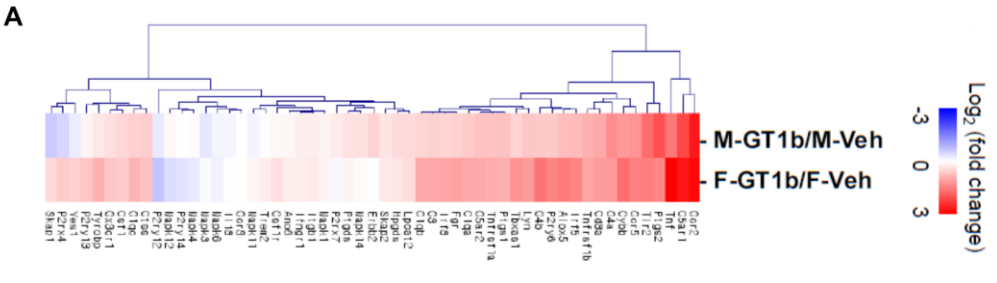


Figure 6. Gene expression related to pain sensitization between sexes were comparably regulated by GT1b administration.

(A) Heat map of RNA-seq transcriptome analysis for genes putatively involved in pain sensitization at the spinal cord level (e.g., proinflammatory cytokines, chemokines, etc.), 53 gene expressions associated with neuropathic pain following GT1b administration. Genes are grouped by hierarchical clustering analysis (HCL) using Euclidean distance.

3.3 GT1b-induced pain central sensitization is dependent on estrogen

To test whether estrogen plays a role in the GT1b-induced pain central sensitization in female mice, we systemically reduced estrogen levels in female mice by OVX. Two weeks after removing the ovaries of 8-week-old adult female mice, uterine shrinkage, as well as an increase in body weight were detected (Figure 7A–C). We also confirmed decrease in estrogen levels in the OVX females, and that this decrease was rescued by estrogen supplementation (OVX−E2) (Figure 8A). While female mice were resistant to GT1b-induced pain central sensitization, OVX rendered female mice susceptible to GT1b-induced mechanical allodynia (Figure 8B). Then, we tested whether exogenous estrogen supplementation could restore the resistance to GT1b-induced pain central sensitization. We administered 17β-estradiol (OVX-E2, 5 μg/kg, 50 μL daily) through intraperitoneal injected 1 day prior to GT1b injection and confirmed estrogen recovery (Figure 9A, B). When we supplemented 17β-estradiol to OVX female mice, the OVX mice became resistant againist to GT1b-induced mechanical allodynia (Figure 9C).

These data indicate that high estrogen levels in female mice are responsible for the sexual dimorphism of GT1b-induced pain central sensitization. We then tested if male sex hormones affect GT1b-induced pain sensitization. To this end, we subjected male mice to ORX. Unlike OVX female mice, ORX male mice exhibited comparable levels of mechanical sensitivity upon GT1b administration (Figure 10), indicating that male sex hormones are not involved in sexual dimorphism.

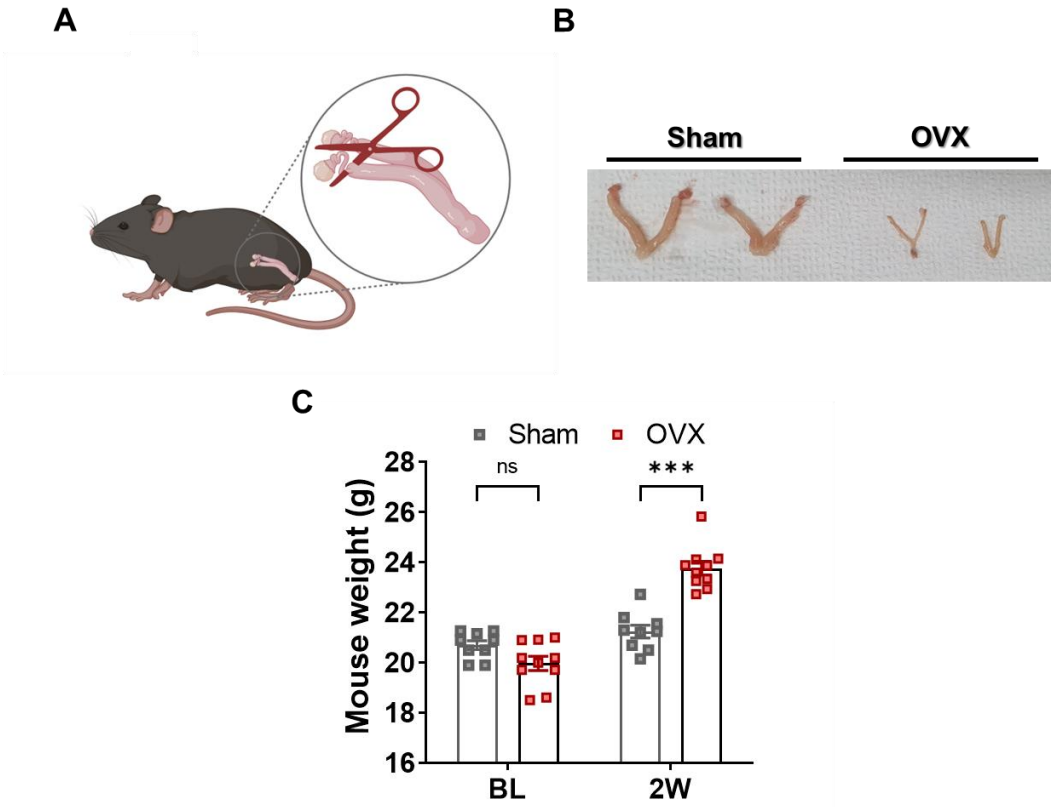


Figure 7. Characteristics of female mice after ovariectomy.

(A) Schematic diagram of bilateral ovariectomy. **(B)** Representative images of uteri in sham and OVX mice 2 weeks after surgery. **(C)** Mouse weight (n = from 9 to 10/group, two-way ANOVA with post hoc Bonferroni's multiple comparison test) ns: not significant, ***$P < 0.001$. OVX: ovariectomy. Sham: control for ovariectomy. BL: basal level. 2W: 2 weeks.

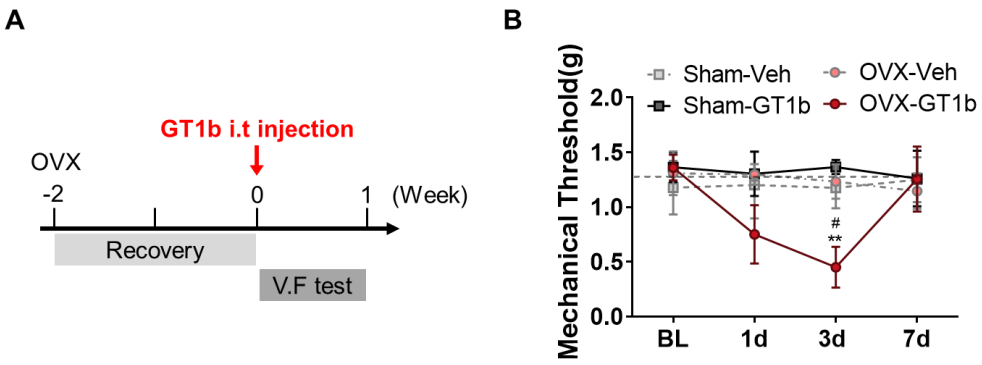


Figure 8. Behavior test after GT1b administration in ovariectomized female mice.

(A) Experimental scheme of the behavior test after GT1b i.t. injection in OVX. **(B)** Mechanical threshold of orchiectomized mice after GT1b administration (n = 4 to 7/group, two-way ANOVA with post hoc Tukey's multiple comparison test, * vs Sham-GT1b, # vs GNX-GT1b). Data are presented as mean ± SEM. ns: not significant, ** $P < 0.01$, # $P < 0.05$. OVX: ovariectomy. Sham: control for ovariectomy. BL: basal level. 1, 3, 7d: 1, 3, 7 days after injection.

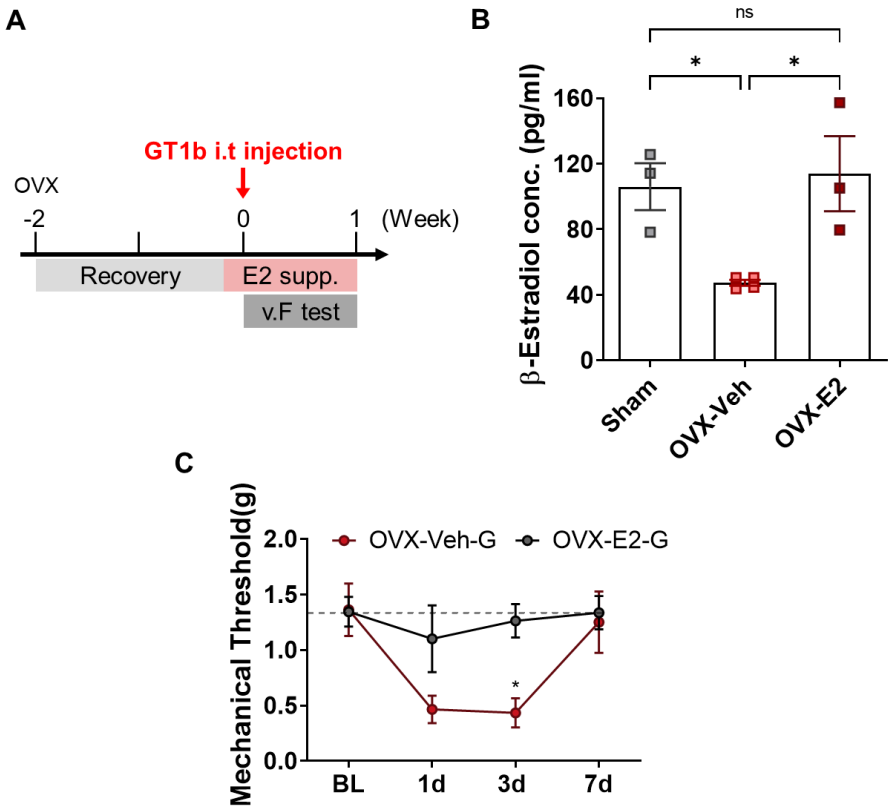


Figure 9. Effects of estrogen supplement in GT1b administration in ovariectomized female mice.

(A) Experimental scheme of the behavior test after GT1b i.t. injection and estrogen supplement in OVX females. **(B)** Serum levels of estradiol were measured 2 weeks after OVX (n = from 3 to 4/group, one-way ANOVA with post hoc Tukey's multiple comparison test). **(C)** Mechanical allodynia was measured after GT1b i.t injection in animals with OVX-E2 supplementation (n = from 3 to 5/group, two-way ANOVA with post hoc Bonferroni's multiple comparison test, # vs. OVX-Veh-V, * vs. OVX-E2-G). Data are presented as mean ± ns: not significant, *P < 0.05. OVX: ovariectomy. Sham: control for ovariectomy. OVX-Veh-G: GT1b administered vehicle control group of ovariectomized female. OVX-E2-G: GT1b administered E2. supplement group of ovariectomized female. BL: basal level. 1, 3, 7d: 1, 3, 7 days after injection.

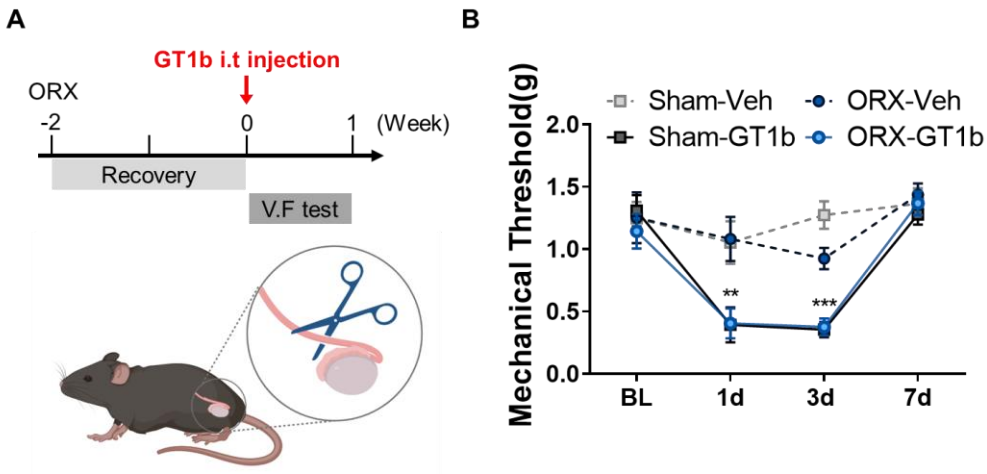


Figure 10. Comparison of pain behavior by orchiectomy of GT1b-induced pain sensitization.

(A) Experimental scheme and schematic diagram of the behavior test after GT1b i.t. injection in orchiectomized male mice. **(B)** Mechanical threshold of ORX mice after i.t. GT1b administration. Data are presented as mean ± ns: not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. ORX: orchiectomy. Sham: control for orchiectomy. OVX-Veh-G: GT1b administered vehicle control group of ovariectomized female. OVX-E2-G: GT1b administered E2 supplement group of ovariectomized female. BL: basal level. 1, 3, 7d: 1, 3, 7 days after injection.

3.4 17β-Estradiol inhibits GT1b-induced inflammasome activation and IL-1β release

Previously, we showed that i.t. GT1b administration induced microglia activation and IL-1β expression in the spinal cord, which results in the development of central sensitization [60]. Since GT1b upregulated IL-1β transcripts not only in male mice but also in female mice (Figure 11), we tested if estrogen affects the post-translational modification of IL-1β. IL-1β is initially expressed in its pro-form (pro-IL-1β) and then released upon cleavage by NLRP3- and Caspase-1-containing inflammasomes [63].

Therefore, we compared the gene expression of NLRP3 and Caspase-1 in male and female mice upon GT1b stimulation. Though GT1b administration induced NLRP3 expression in both sexes, Caspase-1 was upregulated only in male mice (Figure 11B, C), indicating sexually dimorphic inflammasome activation. Then, we examined estrogen effects on primary mixed glia in vitro (Figure 12A). We treated cells with GT1b (10 μg/mL) for 16 h, following pre-treatment with 17β-estradiol (100 nM) for either 16 h or 0.5 h. To measure the IL-1β secreted from mixed glia, we pretreated all groups, including the control, with ATP (5 mM) 30 min prior to collecting the supernatant. ATP stimulates P2X7 on microglia leading to K^+ efflux and increase of $Ca2^+$, which, in turn, induces secretion of IL-1β. While 17β-estradiol pretreatment did not affect GT1b-stimulated Caspase-1 and IL-1β transcript expression, it significantly inhibited the IL-1β release from the primary mixed glia (Figure 12B-D). Regarded together, these results suggest that estrogen inhibits IL-1β release in the spinal cords of female mice, which underlies the sexual dimorphism of GT1b-induced pain central sensitization.

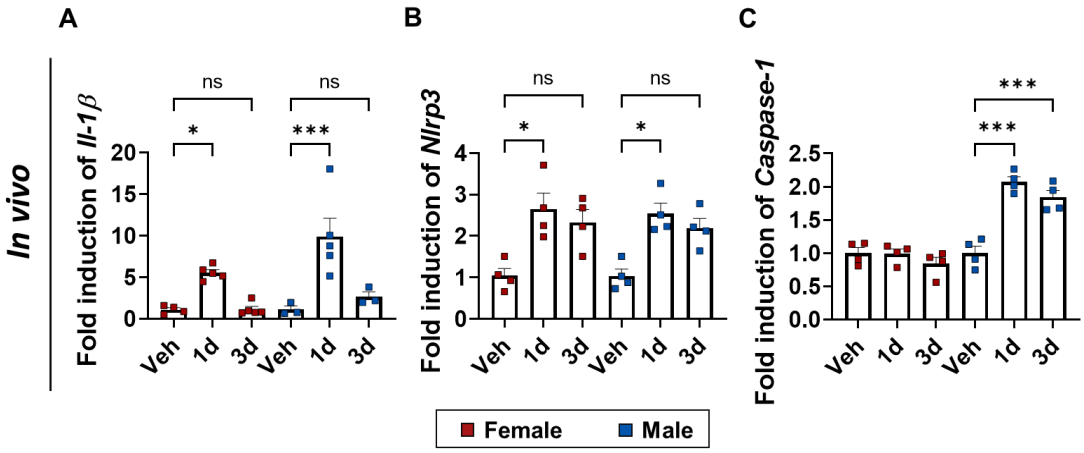


Figure 11. Comparing transcript levels related to NLRP3 inflammasome between GT1b-induced female mice and male mice.

Transcript levels of (**A**) IL-1β, (**B**) NLRP3, and (**C**) Caspase-1 in the spinal cord after GT1b i.t. injection (n = from 3 to 5/group, two-way ANOVA with post hoc Tukey's multiple comparison test). Data are presented as mean ± SEM. ns: not significant, *$P < 0.05$, and ***$P < 0.001$.

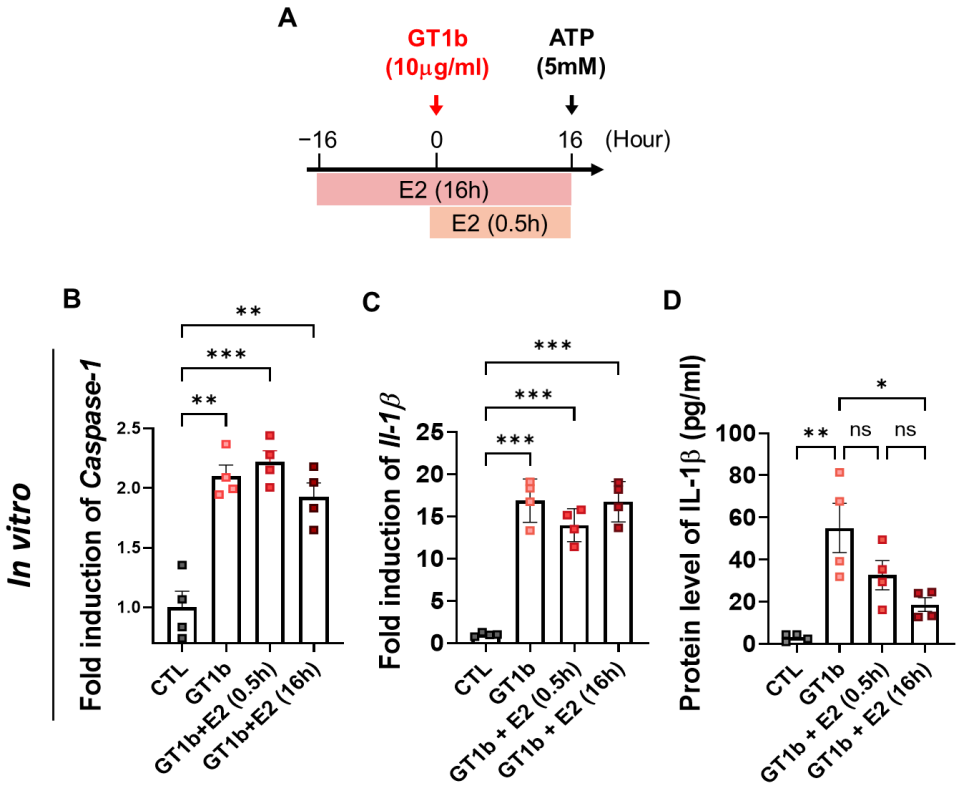


Figure 12. 17β-estradiol regulates post-translational IL-1β modification. **(A)** Experimental scheme of the IL-1β release assay from primary glia. **(B)** Transcript levels of Caspase-1, **(C)** IL-1β, and **(D)** protein levels of released IL-1β from primary glia (n = 4/group, one-way ANOVA with post hoc Tukey's multiple comparison test). Data are presented as mean ± SEM. ns: not significant, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

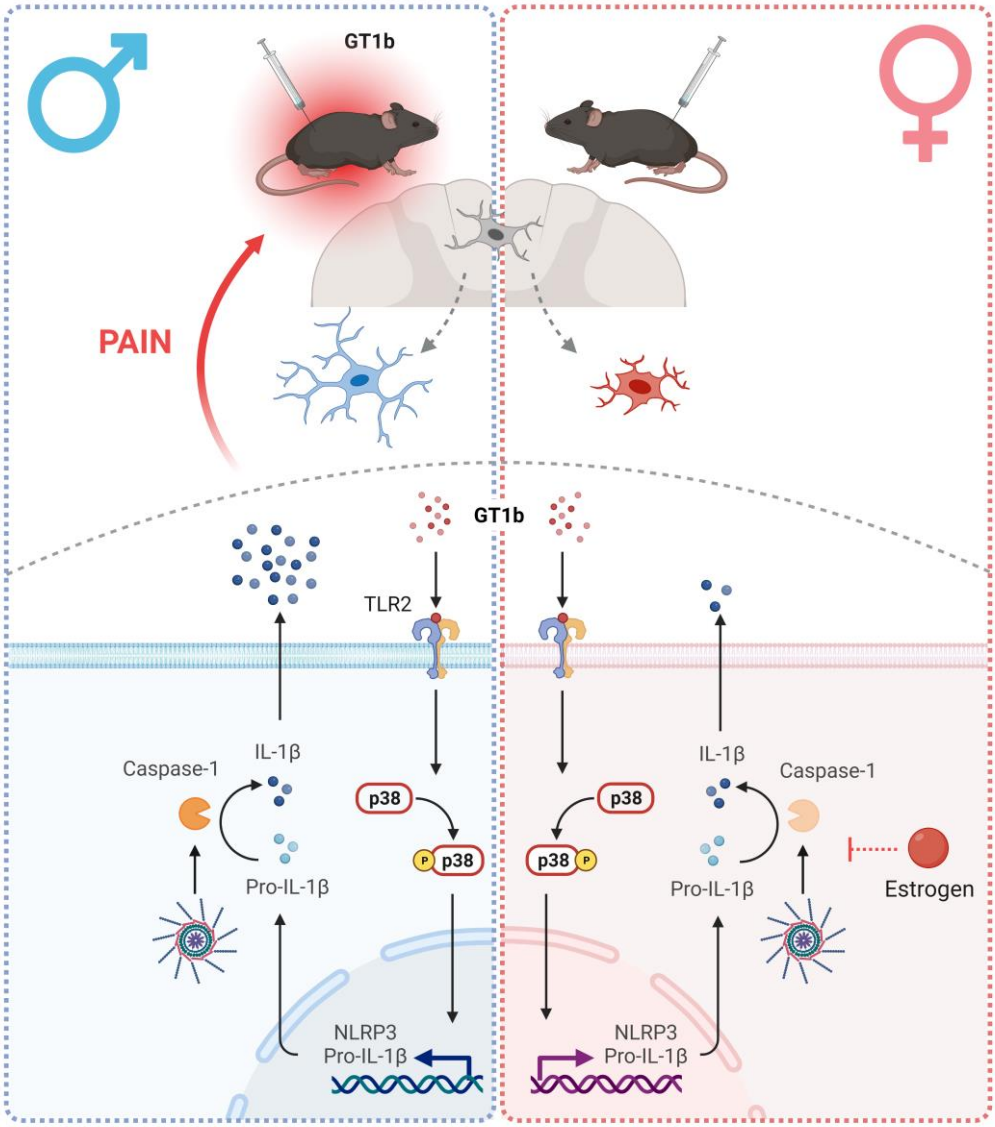


Figure 13. Graphical summary of sexual dimorphic mechanism of GT1b-induced pain central sensitization in male and female mice.

4. Discussion

In this study, we uncovered a sex difference in the involvement of GT1b, endogenous TLR2 agonist in neuropathic pain, induces sexually dimorphic in the development of mechanical pain hypersensitivity only in male but not in female mice. In addition, we found that estrogen has analgesic effects by inhibiting the release of IL-1β.

Sexual dimorphism in pain central sensitization has been reported in several other animal models. For example, in the administration of LPS in spinal cord, the immune response and mechanical allodynia were induced through activating TLR4. However, pain allodynia observed only in male group. Our findings are in line with these previous studies. Considering TLR2 and TLR4 share most of their downstream intracellular signaling pathways mediated by MyD88 [64], it is not surprising that the GT1b-mediated activation of microglial TLR2 induces central sensitization only in male mice, not female mice. Our data, along with previous reports, indicate that microglia activation in female mice is not sufficient to induce pain central sensitization.

Recent studies indicate that morphological phenotypes of microglia, such as ramified, rod-like, activated, and amoeboid forms, represent the state of microglia and can be used as indicator of the CNS physiological environment [27; 38]. Here, we report that GT1b-induced microglial activation occurs in both males and females, but with different kinetics and morphology. In general, there are several functional and morphological differences reported between male and female microglia. Male microglia, for instance, show an enlarged cell body volume and density in brain. It have higher antigen presenting capacity which caused higher expression of MHC1, MHC2 and enhanced P2X receptor mediated signaling [65; 66]. Moreover, male microglia have higher immunoreactivity and increased proinflammatory gene expression [67]. Thus, even under the same

physiological conditions, male microglia can react more reactively than female microglia.

In addition, microglia-specific molecules such as P2X4 and p38 MAPKs in spinal cord microglia which associated with neuropathic pain operate differently depending on the sex [68; 69]. In males, microglial p38 deficiency led to the transition of microglia from homeostatic to disease associated. On the other hand, the effect of p38 deficiency was divergent in female, upregulating small subsets of both tissue protective genes and inflammatory genes. These male-specific p38α-dependent molecular pathway in CNS auto-immunity suggest that female have differential cellular and molecular pathway for protection in microglia [70].

Moreover, the remarkable results indicating that the nociceptive process in female operates through a microglia-independent pathway [42]. When microglia are specifically depleted in pain-induced mice, pain hypersensitivity was reversed only in males, whereas allodynia was still present in females. Additionally, blocking signaling mediators related to the microglia-neuron nociception pathway, such as P2X4 receptor or p38 MAP kinase, reversed allodynia only in males [42; 71].

Then, to identify which mechanism transmit pain in female, the researchers compared changes in allodynia between male and female nude mice deficient in T and B cells, and recombination-activating gene 1-knockout (Rag1-/-) mice. Both nude and Rag1-/- mice of both sexes were sensitive to glial inhibitors and showed a reversal of SNI-induced allodynia [42; 43; 45]. These results explain that the nociception process in female mice may primarily involve T cells dealing with injury, rather than microglia [72].

However, this fact does not mean that female microglia cannot contribute to pain central sensitization. Recent studies have demonstrated that in Treg-depleted female mice, the immune pathway switches to a microglia-dependent pathway that mediates hypersensitivity. These findings

provide evidence that T cells play a pivotal role in the activation of inflammatory responses in females, whereas the absence of T cells leads to the involvement of microglia in pain hypersensitivity. In this regard, it is conceivable that i.t. administration of GT1b selectively triggers the activation of microglia in the spinal cord, without recruiting T cells to the same region. Consequently, this phenomenon could result in the failure to induce central sensitization of pain in female mice.

To better understand the functional differences in the activation of male and female spinal cord microglia, we conducted a study to investigate sex-specific transcriptome profiles in the spinal cord after GT1b injection. Previous transcriptome analyses to identify DEGs in microglia responsible for sexual dimorphism have provided minimal insight into the mechanisms [73]. According to this study, while in CCI, most genes involved in microglia activation and implicated in pain central sensitization, such as proinflammatory cytokines, were also upregulated in female microglia. Though it failed to identify specific genes that were selectively upregulated in male microglia and rendered pain central sensitization, male microglia displayed more prominent global transcriptional shifts and increased phagocytic activity compared to female microglia [73]. Likewise, our RNA-seq results revealed that GT1b regulates genes involved in neuropathic pain in both sexes. Meanwhile, our DAVID gene ontology analysis indicated that most enriched DEGs in biological processes are associated with the response to estrogen, including F7, Tph2, Cyp27b1, Krt19, Abcc2, Mstn, Dhh, Ghrl, Smad6, Agtr1a, Tshb, and Agtr1b, which suggests the putative involvement of estrogen in the sexual dimorphism of GT1b-induced pain central sensitization. Therefore, we focused on the estrogen response, and our study using OVX mice demonstrated that estrogen was indeed responsible for the lack of pain-sensitizing effects of i.t. GT1b administration in female mice.

Estrogen is well-known for its anti-inflammatory and neuro-

protective effects on the nervous system. Studies have documented that estrogen has a neuroprotective effect in various diseases such as Alzheimer's disease, Parkinson's disease, stroke, and brain trauma. It also protects against injured brain and spinal cord injury [74; 75]. In stroke and ischemic brain injury, estrogen exerts neuroprotective functions by inhibiting inflammasome activation and proinflammatory cytokine expression in the brain [76; 77]. In addition, estrogen attenuates the spinal-cord-injury-induced inflammatory response by regulating inflammasomes [63].

Studies indicate that estrogen mediates its effects by affecting glia activation. For example, estrogen inhibits global cerebral ischemia-induced NLRP3 inflammasome activation and proinflammatory cytokines expression in glia [78]. It also significantly reduces superoxide dismutase production and phagocytic activity, ultimately attenuating neuro-degeneration [79; 80]. Besides, p-p38MAPK and p-ERK, which activate microglia, and astrocyte JNK activation are decreased by estrogen, thereby mediates anti-inflammatory effects in the spinal cord [75]. In particular, microglia are more sensitive to estrogen because they physiologically express steroid hormone receptors [65]. This suggests that estrogen can inhibit inflammation and promote functional recovery while attenuating activated microglia and astrocyte during pain.

In line with these previous studies, our data revealed that estrogen inhibits GT1b-induced IL-1β production in primary mixed glia. It was well-known that IL-1β expression in the spinal cord renders pain central sensitization [81; 82]. Of note, IL-1β transcripts in the spinal cords of female mice were comparable to those of male mice. Instead, IL-1β released into the media was inhibited by estrogen, and Caspase-1 induction by GT1b was blocked in the female mice. It needs to be noted that GT1b-stimulated Caspase-1 expression was not inhibited by E2. We speculate that E2 may indirectly suppress glial Caspase-1 transcript *in vivo*. Although, we revealed

an essential role of E2 in the sexual dimorphism of the GT1b-induced central sensitization, the functional and mechanistic link between the results obtained in primary glial cultures and *in vivo* remains weak, which is a limitation of this study. Therefore, further studies are needed on the regulation of GT1b-induced inflammasome activation by E2 to completely elucidate the mechanism of sexual dimorphism in the GT1b-induced central sensitization.

While estrogen is known to anti-inflammatory and neuroprotective effects on the nervous system, it has also been demonstrated to have a pain-enhancing role through the modulation of gene expression [83] and neuronal activation in dorsal root ganglia (DRG) of female [84]. Specifically, low estrogen levels have been associated with chronic pain conditions in females. While in male, chronic pain conditions are often accompanied by low level testosterone levels in contrast to normal conditions [85]. These observations suggest that the effects of sex hormones on pain are more complicated, with hormone levels influencing immunological variation and pro- and anti-inflammatory responses. Consequently, further investigations are necessary to fully elucidate the underlying mechanisms the drive the biphasic effects of estrogen on pain modulation in different contexts. Anyhow, our study utilized an intrathecal GT1b-induced pain model, which primarily involves upper circuits and may not directly impacts the DRGs.

The mechanism behind the sexual dimorphism in GT1b-induced pain central sensitization is distinct from that of TLR4-induced pain. According to a study by Sorge et al., TLR4-mediated pain is dependent on testosterone [50]. However, in our study, we observed comparable pain induction by GT1b in ORX mice, indicating that GT1b-induced pain sensitization is independent of testosterone. The reason for this discrepancy remains unclear. Although TLR2 and TLR4 share MyD88 as their intracellular signaling molecule, TLR4 induces an additional intracellular signal via IRF3 [33]. Hence, it is speculated that IRF3-dependent microglia

activation is affected by testosterone, which needs to be tested in the future.

Furthermore, hormonal imbalances of estrogen and progesterone are thought to contribute to menstrual cramps experienced by many women [86; 87]. During the menstruation, the downregulation of estrogen levels may lead to the sensitization of pain receptors in the central nervous system, resulting in more intense and painful cramps. Understanding the association between estrogen downregulation and heightened pain response provides valuable insights into the mechanisms underlying menstrual cramps. Targeting estrogen as a therapeutic approach could potentially alleviate the symptoms of various women's health conditions [88].

Conclusively, our findings reveal that estrogen is responsible for sexual dimorphism in the GT1b-induced pain central sensitization, and that estrogen ameliorates GT1b-induced IL-1β production in the spinal cord by inhibiting inflammasome activation as an underlying mechanism (Figure 13).

5. Limitation and future research

Although our study has shed light on the relationship between estrogen and Caspase-1 in pain central sensitization, there are still some limitations that need to be addressed in future research.

First, we need to conduct behavior test on male mice after administering estrogen to determine the specific impact of this hormone on GT1b-induced pain central sensitization. This will help us clarify any potential sex differences in the effects of estrogen on pain sensitization.

Second, while our study has established a link between Caspase-1 and pain central sensitization, the specific inhibitory mechanisms mediated by this enzyme are not yet fully understood. To address this, we need to explore whether estrogen plays a role in decreasing Caspase-1 levels in vivo, through supplementing estrogen in GT1b-induced mice and comparing cytokine levels *in vivo* to determine the specific impact of estrogen on Caspase-1 levels. Additionally, we need to investigate whether inhibiting Caspase-1 activity or expression can make male mice resistant to GT1b-induced mechanical allodynia.

By answering these questions, we can gain a better understanding of the sexual dimorphic mechanism and role of Caspase-1 in pain.

6. Conclusion

A comprehensive understanding of sexual dimorphism in pain processing is essential for advancing our knowledge and developing targeted interventions in neurological diseases and disorders, as highlighted by the findings of our study. These sex differences originate from cellular-level variances that go beyond environmental influences, hormonal factors, and transcriptional profiles. While the most current studies have exhibited a bias towards males, there is a growing recognition of the significance in exploring sexual dimorphism in the context of neurological diseases and disorders.

In this study, we explored the sexual dimorphism of GT1b-induced microglia activation and mechanical pain. Our findings revealed that intrathecal administration of GT1b induced mechanical pain exclusive in male, demonstrating a sexual dimorphism in the pain central sensitization. Additionally, we identified distinct mechanisms underlying GT1b-induced pain between male and female. Specifically, GT1b activates microglia in both sexes, but the morphology of microglia activation is slightly different between male and female. In addition, we found that estrogen blocks Caspase-1, which mediates downregulation of IL-1β release, resulting in an inhibition of GT1b-induced inflammasome activation and IL-1β production.

Our findings suggest that estrogen plays a pivotal role in the sexual dimorphism of GT1b-induced pain central sensitization, while also demonstrating its potential in ameliorating GT1b-induced IL-1β production in the spinal cord through the inhibition of inflammasome activation. These mechanistic insights have significant implications for developing sex-specific therapeutic strategies aimed at alleviating pain central sensitization using estrogen.

Further research is necessary to fully elucidate the specific inhibitory mechanisms of GT1b-induced pain upon E2, as well as their in

vitro and in vivo connections. Nonetheless, our results provide a novel understanding of sexual dimorphism in medical research and offer valuable insights for guiding future therapeutic interventions.

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국문초록

GT1b에 의한 통증 중추 감작의 성적 이형성 기전

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정 서 현

통증 중추 감작이란 만성통증에서 통증자극에 의해 자극되는 생리학적 증상으로, 통각수용체에 의한 반응성이 증가하고 환자의 신경계가 지속적으로 활성화되는 상태를 말한다. Ganglioside의 한 종류인 GT1b는 척추강내(i.t.) 주사를 통해 투여할 때 통증 중추 감작을 유도한다. 이는 소교세포에 대한 Toll-유사 수용체 2의 내인성 작용제로 작용하여 소교세포 활성화를 시키며 통증 감작을 유도하게 된다.

해당 연구에서 우리는 GT1b 유발 중추 통증 감작의 성적 이형성과 관련 메커니즘을 조사했다. GT1b 투여 결과, 수컷 쥐에서는 중추 통증 감작이 유도되는 반면 암컷 쥐에서는 통증이 전혀 유도되지 않았다. GT1b 주입 후 수컷과 암컷 쥐 사이 척추 조직의 유전자 전사체를 비교한 결과, 에스트로겐 매개 신호 전달의 관련 가능성을 확인했다. 난소 절제술을 통한 에스트로겐 감소 시, 암컷 쥐는 GT1b 유도 중추 통증 감작이 유의미한 수준으로 관찰됐으며, 에스트로겐 보충 시에는 완전히 역전되었다. 한편, 수컷 쥐의 고환 절제술은 통증 감작에 영향을 미치지 않았다. 성적 차이가 유발되는 기전의 차이를 확인하기 위해 NLRP3 관련 유전자들의 발현을 비교분석한 결과, 에스트로겐이 GT1b에 의해 유발된 인플라마좀 활성화 및 IL-1β 생성을 억제하는 것을 확인했다.

위 연구결과는 에스트로겐의 발현 조절이 통증 중추 감작을 치료하는 핵심 치료 전략임을 증명했다.