High-Frequency Spinal Stimulation Suppresses Microglial Kaiso-P2X7 Receptor Axis-Induced Inflammation to Alleviate Neuropathic Pain in Rats

Yu, Jing; Wong, Stanley; Lin, Zhihan; Shan, Zhiming; Fan, Chaoyang; Xia, Zhengyuan; Cheung, Martin; Zhu, Xiaowei; Liu, Jessica Aijia; Cheung, Chi Wai

Published in:
Annals of Neurology

Published: 01/05/2024

Document Version:
Final Published version, also known as Publisher's PDF, Publisher’s Final version or Version of Record

License:
CC BY-NC

Publication record in CityU Scholars:
Go to record

Published version (DOI):
10.1002/ana.26898

Publication details:

Citing this paper
Please note that where the full-text provided on CityU Scholars is the Post-print version (also known as Accepted Author Manuscript, Peer-reviewed or Author Final version), it may differ from the Final Published version. When citing, ensure that you check and use the publisher's definitive version for pagination and other details.

General rights
Copyright for the publications made accessible via the CityU Scholars portal is retained by the author(s) and/or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights. Users may not further distribute the material or use it for any profit-making activity or commercial gain.

Publisher permission
Permission for previously published items are in accordance with publisher's copyright policies sourced from the SHERPA RoMEO database. Links to full text versions (either Published or Post-print) are only available if corresponding publishers allow open access.

Take down policy
Contact lbscholars@cityu.edu.hk if you believe that this document breaches copyright and provide us with details. We will remove access to the work immediately and investigate your claim.
High-Frequency Spinal Stimulation Suppresses Microglial Kaiso-P2X7 Receptor Axis-Induced Inflammation to Alleviate Neuropathic Pain in Rats

Jing Yu, PhD, 1 Stanley Wong, MD, 1 Zhanan Lin, MSc, 2 Zhiming Shan, MD, 1 Chaoyang Fan, MSc, 2 Zhengyuan Xia, MD, PhD, 1,3 Martin Cheung, PhD 4, Xiaowei Zhu, PhD 2, Jessica Aijia Liu, PhD 1,2 and Chi Wai Cheung, MD 1,5

Objective: Neuropathic pain poses a persistent challenge in clinical management. Neuromodulation has emerged as a last-resort therapy. Conventional spinal cord stimulation (Con SCS) often causes abnormal sensations and provides short analgesia, whereas high-frequency spinal cord stimulation (HF SCS) is a newer therapy that effectively alleviates pain without paresthesia. However, the modes of action of 10kHz HF SCS (HF10 SCS) in pain relief remain unclear. To bridge this knowledge gap, we employed preclinical models that mimic certain features of clinical SCS to explore the underlying mechanisms of HF10 SCS. Addressing these issues would provide the scientific basis for improving and evaluating the effectiveness, reliability, and practicality of different frequency SCS in clinical settings.

Methods: We established a preclinical SCS model to examine its effects in a neuropathic pain rat model. We conducted bulk and single-cell RNA sequencing in the spinal dorsal horn (SDH) to examine cellular and molecular changes under different treatments. We employed genetic manipulations through intrathecal injection of a lentiviral system to explore the SCS-mediated signaling axis in pain. Various behavioral tests were performed to evaluate pain conditions under different treatments.

Results: We found that HF10 SCS significantly reduces immune responses in the SDH by inactivating the Kaiso-P2X7R pathological axis in microglia, promoting long-lasting pain relief. Targeting Kaiso-P2X7R in microglia dramatically improved efficacy of Con SCS treatment, leading to reduced neuroinflammation and long-lasting pain relief.

Interpretation: HF10 SCS could improve the immunopathologic state in the SDH, extending its benefits beyond symptom relief. Targeting the Kaiso-P2X7R axis may enhance Con SCS therapy and offer a new strategy for pain management.

ANN NEUROL 2024;95:966–983
(eg, tumor necrosis factor α [TNF-α] and interleukin [IL]-1β), driving persistent central sensitization. However, microglia also have protective roles in resolving pain through anti-inflammatory activation, making the depletion of microglia an impractical approach in patients.

Epidural spinal cord stimulation (SCS) is a US Food and Drug Administration-approved therapy introduced to manage pain when pharmacological interventions are insufficient. Conventional SCS (Con SCS), first-generation dorsal columns neuromodulation, adopts a stimulation frequency ranging from 30 to 80Hz in various indications. Con SCS operates on the principles of Gate Control Theory, where electrical pulses delivered by Con SCS activate amyloid β (Aβ) fibers in the dorsal horn, promoting inhibitory interneuron activities. This results in increased inhibitory neurotransmitter γ-aminobutyric acid (GABA), which blocks transmission of nociceptive signals, but often leads to paresthesia (abnormal sensation) and diminished analgesic effects over time. High-frequency (HF) SCS is an emerging new paradigm, utilizing a high frequency varying from 1k to 10kHz, which delivers more charge per second compared to Con SCS. HF SCS is considered a promising therapy, as it offers long-lasting analgesic effects without paresthesia or drug addiction risk and also relieves pain in patients refractory to Con SCS. Some preclinical model studies that mimic clinical SCS features have suggested distinct mechanisms underlying the pain-relieving effects of HF SCS compared to Con SCS, evidenced by a lack of dorsal columns activation, a weak inhibition of wide dynamic range neurons in the deep dorsal horn, and a slower onset of pain inhibition. Thus, it is important to investigate the cellular and molecular changes underlying the analgesic effects of HF SCS, specifically its link to the immunopathologic state in the SDH. Gaining insights into this knowledge gap would provide a scientific basis for improving treatment efficacy, generating a biological basis for increasing indications, and advancing targeted/personalized therapy development clinically.

Kaiso, a transcription factor, acts as a key regulator in promoting immune responses across various tissues. Studies have shown that transgenic mice with ectopic expression of Kaiso (KaisoTg+) experienced extensive inflammation in multiple tissues, highlighting its significance as a master regulator of immune signaling. Furthermore, the upregulation of Kaiso has been linked to the establishment of an inflammatory microenvironment in intestinal and breast cancer. P2X7 receptor (P2X7R) is an adenosine triphosphate-binding purinergic receptor primarily expressed in microglia and is highly involved in the release of proinflammatory cytokines.

It has been identified as a key regulator for proinflammatory (also referred to as M1) microglia activation in pain. The blockade or deletion of P2X7R ameliorates neuropathic pain, whereas stimulation of P2X7R induces intermediate hypersensitivity via the release of inflammasome-dependent cytokines, such as IL-1β and IL-18.

In this study, we uncovered the therapeutic mechanisms of HF SCS using 10kHz (HF10 SCS) for prolonged pain relief, which involves the repression of Kaiso-P2X7R-mediated microglia activities. Through bulk and single-cell RNA sequencing (scRNA-seq), we found that HF10 SCS significantly improved immunopathologic conditions in the SDH by modulating microglia activity. In contrast, Con SCS led to early pain relapse, potentially exacerbating neuroinflammation by increasing disease-associated microglia properties. At the molecular level, elevated Kaiso following the injury induced detrimental microglia activation by transcriptional activation of P2X7R, which results in robust inflammation in the dorsal horn. HF10 SCS exerts its effects on microglia properties to specifically inactivate this pathological axis, reducing microglia activation and promoting their shift to anti-inflammatory status. Targeting the Kaiso-P2X7R pathway in Con SCS could ameliorate early pain relapse and enhance its therapeutic efficacy. In summary, our study unravels the mode of action and molecular basis in using high-frequency neuromodulation for pain management, demonstrating its beneficial effects in resolving pathophyiology rather than mitigating pain symptoms. Moreover, our findings provide potential therapeutic targets for treating pain and a viable strategy for improving Con SCS therapy.

Materials and Methods

Animals
All experiments were conducted following the guidelines of the Center for Comparative Medicine Research, an American Association for Accreditation of Laboratory Animal Care internationally accredited service center at the University of Hong Kong, for the care and use of laboratory animals. Adult male Sprague Dawley rats weighing between 250 and 300g were used in the experiment. The rats were housed in a controlled environment with a temperature of 23°C, humidity between 25% and 45%, and a 12-hour light/dark cycle (lights on at 07:00). They had free access to food (laboratory diet 5012, containing 1.0% calcium, 0.5% phosphorus, and 3.3IU/g of vitamin D3) and water. The study was approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong (Approval No. 5687-20).

Spared Nerve Injury Model and Electrode Implantation
The spared nerve injury (SNI) model was performed following previously described methods. Briefly, under isoflurane...
anesthesia, a skin incision was made on the lateral surface of the thigh, and a direct section was made through the biceps femoris muscle to expose the sciatic nerve and its 3 terminal branches: the sural, common peroneal, and tibial nerves. The common peroneal and tibial nerves were ligated using 5-0 silk, and the distal ligation was then incised. For the sham group, the right lateral surface was exposed without any nerve injury. The incision was closed using a 4-0 nylon suture and disinfected with ethanol. Following the surgery, the rats were carefully monitored in an intensive care unit cage until they recovered from anesthesia. The electrodes were placed epidurally through a small laminectomy as described previously. In brief, after a small laminectomy at the level of T13, the electrode was inserted epidurally in the rostral direction. We utilized custom-made 4-contact electrodes with a contact diameter of 1.0–1.2mm and a center spacing of 1.0mm (Kedou Brain Computer Technology, Suzhou, China). The leads were positioned at the T11–13 vertebral level, corresponding to the upper lumbar spinal cord region. To facilitate connection with the external pulse stimulator (Master-9, A.M.P.I., Jerusalem, Israel), the proximal end of the electrode was tunneled subcutaneously and placed in the upper thoracic region, where it exited the skin. Motor threshold (MT) was determined in each rat before SCS by slowly increasing the amplitude of the indicated frequency until a motor twitch was observed (often in the hindpaw or mid-lower trunk, as reported in previous studies). In rats, the sensory threshold is typically approximately 50% of the MT (as per previous findings). For this study, we used a subsensory threshold intensity of 40% MT for SCS. SCS was delivered for 4 days beginning 3 days post-surgery and adopted the following parameters: HF10 SCS: biphasic, frequency = 10kHz, pulse duration = 100 microseconds, pulse width = 20 microseconds, amplitude = 0.13mA (40% MT); Con SCS: biphasic, frequency = 40Hz, pulse duration = 25 milliseconds, pulse width = 20 microseconds, amplitude = 0.23mA (80% MT).

**Behavioral Tests**
All tests were performed by an experimenter blinded to treatments.

**Von Frey Test.** The mechanical nociceptive threshold was assessed using the von Frey test, following previously described methods. Briefly, rats were individually placed in transparent plastic boxes on a stainless-steel mesh-bottom platform for 30 minutes to acclimate to the environment. The hindpaw withdrawal threshold was measured using calibrated electronic von Frey filaments connected to a von Frey filament aesthesiometer (IITC Life Science, Woodland Hills, CA). The filaments were applied perpendicularly to the plantar surface of the ipsilateral hindpaw (the side of the surgery). A quick withdrawal response or paw licking was considered a positive response. Five repeated measurements were taken per animal during each test session, with 3-minute intervals.

**Cold Avoidance Test.** Cold avoidance was assessed using 2 temperature preference tests. The rats were allowed to acclimate on a plate at ambient temperature (22°C ± 2°C) for 1 week. At the end of the habituation period, 2 temperature-controlled plates were turned on, with one side set at 32°C ± 1°C and the other side at ambient temperature. The locomotion activity of the rats was recorded using a portable digital action camera and analyzed using Smart 3.0 video tracking software.

**Acetone Evaporation Test.** Cold allodynia was assessed using the acetone evaporation test. Rats were individually placed in small cages with a mesh floor. Approximately 20μl of acetone was applied through the mesh-bottom platform onto the plantar surface of the hind paw using a 1ml syringe barrel without a needle tip. Care was taken to gently apply the acetone bubble to the paw skin without inducing mechanical stimulation. The paw withdrawal time within a 60-second observation period after acetone application was recorded. Acetone was applied to the hindpaw, and the response was counted 5 times. The ratio of paw withdrawal times was calculated.

**Randall–Selitto or Paw Pressure Test.** Mechanical hyperalgesia was measured using the Randall–Selitto or paw pressure test. Rats were assessed using held devices (Paw Pressure Test Apparatus, IITC Life Science) while restrained in a hammock that provided access to the hind paws. To obtain reliable data, animals were habituated to the restraint method and experimental apparatus for 7 days.

**RNA Isolation and Library Preparation for RNA Sequencing**
Spinal cord dorsal horn was harvested and immediately stored in 1ml of Trizol (Invitrogen, Carlsbad, CA) at −80°C. Total RNA was extracted from homogenized spinal cord segments in Trizol as described previously. cDNA was synthesized from mRNA using a reverse transcriptase Kit (Takara Bio, Kusatsu, Japan). Prepared cDNA samples were stored at −20°C until further use. RNA sequencing and the bioinformatic analyses were performed by BGI Genomics (Shenzhen, China).

**Single-Cell RNA-Sequencing Analysis**
Rats were perfused, and dorsal spinal cord tissue was harvested on ice and rapidly chopped into small pieces using a sharp scalpel in ice-cold phosphate-buffered saline (PBS). The tissue fragments were then transferred into 15ml Falcon tubes containing 1ml of digestion solution (Neural Tissue Dissociation Kit [P] 130-092-628) and incubated for 15–20 minutes at 37°C in a tube rotator under slow continuous rotation. For the mechanical dissociation step, P200 pipettes were applied 15–20 times every 5–10 minutes. The final cell suspension was passed through a 70μm cell strainer, and the strainer was washed with
10ml of PBS. The suspension was collected in a 50ml Falcon tube and centrifuged at 450 × g at 4°C for 5 minutes. The supernatant containing dead cells and debris was discarded, and the pellet was resuspended in 7ml of PBS. Then, 1.2ml of fetal bovine serum (FBS) and 3.6ml of 100% Percoll were added to the suspension. The tubes were spun at 800 × g for 15 minutes at 4°C, and the pellet was collected in a new 15ml tube and resuspended in 0.5ml of fluorescence-activated cell sorting (FACS) buffer for cell sorting to remove cell debris and dead cells by 4,6-diamidino-2-phenylindole staining. Samples with a viability higher than 60% were used for downstream scRNA-seq analysis.

All the scRNA-seq analysis was performed in R (version 4.2.2) using the following packages: Seurat (version 4.3.0), DoubletFinder (version 2.0.3), clusterProfiler (version 4.6.2), clustree (version 0.5.0), and ggplot2 (version 3.4.2). The Seurat package was utilized for filtering, normalizing, integrating, and clustering the dataset, whereas the DoubletFinder package was used for doublet removal. Low-quality cells were removed if they had <200 genes detected per cell, >10% of reads coming from mitochondrial genes, <5% of reads coming from ribosome genes, or >5% of reads coming from hemoglobin genes. Genes that were present in <3 cells were also excluded from the analysis. The samples from the 4 conditions were integrated and normalized using SCTransform. Clustering was performed using 50 principal components (PCs) and a resolution of 0.3. After identifying microglia and other cell types using specific markers, the microglia were subclustered using 12 PCs and a resolution of 0.7. The clusters were visualized using Uniform Manifold Approximation and Projection (UMAP) for dimension reduction. To assess the enrichment of marker genes, gene set enrichment analysis (GSEA) was performed for differentially expressed genes (DEGs), comparing the injury conditions (with or without treatment) to the sham condition. The DEGs were identified using FindMarkers (Seurat) with the DESeq2 test, and GSEA was performed.

**Western Blot**

L4 to L5 ipsilateral spinal dorsal horn tissues were rapidly collected and lysed in cold lysis buffer (Cell Signaling Technology, Danvers, MA) containing a complete cocktail and phosphorylated cocktail tablets (Roche, Mannheim, Germany). The protein samples were then separated by 8%–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was incubated for 1 hour in blocking buffer (5% nonfat milk in Tris-buffered saline with Tween [TBST]). Primary antibodies (see Table S5) were added to the blocking buffer and incubated overnight at 4°C. Afterward, the membranes were washed 3 times in TBST and incubated with horseradish peroxidase–conjugated secondary antibodies (anti-rabbit IgG or anti-mouse IgG; Cell Signaling Technology) at a 1:3,000 dilution. Immunoreactive protein bands were visualized using enhanced chemiluminescence (Bio-Rad, Hercules, CA) with exposure to x-ray films.

**Quantitative Real-Time Polymerase Chain Reaction**

The RNA from sorted cells was extracted using the PureLink miRNA Isolation Kit (K157001; Thermo Fisher Scientific, Waltham, MA), and cDNA samples were synthesized using a reverse transcriptase kit (Takara Bio) and stored at −20°C. Quantitative polymerase chain reaction (qPCR) was performed using the TB Green Premix Ex Taq II (Tli RNase H Plus; RR820B; Takara Bio) on an ABI 7900HT quantitative PCR system (Applied Biosystems, Foster City, CA). GAPDH was used as the housekeeping gene. The primer sequences used are provided in Table S5. The relative mRNA expression was normalized to GAPDH, and the fold change was calculated using the comparative CT (ΔΔCT) method, normalized to GAPDH. Mean fold change and standard deviation were calculated using Excel (Microsoft, Redmond, WA) and Prism (GraphPad Software, San Diego, CA).

**Luciferase Assay**

*Escherichia coli* competent bacterial strain DH5α (Tiangen Biotech, Beijing, China) was used for plasmid transformation. The lentiviral vector containing the full-length sequence of Kaiso was constructed. The promoter region of *P2X7* was constructed into the pmirGLO dual-luciferase reporter vector (Singke Company, Suzhou, China) and dissolved in pure water at a concentration of 1μg/μl. BV2 cells were cultured in Dulbecco modified Eagle medium (HyClone, Logan, Utah) supplemented with 10% FBS and maintained at 37°C and 5% CO2 in a humidified incubator. Cells were seeded at a density of 2.0 × 105/cm2 in 6-well and 48-well plates and cultured overnight before transfection. Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific) was used to transfect the cells following the manufacturer’s protocol. BV2 cells were cotransfected with a mixture of 200ng of pmirGLO–*P2X7*–promoter–reporter luciferase vector and/or PLVX-Kaiso plasmid at a final concentration of 200nM using Lipofectamine. After 48 hours, the luciferase activity of the cells was measured using a dual-luciferase reporter assay kit (Promega, Madison, WI). The results were normalized to the ratio between firefly activity and Renilla luciferase activity.

**Chromatin-Immunoprecipitation Assay**

Chromatin-immunoprecipitation (ChIP) assays were performed using the ChIP kit (17-610; Millipore, Billerica, MA). The L4 and L5 segments of the SDH were quickly removed and placed in 1% formaldehyde for 10 minutes to crosslink the proteins and DNA. Formaldehyde was inactivated by adding glycine. Sonicated chromatin extracts containing DNA fragments were immunoprecipitated overnight at 4°C with the Kaiso antibody or normal rabbit IgG, along with pre-blocked protein G-Sepharose beads. The next day, the chromatin complexes were eluted, and the DNA was extracted. The precipitated DNA was resuspended in nuclease-free water, and qPCR was performed as described above. Finally, the ratio of ChIP to input in the SDH was normalized and calculated.

May 2024
Flow Cytometry and Cell Sorting

Animals were perfused with 0.9% saline, and the L4–L5 ipsilateral side of the dorsal spinal cord was harvested on ice and quickly dissociated in Hank balanced salt solution containing 2.5% trypsin for 5 minutes at 37°C. The digestion was stopped by adding 20% FBS, and the dissociated cells were filtered through a 70μm filter and spun down at 700 × g for 5 minutes. The supernatant, containing dead cells and debris, was discarded, and the pellet was resuspended in 1ml of 30% Percoll (45-001-748; GE Healthcare, Chicago, IL). The tubes were centrifuged at 700 × g for 15 minutes at 4°C with slow acceleration and termination, and the pellet was collected in a new 15ml tube. It was then incubated in 0.2ml of blocking buffer (1% bovine serum albumin in PBS) containing primary antibodies for 30 minutes at 4°C. The primary antibodies listed in Table S5 were used to stain different microglial populations with different phenotypes at a 1:100 dilution for 60 minutes at 4°C. Data were analyzed using FlowJo 10.0.8 (FlowJo, Ashland, OR).

Lentivirus Production

Plasmids were constructed by VectorBuilder. Cells were seeded at a density of 2.0 × 10⁴ cells/cm² in each well of a 6-well plate and incubated for 24 hours at 37°C in a humidified incubator with 5% CO₂ before transfection. A 500μl mixture of psPAX2, pMD2.G, and the different plasmids, diluted in Opti-MEM, was prepared according to Addgene’s protocol. This mixture was further mixed with diluted Lipofectamine 2000 in Opti-MEM and incubated at room temperature for <20 minutes. The plasmid mixture with Lipofectamine 2000 was slowly added to the cells. The medium was harvested 48–72 hours posttransfection only if the transfection efficiency was >80% as indicated by (green fluorescent protein) GFP+ cells.

Intrathecal Injections

Intrathecal injections were performed under anesthesia. Rats were gently but firmly held by the pelvic girdle using the thumb and forefinger of the nondominant hand. The skin above the iliac crest was pulled taut to create a horizontal plane for needle insertion. With the other hand, the experimenter traced the spinal column of the rats, slightly rounding or curving it to open the spaces between the vertebrae. A 30-gauge needle connected to a 10ml Hamilton syringe was inserted between the 5th and 6th lumbar vertebrae. The accuracy of needle placement was determined by a quick tail flick from the animal. After injection, the syringe was rotated and removed, and the posture and locomotion of the rat were checked.

Immunohistochemistry

Rats were anesthetized with pentobarbital and perfused with 0.9% saline, followed by 4% paraformaldehyde (PFA) in a 0.1M phosphate buffer. The lumbar spinal cords were dissected out, fixed in 4% PFA overnight, and then transferred to a 30% sucrose solution in phosphate buffer at 4°C for at least 24 hours. Spinal cord sections were cut on a freezing microtome set at 20–40μm. For fluorescent immunohistochemistry, the sections were incubated with primary antibodies (see Table S5) overnight at room temperature. Direct secondary antibody was used at a concentration of 1:500 (Alexa Fluor 488, 594 or 647). All fluorescent sections were transferred to glass slides, coverslips were applied using Gel Mount Aqueous Mounting Medium (Sigma-Aldrich, St Louis, Mo) to prevent fading, and they were stored in dark boxes at 4°C. For fluorescence quantification, a region of interest (ROI) was located over laminae I/II. Fluorescence was measured for 6 sections per animal using the same ROI. Readings were taken from the side of the spinal cord contralateral to the inflamed paw or nerve lesion. Contrast enhancement and fluorescence threshold were kept constant.

Statistical Analysis

Statistical testing was performed using GraphPad Prism v8. Values are presented as means ± standard error. For comparing 2 groups, data were analyzed using two-tailed, unpaired t test, which was referred to as unpaired t test in the text. One-way or two-way analysis of variance (ANOVA) or two-way repeated-measures followed by one-way ANOVA was applied for comparison of multiple groups with Tukey post hoc test or Bonferroni correction (which was applied to examine a priori hypotheses for comparisons of specific pairs). The tests used are reported in the text and figure legends. In all cases, values of p < 0.05 were regarded as significant.

Results

Continuous 4-Day HF10 SCS Produces Long-Lasting Analgesic Effects Compared to Con SCS

HF10 SCS has shown superior analgesic effects compared to Con SCS for treating neuropathic pain.9–13 However, the specific mechanisms underlying the differential effectiveness of HF10 SCS and Con SCS in pain relief are still not fully understood. To address the issue, we used a preclinical SNI neuropathic pain model, in which the common peroneal and tibial nerves are injured to consistently induce long-lasting hypersensitivity (Fig 1A,B).21 Following nerve injury, neuropathic pain symptoms, including mechanical and thermal hypersensitivity, developed from 1 day post injury and persisted for >3 weeks. Con SCS dramatically improved mechanical allodynia, peaking at 60 minutes poststimulation, but exhibited very short analgesic periods with refractory pain developing <24 h after the stimulation (Fig 1C). In contrast, HF10 SCS, using a similar time frame of stimulation but at a frequency of 10kHz, amplitude of 40% MT, and pulse width of 20 microseconds, resulted in much longer pain relief. The antiallodynic effects of HF10 SCS lasted for 15 days poststimulation (Fig 1D). Furthermore, HF10...
FIGURE 1: Ten-kilohertz high-frequency spinal cord stimulation (HF10 SCS) promotes long-lasting analgesic effects. (A) Experimental setup for SCS, electrode design, and neuropathic pain induction. Panel a’ indicates the parameters for conventional SCS (Con SCS) and HF10 SCS. Panel a” shows the design of the implanted electrode along the L4–L6 epidural space for SCS. Panel a’’ shows the surgical procedure of spared nerve injury (SNI) for neuropathic pain induction. Red color indicates severely affected area upon the SNI. (B) Experimental paradigm showing time frame for nerve injury, implantation of electrode, and SCS. (C) Von Frey tests of sham, SNI, and Con SCS. (D) Von Frey tests of sham, SNI, and HF10 SCS. (E) Randall–Selitto test (mechanical hyperalgesia) of sham, SNI, and Con SCS. (F) Randall–Selitto test of sham, SNI, and HF10 SCS. (G) Assessments of cold allodynia of sham, SNI, and Con SCS. (H) Assessments of cold allodynia of sham, SNI, and HF10 SCS. (I) Representative images of 2 temperature preference test (20°C–30°C) for all treatment groups. (J) Unpaired Student t test. BL = baseline; dpi = days post injury; HF SCS = high-frequency SCS; PWT = paw withdrawal threshold. [Color figure can be viewed at www.annalsofneurology.org]
SCS showed a gradual higher tolerance to mechanical pressure stimulation compared to the SNI group, whereas Con SCS-induced tolerance occurred only at 60 minutes poststimulation and returned to pretreatment levels within 24 hours, suggesting reversed mechanical hyperalgesia after HF10 SCS (Fig 1E,F). Additionally, HF10 SCS exhibited superior efficacy in alleviating thermal hypersensitivity compared to Con SCS (Fig 1G). Overall, our results confirmed the long-lasting analgesic effects of HF10 SCS when compared to Con SCS, with less frequent pain relapse.

Differential Immune Responses after HF10 SCS and Con SCS Treatment

To investigate the cellular and molecular changes underlying differential analgesic effects of HF10 SCS and Con SCS, we performed bulk RNA-seq analysis on the ipsilateral side of the SDH in sham, SNI, SNI + HF10 SCS, and SNI + Con SCS conditions 8 days post injury (2 days following the 4th SCS), a time point when Con SCS and HF10 SCS exhibited the most significant differences in attenuating neuropathic pain symptoms (see Fig 1B). Hierarchical gene clustering analysis revealed distinct gene expression profiles among all treatment groups (Fig 2A). There are 573 differentially expressed genes (DEGs) (false discovery rate < 0.05, log2 [Fold Change] > 1) between HF10 SCS and Con SCS, with 466 genes upregulated and 107 genes downregulated following HF10 SCS treatment (Fig 2A). Gene ontology (GO) analysis indicated that these DEGs were significantly enriched in immune response, glial cell development, and response to cytokines, indicating the functional link between SCS and inflammation. Subclustering confirmed a profound inflammatory induction in the SDH in the SNI group (Fig S2B), which is a primary cause of neuropathic pain. Although Con SCS produced analgesic effects, we did not observe any significant alterations in proinflammatory gene profiles. GO analysis between Con SCS and SNI revealed that the majority of genes are associated with neuronal activity, which is in line with previous studies showing that Con SCS acts on the Aβ fibers in the SDH, promoting inhibitory interneuron activity (Fig S1D). In contrast, HF10 SCS exerted potent inhibitory effects on inflammatory progression, as evidenced by a significant downregulation of immune response genes (eg, Csf1, Gpr132, Ift7) and proinflammatory factors associated with chemokines, interleukin, and tumor necrosis factor superfamily (see Fig 2B,C). Notably, some proinflammatory genes upregulated in response to HF10 SCS also exhibited anti-inflammatory functions (labeled with asterisks), implying that they may have beneficial effects in resolving pain following HF10 SCS. However, we did not observe significant differences in the subclusters of anti-inflammatory genes between HF10 SCS and Con SCS (Fig S1C). This might be due to the presence of mixed cell populations, where the expression levels of anti-inflammatory genes are low compared to proinflammatory genes. As a result, the effects of anti-inflammatory profiles might be masked.

To better understand the heterogeneity of immune cells under pain conditions following different treatments, we performed single-cell RNA sequencing (scRNA-seq; 10× Genomics, Pleasanton, CA) analysis on the ipsilateral side of the SDH from SNI treated with either Con SCS or HF10 SCS, in which the SNI group was used as a reference. Through unsupervised clustering, we identified 7 distinct cell populations, visualized by UMAP (see Fig 2D). The largest cluster was composed of microglia (Tmem119), whereas other cell types included oligodendrocytes (Mog), macrophages (Ly6c2), endothelial cells (igfbp7), T cells (cd8b), NK cells (Gzmna), neutrophils (S100a8), and B cells (cd49a; see Figs 2D and S1E). Notably, the cluster frequency revealed a significant reduction in the microglia population following HF10 SCS compared to the injury and Con SCS, suggesting that HF10 SCS may modulate inflammatory responses by regulating microglial activity (see Fig 2E). Further analysis of microglial DEGs from different treatment groups identified a dramatic reduction in neurologic/immune disease-associated factors, also known as disease-associated microglial (DAM) genes, such as C1qa, Ier2, Ift1, Rps2, Plac8, Ctsd, Cxcl2. Furthermore, HF10 SCS significantly downregulated immune response genes. As a result, the effects of microglia are low compared to proinflammatory genes. HF10 SCS may reduce clusters 2, 3, and 5 compared to both injury and Con SCS (Fig S2D). Notably, clusters 2 and 5 may take up largely signaling molecules associated with neuroinflammation during pain development. Cluster 2 expresses immune-related genes (eg, Cd3, Cd4, Cx32, Cx42, Tnf, Il1b, Nfkbia) involved in regulating chemokine/cytokines, inflammatory responses, and N-methyl-D-aspartate receptor activity that is highly associated with central sensitizations.

HF10 SCS Modulates the Activation Status of Microglia in Neuropathic Pain

Microglia can be further partitioned into 8 subclusters, with clusters 0–5 being the most abundant (see Fig 2F). Notably, HF10 SCS significantly reduced clusters 2, 3, and 5 compared to both injury and Con SCS (Fig 2G). Clusters 2 and 5 take up largely signaling molecules associated with neuroinflammation during pain development. Cluster 2 expresses immune-related genes (eg, Cds, C4d, Cx32, Cx42, Tnf, Il1b, Nfkbia) involved in regulating chemokine/cytokines, inflammatory responses, and N-methyl-D-aspartate receptor activity that is highly associated with central sensitizations.
FIGURE 2: Differential immune responses following conventional spinal cord stimulation (Con SCS) and 10kHz high-frequency SCS (HF10 SCS) treatment. (A) Heatmap of differential gene expressions of bulk RNA sequencing from the ipsilateral side of the dorsal horn of all treatment groups. Upregulated and downregulated genes are colored in red and blue, respectively. (B) Subclustering of genes from the top 2 Kyoto Encyclopedia of Genes and Genomes (KEGG) MSigDb canonical pathways related to immune responses. (C) Subclustering of inflammatory genes from top 10 KEGG MSigDb canonical pathway collections. Asterisks denote genes with both pro- and anti-inflammatory functions. (D) Uniform Manifold Approximation and Projection (UMAP) plot from single-cell RNA sequencing showing 7 cell type clusters identified in the ipsilateral side of dorsal horn (total n = 23,829 cells integrated from three experimental conditions). (E) Cluster analysis based on percent composition in different conditions. (F) Unbiased subclustering UMAP of microglia (n = 17,944 microglia from all conditions combined). (G) Frequency heatmaps of subclusters relative to all microglial cells under each condition. (H) Significantly enriched gene ontology terms associated with the top differentially expressed markers of indicated microglia subcluster. Red represents positively enriched. Blue represents negatively enriched. (I) Violin plots showing expression of indicated markers related to the pathway ontologies indicated above. BMP = bone morphogenetic protein; FC = fold change; IFN = interferon; IL = interleukin; NMDA = N-methyl-D-aspartate; SNI = spared nerve injury; TNF = tumor necrosis factor. [Color figure can be viewed at www.annalsofneurology.org]
Interestingly, Con SCS expanded clusters 3 and 4, with more prominent expression in microglia clusters 2, 4, and 6. Notably, HF10 SCS led to diminished expression of these markers in the microglia population, particularly in clusters 2 and 4, indicating Kaiso could be a downstream effector of HF10 SCS in regulating microglia-mediated immune responses (Fig 4B). Subsequently, we validated the expression profile of Kaiso in different treatment groups. Following nerve injury, Kaiso expression was robustly induced at both mRNA and protein levels at 6 days post injury (dpi). Although both Con SCS and HF10 SCS attenuated neuropathic pain at 6 dpi (4th stimulation), Kaiso repression was specific to HF10 SCS treatment (Fig 4C–E). Importantly, the expression levels of Kaiso showed a negative correlation with the analgesic effects of HF10 SCS. Pain relapse observed at 22 dpi (15 days after 4th HF10 SCS) coincided with the restored Kaiso expression, in contrast to 2 dpi (2 days after HF10 SCS) (Fig 4F–H). Immunofluorescence analysis further confirmed high enrichment of Kaiso in microglia populations in the SNI and Con SCS groups, whereas HF10 SCS significantly reduced Kaiso expressions in Iba1-marked microglia (Fig 4I,J).

**Nerve Injury-Induced Kaiso in Microglia Is Negatively Correlated with Analgesic Effects Produced by HF10 SCS**

These findings prompted us to investigate the molecular events underlying the altered status of microglia activation and inflammation during HF10 SCS treatment. In search of key effectors specifically regulated by HF10 SCS, we conducted analyses of both bulk RNA sequencing and scRNA-seq to identify genes differentially expressed in microglia following the treatment of HF10 SCS and Con SCS. Among the top 10 differential expressed genes, we found that Kaiso showed significant induction following nerve injury and was downregulated specifically by HF10 SCS but not by Con SCS (Fig 4A). Furthermore, Kaiso was found to be highly enriched in microglia populations, highlighting its potential role in controlling microglia activities. Importantly, Kaiso is known to be a master regulator in promoting robust inflammation in various tissues and cancers. UMAP plots revealed a similar expression pattern of Kaiso and proinflammatory factors, Tnf and Il1b, with more prominent expression in microglia clusters 2, 4, and 6. Notably, HF10 SCS led to diminished expression of these markers in the microglia population, particularly in clusters 2 and 4, indicating Kaiso could be a downstream effector of HF10 SCS in regulating microglia-mediated immune responses (Fig 4B).

To confirm that transcriptome changes are associated with the functional effects of HF10 SCS on microglia activity, we performed immunohistochemistry and FACS analyses on the ipsilateral side of the SDH. Our results showed that HF10 SCS significantly reduces the activated status of microglia compared to SNI and Con SCS (Fig 3A–D). CD11b+CD45high macrophages remained low and unchanged across treatments, indicating the predominant role of microglia in mediating altered immune responses. Intriguingly, despite HF10 SCS reduced CD11b+CD45high microglia populations, we still observed distinct differences in their activated states. HF10 SCS decreased proinflammatory properties (CD86+CD206−) and increased anti-inflammatory phenotype (CD206+CD86−) (Fig 3E,F). qPCR analysis of CD11b+CD45high microglial populations revealed a pronounced downregulation of proinflammatory genes and upregulation of markers associated with anti-inflammatory microglia activation in HF10 SCS when compared to SNI and Con SCS (Fig 3G). These results indicated that HF10 SCS not only achieves global immune repression by reducing microglia activation but also promotes a "beneficial" activation state in microglia, eliciting anti-inflammatory responses that ameliorate pathological conditions.
group robustly induced Kaiso expression at 10 days post injury (Fig 5A,B). Immunofluorescence analysis confirmed elevated Kaiso levels in Iba1+ populations in the SDH of HF10 SCS following the Kaiso OE (Fig 5C,D). To explore the functional link between Kaiso and inflammatory responses mediated by microglia after SCS in the context of neuropathic pain, we examined microglia status by flow cytometry. The results showed that restoring Kaiso in HF10 SCS markedly increased the CD11b+CD45int microglial population compared to...
vehicle + HF10 SCS, indicating enhanced microglia activation (Fig 5E,F). Among CD11b+CD45int populations, Kaiso restoration resulted in a substantial increase in proinflammatory properties (CD86+CD206−) and a decrease in anti-inflammatory properties (CD206+CD86−) in the HF10 SCS group (Fig 5G,H). qPCR analysis of...
FACS-sorted microglia (CD11b+CD45int) further revealed upregulated proinflammatory gene expression in the Kaiso OE + HF10 SCS group compared to the vector control group, although with less impact on anti-inflammatory genes (Fig 5I). Importantly, Kaiso OE led to significant mechanical allodynia development from 10 dpi (4 days after 4th SCS) that had previously been ameliorated by HF10 SCS (Fig 5J). We then investigated whether reducing microglial Kaiso would be sufficient to alleviate neuroinflammation and sustain analgesic effects in Con SCS. To explore this, we used lentiviral-shRNA driven by the Iba1 promoter to knockdown Kaiso expression in the SDH of Con SCS at 0 dpi (Fig S2A–C). Inhibition of Kaiso in Con SCS reduced the activated status of microglia and proinflammatory properties (Fig S2F–I). Remarkably, Kaiso knockdown (KD) resulted in a notable shift in activated proinflammatory phase (CD86+CD206–) and anti-inflammatory phase (CD206+CD86–).
Our RNA dataset analysis showed upregulations of 6K UMAP plots of scRNA-seq. We proceeded to investigate how Kaiso, acting as a transcriptional regulator, could exert control over microglia phenotypes and inflammatory responses. It is well established that the function of microglia is influenced by a range of purinergic receptors, such as P2Y12, P2Y6, P2Y4, P2X4, and P2X7, in various neurological disorders. Our RNA data analysis showed upregulations of several purinergic receptors, such as P2X2, P2X6, P2X4, and P2X7, following the nerve injury (Fig 6A). Notably, only P2X4 receptor (P2X4R) and P2X7R were specifically downregulated in HF10 SCS treatment. Considering the sex specificity of microglial P2X4R in evoking neuropathic pain, we focused on P2X7R, which exhibits pronounced expression in spinal microglia and was previously shown to promote proinflammatory microglia polarization (M1 polarization) in pain. UMAP plots of scRNA-seq and subsequent validation confirmed a similar expression pattern of P2X7R in microglia to that of Kaiso and proinflammatory factors, with reduced expressions in specific clusters 2 and 4 following HF10 SCS (Fig 6B–E).

We next examined the functional importance of decreased expression of P2X7R in HF10 SCS-mediated long-lasting analgesic effects. To explore this, we intrathecally injected a lentiviral vector coding for P2X7R or vehicle driven by Iba1 promoter into HF10 SCS at 0 dpi. We observed a significant increase in protein and mRNA levels of P2X7R, with prominent expression in Iba1+ cells at 8 dpi (2 days after 4th stimulation; see Fig 6F,G). Immunofluorescence analysis revealed a notable increase in microglial properties in the SDH accompanied by P2X7R upregulation following P2X7R OE in HF10 SCS (see Fig 6H). In response to elevated P2X7R expression, activated microglia in the HF10 SCS group exhibited increased proinflammatory properties with reduced anti-inflammatory microglia (Fig 6I,J). qPCR analysis of CD11b+CD45imicroglia further confirmed a significant upregulation of proinflammatory cytokines, with a subtle reduction in anti-inflammatory factor il-10 (Fig 6K). Importantly, restoration of P2X7R abolished the analgesic effects of HF10 SCS, resulting in mechanical allodynia development from 8 dpi (Fig 6L). These observations suggest that P2X7R could function downstream of Kaiso in regulating microglia-mediated inflammation.

Based on the above findings, we further investigated whether Kaiso possesses the ability to transcriptionally activate P2X7R expression. Overexpression of Kaiso in HF10 SCS induced P2X7R expression, whereas Kaiso KD in Con SCS reduced P2X7R expression (Fig 7A,B). Bioinformatics analysis (JASPAR) predicated 2 potential Kaiso binding sites within the 2 kb promoter region of P2X7R (Fig 7C). ChIP-qPCR assay using primers against binding fragments indicated that Kaiso can directly bind to the promoter region of P2X7R, whereas HF10 SCS significantly reduced the recruitment of Kaiso to the P2X7R promoter region compared to SNI and Con SCS treatment (Fig 7D). To further validate that the binding of Kaiso to the P2X7R promoter could lead to transcriptional activation, a 2kb P2X7R promoter fragment was fused to the luciferase reporter and assayed its activity in BV2 cells. Overexpression of Kaiso enhanced luciferase activity compared to the vector control, whereas mutations of bindings sites abolished transcriptional activations upon Kaiso overexpression (Fig 7E). These data demonstrated that Kaiso could directly transactivate P2X7R expression via promoter bindings.

To confirm the P2X7R function downstream of Kaiso in mediating differential analgesic effects between HF10 SCS and Con SCS, P2X7R was knocked down by siRNA intrathecal administration in HF10 SCS with or without Kaiso overexpression. Consistent with previous observations, overexpression of Kaiso abolished analgesic effects produced by HF10 SCS, whereas reduced P2X7R expression levels could restore the pain relief abolished by high levels of Kaiso from 10 dpi (see Fig 7F,G). These data indicated that HF10 SCS effectively inactivated the Kaiso-P2X7R axis in the SDH to resolve neuroinflammation by repressing proinflammatory microglia polarization, thus producing long-lasting analgesia.

**Discussion**

The spinal neuroimmune response mediated by microglia activation following nerve injury is a key mechanism driving pain induction and development. However, directly targeting microglial activation to modulate neuroinflammation may lead to side effects such as infection and impaired anti-inflammatory effects, particularly with long-term use. Epidural SCS offers a nonpharmacological approach for chronic pain management that addresses the drawbacks associated with long-term pharmacological treatments, including drug tolerance, abuse, and...
FIGURE 6: Downregulation of P2X7R is required for suppressing proinflammatory microglial activation and producing analgesic effects. (A) Differential expressions of purinergic receptors in all treatment groups. Upregulated and downregulated genes are colored in red and blue, respectively. (B) Microglial scatterplots show the expressions of P2X7R in the indicated treatments. (C) Western blot analysis showing the expressions of P2X7R in sham, spared nerve injury (SNI), 10kHz high-frequency spinal cord stimulation (HF10 SCS), and conventional SCS (Con SCS). (D) Relative band intensity ratio of P2X7R/GAPDH, unpaired Student t test. (E) mRNA expressions of P2X7R in all treatment groups (unpaired Student’s t test). (F) Western blot analysis and quantification showing the expressions of P2X7R in HF10 SCS + vector control and HF10 SCS + P2X7R OE. Quantification of the ratio of P2X7R/GAPDH, normalized to vector control, unpaired Student’s t test. (G) mRNA expressions of P2X7R in HF10 SCS + vector control and HF10 SCS + P2X7R OE. (H) Confocal images of microglia labeled by ionized calcium-binding adaptor molecule 1 (Iba1; green) and P2X7R (red) in the L4 spinal dorsal horn (SDH) in HF10 SCS + vector and HF10 SCS + P2X7R OE (scale bar = 100μm). Right panel: Quantification of percentage of P2X7R in Iba1+ cells in different treatments. (I) Cytometric analysis of CD11b+CD45+CD11c+ microglia from the SDH in HF10 SCS + vector and HF10 SCS + P2X7R OE. Right panel: Quantification of cell percentages (CD11b+CD45+CD11c+) of microglial proinflammatory phase (CD86+CD206−) and anti-inflammatory phase (CD206+CD86−) from the SDH in HF10 SCS + vector and HF10 SCS + P2X7R OE groups. Right panel: Quantification of indicated cell population (unpaired Student’s t test). (J) Quantitative polymerase chain reaction analysis of proinflammatory and anti-inflammatory genes in CD11b+CD45+CD11c+ microglia from the SDH in HF10 SCS + vector and HF10 SCS + P2X7R OE groups (n = 6) was performed in 2 independent experiments (unpaired Student t test). (L) Von Frey tests in HF10 SCS + vector and HF10 SCS + P2X7R OE. All behavioral tests are two-way repeated-measures ANOVA with Bonferroni correction. Each bar represents mean ± standard error of the mean. *p < 0.05, **p < 0.01, ***p < 0.001. APC = allophycocyanin; KD = knockdown; OE = overexpression; PE = phycoerythrin; PWT = paw withdrawal threshold. [Color figure can be viewed at www.annalsofneurology.org]
addiction. Con SCS with a stimulation frequency in the range of 40 to 60Hz is widely used clinically but has short analgesic effects.\textsuperscript{9,22,23} HF SCS is a novel system delivering tonic pulses with frequencies ranging from 1 to 10kHz, providing greater charge per second and better pain relief. However, the effects of SCS on immune cell-mediated neuroinflammation in the SDH, which is associated with pain states, are not well understood. Our

FIGURE 7: P2X7 function downstream of Kaiso upon spinal cord stimulation (SCS). (A) Western blot analysis showing the expressions of P2X7R in 10kHz high-frequency SCS (HF10 SCS) + vector and HF10 SCS + Kaiso OE. (B) Western blot analysis showing the expressions of P2X7R in conventional SCS (Con SCS) + scramble and Con SCS + Kaiso knockdown (KD). (C) In silico analysis showing 2 potential Kaiso binding sites in the promoter region of P2X7R. (D) Chromatin immunoprecipitation assays were performed using Kaiso antibody in sham, spared nerve injury (SNI), HF10 SCS, and Con SCS. IgG was used as a negative control (***p < 0.001 two-tailed one-way ANOVA, n = 3). (E) Kaiso overexpression enhanced luciferase (Luc) activities with P2X7R promoters (**p < 0.01 vs vector control). (F) Western blot analysis showing the expressions of P2X7R and Kaiso in HF10 SCS + scramble, HF10 SCS + Kaiso OE, and HF10 SCS + Kaiso OE + P2X7R KD. One-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001. (G) Von Frey tests in HF10 SCS + scramble, HF10 SCS + Kaiso OE, and HF10 SCS + Kaiso OE + P2X7R KD. ***p < 0.001, ****p < 0.0001, HF10 SCS + Kaiso OE versus HF10 SCS + Kaiso OE + P2X7R KD. All behavioral tests are 2-way repeated-measures ANOVA with Bonferroni correction. Each bar represents mean ± standard error of the mean. GAPDH = glyceraldehyde-3-phosphate dehydrogenase; GFP = green fluorescent protein; MUT = mutants; PWT = paw withdrawal threshold; OE = overexpression; R-luc = Renilla Luciferase; TSS = transcription start sites. [Color figure can be viewed at www.annalsofneurology.org]
study revealed cellular changes and molecular mechanisms underlying the differential analgesic effects of Con SCS and HF10 SCS. We provided the first evidence that HF10 SCS can improve the pathological conditions in the SDH after injury, rather than simply blocking pain transmission.

In response to nerve injury, microglial activation predominates with a proinflammatory phenotype, inducing central sensitization and pain chronification by releasing proinflammatory mediators, such as IL-1β, IL-6, and TNF-α, that modulate inhibitory synaptic transmission in the SDH. Targeting cytokines using multiple inhibitors has shown promise but has limited clinical efficacy in treating neuropathic pain. This could be due to the complex immune actions on SDH neurons via pre-, post-, and extrasynaptic mechanisms or the induction of reactive astrocytes by microglia that contribute to chronic neuroinflammation. Proresolution strategies that can control “deleterious” microglial activation by identifying new targeting pathways or alternative therapy, such as neuromodulation, should be explored. Con SCS utilizes high current amplitudes to activate GABA inhibitor neurons, blocking nociceptive transmission in the SDH, but often causes sensory side effects, such as paresthesias. HF SCS, adopting low current amplitudes below the sensory threshold, has demonstrated superiority in terms of cost and analgesic effects in several randomized clinical trials, especially for patients with chronic pain refractory to Con SCS. Notably, previous studies have reported that HF SCS with subthreshold lacks dorsal column activation and exhibits a weak inhibition of wide dynamic range neurons in the deep dorsal horn. In addition, our study and others have shown that pain relief from HF10 SCS often has a late onset compared to Con SCS in rats after nerve injury, and sometimes requires days in patients. These findings implicated that the effects of HF10 SCS may involve modulation of supportive cells (e.g., electrically nonexcitable cells) that influence neuronal activity through the release of various molecules, including cytokines, rather than directly regulating nociceptive transmission in specific neurons. In line with these hypotheses, our further investigations revealed that HF10 SCS profoundly suppresses immune responses induced by nerve injury by modulating proinflammatory microglia in the SDH. This finding indicated that microglia are highly responsive to HF10 SCS, possibly due to the delivery of a higher charge per second compared to Con SCS, which alters membrane potentials and intracellular chemical signaling in microglia, such as Ca2+, an important messenger that triggers multiple downstream signaling pathways (e.g., Kaiso-P2X7R in our case). Other studies investigating electric stimulation for treating neurodegenerative disorders have also demonstrated direct modulatory effects on microglia, previously considered “nonresponsive” by using different stimulation parameters, which showed reduced immune responses and increased anti-inflammatory effects. Microglia can undergo “alternatively activation” in addition to classic proinflammatory activation, releasing anti-inflammatory factors (e.g., TGF-β, IL-4, and IL-10) to resolve neuroinflammation and promote antinociceptive effects. Previous studies have demonstrated that eliminating microglia populations results in an increased duration of pain or a relapse of pain hypersensitivity during the recovery stage. An intriguing finding in our study is that HF10 SCS can modulate the activated status of microglia in the SDH, globally reducing microglial activation to achieve extensive immune repression and promoting a beneficial activation state in microglia that exerts anti-inflammatory responses. This modulation is essential for ameliorating pathological conditions, providing long-term benefits for nerve regeneration and homeostatic re-establishment. The finding is consistent with recent observations from a clinical trial using HF10 SCS on diabetic peripheral neuropathy patients, who often experience a loss of protective sensation due to nerve degeneration and continuous infections. Most of the patients in the trial showed improvements in proprioception at the end of the HF10 SCS stimulation period, and this improvement persisted over the course of follow-up.

Molecularly, we further uncovered a novel pathological axis, Kaiso-P2X7R, providing mechanistic insight into the differential immune responses mediated by Con SCS and HF10 SCS. Kaiso, a master regulator of inflammatory responses in various tissues and cancers, has been found to control immune cell functions by transcriptionally regulating receptors or signaling molecules. However, its role in pain-related immune functions has not been previously explored. In our study, Kaiso emerged as one of the top DEGs in HF10 SCS, displaying a positive correlation with pain development and a negative correlation with the analgesic effects of HF10 SCS. Following the injury, Kaiso was significantly upregulated in microglia and sustained even with Con SCS treatment. In HF10 SCS, the repression of Kaiso lasted >15 dpi, which started to elevate from day 17 dpi, coinciding with gradual pain relapse. To explore how Kaiso can regulate microglia activities, we focused on P2X7R, a purinergic receptor highly expressed in microglia, which has previously been identified as a key regulator promoting pro-inflammatory microglia activation in chronic pain. Our results demonstrated that P2X7R is a direct transcriptional target of Kaiso. First, Kaiso and P2X7R exhibit a similar expression pattern in response to nerve injury and SCS. Functionally, P2X7R...
also shifts microglia from an anti-inflammatory state to a proinflammatory state in HF10 SCS, thus eliminating the prolonged analgesic effects. Importantly, P2X7R expression was positively correlated with Kaiso after genetic manipulations. Through ChIP-qPCR and luciferase assays, we confirmed that Kaiso directly binds to the promoter region of P2X7R, thus transcriptionally regulating its expressions in microglia. Finally, by knocking down P2X7R, we effectively restored analgesia in HF10 SCS that had been abolished by Kaiso overexpression.

In conclusion, we demonstrate how HF10 SCS acts on spinal cord tissues as a treatment for neuropathic pain by defining a functional regulatory axis and underlying molecular mechanisms. Unlike current pharmacological treatments or Con SCS, which only alleviate pain symptoms without addressing the underlying pathophysiology, HF10 SCS significantly improves immunopathogenic conditions without negatively affecting normal neuronal transmission. Our findings also offer alternative therapeutic targets, particularly beneficial for patients experiencing pain relapse, loss of protective sensation, or low response to SCS. However, it is important to acknowledge the limitations of this study. Although HF10 SCS provides longer lasting pain relief compared to Con SCS, its analgesic effects still gradually diminish with the intrinsic restoration of the pathological axis in our preclinical models. Further investigation is needed to determine whether repetitive stimulation would weaken or strengthen the repression of this functional axis.

Acknowledgments
We thank Prof E. A. Joosten for his valuable advice of SCS on this work, Prof Michael Irwin for providing comments on the manuscript, the BGI for RNA sequencing and bioinformatics services, and the Core facility of the University of Hong Kong for its support and technical assistance with ScRNA sequencing. This work was supported by a GRF grant (17116621) and funding from the Peter Hung Professorship in Pain Research granted to C.W.C.

Author Contributions
C.W.C., J.Y., and J.A.L contributed to the conception and design of the study. All authors contributed to the acquisition and analysis of data. J.Y., C.W.C., Z.X., Z.L., M.C., X.Z., and J.A.L contributed to drafting the text or preparing the figures.

Potential Conflicts of Interest
Nothing to report.

Data Availability Statement
All data needed to evaluate the conclusions are present in the paper and/or the Supplementary Materials.

References


