TEXAS TECH UNIVERSITY HEALTH SCIENCES CENTER
School of Medicine

DEPARTMENT OF PHARMACOLOGY & NEUROSCIENCE

Hmgb1 silencing in the amygdala reduces pain-related behaviors in a sex-specific manner in a chronic neuropathic pain model

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Introduction

Chronic pain is a prevalent national healthcare issue, yet many knowledge gaps exist with regard to brain mechanisms of pain, particularly including potential sexual dimorphisms. An intricate interplay between sensory, cognitive, and emotional-affective dimensions forms the complex experience of pain, presenting a challenge to identifying effective treatment options. Maladaptive neuroplasticity is a key contributor in the transition from acute protective to chronic pathological pain. Mechanistic investigation of this transition is of particular interest for the potential discovery of new therapeutic strategies. This transition may be associated with neuroimmune mechanisms in the brain, which have not yet been elucidated. The amygdala is a limbic brain center that is a key player in the emotional-affective dimensions of pain and pain modulation. The role of neuroimmune signaling in the amygdala in chronic pain states is an important knowledge gap. High motility group box 1 (Hmgb1) is a proinflammatory signaling molecule involved in pain-related crosstalk between neurons and glial cells in the spinal cord, yet its role in the amygdala in pain states has yet to be explored. Here we tested the hypothesis that Hmgb1 is involved in pain-related amygdala plasticity and that inhibition of this molecule can reduce neuropathic pain behaviors, potentially through sexually dimorphic mechanisms. Identification of brain mechanisms of pain will aid in the development of sex-specific therapeutic strategies for chronic neuropathic pain relief.

Methods

Animals:
Male and female Sprague–Dawley rats (150-200g) were housed in a temperature-controlled room and maintained on a 12-hour day/night cycle with unrestricted access to food and water.
Neuropathic pain model:
Rats were anesthetized with isoflurane (2-3%) and underwent surgery for left L5 spinal nerve ligation (SNL). Sham rats underwent a similar procedure without nerve ligation.
RNA isolation and bulk sequencing:
4 weeks after SNL surgery, male rats were briefly anesthetized with isoflurane and perfused with phosphate-buffered saline (PBS). Brains were extracted and the central nucleus of the amygdala (CeA) was dissected out. Tissue was dissociated using the Neural Tissue Dissociation Kit with Papain (Miltenyi Biotec). RNA was isolated using the MagMAX-96 Total RNA Isolation Kit (Life Technologies) and examined on the Bioanalyzer (Agilent Technologies). A minimum of 1 ng RNA for each sample was submitted to the Genomic Sequencing and Analysis Facility at the University of Texas at Austin for mRNA selection with the MicroPoly(A) Purist Kit (Life Technologies) and library preparation with NEBNext Module Components (New England Biolabs). Samples were sequenced on the Hi-Seq 4000 (Illumina) at a depth of approximately 20 million paired-end reads (150 base). Read quality was assessed using FASTQC.
RNA isolation, cDNA amplification, and qRT-PCR:
1 week (acute phase) or 4 weeks (chronic phase) after SNL surgery, male and female rats were euthanized by decapitation. Brains were extracted and the CeA dissected out for mRNA analysis. RNA was extracted using the MagMAX-96 Total RNA Isolation Kit and quantified on a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific). Reverse transcriptase reactions were performed on total RNA using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems). Gene expression was measured with quantitative real-time polymerase chain reaction (qRT-PCR) using TaqMan Gene Expression Assays (Applied Biosystems) and primers for genes of interest (Thermo Fisher Scientific). Results were analyzed using RQ Manager and DataAssist Software (Applied Biosystems). The data were normalized to the geometric mean of beta-actin (ActB), ribosomal protein L3 (Rpl3), and ribosomal protein L29 (Rpl29), and delta-delta Ct was calculated.
Experimental protocol:
Male and female rats were anesthetized with isoflurane (2-3%) and a small unilateral craniotomy was performed two weeks before SNL surgery in the pre-treatment group or one week after surgery in the post-treatment group. Using a stereotaxic apparatus (David Kopf Instruments), Hmgb1 siRNA (pooled AAV vector) or scrambled siRNA (control vector) (1.0 µL) was delivered into the right CeA using a 5 µL Hamilton syringe (33 gauge) with the following coordinates: 2.3—2.8 mm caudal to bregma, 3.8—4.2 mm lateral to midline, 7—8 mm deep. Behavioral assays were performed 4 weeks after SNL induction. Sensory withdrawal thresholds and emotional-affective responses were measured. The brain was then extracted and the CeA dissected out for mRNA analysis.
Pain-related behavioral tests
•Mechanical withdrawal thresholds were measured using a plantar electronic von Frey anesthesiometer (IITC Life Sciences) with the tip applied perpendicularly to the base of the 3rd or 4th toe of the left hind paw. Withdrawal thresholds were also measured using a calibrated forceps on the left hind paw until a withdrawal reflex was produced.
•Vocalizations in the audible (20Hz - `16kHz; supraspinally organized nocifensive responses) and ultrasonic (22kHz; limbic-driven negative emotional responses) ranges were measured with our patented computerized recording system. Brief (10 s) noxious (400-600 g/6 mm^2) stimuli were applied to the left hind paw of rats using a calibrated forceps. Vocalizations were recorded for 1 min and analyzed using Ultravox 2.0 software (Noldus).
Statistics:
Significance was accepted at the level P < 0.05. All averaged values represent means ± SEM.

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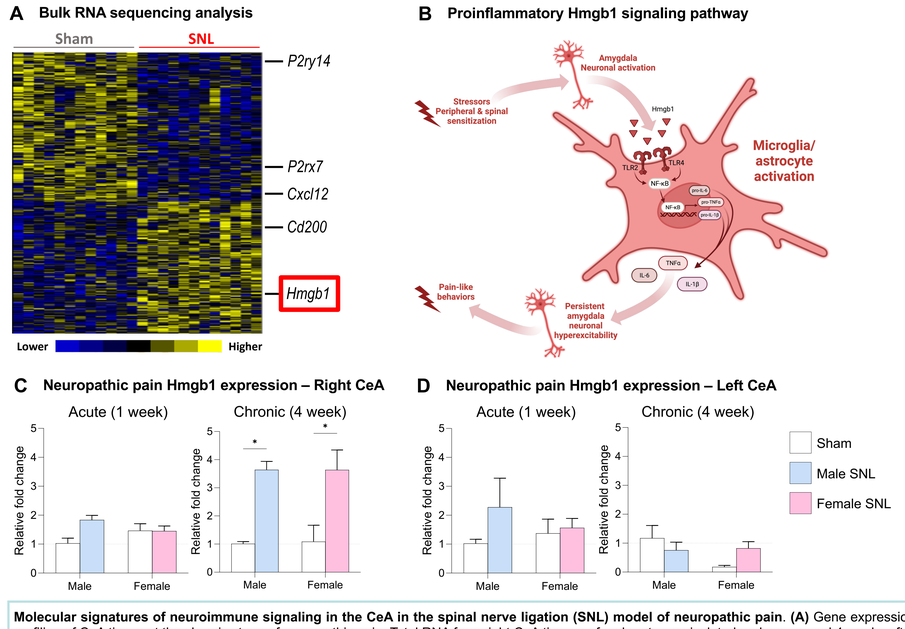
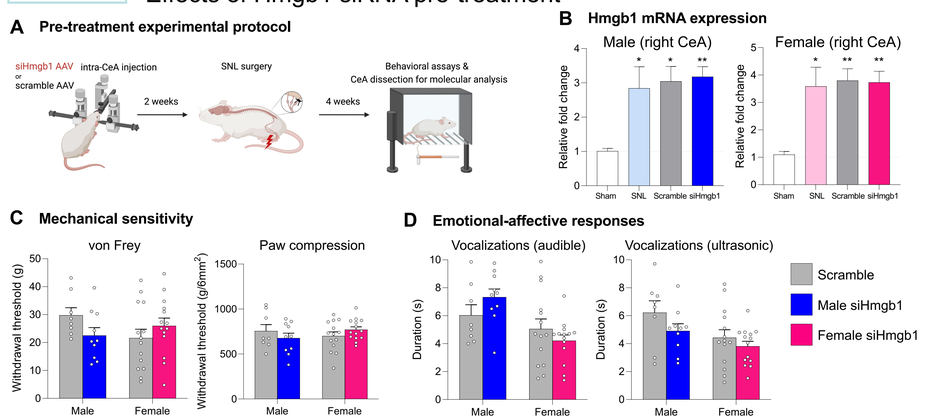
Results

Figure 1 Neuroimmune signaling in the CeA in neuropathic pain



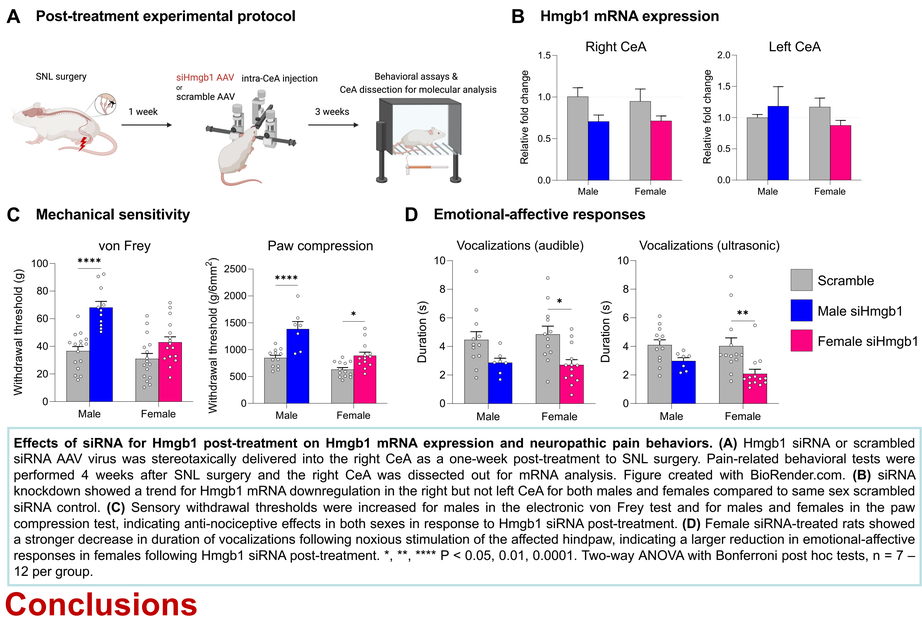
Molecular signatures of neuroimmune signaling in the CeA in the spinal nerve ligation (SNL) model of neuropathic pain. **(A)** Gene expression profiling of CeA tissue at the chronic stage of neuropathic pain. Total RNA from right CeA tissues of male rats was isolated and sequenced 4 weeks after SNL or sham surgery (n = 6 per group). 571 differentially expressed genes were identified, many of which were involved in neuroimmune signaling. Hmgb1 was upregulated in the right CeA of SNL rats compared to sham. **(B)** Proposed amygdala neuroimmune mechanisms in neuropathic pain. In response to increased synaptic drive from stressors and peripheral/spinal sensitization, activated amygdala neurons may release factors that trigger glial activation, such as Hmgb1. Hmgb1 is known to activate TLR2 and TLR4, which causes a translocation of NF-xβ into the nucleus to initiate transcription of various proinflammatory cytokines, including IL-6, IL-1β, and TNFα. Release of these molecules in turn may cause persistent amygdala neuronal hyperexcitability and pain-like behaviors. Figure created with BioRender.com. **(C)** qRT-PCR validation of Hmgb1 upregulation in the CeA in neuropathic pain. For both the acute (1 week) and chronic (4 week) phases of the SNL model, brains were extracted and the CeA dissected out for mRNA analysis. In the right CeA, Hmgb1 mRNA expression was higher in male SNL rats than female SNL rats at the acute stage but upregulated at the chronic phase in both sexes compared to sham. **(D)** No significant differences in Hmgb1 expression were seen for either sex in the left CeA at the acute or chronic phases. * P < 0.05 compared to same sex sham. Two-way ANOVA with Bonferroni post hoc tests, n = 4 - 6 per group.

Figure 2 Neuropathic pain behaviors and mRNA expression - Effects of Hmgb1 siRNA pre-treatment



Effects of siRNA for Hmgb1 pre-treatment on Hmgb1 mRNA expression and neuropathic pain behaviors. (A) Hmgb1 siRNA or scrambled siRNA AAV virus was stereotaxically delivered into the right CeA as a two-week pre-treatment to SNL surgery. Pain-related behavioral tests were performed 4 weeks after SNL surgery and the right CeA was dissected out for mRNA analysis. Figure created with BioRender.com. **(B)** In the right CeA, Hmgb1 mRNA expression was upregulated in SNL compared to sham, but there was no effect of siRNA pre-treatment in male or female rats. *, ** P < 0.05, 0.01 compared to same sex sham. One-way ANOVA with Dunnett post hoc tests. **(C)** Sensory withdrawal thresholds (measured by electronic von Frey and paw compression) were not significantly affected by siRNA pre-treatment in male or female rats. **(D)** Emotional-affective responses (measured by duration of audible and ultrasonic vocalizations in response to noxious stimulation of the affected hindpaw) were not significantly affected by siRNA pre-treatment in male or female rats. Two-way ANOVA with Bonferroni post hoc tests, n = 8 - 14 per group.

Figure 3 Neuropathic pain behaviors and mRNA expression- Effects of Hmgb1 siRNA post-treatment



Effects of siRNA for Hmgb1 post-treatment on Hmgb1 mRNA expression and neuropathic pain behaviors. (A) Hmgb1 siRNA or scrambled siRNA AAV virus was stereotaxically delivered into the right CeA as a one-week post-treatment to SNL surgery. Pain-related behavioral tests were performed 4 weeks after SNL surgery and the right CeA was dissected out for mRNA analysis. Figure created with BioRender.com. **(B)** siRNA knockdown showed a trend for Hmgb1 mRNA downregulation in the right but not left CeA for both males and females compared to same sex scrambled siRNA control. **(C)** Sensory withdrawal thresholds were increased for males in the electronic von Frey test and for males and females in the paw compression test, indicating anti-nociceptive effects in both sexes in response to Hmgb1 siRNA post-treatment. **(D)** Female siRNA-treated rats showed a stronger decrease in duration of vocalizations following noxious stimulation of the affected hindpaw, indicating a larger reduction in emotional-affective responses in females following Hmgb1 siRNA post-treatment. *, **, **** P < 0.05, 0.01, 0.0001. Two-way ANOVA with Bonferroni post hoc tests, n = 7 – 12 per group.

Conclusions

Neuroimmune signaling mechanisms in the CeA in neuropathic pain

•Many genes related to neuroimmune signaling were differentially expressed in the right CeA of male rats following SNL or sham surgery.

Hmgb1 as a molecular factor in pain-related crosstalk between neurons and glia

•Hmgb1 was differentially expressed in the right CeA of SNL and sham rats.
•Hmgb1 may be released by CeA neurons to trigger a proinflammatory signaling cascade in glial cells that leads to maladaptive CeA neuroplasticity and persistent pain behaviors.
•Hmgb1 is increased in the right CeA of males at the acute stage but in the right CeA of males and females at the chronic stage. No changes were seen in the left CeA. The data suggests a right-hemispheric lateralization of Hmgb1 that may play a more important role in chronic neuropathic pain states.

Effects of Hmgb1 siRNA as a pre-treatment to SNL surgery

•Pre-treatment with Hmgb1 siRNA did not significantly affect Hmgb1 mRNA levels in the right CeA, sensory withdrawal thresholds, or emotional-affective responses in either sex at the chronic stage of neuropathic pain. The data suggests that Hmgb1 upregulation may not occur in the early stages of the pain condition.

Effects of Hmgb1 siRNA as a post-treatment to SNL surgery

•Post-treatment with Hmgb1 siRNA decreased Hmgb1 mRNA levels in the right but not left CeA of both males and females, supporting the effectiveness of the AAV viral vector and suggesting that upregulation of Hmgb1 occurs at the later stages of the pain condition.
•Post-treatment with Hmgb1 siRNA inhibited mechanical hypersensitivity in males and females at the chronic neuropathic pain stage, indicating an anti-nociceptive effect in both sexes.
•Post-treatment with Hmgb1 siRNA inhibited audible and ultrasonic vocalizations in response to a noxious stimulus more strongly in females at the chronic neuropathic pain stage, suggesting potential sex differences in the regulation of emotional-affective responses.

The data suggests that Hmgb1 may play an important role in amygdala neuroimmune mechanisms that are involved in the transition from acute to chronic neuropathic pain, though this role may differ in males and females. Subacute inhibition of Hmgb1 signaling may serve as a novel therapeutic strategy for chronic neuropathic pain relief.

Future Directions

Cell type-specific (astrocytic, microglial, and neuronal) bulk and single cell RNA sequencing from neuropathic male and female rats will be used to determine the source of Hmgb1 release. Differential expression analyses of these tissues compared to sham controls will help determine additional molecular targets that may impact both neuronal and non-neuronal signaling in a neuropathic pain state. These molecular targets may differ between the sexes and across different stages of neuropathic pain.