Understanding The Role Of Inflammatory And Mechanically Sensitive Ion Channels In A Disc-Associated Chronic Low Back Pain Model

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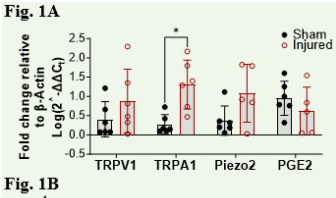
INTRODUCTION: Chronic low back pain (cLBP) is a leading source of disability worldwide with limited treatments [1]. Patients with disc-associated cLBP often have discs characterized by aberrant innervation of pain-sensing neurons (whose cell bodies reside in dorsal root ganglia (DRG)), increased inflammatory factor levels (for example, prostaglandin E2 (PGE2)) [2], and altered mechanical loading [3]. Pain-sensing neurons can be stimulated by exposure to pro-inflammatory factors as well as mechanical agitation [2]. For example: TRPV1 (chemically sensitive), TRPA1 and TRPV4 (chemically and mechanically sensitive), and Piezo2 (mechanically sensitive) are ion channels often upregulated in pain models [4-5]. Repeated stimulation of these ion channels with inflammatory factors, such as PGE2, can lead to neuronal sensitization, or maladaptive changes resulting in both an upregulation of ion channels and a lowered firing threshold. However, no studies have examined if inflammatory or mechanical stimuli are the key driver of neuronal sensitization in cLBP. Therefore, the **overarching goal** of this work is to determine the contributions of inflammation and mechanical stimuli in the initiation and progression of cLBP. As a step towards this understanding, the **objective** of this work is to characterize changes in mechanosensitive ion channel gene expression of DRGs in our rodent model of disc-associated cLBP compared to sham animals [6]. Another **goal** of this work is to validate that unique ion channel agonists and antagonists have efficacy in rat DRGs thereby allowing direct probing into the contribution of inflammatory or mechanical stimuli in our cLBP model.

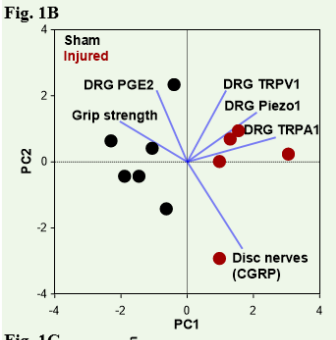
METHODS: All experiments were approved by the University of Nebraska-Lincoln's Institutional Animal Care and Use Committee. **Animal model**: 17-week-old female Sprague Dawley rats (Envigo) were randomly assigned into sham or injured groups (n=12 per group). After six weeks, L5-L6 discs were scraped six times bilaterally with a dissecting needle at a 3 mm depth [7]. Sham animals L5-L6 discs were visualized before surgical closure. Grip strength was performed biweekly to assess axial hypersensitivity. 16 weeks post-injury, rats were euthanized and L5-L6 motion segments were prepared for immunohistochemistry to access pain-sensing calcitonin gene-related peptide (CGRP+) neurons in the disc. T13-L6 left DRGs were removed and snap frozen. Grip strength data was transformed using log2 and baseline corrected. Statistical analysis was performed using a Two-Way ANOVA with Sidak's correction. CGRP immunohistochemistry data was transformed with log2, and statistical analysis was performed using a Kruskal-Wallis test with Dunn's correction. A principal component analysis (PCA) and correlation matrix were conducted to assess the contribution of grip strength, CGRP in the disc, and DRG expression of TRPV1, TRPA1, Piezo2, and PGE2 on animal outcome. **qPCR**: T13-L3 left DRGs per animal (n=6 sham and injured animals) were homogenized and cells were lysed prior to phase separation. RNA was precipitated and then cDNA was synthesized using iScript cDNA Synthesis Kit (BioRad) according to the manufacturer's protocol. Expression of TRPV1, TRPA1, Piezo2, and PGE2 were measured, and all genes were normalized to β-Actin. Statistical analysis was performed using a Two-Way ANOVA with Sidak's correction. **Agonizing ion channels**: Intact DRGs were harvested, trimmed, and cut from adult Sprague Dawley rats after humane euthanasia. DRG pieces were embedded in 250 µL of hydrogel consisting of laminin (0.75 mg/mL), type I collagen (4.5 mg/mL), and methacrylated hyaluronic acid (1.25 mg/mL). Hydrogels were thermally crosslinked (30-minute incubation at 37˚C) and UV-photo-crosslinked (90 seconds), then cultured with neuronal culture media. After robust DRG outgrowth in the hydrogel, BAPTA Oregon Green 488 (2 µM, Fisher) florescent dye was added to each hydrogel and incubated at 37˚C (one hour). Hydrogels were spiked with various concentrations of capsaicin (TRPV1 agonist) and GSK1016790A (TRPV4 agonist) and the neurites were imaged using high-speed confocal microscopy. All pixels within a region of interest (ROI) on a neurite were averaged. Data shown as the relative change in fluorescence (ΔF/F0), where F0 is baseline fluorescent signal intensity and ΔF= F - F0. N=3 DRGs per condition with three ROIs averaged per DRG. Statistical analysis was performed using a One-Way ANOVA with Dunnett's correction. Work is underway to establish standard curves using chemical agonists for mechanically sensitive ion channels TRPA1, TRPC5, and Piezo1.

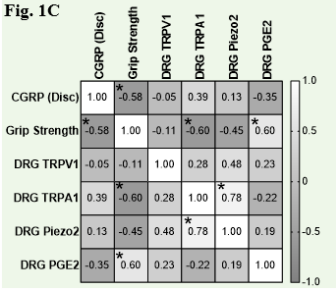
RESULTS: **Animal model**: Injured animals showed significantly lowered grip strength threshold (mean: 84 percent of baseline ± 14.26%) compared to sham (mean: 96 percent of baseline ± 9.1%) at week 15 post-injury. Injured animals showed significantly increased CGRP+ neuron staining in discs (mean: 1.65 ± 1.22%-disc area) compared to sham (mean: 0.49 ± 0.24%-disc area). **qPCR**: Injured animals showed a significant increase in DRG gene expression of TRPA1 compared to sham (**Figure 1A**). PCA showed TRPA1 and grip strength have strongest contribution to PC1 and TRPV1 and PGE2 have the strongest contribution to PC2. Clustering was observed between sham and injured animals (**Figure 1B**). Correlation matrix showed significant relationships between grip strength and CGRP, grip strength and TRPA1, and grip strength and PGE2 (**Figure 2C**). **Agonizing ion channels**: Fluorescent change shows significant differences between 1 µM and 10 µM capsaicin compared to vehicle control and 15 µM and 25 µM GSK1016790A compared to vehicle control, suggesting DRGs have the TRPV1 and TRPV4 channels and we have validated known agonists that can be translated to our in vivo model. (**Figure 2A-B**).

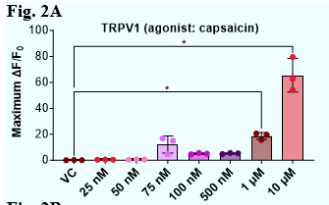
DISCUSSION: Interestingly, TRPA1 is highly correlated with grip strength suggesting an increased expression of TRPA1 may influence pain threshold. Experiments are currently underway to characterize gene expression of an additional n=6 DRGs per group to bolster these data. Further, these data demonstrate TRPV1 and TRPA1 are present in DRGs and can be stimulated using capsaicin and GSK1016790A, respectively. Future work in this platform will also include validation of additional agonists and antagonists, so we can modulate inflammatory and mechanical stimulation of neurons in vivo in our cLBP model.

SIGNIFICANCE/CLINICAL RELEVANCE: Currently, there are no long-term clinically effective therapeutics for disc-associated cLBP, and the mechanisms of neuronal sensitization are not well understood. These data will be translated into our in vivo model of disc-associated chronic low back pain to parse the role of mechanics and inflammation in the development of pain. We believe the novel approach of decoupling the contribution of inflammatory and mechanical stimulation on pain-sensing neurons can inform novel therapeutic targets to improve clinical translation in disc-associated cLBP.









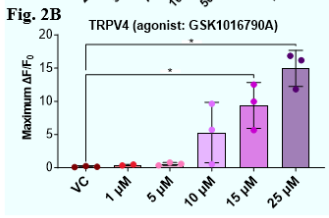


Figure 1A. DRG gene expression of TRPV1, TRPA1, Piezo2, and PGE2 for sham and injured animals. Error bars = ± SD. **1B**. PCA biplot showing eigenvectors with PC scores of each animal overlayed. Sham = black, injured = red. **1B**. Correlation matrix. N=6 animals per group. *p=≤0.05.

Figure 2A. Fluorescent response to various concentrations of capsaicin to stimulate TRPV1. **2B**. Fluorescent response to various concentrations of agonist GSK1016790A to stimulate TRPV4. VC = vehicle control, 0.055% EtOH. *=p<0.05. Error bars = ± SD. N=3 DRGs per condition.

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