Dynamic Changes in Dorsal Root Ganglion Neurons Following Bone Fracture

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INTRODUCTION: Bone fractures are one of the most common global injuries associated with both a high degree of acute pain and a significant risk for transitioning to chronic pain. Fracture pain represents a complex state that involves both nociceptive and neuropathic components, yet the underlying neural mechanisms remain poorly understood. In particular, the dynamic changes of dorsal root ganglion (DRG) neurons during fracture healing have not been fully elucidated. In this study, we cross-sectionally investigated neural injury responses and sensitization-related peptides in DRG neurons using a mouse fracture model. We hypothesize that neuronal changes in the DRG will map to behavioral changes related to functional recovery from fracture.

METHODS: Adult (10-14 week old) male C57BL/6 mice were used to minimize variability related to sexual dimorphism. A stabilized tibial fracture was generated by right tibial osteotomy with intramedullary pin fixation. Animals were socially housed in a climate and light controlled facility with open access to food, water, and enrichment at all points in the study. For retrograde tracing, adeno-associated virus (AAV) was injected into the tibial proximal periosteum prior to fracture surgery. L1–L6 DRG were harvested at postoperative day 5 for quantification of AAV-labeled cells (N=6, three sections averaged per DRG). In a separate cohort, L3 DRG were collected at postoperative days 5, 10, and 21 for fluorescent immunohistochemistry to evaluate Activating Transcription Factor 3 (ATF3), a stress induced marker, and the neuropeptides Calcitonin Gene-Related Peptide (CGRP) and Substance P (SP) (N=5 per group, three sections averaged per DRG). Quantification included integrated density (shown) and positive cell ratios, calculated using Otsu thresholding (Otsu, 1979). Longitudinal functional recovery from fracture was assessed using the BlackBox. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Francisco, and were conducted in accordance with national regulations and ARRIVE Guidelines. Statistical analyses were performed using two-way ANOVA with appropriate post hoc comparisons, with significance defined as $p<0.05$.

RESULTS SECTION: Semi-quantifications of AAV neural retrograde showed that the highest localization of staining in both an unfractured and fractured tibia was L3 with fracture causing a significant increase in labeling at L4 and L5 compared to sham (**Fig. 1**). Immunohistochemistry demonstrated that both ATF3 (**Fig. 2A**) and SP (**Fig. 2B**) expression peaked at day 5 in fractured mice and remained significantly elevated above sham even at day 21 post-fracture. CGRP expression in fracture mice was only significantly elevated at day 5 compared to sham (**Fig. 2C**). Co-localization analysis revealed that there was minimal overlap between ATF and SP positive cells and that only 13% of ATF3$^+$ cells contained CGRP at day 5 (**Fig. 2D-E**). Behavioral phenotyping shows that most weight-bearing and kinematic outcomes decline by 5 and 7 days post-surgically, but return to levels comparable to naïve controls by day 10 (**Fig. 3**).

DISCUSSION: Despite behavioral recovery by day 10, ATF3 expression persisted, indicating that neuronal injury responses follow a slower temporal course than fracture-related behavior. In contrast, there was an overall increase in CGRP expression in fractured mice compared to sham that is consistent with prior evidence that peptidergic CGRP$^+$ C-fibers are involved in inflammatory sensitization at the injury site. Despite broadly elevated CGRP and ATF3, co-localization analysis showed that only 13% of ATF3$^+$ cells also contained CGRP at day 5, indicating minimal overlap that may highlight injury related neuron subtypes. Persistent DRG expression of SP at 21 days after fracture compared to sham indicates SP is not limited to the acute phase, and may play additional roles beyond early inflammation. The lack of overlap between SP and ATF3 further indicates that SP and ATF3 label separate DRG neuronal populations. Taken together, these results suggest distinct sensory nerve responses due to tibia fracture that are mediated by different DRG subtypes and have unique temporal profiles.

SIGNIFICANCE/CLINICAL RELEVANCE: Our findings indicate that fracture pain arises from distinct DRG neuronal subtypes mediating nociceptive versus neuropathic components. The mismatch between pain behavior recovery and sustained ATF3 expression highlights the need for separate strategies targeting acute nociceptive pain and neuronal repair in post-fracture management.

IMAGES AND TABLES:

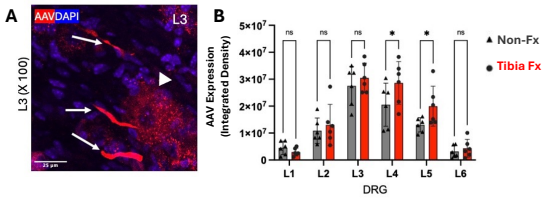

FIG 1. Retrograde Tracing of DRG Signaling Following Tibia Fracture. (A) Represent-ative Z-stack image of L3 following fracture (scale 25 µm), **(B)** Semi-quantification of AAV expression L1-L5 in DRG without fracture (grey) or 5 days following fracture (red).

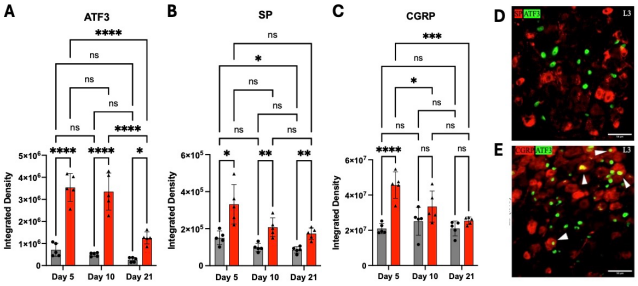

FIG 2. Immunohistochemical investigation of neuronal injury and sensitization in DRG following fracture. (A) ATF3, **(B)** Substance P, **(C)** CGRP in L3 in DRG without fracture (grey) or 5 days following fracture (red). Representative Z-stack images of **(D)** ATF (green) and SP (red), and (E) ATF green and CGRP (green), scale 50 µm.

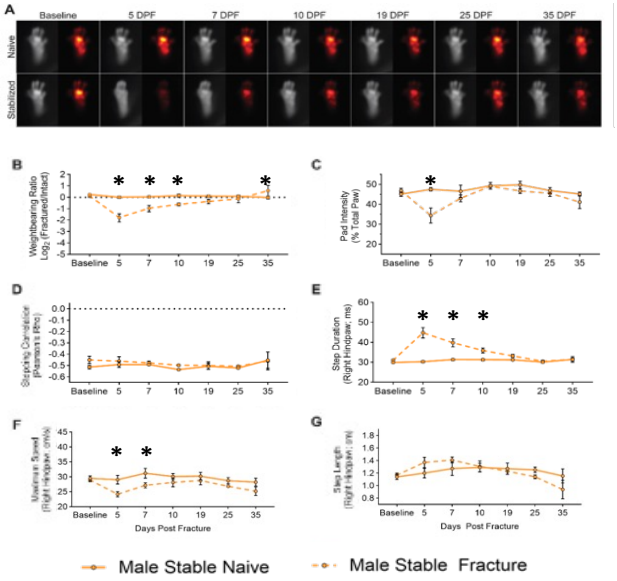

FIG 3. Functional recovery following stabilized tibia fracture. (A) Average images of paw placement and weightbearing of naïve and stabilized fracture males at baseline and 5-, 7-, 10-, 19-, 25-, and 35-days post injury (DPI). Longitudinal analysis of weightbearing and kinematic parameters: **(B)** Weightbearing ratio of the fractured to the intact hindlimb. **(C)** Percentage of FTIR intensity localized to the pad of the hindpaw of the fractured hindlimb. **(D)** Hindlimb stepping correlation. **(E)** The average full-width, half-max duration of a single step, in milliseconds. **(F)** Average maximum speed (cm/s) of right hindpaw during stepping. **(G)** Average distance of each step of the right hindlimb in centimeters. Significant differences ($p<0.05$) indicated with *.