## Annulus Fibrosus Injury Induces Acute Dorsal Root Ganglion Sensitization and Neuroinflammation, and Chronic Neuronal Remodeling and Disc Inflammation in an In Vivo Rat Model

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INTRODUCTION: Chronic lower back pain (CLBP) is a leading cause of disability worldwide, and major socioeconomic burden with more than \$100 billion annual health care costs in the US [1-2]. Intervertebral disc (IVD) degeneration, characterized by matrix breakdown, disc height loss and inflammation, is a key contributor to discogenic CLBP [3]. There is a need for greater understanding of CLBP to develop improved disease modifying treatments, yet lack of efficacy of spinal fusion for discogenic CLBP indicates degenerated IVDs are not the sole source of pain [3,4]. IVDs are innervated with nociceptive fibers connecting to the dorsal root ganglia (DRGs). Inflammatory and neuropathic pain models demonstrated sensitization and neuroinflammation of DRGs play important roles initiating and progressing painful conditions [3,5,6]. IVD degeneration can induce DRG sensitization and neuroinflammation at chronic time points [5], yet a deeper understanding is needed on the kinetics of DRG changes following IVD injury in order to identify more optimal treatment times and targets. This study determined the kinetics of spine and DRG changes at acute (3d - 2wks) and chronic (8wks) timepoints following AF injury in an in vivo rat model. At all timepoints, the spine was evaluated for inflammation with pan-macrophage marker (CD68); and the DRG was evaluated for pain sensitization with substance P (SubP), neuroinflammation with macrophage marker (Iba1) and neuronal remodeling with satellite glial cells marker (GFAP).

METHODS: All procedures were approved by the IACUC. Thirty-seven skeletally mature Sprague Dawley rats (5-6 months old) were assigned to Naïve or AF injury groups. AF Injury was induced by puncturing the anterior AF of L3-4, L4-5, and L5-6 IVDs with a 26G needle at a depth of 3mm at the midline as well as left and right lateral areas of the IVD (3 punctures/disc) including PBS injection into the midline puncture [6]. Naïve rats had no surgical interventions. Rats were euthanized at 0 days (d) (Naïve), 3d, 1 week (1wk), 2wk, and 8wk post-injury (n=5-8/group). After euthanasia, the lumbar spines (L2-L6) and L2 DRGs were dissected, fixed, paraffin-embedded, sectioned. Sections were stained with Safranin-O/fast-green/hematoxylin for spine morphology, and with immunohistochemistry for CD68. DRG immunostaining involved SubP, Iba, and GFAP with Nissl and DAPI counterstain to visualize neurons and cells. IVD degeneration and spine CD68 were analyzed using semi-quantitative grading schemes [7-8]. DRG sections were imaged and analyzed using a standardized immunoreactivity (ir) threshold to calculate positive pixels. The SubP-ir, Iba1-ir and GFAP-ir relative to total DRG area (average of left and right L2 DRG) was an estimate of numbers of positive cells. One-way ANOVA with Tukey's post-hoc tests were performed with GraphPad Prism9 with α=0.05 as significant.

RESULTS: Naive IVDs exhibited normal IVD morphology, while injured IVDs exhibited acute and chronic severe degenerative changes, including a smaller NP with less glycosaminoglycan, fewer and clustered NP cells, less distinct NP-AF boundary, and disorganized AF with ruptured lamellae along the puncture track (Fig. 1A). Injured IVDs had significantly higher IVD degeneration score than the Naive IVDs for all timepoints with no difference between different timepoints (Fig1B). The spine had significantly increased CD68-ir after AF injury, particularly in the regions of anterior AF, anterior longitudinal ligament (LL) and endplate. The CD68-ir in anterior AF increased at 3d post-injury and remained elevated until the final 8wks; the CD68-ir increased temporarily in LL and endplate with peaks at 1wk and 3d, respectively, that gradually returned to naïve levels (Fig. 2A & B). AF injury induced acute and temporary DRG sensitization and neuroinflammation with SubP- and Iba1-ir levels that peaked at 3d post-injury and then decreased by 8wks (Fig. 3A & B). GFAP-ir also significantly increased at 3d post-injury, but the level remained elevated until the final 8wks time point, suggesting remodeling with chronically increased satellite glial cells (SGC).

DISCUSSION: This study showed acute and chronic spine and DRG changes following an AF injury in a rat discogenic pain model. AF injury caused significant rapid IVD degeneration and dynamic changes in spinal CD68 and DRG SubP, Iba1, and GFAP levels. The spine CD68-ir, DRG SubP- and Iba1-ir were all increased at 3d post-injury, suggesting acute spine-DRG crosstalk with broad spinal inflammation that affects DRG neuroinflammation and sensitization. A similar AF injury model showed increase in spine inflammation (TNFα and IL-6) and DRG nociceptive neuropeptide (CGRP) during acute timepoints (1d-1wk), however they showed the DRG CGRP remained elevated until chronic timepoint (8wk) warranting further research on sex differences as they used female rats [9]. Spine CD68-ir was increased at the anterior LL and AF, which are known to be highly innervated with nociceptive fibers from DRG, suggesting the nerve fibers might be a direct pathway for the spine-DRG crosstalk. On the other hand, GFAP, a filament protein in SGC, also significantly increased at 3d and was chronically increased through 8wks suggest the SGCs could also play a role in this crosstalk, and this rapid increase in is consistent with peripheral nerve injury models showing elevated GFAP acutely (2d-2wk) post-injury [10]. However, GFAP was also elevated chronically (8wks) in this study suggesting roles neuronal remodeling occurs at acute and chronic timepoints in discogenic pain. This AF injury model was previously shown to induce pain-related behavior with hindpaw mechanical allodynia and altered DRG gene expression for neuropeptides [6], indicating the increased SubP is consistent and likely to involve pain. This study highlights importance of macrophages and SGCs in acute neuroinflammatory crosstalk between spine and DRGs from AF injury and identified elevated DRG GFAP at all times suggesting roles of SGCs in DRG matrix remodeling in acute and chronic discogenic pain.

SIGNIFICANCE: IVD injury can cause acute inflammatory crosstalk between IVD and DRG with increased macrophages and chronic DRG remodeling with increased SGC, suggesting therapeutic strategy for discogenic pain must target both the spine and peripheral nervous system with potentially distinct strategies at acute and chronic timepoints.

**REFERENCES:** [1] Vos+ Lancet. 2013; [2] Katz JBone Joint Surg. 2006; [3] Fujii+ JBMR Plus. 2019; [4] Baliga+ Asian Spine J. 2015; [5] Koes+ BMJ. 2006; [6] Mosely+ Sci Rep. 2020; [7] Lai+ Int J Mol Sci. 2023 [8] Lai+ JOR Spine. 2021; [9] Miyagi+ SPINE. 2011 [10] Kim+ Pain. 2009 **ACKNOWLEDGEMENTS:** Supported by NIH/NIAMS grant R01AR078857.

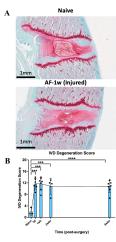


Figure 1. EP Injury induced degeneration. (A) Representative images of rat IVD with safranin-O/Fastgreen/ Hematoxylin staining. (B) AF Injury across all time points exhibited increase in IVD degeneration score.

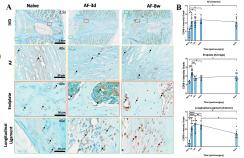


Figure 2. AF injury increased spine CD68-ir. (A) Representative images of CD68-ir with toluidine for whole IVD, AF, EP, and LL. (B) CD68-ir significantly increased in anterior AF and remained elevated until 8wks. CD68-ir temporary increased in anterior LL and EP with peaks at 1wk and 3d.

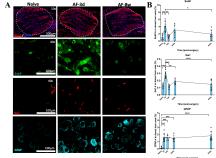


Figure 3. AF Injury increased SubP-, Iba1-, GFAP-ir in DRG. (A) Representative images of SubP-, Iba1-, GFAP-ir in the DRG from naïve to 8 wks. (B) SubP-ir and Iba1-ir significantly peaked at 3d and decreased until 8wks. GFAP-ir significantly increased at 3d and remained elevated until 8wks.