AF Injury Induces Netrin-1/DCC-Associated Nerve Ingrowth Resistant to Combined Etanercept and Duloxetine Treatment in a Rat Discogenic Pain Model

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INTRODUCTION: Chronic back pain is a leading cause of disability worldwide and often linked to intervertebral disc (IVD) degeneration (IVDD). IVDD involves structural breakdown, inflammation, and sensory nerve ingrowth into regions of the IVD that aneural and avascular in health conditions [1]. IVDD degrades the annulus fibrosus (AF) and nucleus pulposus (NP) microenvironment with immune cell infiltration, pro-inflammatory conditions, and extension of pathological ingrowth and sensitization of nerve fibers [2]. This aberrant nerve ingrowth is a key driver of discogenic pain often associated with increased expression of nerve growth factor. Recent studies implicate axon guidance cue, Netrin-1, and its attraction receptor, Deleted in Colorectal Cancer (DCC), as mediators of sensory nerve attraction and angiogenesis, yet with very few studies in discogenic pain [3]. We previously determined AF injury induced IVDD caused chronic pain and spinal cord sensitization in a rat model that was ameliorated with combined treatment of Etanercept, a TNFα inhibitor, and Duloxetine, a serotonin-norepinephrine receptor (SNRI) [4,5]. This study determined if AF injury induced IVDD caused by Netrin-1/DCC-dependent innervation in a rat discogenic model and whether the combined treatment modulates this pathway. We hypothesized AF injury would increase nerve ingrowth and correlate with Netrin-1/DCC and be affected by macrophage presence but not systemic Etanercept and Duloxetine Combined treatment.
METHODS: All procedures were approved by the IACUC at the Icahn School of Medicine at Mount Sinai. Thirty-eight skeletally mature male Sprague-Dawley rats (5-6 months old) were split into 3 groups: Naïve (n=12), Vehicle (n=13) or Combined (n=13). Only males were analyzed in this study to ensure cohort consistency; however, current work is extending these analyses to female spines to evaluate sex-specific differences in nerve ingrowth. Vehicle and Combined groups underwent a three-level AF puncture injury at the lumbar IVD levels L3-L4, L4-L5, and L5-L6, to induce IVDD and discogenic pain. Each IVD was punctured at the midline with a 26G needle 3 mm deep and injected with 2.5 µL of sterile PBS, followed by transverse sweeps to further increase the severity of IVDD. Beginning 4 weeks post-injury, injured animals were administered either sterile injections for Vehicle or Combined Etanercept (5 mg/kg subcutaneously every 3 days) and Duloxetine (20 mg/kg, intraperitoneally daily) treatment for 2 weeks. Naïve animals served as uninjured controls. At 8 weeks, lumbar spines were harvested for histology and immunohistochemistry. IVDD was graded using safranin O/Fast Green staining. Inflammatory macrophages, nerve ingrowth, and axonal guidance were assessed by CD68, PGP9.5 and Netrin-1/DCC immunohistochemistry, respectively. Semi-quantitative grading on a scale from 0 to 4 was performed for CD68, PGP9.5, Netrin-1, and DCC across all injured IVD regions. Regions of interest included the anterior and posterior longitudinal ligament, granulation tissue, outer and inner anterior AF, NP, posterior AF, and endplates. Statistical comparisons between groups were made using one-way ANOVA or Kruskal-Wallis test with Dunn's post-hoc correction, where needed. Spearman and multiple regression analysis determined if PGP9.5 was driven by Netrin-1/DCC, and if Netrin-1/DCC was modulated by macrophages.
RESULTS: Histological grading confirmed severe IVDD across all injured groups, and Combined treatment did not lead to an improvement of degeneration score or reduced macrophage infiltration (Fig. 1). CD68 and TNFα immunoreactivity were elevated in the anterior ligament and granulation tissue regions and was not alleviated in the treatment group, indicating persistent macrophage-driven infiltration at the site of injury (Fig. 1). This AF injury also induced significant aberrant nerve ingrowth within the injured IVDs that was not reduced with treatment, as shown by significantly increased PGP9.5 immunoreactivity compared to Naïve (Fig. 2). PGP9.5-positive nerve fibers were also abundant in the anterior and granulation tissue regions of the injured IVDs, indicating robust nerve infiltration (Fig. 2). Expression of Netrin-1 and DCC was also significantly increased in all injury groups compared to Naïve with no significant difference between Vehicle and Combined, indicating that Combined Duloxetine and Etanercept did not alter the Netrin-1/DCC signaling pathway (Fig. 2). To explore drivers of this pathway, while all markers were significant, correlation analysis showed that PGP9.5 was most significantly correlated with DCC and Netrin-1 (Fig. 3A), highlighting Netrin-1/DCC as the largest factor in nerve ingrowth. Strong positive correlations also showed Netrin-1 expression was increased by DCC, IVDD, CD68, and TNFα (Fig. 3B). Multivariate regression analysis confirmed that these markers act in coordination, indicating that nerve ingrowth arises from the combined activity of inflammatory and axonal guidance pathways, rather than a single driver. Female spinal measurements are in progress.
DISCUSSION: AF injury induced nerve ingrowth and activation of the Netrin-1/DCC signaling pathway within degenerated IVDs. Increased PGP9.5 expression confirmed aberrant nerve innervation at 8 weeks post-injury. The coordinated relationship of PGP9.5 with Netrin-1 and DCC observed in multiple regression analysis demonstrated their roles in axon guidance in IVDD. Netrin-1 was significantly increased in human IVDD, especially in granulation tissue regions where nerve and vessel ingrowth occurs [3,5], supporting the finding that Netrin-1 is important in IVDD in multiple model systems. PGP9.5, Netrin-1, and DCC expression persisted despite Combined treatment, which may indicate a Netrin-1/DCC-specific therapeutic target is needed. However, it is possible that the systemic drug treatments did not make it to the injured IVDs at sufficient dose. We showed macrophage infiltration persists chronically after AF injury, as reported previously [5]. TNFα is also known to upregulate Netrin-1 by suppressing transcriptional repressors, enabling Netrin-1 to promote nerve ingrowth [6]. This study, together with prior literature, supports that IVDD involves macrophage-driven TNFα signaling that enhances Netrin-1 production, attracting DCC-expressing axons. In this context, it is not surprising that systemic anti-inflammatory and neuromodulator treatments failed to inhibit nerve ingrowth in rat discogenic pain, and data point to Netrin-1/DCC as a molecular target to more specifically reduce nerve ingrowth associated with chronic IVDD.
SIGNIFICANCE: Current therapies primarily target central sensitization but fail to address intradiscal mechanisms contributing to chronic nociception. The persistence of PGP9.5, Netrin-1, and DCC despite systemic combined treatment identifies Netrin-1/DCC signaling as a potential therapeutic target to inhibit nerve ingrowth associated with IVDD in chronic back pain.
REFERENCES: [1] Nicol+ J Clin Med 2023; [2] Freemont+ Lancet 1997; [3] Ni+ Nature Commun 2019; [4] Iliff+ Trans ORS 2026, Podium #286; [5] Lai+ Int J Mol Sci 2024; [6] Zheng+ J Ortho Transl 2023.
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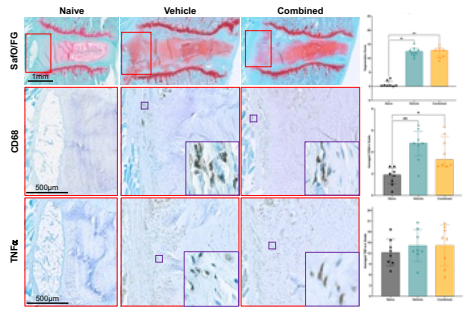

Fig. 1: AF-injury induced severe IVDD and increased CD68, and TNFα in the anterior ligament and granulation region by experimental group.

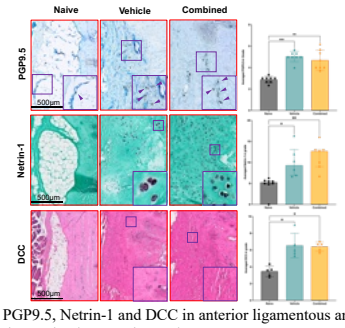

Fig. 2: PGP9.5, Netrin-1 and DCC in anterior ligamentous and granulation region by experimental group.

Correlation Analysis			
Dependent Var.	**Independent Var.**	**Spearman ρ**	**P-value**
Spine PGP9.5	Spine Netrin-1	0.8	p < 0.001
	Spine DCC	0.785	p < 0.001
	IVD Degeneration	0.69	p < 0.001
	Spine CD68	0.668	p < 0.001
	Spine TNFα	0.482	0.023

Correlation Analysis			
Dependent Var.	**Independent Var.**	**Spearman ρ**	**P-value**
Spine Netrin-1	Spine DCC	0.8	0.002
	IVD Degeneration	0.651	0.003
	Spine CD68	0.602	0.008
	Spine TNFα	0.547	0.02

Fig. 3: Multiple correlation analysis showing drivers of (A) PGP9.5 and (B) Netrin-1.