Identifying the transcriptional profile of neurons specifically associated with calvarial bone injury

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INTRODUCTION: Skeletal sensory nerves play critical roles in bone healing. After injury, early nerve infiltration stimulates bone formation followed by nerve retraction to baseline levels in healing injuries.[1] Previous studies identified bone-forming roles for nerve-secreted factors, such as calcitonin-gene related peptide (CGRP).[2] In vivo, these factors may function indirectly (e.g. via vasculature) or directly through osteoprogenitor signaling to promote bone regeneration.[3,4] In non-healing injuries, however, nerves do not retract, suggesting that nerves and nerve factor persistence may have a negative impact on bone healing.[5] As such, understanding the complex interplay between nerves, vasculature, osteoprogenitor differentiation, and, ultimately, mineral deposition is important for developing therapeutic strategies for the treatment of non-healing bone injuries. In craniofacial bones, such as the calvaria, there has been a dearth of studies that have elucidated the neural signaling interactions that govern bone healing. One barrier has been that calvarial nerve cell bodies are located in distal trigeminal ganglia which innervate multiple craniofacial regions and tissues in addition to bone,[6] resulting in difficulty identifying bone-specific nerves that secrete factors to signal osteoprogenitors in the defect region. To address this challenge, we propose to use viral retrograde tracing to label and subsequently isolate nerve nuclei specifically associated with calvarial sub critical- and critical-sized defect injuries to temporally investigate nerve phenotypes and interactions with osteoprogenitors during bone healing.

METHODS: We performed sub critical- (1-mm) and critical-sized (4-mm) calvarial defect injuries on the right parietal bones of C57BL/6J mice using a trephine drill bit without disturbing the underlying dura mater (**Figure 1A**). Following injury, we harvested calvaria at post fracture day (PFD) 7, 14, 28, 56, and 112 to observe infiltration and retraction patterns of calvarial nerves at early and late timepoints after injury. We used quantitative light sheet microscopy (QLSM) and confocal imaging to characterize the densities of TUBB3+ nerves in both defects. Immunofluorescence staining of neurovascular factors, including CGRP, VEGF-A, and VEGF-D, was used to quantify the expression levels of known factors that regulate bone formation in the ipsilateral trigeminal ganglia that innervates the defect region. To better understand the transcriptional signature of the nerve cell bodies that innervate the defect, we developed a retrograde tracing method that will be paired with single cell RNA-sequencing (scRNA-seq) to isolate the gene signature and signaling interactions that correspond exclusively to nerves that innervate the defect region. To validate this method and identify the timing required for complete tracing, AAV-PHP.S (tdTomato+) (Addgene #59462) was injected with Fast Blue stain into the right parietal bone. Ipsilateral and contralateral trigeminal ganglia were harvested and imaged via whole mount confocal imaging at D0, D3, D7, D14, and D21 after injection. In addition, ganglia were also collected for flow cytometry to quantify the tdTomato+ population. Subsequent studies will harvest cells from the tdTomato+ ipsilateral trigeminal ganglia and sub critical- and critical-sized defect regions for scRNA-seq at PFD 7 and 56 following 1-mm and 4-mm defect injury and subsequent analysis. All statistical tests were performed in GraphPad Prism with either a two-way ANOVA with Bonferroni's post-hoc test or a two-tailed t-test.

RESULTS: At early timepoints following injury, nerves rapidly infiltrate into 1-mm and 4-mm defects at similar densities; however, at late timepoints, nerves retract to baseline levels in 1-mm defects yet fail to retract in 4-mm defects (**Figure 1B**). At PFD 112, nerve length and diameter are increased in 4-mm defects. In the ipsilateral trigeminal ganglia, there are no differences in neurovascular factor expression between 1-mm and 4-mm defects ($p > 0.5$) (**Figure 1C-D**). AAV-PHP.S tdTomato and Fast blue retrograde tracing initially reaches the trigeminal ganglia at D3 but continues to increase in intensity until D14. At D14 and D21, tdTomato+ signaling intensity and cell number remains constant, suggesting that retrograde tracing has been completed (**Figure 1E-F**). tdTomato labeling is restricted to the injection site and nerve cell bodies, with some labeling of the skin surrounding the calvaria. Although contralateral ganglia demonstrate low levels of retrograde tracing, there is a higher population of tdTomato+ nerves in ipsilateral ganglia at D10 (**Figure 1G-H**). In total, ~1000 tdTomato+ cells were isolated per ganglia, demonstrating a large population of labeled cells for subsequent transcriptomic analysis.

DISCUSSION: These data demonstrate nerve infiltration and retraction in healing and non-healing calvarial defects. As nerves do not fully retract to baseline levels in 4-mm defects yet are known to be required at early stages of healing, we propose to investigate whether their persistence may be inhibitory to complete bone formation. Although differences in neurovascular factors in the trigeminal ganglia were not observed, we hypothesize that nerve cell bodies that innervate the defect region will demonstrate signaling differences between healing and non-healing injuries. Since trigeminal ganglia innervate several craniofacial regions, a method to isolate calvarial nerves from other nerves in the ganglia is needed to clearly elucidate neuroskeletal signaling interactions. Thus, to further understand the roles of calvarial nerves in coordinating bone formation, we developed a novel high throughput method to identify the transcriptional signatures of nerves that innervate calvarial defects using both retrograde tracing and scRNA-seq. Limitations of this method include high animal numbers required to isolate enough tdTomato+ cells for sequencing and potential contamination from labeling tdTomato+ nerves in the skin overlying the defect region. Taken together, we have spatiotemporally mapped neural patterns in healing and non-healing defects and developed a method to study signaling interactions between calvarial nerve cell bodies located in the distal ganglia and cells in the defect region of the right parietal bone, allowing us to identify potential targets to modulate the healing environment and develop therapies for improved bone formation.

SIGNIFICANCE/CLINICAL RELEVANCE: Bone is one of the most widely transplanted tissues in the world, however, the complex mechanisms that underlie bone formation after injury remain incomplete. Nerves have been shown to play a role in modulating bone healing, yet their interactions with vasculature and osteoprogenitors have not been fully elucidated. By understanding these interactions, we can identify potential therapeutic targets to encourage bone formation following injury or disease.

REFERENCES: 1. Li et al. J. Clin. Invest. 129:5317-5150 (2019). 2. Xu et al. Curr. Osteo. Rep. 18:621-632 (2020). 3. Qin et al. Bone Res. 10:43 (2022). 4. Cherief et al. Stem Cells Trans. Med. 11:1165-1176 (2022). 5. Kasai et al. J. Pain. Res. 14:3565-3571 (2021). 6. Zhao et al. Pain 155:1392-1400 (2014).

Figure 1: Calvarial nerves innervate defect regions after injury, but do not retract from non-healing defects. A) Experimental design **B**) TUBB3+ nerves in 1-mm and 4-mm defect regions at PFD 112 **C**) CGRP expression in trigeminal ganglia from 1-mm and 4-mm defects at PFD 56 **D**) Quantification of CGRP+ signal in trigeminal ganglia. **E)** MIP of Fast blue (blue) and AAV-PHP.S tdTomato+ (red) signal at various timepoints after injection. **F)** Number tdTonato+ cells per region oever time. **G)** Flow cytometry analysis of tdTomato+ nerves from ipsilateral trigeminal ganglia. **H)** Count of tdTomato+ nerves in ipsilateral and contralateral ganglia per 10,000 cells. (Scale bars: B: 200 μm, C: 330 μm, and E: 100 μm, respectively)