A quantitative atlas of the murine calvaria throughout mouse lifespan

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INTRODUCTION: Peripheral nerves have been known to innervate bone tissue for over 50 years, however, a complete understanding of bone nerve distributions and patterning remains limited. Further, nerves play complex roles in coordinating bone development and maturation, thus, an understanding of changes in nerve distributions and patterns throughout a mouse lifespan lends insight into changes in the bone microenvironment that occur during development and aging. In long bone, previous studies have mapped the distributions of sensory and sympathetic nerve throughout the periosteum, where several patterns of innervation were identified.[1] In the calvaria, however, there has yet to be a comprehensive atlas exploring the innervation patterns of the periosteum and dura mater, specifically focusing on the distinct patterns of the parietal and frontal bones. While nerve mapping studies have previously identified nerve patterns through traditional histological methods, such as 2D immunofluorescence, these studies are limited in their capacity to effectively visualize 3D nerve structures and their interactions with other 3D structures, such as blood vessels. Thus, in this study, we have developed a 3D quantitative atlas of the murine calvaria throughout the complete mouse lifespan, including neonatal P0 to 80 weeks of age. Taken together, these data provide a complex understanding of innervation in intramembranous bones and can act as a baseline for alterations to the nerve microenvironment following disease or injury.

METHODS: We developed Quantitative Light Sheet Microscopy (QLSM) to characterize the murine calvarial vasculature and osteoprogenitors,[2] and used it to map beta-3 tubulin+ (TUBB3+) nerves with CD31+ and Endomucin+ (Emcn+) blood vessels throughout the mouse lifespan. Briefly, P0, 4-wk-old, 12-wk-old, 40-wk-old, and 80-wk-old mice underwent heparinized saline perfusion and calvarial harvest. Following overnight fixation, samples were blocked and whole mount immunostained with primary and secondary antibodies and a streptavidin amplification. Following staining, samples were cleared with 2,2'-thiodiethanol for refractive index matching. Calvaria were imaged with light sheet microscopy using a LaVision Ultramicroscope II. Tiles were converted and stitched using the Imaris Converter and the Imaris Stitcher, Following file conversion and stitching, the QLSM platform results in a complete 3D image of the murine calvaria with up to three fluorescent markers. To generate statistics regarding distribution and spatial association of labeled cell types, we segmented cell types using a Gaussian filter background subtraction to reduce segmentation of background signal. For nerves and blood vessels, the surfaces segmentation object is used, while the spots segmentation object is used for osteoprogenitors and other punctate-like cell types. First, initial segmentations are performed for each of the markers using either the surfaces or spots algorithm in the parietal, coronal, and frontal regions. Next, the image is down sampled by a factor of two to proceed with the remainder of the analysis. For nerves and blood vessels, a binary mask is created based on the initial segmentations. This mask is used to create a secondary segmentation where nerve and blood vessel surfaces are split into 10 μm sections, which allows quantification of the spatial relationships with other cell types. Finally, nerves are segmented into the periosteal and dura mater regions using the machine learning classification algorithm. For each sample, a training data set is selected from each region and the algorithm predicts additional labeling based on internally selected factors. Final statistics can be exported in Excel and imported into a graphing software.

RESULTS: We found that nerves remain distributed throughout all regions of the calvaria for up to 40 weeks and then significantly decrease in all three regions in 80-wk-old samples. From 4-wk-old to 40-wk-old, a network of nerves was still found throughout the periosteum and dura mater and most nerves seemed to originate from the suture regions **(Figure 1A)**. In P0 samples, a network of nerves had not been established and few nerves were found in the parietal region. Most nerves originated from the sides of the calvaria, followed by growth towards the center of both the parietal and frontal bones. In 80-wk-old samples, the network was also not found with few nerves remaining in the parietal bone region. We were still able to visualize nerves in transcortical canals in all ages. When nerve densities were quantified, a significant increase between P0 and all other time points was found (**$p < 0.01$). No significant difference was found between 4-wk-old and 12-wk-old samples, while there was a significant increase at 40 weeks of age (**$p < 0.01$). 80-wk-old samples also demonstrated a decrease compared to 40-wk-old samples (**$p < 0.01$), correlating with the changes in innervation patterns **(Figure 1B)**.

DISCUSSION: We imaged the pan-neural marker, TUBB3, and mapped its expression in the various regions of the calvaria throughout the mouse lifespan. We also expanded on our previous work[1] that mapped the changes in vascular phenotypes with postnatal development by tracking these changes following aging. In 80-wk-old mice, $CD31^{hi}Emcn^{hi}$ vessel fraction decreased as compared to 12-wk-old mice, which is in agreement with previous studies in long bone.[3] These changes are associated with a decrease in remodeling capacity and bone integrity that is associated with aging. For nerves, we found changes in nerve distributions between ages groups, however, as consistent decrease over time was not found. In mice, Chartier et al. identified a decrease in nerve number in the periosteum, but no decrease in nerve density, due to the thinning periosteum with age.[4] In addition, they did not identify any differences with aging in nerves in any other regions of the bone. In human bone, Steverink et al. used multivariate analysis to find a decrease in nerves with aging, which was most significant in the periosteum.[5] Due to the resolution of light sheet imaging, while we can visualize the periosteal region, we cannot accurately quantify changes in its thickness, thus, the decrease in nerve number may not be accounted for through our volume fraction measurements. Further, TUBB3 is a pan-neural marker and stains for both sensory and sympathetic nerve populations, which may not demonstrate the same trends with aging. Therefore, to further clarify the changes in nerves with aging, additional subtype markers should be used to identify the individual roles of nerves in aging. These data will be further analyzed to explore regional changes in nerve densities as well as alterations in nerve tortuosity and directionality. We will further validate our results by applying additional segmentation approaches that use machine learning based segmentation, rather than intensity-based segmentation, and we will quantitatively explore changes in blood vessel phenotypes over the mouse lifespan to determine if a correlation exists between nerve and blood vessel data.

SIGNIFICANCE/CLINICAL RELEVANCE: Using the 3D QLSM method to study neurovascular interactions in bone allows accurate visualization and quantification of nerves and blood vessels in both physiological and pathophysiological conditions. With this platform, we can systematically study the role of nerves in modulating bone growth and development and aging to better develop functional therapies that target bone healing and disease mechanisms.

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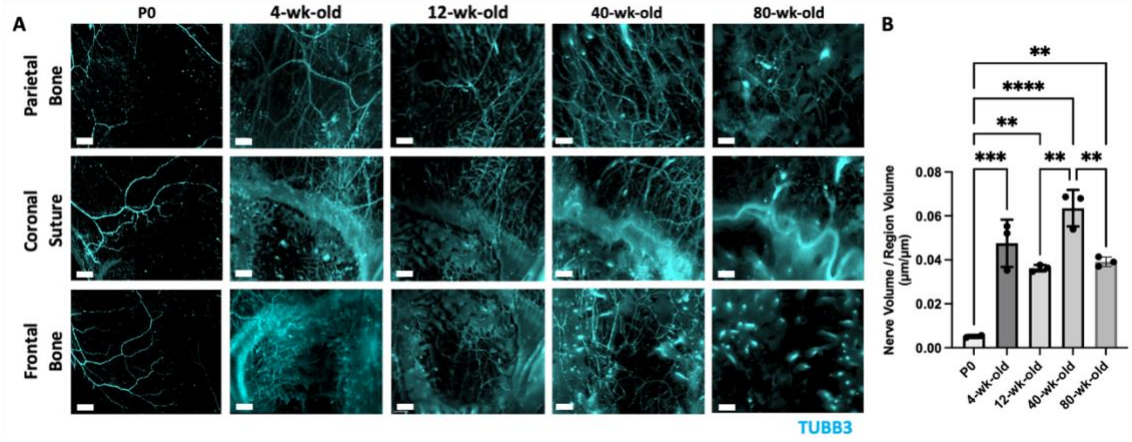

Figure 1: Changes in calvarial nerves throughout murine postnatal development and aging. A) Maximum intensity project of TUBB3+ (blue) nerves in P0, 4-wk-old, 12-wk-old, 40-wk-old, and 80-wk-old murine calvaria imaged via QLSM. Scale bar is 300 μm. B) TUBB3+ nerve volume fraction (μm/μm) quantification for all ages. Statistics calculated with a one-way ANOVA with multiple comparisons. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.