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Bone Up: Craniomandibular Development and Hard-Tissue Biomineralization in Neonate Mice

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Abstract

The presence of regional variation in the osteogenic abilities of cranial bones underscores the fact that the mechanobiology of the mammalian skull is more complex than previously recognized. However, the relationship between patterns of cranial bone formation and biomineralization remains incompletely understood. In four strains of mice, micro-computed tomography was used to measure tissue mineral density during perinatal development in three skull regions (calvarium, basicranium, mandible) noted for variation in loading environment, embryological origin, and ossification mode. Biomineralization levels increased during perinatal ontogeny in the mandible and calvarium, but did not increase in the basicranium. Tissue mineral density levels also varied intracranially, with the mandible being greatest, the basicranium intermediate, and the calvarium lowest. Perinatal increases in, and elevated levels of, mandibular biomineralization appear related to the impending postweaning need to resist elevated masticatory stresses. Similarly, perinatal increases in calvarial biomineralization may be linked to ongoing brain expansion, which is known to stimulate sutural bone formation in this region. The lack of perinatal increase in basicranial biomineralization could be a result of earlier developmental maturity in the cranial base relative to other skull regions due to its role in supporting the brain's mass throughout ontogeny. These results suggest that biomineralization levels and age-related trajectories throughout the skull are influenced by the functional environment and ontogenetic processes affecting each region, e.g., onset of masticatory loads in the mandible, whereas variation in embryology and ossification mode may only have secondary effects on patterns of biomineralization. Knowledge of perinatal variation in tissue mineral density, and of normal cranial bone formation early in development, may benefit clinical therapies aiming to correct developmental defects and traumatic injuries in the skull, and more generally characterize loading environments and skeletal adaptations in mammals

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by highlighting the need for multi-level analyses for evaluating functional performance of cranial bone.

Keywords

biomineralization; mammalian cranial bone; perinatal ontogeny; intracranial variation

1. Introduction

Recent studies of the developing mammalian skull suggest that the mechanobiology of cranial cortical bone is more complex than previously recognized. This discovery contrasts with prior notions that bone throughout the skeleton generally has similar peak-strain magnitudes during forceful activities (Biewener, 1982, 1993; Rubin and Lanyon, 1984; Lanyon and Rubin, 1985). For instance, circumorbital and calvarial strains during biting and chewing are lower than along the mandible and other masticatory structures (Hylander et al., 1991; Hylander and Johnson, 1997; Herring and Teng, 2000; Ravosa et al., 2000, 2010). Also, while mammalian jaw and limb elements exhibit marked osteogenic responses in terms of initiating processes of resorption and formation of bone tissue under altered loading conditions, calvarial, circumorbital and basicranial hard tissues are minimally responsive to similar changes in loading stimuli (Bouvier and Hylander, 1981, 1996; Biewener et al., 1986; Rawlinson et al., 1995; Franks et al., 2017). Such regional differences in mechanosensitivity may indicate that non-masticatory regions of the skull may be under greater genetic control than elsewhere in the skeleton to ensure sufficient bone exists to resist traumatic loads that could injure the underlying brain (Hylander et al., 1991; Rawlinson et al., 1995; Hylander and Johnson, 1997; Ravosa et al., 2000). Alternatively, lack of proximity of the calvarium and basicranium to the masticatory apparatus may also explain lower responsiveness to masticatory loading, as chewing stresses may differentially affect cranial elements that are more directly involved in feeding behaviors, such as the maxilla and mandible (Franks et al., 2017).

Research on cranial osteoblasts has further revealed the presence of comparable complexity at the cellular level. Specifically, differences in embryological origin (neural crest vs. paraxial mesoderm) and mode of ossification (intramembranous vs. endochondral) among cranial sites have been implicated as factors in intracranial variation of osteoblast mechanobiology. For example, osteoprogenitor cells from the neural crest-derived frontal bone have higher rates of proliferation and differentiation as well as lower rates of apoptosis than osteoblast precursor cells from the paraxial mesoderm-derived parietal bone (Quarto et al., 2010; Li et al., 2013). Likewise, Rawlinson et al. (1995) have demonstrated that osteoblasts from the intramembranous calvarium exhibit unique osteogenic behavior compared to cells obtained from long bones, which ossify endochondrally. Furthermore, differences in bone matrix production between intramembranous and endochondral grafts (Wong and Rabie, 1999) suggest that site-specific patterns of bone formation may depend on a cranial bone's ossification mode.

Despite growing appreciation of complexity among cranial bones, the relationship between cranial osteogenesis and hard-tissue biomineralization has yet to be fully characterized. Biomineralization, or tissue mineral density, refers to an organism's production of minerals to fortify its own biological tissues, such as bone, to confer resistance against compressive forces (Burger et al., 1992; Beniash, 2011). Postnatal cranial bone biomineralization has rarely been quantified in mammals, though some studies have quantified tissue mineral density of skull bones as a function of altered loading in growing animals (Ravosa et al., 2007a,b; Franks et al., 2017). While cortical bone mineralization patterns have been previously evaluated in mouse parietal bone during perinatal ontogeny (Tarnowski et al., 2002), no such assessments of cortical tissue mineral density during early development exist for other cranial bones. Although prior research has evaluated mandibular bone formation during postnatal development (Martinez-Maza et al., 2012), it is unknown how matrix mineralization relates with trends of bone deposition in this region, particularly in pre-weaning stages before the ontogenetic shift to adult feeding behavior influences tissue mineral density patterns (Ravosa et al., 2007a,b; Franks et al., 2017). Studies have also shown that bone formation in the posterior basicranium is notably delayed relative to the rest of the skull during postnatal development (Jeffery and Spoor, 2002), but the corresponding lack of insight into basicranial biomineralization patterns highlights a gap in our understanding of the cranial base's role in coordinating skull ontogeny and supporting the growing brain (Opperman, 2000; Jeffery and Spoor, 2002; Nie, 2006). Additionally, while it is known that neurogenesis stimulates new bone growth in the developing skull (Opperman, 2000; Nyugen et al., 2012), it is unclear if tissue mineral density patterns differ between the two calvarial bones (frontal and parietal) as do *in vitro* osteogenic potentials (Quarto et al., 2010; Li et al., 2013) since only parietal biomineralization has been characterized (Tarnowski et al., 2002). Furthermore, as biomineralization patterns are seldom compared among skull sites, it has been difficult to evaluate the respective roles of functional and developmental factors underlying intracranial variation in bone formation among mammals (Ravosa et al., 2016).

To this end, we analyzed and compared cranial tissue mineral density in mice throughout the first two postnatal weeks in three skull regions (calvarium, basicranium, mandible) noted for variation in loading environment, embryological origin, and ossification mode (Hylander et al., 1991; Hylander and Johnson, 1997; Ravosa et al., 2000; Tortelli et al., 2010; Quarto et al., 2010; Li et al., 2013). These data will provide further insight into differences in developmental and adaptive patterns among the calvarium, basicranium and mandible, complementing recent research on diet-induced plasticity in the skull (Franks et al., 2017; Menegaz and Ravosa, 2017). It is hypothesized that craniomandibular biomineralization will increase across all three regions during perinatal development as new bone is deposited at each site, given that cortical bone mineralization has been shown to increase following bone deposition in young animals elsewhere in the body (Boivin and Meunier, 2003; Bala et al., 2010). Additionally, tissue mineral density patterns will be expected to vary intracranially among the calvarium, basicranium, and mandible, with mandibular biomineralization predicted to be greater than that of the two non-masticatory regions (calvarium, basicranium). Such elevated mandibular tissue mineral density is anticipated due to higher strains and load-induced osteogenic responses in the postweaning mammalian jaw (cf.,

Bouvier and Hylander, 1981, 1996; Hylander et al., 1991; Hylander and Johnson, 1997; Ravosa et al., 2007a,b).

Findings from this study will provide insight into age-related and regional patterns of hard-tissue biomineralization of cranial bones throughout the skull, in addition to further evaluating potential effects of differing embryological origins and ossification modes on bone development. Such information is important for understanding the relationship between biomineralization and the functional properties of cranial bones in living and fossil mammals (Ravosa et al., 2007a,b, 2016; Scott et al., 2014a, b) and may provide further context for the use of *in silico* models to study morphological variation. Additionally, increased knowledge of biomineralization patterns across the mammalian skull may aid experimental approaches that seek to mimic natural processes of bone growth in different sites during craniofacial development for clinical treatment of skeletal defects and pathologies.

2. Materials and Methods

2.1 Animal Model

Mice were selected as a model organism because they have comparable skull elements to other mammals, such as humans. All mouse specimens used herein were obtained opportunistically from breeding colonies at the Freimann Life Sciences Center at Notre Dame. Pups that were not needed for further breeding or analyses by other research groups were euthanized and then collected immediately for use in this study. Thus, the mice were not manipulated in any way prior to dissection and were not part of any experiment. All animal care and euthanasia followed guidelines approved by the University of Notre Dame Institutional Animal Care and Use Committee. One hundred (N=100) total mice from four strains (EPCR, SPHK1, S₁P₁-lox, HPC) were analyzed, with five mice per five neonate age groups (N1, N3, N5, N9, N13; N1—postnatal day 1) sampled for each of the four strains (n=25 per strain; n=20 per age group overall). The four strains were used as a means to independently test hypotheses about postnatal patterns of biomineralization throughout the skull.

The five neonatal age groups (N1, N3, N5, N9, N13) were selected in order to study changes in cranial growth and biomineralization throughout the mouse perinatal period. N1, N3, and N5 mice all display similarly underdeveloped ossification (Holzgraefe and Spoerri, 1978). N9 mice represent a later stage of cranial growth and ossification comparable to that of previously described N10 mice (Holzgraefe and Spoerri, 1978). N13 mice represent the latest ossification stage available in our postnatal samples, where the calvarial bones begin to draw almost completely together (Holzgraefe and Spoerri, 1978; Bradley et al., 1996). This murine age is comparable to the human age of approximately 18 months old—about the time that the metopic suture closes along the frontal bone (Opperman, 2000).

Strains were selected based primarily on whether they contained sufficient sample sizes for each age group to facilitate the assessment of perinatal and craniomandibular variation in biomineralization. All of these strains exhibit genetic modifications that affect the vascular endothelium of the animals during embryonic development. EPCR (endothelial cell protein

C) mice have disrupted EPCR genes that normally augment activation of protein C—which prevents blood clots—in endothelial cells that form the embryonic placenta (Gu et al., 2002). SPHK1 strains involve modifications to the protein coding gene *SPHK1*, which affects cell proliferation and apoptosis in various organs (Shida et al., 2008). S₁P₁-lox strains have defects in S₁P₁ receptor genes, which interrupt proliferation of endothelial cells and their differentiation into vascular structures (Lee et al., 1999). HPC mice have been treated with transplanted hematopoietic progenitor cells (HPCs), which are used to promote reconstitution of blood cells in bone marrow, kidneys, or the peripheral blood stream (Levesque et al., 2004). Though previous studies have indicated effects of genetic variation on limb bone morphology in mice (Li et al., 2005; Jepsen, 2009; Wallace et al., 2009; Wallace et al., 2010), none of the transgenic changes represented in our mice are known to influence changes in skeletal growth and form. As such, we did not expect any strain-related differences in cranial biomineralization. Hereon, the strains will be referred to numerically (EPCR—Strain 1; Sphk1—Strain 2; S₁P₁-lox—Strain 3, HPC—Strain 4).

2.2 Samples

Calvarial (frontal and parietal), basicranial (basisphenoid and basioccipital), and mandibular regions were surgically isolated from each specimen for study (Figure 1). After dissection, these specimens were then fixed in 10% formalin solution and stored at room temperature until imaged via microcomputed tomography (micro-CT). These cranial regions were chosen for study because they exhibit variation in embryological origin, ossification mode, and loading environment (Figure 1). Frontal and mandibular bones are derived from neural crest cells. Parietal and basicranial bones are derived from paraxial mesoderm. The basicranium is derived from neural crest and, mostly, paraxial mesoderm. Calvarial and mandibular bones experience intramembranous ossification, while the basicranium primarily undergoes endochondral ossification, particularly in the posterior portion studied herein. Lastly, in relation to other skull elements examined, the mandible experiences the highest peak strain levels during biting and chewing.

2.3 Measurements

Each cranial region (calvarium, basicranium, mandible) was imaged using micro-CT (Albira Micro-CT, Notre Dame Integrated Imaging Facility). Micro-CT settings, as well as materials used to support samples while scanning, were held constant during each scan. All scans were performed at high dose (400 μ A) and high voltage (45 kVP) with 600 projections and 125 μ m voxel size. Voxel attenuation was calibrated to the Hounsfield scale, which ranges from -1000 (density of air) to 3000 (density of cortical bone). Because soft tissues were removed during sample preparation, leaving only bony tissue, no threshold techniques were applied to scans to eliminate background or illuminate specific ranges of attenuation, such as filtering out soft tissues. Within each of the three aforementioned cranial regions, two bone sites (6 sites overall) were selected for biomineralization analysis: frontal and parietal (calvarium); basisphenoid and basioccipital (basicranium); and lateral and medial mandible (mandible). Cranial images were reconstructed using PMOD 3.3 (PMOD Technologies Ltd, Zurich, Switzerland). All images were measured and analyzed while oriented in the coronal plane, though sagittal and transverse planes were used to optimally locate regions of interest within the coronal plane in preparation for measurement. Once the image was oriented

properly, volumes of interest (VOIs) were drawn at the desired location of each bone (Figure 2); only non-sutural areas of cortical bone were measured. Attenuation values of pixels contained within each VOI were averaged together to yield mean biomineralization for each site. VOIs were not standardized to include the same number of pixels due to the varying dimensions and thicknesses of each bone site of interest, but all VOIs contained 3 pixels (calvarial VOIs—3 pixels; basicranial and mandibular VOIs—6 pixels). However, preliminary analyses indicated that averaging fewer pixels per VOI in the calvarium did not result in greater standard deviations among calvarial pixels than were observed within mandibular or basicranial VOIs. As variation in pixel attenuation within all VOIs were similar, we determined that our VOI methods adequately represented variation in biomineralization within cranial cortical bone among all sites measured despite variable numbers of pixels among VOIs. Frontal and parietal bone biomineralization was measured between the midline and the lateral border (left to right) and midway between the anterior and posterior borders of their respective bones in the coronal plane (Figure 2D). While calvarial bones typically contain an intervening layer of trabecular bone (diploe) between cortical bone layers later in ontogeny, as do other mammals (Tubbs et al., 2012), no such layer of diploe was observed in the frontal and parietal bones of these specimens, suggesting that measurements are not confounded by the presence of diploe. Basisphenoid and basioccipital biomineralization was measured at the midpoint of their respective left and right wings and midway between their respective anterior and posterior borders (Figure 2E). Mandibular biomineralization was measured at the midpoint between the alveolar margin of the first tooth and the ventral border of the mandible on the buccal (lateral) and lingual (medial) surfaces of the mandible (Figure 2F). Left and right measurements were taken for each bone and averaged to represent a single datum for that site; in the case of the basicranium, the left and right wings of each bone were averaged together. Biomineralization, or tissue mineral density, at all six bone sites was measured in dimensionless Hounsfield units (HU: -1000–3000) via the PMOD program (Lang, 2010). All values for Hounsfield units were obtained by averaging attenuation values across all of the pixels contained within each VOI. Partial volume effects (i.e., lower attenuation values due to inclusion of non-skeletal pixels) were eliminated by increasing magnification of images and adjusting brightness/contrast ratios to better illuminate cortical bone. These calibrations did not alter attenuation values of pixels and facilitated selection of VOIs that included only cortical bone, as VOIs located too near the surface of bone resulted in outliers (standard deviations among pixels greater than 100 HU) through averaging of cortical bone and background pixels (air: -1000 HU). By contrast, pixels within VOIs located in the center of cortical bone layers differed by less than 100 HU, for which reason 100 HU was selected as the threshold for acceptable standard deviations within VOIs. VOIs for which standard deviations among pixels were greater than 100 HU were subsequently re-drawn to exclude affected pixels.

2.4 Statistical Analysis

Perinatal variation in biomineralization was assessed via correlations for least-squares bivariate regression lines ($\alpha=0.10$) comparing Hounsfield units across age group means (N1, N3, N5, N9, N13) at each of the six cranial sites in each of the four strains. Due to limited sample sizes, the threshold for significance $\alpha=0.10$ was used in order to more clearly

identify age-related changes in craniomandibular biomineralization that may otherwise have been overlooked using a more stringent threshold ($\alpha=0.05$). Though this convention was mainly intended for bivariate regressions evaluating changes in biomineralization with age for each site within each strain, the $\alpha=0.10$ threshold was also applied to analyses of within- and among-region variation to maintain continuity of statistical methods throughout the study. Intracranial variation in biomineralization was assessed in each of the four strains via pairwise comparisons of perinatal regression lines for the biomineralization of bone regions and sites against one another, e.g., N1–N13 Strain 1 frontal vs. N1–N13 Strain 1 parietal (ANCOVA, $\alpha=0.10$), coupled with visual examination of the data scatter. First, regression lines for sites from the same cranial region, e.g., frontal and parietal (calvarium), were compared to one another to assess whether ontogenetic variation in biomineralization levels differs. If within-region variation was not found (i.e., slopes and intercepts for regression lines did not differ significantly), then perinatal data from similar regions were pooled and compared to pooled data of other regions (e.g., N1–N13 Strain 1 calvarium vs. N1–N13 Strain 1 mandible). If perinatal regression lines differed significantly between sites within a region, then regression lines for each cranial site were compared to one another individually (e.g., N1–N13 Strain 1 frontal vs. N1–N13 Strain 1 lateral mandible) to assess site-specific differences in perinatal mineralization. Hierarchical relationships (greater than/less than) between cranial regions or sites were assessed by comparing regression values for individual lines and by visual inspection of the bivariate plots.

3. Results

3.1 Age-related Variation

Mean biomineralization levels increased significantly with age for seven of eight calvarial measurements (Table 1; Figure 3). Frontal tissue mineral density increased significantly in all four strains, and mineralization of the parietal bone increased in three of four strains. Similar age-related increases in mean biomineralization levels were noted in six of eight mandibular sites (Table 1; Figure 3). Mineralization in the lateral mandible site increased with age in all four strains, while tissue mineral density of the medial mandible site increased in only two of four strains (Strain 1, Strain 4). In contrast, basicranial sites exhibited no significant changes in biomineralization with age for any of the four strains (Table 1; Figure 3). The use of $\alpha=0.10$ as a threshold for significance identified two comparisons in calvarial sites and one in mandibular sites that would have been non-significant at $\alpha=0.05$.

3.2 Regional Variation

In all four strains, biomineralization patterns for the lateral and medial mandible were similar across ages. Frontal and parietal mineralization patterns were also coincidental, as were perinatal trajectories for the basisphenoid and basioccipital sites (Table 2; Figure 3). Calvarial mineralization patterns were consistently transposed below mineralization trajectories for the basicranium and mandible at a given age in all four strains (Table 3; Figure 3). Perinatal biomineralization patterns for the mandible and basicranium also differed significantly in each strain. For mice in Strains 1, 3, and 4, mandibular trajectories were more notably positioned above those of the basicranium in older animals than in

younger specimens. In Strain 2 mice, mandibular mineralization trajectories were more elevated relative to basicranial patterns earlier in perinatal ontogeny than at later ages (Table 3; Figure 3). Neither within-region comparisons nor among-region comparisons of biomineralization patterns were affected by the consistent use of $\alpha=0.10$ as a threshold for significance. No within-comparisons were significant at $p<0.10$ (Table 2), while all among-region comparisons were significant at $p<0.05$ (Table 3).

4. Discussion

This study addressed the incomplete understanding of the relationship between non-sutural cranial bone formation and matrix mineralization in the mammalian skull by evaluating perinatal craniomandibular biomineralization patterns in three cranial regions of interest (calvarium, basicranium, mandible). Additionally, comparisons of the tissue mineral density patterns among these cranial regions evaluated potential intracranial variation in biomineralization. Measured attenuation values revealed that mandibular and calvarial biomineralization trajectories increased during perinatal ontogeny while basicranial patterns surprisingly demonstrated no change. Furthermore, biomineralization levels varied regionally throughout the skull. Mandibular biomineralization was greatest among the three cranial regions during the perinatal period, especially in later stages, followed by intermediate values in the basicranium and markedly lower tissue mineral density in the calvarium. These data suggest continued elevation of the mandible's biomineralization relative to the other cranial regions during development.

4.1 Mandible

Age-related increases in, and high levels of, perinatal mandibular biomineralization found herein could be indicative of the developing jaw's eventual need to resist elevated masticatory stresses later in ontogeny (Ravosa et al., 2007b). Previous research on mouse cranial development shows that mandibular bone formation is elevated in the first 3–4 postnatal weeks prior to weaning (Martinez-Maza et al., 2012), which parallels increasing perinatal biomineralization trajectories observed in this study. Interestingly, though such elevations in tissue mineral density and bone formation occur before the transition toward adult-like masticatory behaviors, previous studies indicated that mammals have demonstrated the ability to process mechanically challenging food items prior to the onset of weaning (Herring, 1985; Huang et al., 1994). Given that mandibular strains are the highest experienced along the mammalian skull (Hylander et al., 1991; Ravosa et al., 2000, 2010; Ross and Metzger, 2004; Franks et al., 2016), increases in bone formation and biomineralization, which are implicated in resisting elevated compressive loads (Ravosa et al., 2007b; Pal, 2014) in the pre-weaning jaw, may be attributed to the ongoing functional shift associated with weaning. Indeed, lower amounts of tissue mineral density in the calvarium and basicranium vs. the mandible are likely related to both rapid postnatal bone growth and higher-magnitude loads related to feeding in the oral cavity (Ravosa et al., 2007b; Martinez-Maza et al., 2012; Franks et al., 2017).

4.2 Calvarium

Increases in biomineralization trajectories of calvarial bone coincide with bone formation in the calvarium during the perinatal period, both of which may be stimulated by neurogenesis (Opperman, 2000; Henderson et al., 2004; Nguyen et al., 2012). Brain expansion—which occurs until postnatal week 10 in mice (Nguyen et al., 2012)—induces new bone deposition at calvarial sutures (Opperman, 2000), which continually relocates excess osteoblasts into non-sutural regions where they mature and secrete bone matrix (Morriss-Kay and Wilkie, 2005). Formation of mature bone in the non-sutural calvarium as a consequence of brain growth may, therefore, drive the increased calvarial biomineralization observed in mice throughout the perinatal period. Additionally, low biomineralization levels found in the calvarium relative to the basicranium may be a result of more advanced development in the posterior basicranium (see also below), which matures earlier in ontogeny than much of the cranium (Jeffrey and Spoor, 2002; Nie, 2006).

4.3 Basicranium

The lack of increase in basicranial biomineralization in perinatal mice may be related to an earlier onset of developmental maturity and mineralized matrix formation than in other cranial regions, a pattern noted in humans (Nie, 2006). Our data for non-sutural basicranial bone are consistent with prior observations that mineralization rates are higher at the spheno-occipital synchondrosis, the main bone growth center from which the basisphenoid and basioccipital are formed, early in neonatal mouse ontogeny (day 0–2) compared to days 10–15 at the same site (Wealthall and Herring, 2006). These findings also agree with observations that the posterior cranial base, consisting mainly of the basisphenoid and basioccipital, exhibits slower postnatal growth relative to other bones of the skull, including the anterior basicranium (Jeffrey and Spoor, 2002; Nie, 2006). In contrast, the posterior basicranium grows at an increased rate, relative to the anterior portion, prior to birth and reaches adult size sooner than the facial skeleton, which grows more rapidly postnatally than the cranial base (Dobbing and Sands, 1979; Bulygina et al., 2006). This early structural development of the posterior basicranium, as well as elevated perinatal mineralization in the basicranium relative to the calvarium, may occur due to the cranial base's role in supporting the enlarging brain, which is known to markedly increase in mass just prior to birth in humans (Dobbing and Sands, 1979). Basicranial tissues are known to be responsive to altered loads (Tang and Mao, 2006). Assuming that low-magnitude loads drive mineralized matrix formation in the cranial base as in the calvarium (Opperman, 2000), such early brain growth may explain biomineralization levels in the basicranium being comparable to the mandible early in perinatal development prior to the jaw's later period of accelerated growth (Martinez-Maza et al., 2012).

4.4 Embryological Origin

Contrary to prior evidence regarding osteogenic differences between the frontal and parietal bones (Quarto et al., 2010; Li et al., 2013), our study shows that regional variation in biomineralization patterns may only be partially related to differences in embryological origin among cranial bones. Though mandibular (neural crest) biomineralization was greater than that of the basicranium (paraxial mesoderm), the lack of disparity between the frontal

(neural crest) and parietal (paraxial mesoderm) bones, as well as elevated posterior basicranial biomineralization relative to the frontal bone, indicate that neural crest-derived bones did not consistently have greater tissue mineral density than paraxial mesoderm-derived sites. It is important to note, however, that the reported disparate behavior of neural crest and paraxial mesoderm calvarial cells addressed the osteogenic and mineralization potential of osteoblasts rather than hard-tissue mineralization of fully formed bone. It is therefore possible that the behavior of cells in culture may not completely track larger-scale processes *in vivo*. Additionally, the lack of statistically significant differences found between frontal and parietal biomineralization may not account for small qualitative disparities in osteogenic and matrix mineralization potential that have been noted between frontal and parietal bone cells (Quarto et al., 2010; Li et al., 2013). Interestingly, however, tissue mineral density comparisons of bone sites derived from the same embryological origin showed that, while trajectories of paraxial mesoderm bones displayed an inconsistent pattern, i.e., parietal biomineralization increased perinatally while basicranial sites did not, trajectories of both neural crest-derived bones increased during the perinatal period. Therefore, though embryological origin does not fully account for regional variation of biomineralization trajectories during perinatal development, bones of neural crest origin may generally have similar matrix mineralization trends, suggesting a partial effect of embryology on biomineralization patterns in the developing skull.

4.5 Ossification Mode

While embryological origin alone appears not to fully account for intracranial variation in cranial tissue mineral density patterns, these data suggest that biomineralization differences among cranial bones may track ossification mode more completely. Perinatal biomineralization patterns for intramembranous sites increased in this study while tissue mineral density trajectories of the endochondral basicranium exhibited no such increases, suggesting that ossification mode has an effect on the trajectory of biomineralization patterns in the skull. However, it must be addressed that the two bone sites representing endochondral ossification, the basisphenoid and basioccipital, share a common growth site. As such, the two basicranial bones, in addition to forming via the same ossification mode, also experience similar functional demands, thereby making it difficult to distinguish whether loading environment or ossification primarily influences the comparable biomineralization trajectories of endochondral sites. On the other hand, among-region similarities between mandibular and calvarial matrix mineralization trends, which differ in loading patterns but both ossify intramembranously, more clearly suggest a relationship between ossification mode and tissue mineral density. Thus, though preliminary evidence suggests that ossification mode may track regional variation in cranial biomineralization patterns more so than embryological origin, the incorporation of more endochondral sites, such as the ethmoid bone of the skull, into this study may be necessary to further distinguish between effects of functional environment and ossification on tissue mineral density trajectories.

4.6 Implications

Viewed more broadly, perinatal changes in bone ultrastructure, such as the degree of biomineralization, appears to be strongly influenced by the functional environment and

ontogenetic patterns associated with each cranial site, e.g., onset of masticatory loads in the mandible, embryology or ossification mode may only partially affect intracranial patterns of tissue mineral density. Each cranial region addressed herein experiences mechanical loading related to its unique function in the skull—masticatory forces in the mandible, loads from supporting the brain's mass in the basicranium, and brain expansion in the calvarium (Dobbing and Sands, 1979; Opperman, 2000; Ravosa et al., 2007b, 2010; Martinez-Maza et al., 2012; Nguyen et al., 2012). Previous work has demonstrated a relationship between biomineralization and stiffness of bone for resisting elevated stresses (Kohn et al., 2009; Wallace et al., 2009; Pal, 2014), suggesting that hard-tissue biomineralization may be instrumental to maintaining structural integrity of cranial bone during development (Ravosa et al., 2007b, 2016). This connection between biomineralization and skeletal performance also re-emphasizes the importance of studying multiple levels of bone hierarchy and structure when assessing functional adaptations of bone (Franks et al., 2016, 2017). Prior studies of dietary plasticity have found that internal bone architecture, such as cortical bone thickness and biomineralization, display greater load-induced plasticity than external bone measurements (Ravosa et al., 2007b, 2016). As such, inferences based solely on comparisons of external bony dimensions can underestimate functional signals by not accounting for cortical bone distribution and tissue properties, implying that multiple levels of analysis are needed to more precisely evaluate skeletal performance of cranial bone.

Better understanding of age-related and regional variation in biomineralization may also aid future studies that seek to more accurately characterize loading environments in living and fossil mammals and connect morphological adaptations to functional behaviors (Ravosa et al., 2007b,c, 2016; Scott et al., 2014b). Our findings likewise have implications for the use of *in silico* models, such as finite element analysis (FEA), for inferring skull function, especially in extinct animals for which *in vivo* data cannot be obtained. As biomineralization varies within and between masticatory elements and this can affect tissue properties and skeletal performance (Ravosa et al., 2007a–c, 2016; Franks et al., 2017), this challenges FEA model assumptions that cranial tissue is homogenous (Dumont et al., 2005; Strait et al., 2009; Young et al., 2012; Gill et al., 2014). The data presented herein show that biomineralization varies among three distinct skull regions as well as between masticatory and non-masticatory elements, further demonstrating the heterogeneity of cranial elements. This intracranial variation in tissue ultrastructure provides additional evidence that FEA modeling as currently applied is inadequate as a sole means of assessing functional dynamics of the skull. Arguably, a multi-leveled approach for evaluating bone function and structure is necessary for analyzing the role of gross dimensions and tissue properties on skeletal performance.

In addition to providing more complete understanding of cranial hard-tissue biomineralization patterns, our study has implications for characterizing normal developmental processes in the mammalian skull, which may benefit functional and translational studies of cranial growth. Quantitative description of cranial tissue mineral density patterns may provide a context for regulating clinical therapies aimed at restoring natural cranial matrix mineralization that have been altered by bone disease. For example, the characterization of basicranial biomineralization trajectories may better inform treatments for disorders such as chondrodystrophy, which is caused by underexpression of

parathyroid hormone-related protein (PTHrP) and leads to irregular increases in mineralized matrix formation in both the sutural and non-sutural basicranium in neonate mice (Karaplis et al, 1994; Philbrick et al., 1998; Martin, 2016). While abnormally elevated basicranial matrix mineralization in chondrodystrophic mice can be corrected by restoring normal PTHrP expression levels, excessive corrective treatment has the capability to cause PTHrP overexpression, which leads to decreases in cranial base tissue mineral density (Weir et al., 1996; Philbrick et al., 1998). This study's quantitative observations of normal perinatal biomineralization patterns could, therefore, be potentially useful in understanding how to regulate corrective therapy for bone disorders such as chondrodystrophy and maintain appropriate development and function of cranial bones.

Moreover, as cellular behavior is impacted by the mechanical properties of the microenvironment (Duncan and Turner, 1995), an understanding of intracranial variation in biomineralization is important for the choice of scaffold design and the source of tissue implants. Previous studies of craniofacial scaffold design have noted that their mechanical properties should mirror those of native tissue as closely as possible in order to ensure proper integration of the engineered transplant into the host tissue (Hutmacher, 2000). As such, increased understanding of biomineralization in multiple cranial regions demonstrated by this research may facilitate engineering of biomimetic tissues that more precisely model the complex structure of craniofacial bone across the skull. Additionally, these data also provide a broader understanding of cranial bone formation during early ontogenetic stages, which may aid tissue engineering research in pediatric patients. Unlike in adult animals, scaffolds implanted into young patients must be able to adapt and remodel to accommodate increases in bone formation and a changing physiological environment throughout development (Patel and Fisher, 2008), making pediatric tissue engineering particularly challenging. Our quantitative assessments of longitudinal changes in bone microenvironment during perinatal ontogeny may be of use for more accurate engineering of scaffolds that better adjust to growth processes in non-adult patients, thereby improving methods of pediatric tissue reconstruction.

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Highlights

- Perinatal increases in biomineralization are not uniform throughout the mouse skull.
- Tissue mineral density levels vary intracranially in mice.
- Functional role of bone sites is linked to regional variation in biomineralization.
- Embryology and ossification mode may also partially affect tissue mineral density.
- Knowledge of normal cranial biomineralization patterns may aid clinical therapies.

Figure 1. Illustration of regional complexity in craniomandibular bone formation, including loading environment, embryological origin, and ossification mode of isolated sites measured for this study.

Figure 2.

Micro-CT images, photographs, and outlines of six isolated skull bones from N9 mouse.

Left column: Micro-CT scans of craniomandibular sites of interest. Blue and green boxes indicate volumes of interest (VOIs) selected for analysis of tissue attenuation. Insets depict magnified illustrations of VOIs drawn and bone sites. Axes in top panel indicate orientation (left-right, dorsal-ventral), which is the same for all the images. *Center column (panels A–C):* photographs of calvarium (A), basicranium (B), and mandible (C). Red boxes indicate bone sites of interest. Green lines bisecting all images represent the midline. Yellow lines indicate the lateral border of cranial bones; two yellow lines are included for the basicranium as both left and right sides are included in isolated basicranial depiction. Axes in top panel indicate orientation (left-right, anterior-posterior), which is the same for all the images.

Right column (panels D–F): isolated outlines of calvarium (D), basicranium (E), and mandible (F). Midline and lateral border labels same as for photographs. Boxes indicate approximate location of VOI measurements.

Figure 3.

Bivariate plots of age-related changes in biomineralization for all six cranial sites of interest in each mouse strain. Figure legend is representative of all four panels. Biomineralization is represented as “HU,” a value for attenuation based on the Hounsfield scale (-1000–3000 HU).

Table 1

Least-squares bivariate regression statistics describing perinatal variation in tissue mineral density for each cranial site and strain.^a

Strain 1 Site vs. Age (n=5)	R	Y-intercept	Slope	Std. Error	Strain 2 Site vs. Age (n=5)	R	Y-intercept	Slope	Std. Error
Frontal	0.90**	-967.79	46.70	13.39	Frontal	0.85*	-709.49	22.48	8.03
Parietal	0.93**	-993.15	60.25	13.37	Parietal	NS	-739.93	16.12	7.57
Basisphenoid	NS	169.99	8.10	7.48	Basisphenoid	NS	185.99	10.86	9.63
Basioccipital	NS	214.41	13.62	9.54	Basioccipital	NS	231.43	11.30	12.08
Lateral	0.82*	230.25	17.82	7.30	Lateral	0.95**	302.47	10.64	2.03
Mandible					Mandible				
Medial Mandible	0.88**	239.52	21.91	6.81	Medial Mandible	NS	406.46	-6.42	6.90

Strain 3 Site vs. Age (n=5)	R	Y-intercept	Slope	Std. Error	Strain 4 Site vs. Age (n=5)	R	Y-intercept	Slope	Std. Error
Frontal	0.88**	-870.02	48.89	15.27	Frontal	0.84*	-762.54	18.59	6.98
Parietal	0.96***	-894.65	51.07	8.07	Parietal	0.94**	-755.68	22.71	4.81
Basisphenoid	NS	261.41	3.24	7.96	Basisphenoid	NS	223.55	-4.70	6.50
Basioccipital	NS	239.49	3.30	4.20	Basioccipital	NS	275.86	-2.18	4.70
Lateral Mandible	0.97***	187.45	28.20	4.36	Lateral Mandible	0.88**	168.62	33.14	10.33
Medial Mandible	NS	273.98	26.38	13.98	Medial Mandible	0.93**	169.80	32.01	7.48

^a Asterisks denote significance level: *p<0.10; **p<0.05; ***p<0.01; NS-not significant (p>0.10)

Table 2

Within-region ANCOVAs of perinatal biomineralization regression lines for each strain.^{a,b}

Comparison	Strain 1	Strain 2	Strain 3	Strain 4
Frontal vs. parietal	NS	NS	NS	NS
Basisphenoid vs. basioccipital	NS	NS	NS	NS
Lateral vs. medial mandible	NS	NS	NS	NS

^aNS—not significant (p>0.10)

^bNote that lack of intraregional differences supports use of single regression lines for each region when comparing biomineralization trajectories among cranial regions.

Table 3

Between-region ANCOVAs of perinatal biomineralization regression lines for each strain.^{a–e}

Comparison	Strain 1	Strain 2	Strain 3	Strain 4
Calvarium vs Basicranium	C<B***	C<B***	C<B***	C<B***
Calvarium vs. Mandible	C<M***	C<M**	C<M***	C<M***
Basicranium vs. Mandible	B<M***	B<M**	B<M***	B<M***

^a Asterisks denote significance level: *p<0.10; **p<0.05; ***p<0.01

^b “>”, line for first region/site transposed above second region/site at a given age

^c “<”, line for first region/site transposed below second region/site at a given age

^d C—calvarium; B—basicranium; M—mandible

^e Note that slope differences between the basicranium and mandible, rather than intercept differences, are responsible for the transpositions observed in these regions.