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Thyroxine Exposure Effects on the Cranial Base

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Abstract

Thyroid hormone is important for skull bone growth, which primarily occurs at the cranial sutures and synchondroses. Thyroid hormones regulate metabolism and act in all stages of cartilage and bone development and maintenance by interacting with growth hormone and regulating insulin-like growth factor. Aberrant thyroid hormone levels and exposure during development are exogenous factors that may exacerbate susceptibility to craniofacial abnormalities potentially through changes in growth at the synchondroses of the cranial base. To elucidate the direct effect of *in utero* therapeutic thyroxine exposure on the synchondroses in developing mice, we provided scaled doses of the thyroid replacement drug, levothyroxine in drinking water to pregnant C57BL/6 wild-type dams. The skulls of resulting pups were subjected to microcomputed tomography analysis revealing less bone volume relative to tissue volume in the synchondroses of mouse pups exposed *in utero* to levothyroxine. Histological assessment of the cranial base area indicated more active synchondroses as measured by metabolic factors including Igf1. The cranial base of the pups exposed to high levels of levothyroxine also contained more collagen fiber matrix and an increase in markers of bone formation. Such changes due to exposure to exogenous thyroid hormone may drive overall morphological changes. Thus, excess thyroid hormone exposure to the fetus during pregnancy may lead to altered craniofacial growth and increased risk of anomalies in offspring.

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Keywords

Thyroid Hormone; Cranial Base; Craniofacial Growth; Cartilage; Collagen Type

Introduction

Thyroid hormone has been shown to be important for skull bone development and growth [1]. Its contribution has been noted at the cranial sutures [2–4] as well as the synchondroses [5]. Thyroid hormones are responsible for regulation of metabolism and are important for growth and development of the skeleton by acting during bone development and maintenance. Proper bone mineral densities are partially maintained by thyroid hormones interacting with factors such as growth hormone and aiding in the regulation of insulin-like growth factor (Igf) [6, 7]. These interactions effect linear bone growth by targeting the hypertrophic zone of cartilaginous growth plates [8–11]. These same processes are important for the craniofacial skeleton, as the synchondroses of the cranial base, like the growth plate in long bones, contribute to elongation by proliferation and hypertrophy of chondrocytes. Unlike long bone growth plates, the synchondroses produce growth in opposing directions [12].

As thyroid hormones have particularly potent effects on cartilage including the growth plates, it stands to reason that the cranial base synchondroses may be similarly targeted [13]. However, little work has been conducted to address this. Aberrant expression and circulation of thyroid hormones, including exposures via maternal thyroid replacement drug, have been linked to craniofacial growth disorders [14–16]. Further, the cranial base contributes to calvarial growth by proper development and maintenance of the coronal ring, which includes the coronal suture, frontosphenoidal suture, and presphenothmoidal synchondrosis in humans [17–19]. In addition, the dural tracts which underlie the calvarial growth sites including the spheno-occipital synchondrosis of the cranial base, allow for the interpretation that cranial base growth may affect overall calvarial expansion [20]. When cranial base growth is altered, aberrant craniofacial growth results [21–23].

There are many exogenous factors that affect craniofacial growth [14, 24–28]. In the current study, we aim to elucidate the effect of *in utero* levothyroxine exposure on the cranial base and by extension craniofacial growth in mice. We hypothesize exposure to levothyroxine *in utero* will affect the cartilaginous portions of the cranial base in a dose-dependent manner involving specific changes in cranial base morphology, target protein expression, and cartilage cell metabolism.

Materials and Methods

In Vivo Exposure

Adult wild type C57BL6 males and females were utilized to produce *in utero* levothyroxine exposed litters. Animals were bred and separated at ~E13 of pregnancy. At this time, doses of levothyroxine (Synthroid) scaled for use in mice (control = no treatment; low dose ~167ng/day; high dose ~667ng/day) were added to the drinking water provided to pregnant

dams [29–36]. Treatments were timed for a critical period of craniofacial development, assigned randomly with the intention of producing 12 pups per group, and continued until birth of litters (~E20) when all animals were provided un-supplemented water[35]. Mouse pups were grown to postnatal day (P) 15, P20, or P25 when they were sacrificed and whole skulls were fixed with 4% paraformaldehyde for 48 hours, then switched to 70% Ethanol for micro computed tomography (μ CT) and then further processed for histology. These Animal Use Protocols were approved by Georgia Regents University Institutional Animal Care and Use Committee (2011-0365), and the Medical University of South Carolina Institutional Animal Care and Use Committee (AR#3341). All breeding procedures were carried out in an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility where all husbandry and related services are provided by the Division of Laboratory Animal Resources. All procedures and the reporting thereof are in compliance with the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines [37].

Microcomputed Tomography Analysis

μ CT images were obtained on all collected skulls with a SkyScan 1174 (Kontich, Belgium) at a 19.17 μ m voxel resolution (Table 1). Skulls were stabilized using a custom cradle, and reconstructed with NRecon v1.6.4.8 (BrukermicroCT, Kontich, Belgium) as previously described [38]. Threshold settings were set to capture bone volume within the skull. Cranial base length was measured from basion to foramen cecum, anterior and posterior base lengths were determined from spheno-occipital synchondrosis (SOS) to foramen cecum and basion to SOS respectively. Measurements of the lengths, widths, and heights of the cartilaginous regions between ossified centers, SOS and inter-sphenoidal synchondrosis (ISS) at the midline of the cartilaginous segment were recorded. A standardized region of interest that included cartilaginous synchondrosis and abutting bone was used to assess the Bone Volume compared to Tissue volume (BV/TV) (Figure 1A). Measures were compared at each time point by split-plot Kruskal-Wallis by postnatal time-point effects by dose; $p < 0.05$ was considered significant for post-hoc Bonferroni analyses. All statistical analyses were completed using SPSS 23.0 (IBM, Armonk, NY, USA).

Histological Investigation

After μ CT scanning, representative samples ($n=3$) from each group (control, low, high) for each time point (P15, P20, P25) were decalcified in 0.25 M EDTA at pH7.4 for 10 days. Samples were then washed, dehydrated in graded ethanol (70–100%), cleared in xylene, and embedded in paraffin. Histology was performed using 8 μ m sections. Harris' hematoxylin (VWR, Radnor, PA, 10143–606) and Eosin (VWR, 95057-848) staining proceeded by standard protocol. As the SOS is the last synchondrosis of the cranial base to fuse in humans, it is assumed that it has a greater impact on craniofacial development, thus we have focused on this region [12]. Three sections at least 30 μ m apart were used for histomorphometric analysis. Stained sections were photographed using a Motic Inverted Microscope with attached camera (Motic, British Columbia, Canada). Standardized variables including total area including adjacent trabeculae, synchondrosis length and height, as well as the length of the isolated resting, proliferative, and hypertrophic zones were measured using Image J Software (National Institutes of Health) [31]. Measures were compared for effects by dose, $p < 0.05$ was considered significant.

For immunohistochemistry, representative samples (n=3) from each group at P20 were subjected to antigen retrieval (10mM Sodium Citrate Buffer). P20 was identified as being where the most differences between control and exposure groups were identified, thus this time-point was used for the remainder of the investigation. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and then washed sections were blocked in 1% goat serum with 1% bovine serum albumin. Sections were incubated with the following primary antibodies for one hour at room temperature or refrigerated overnight: Htra1 (AbCam Cambridge, MA, ab38611, 1:50), Igf1 (ab9572, 1:50), PCNA (ab18197, 1:3000), Alp (ab108337, 1:250), Active Caspase 3 (ab2302, 1:75), Type II collagen (ab34712, 1:600), Type X collagen (ab49945, 1:25), Aggrecan (ab36861, 1:200). Sections were washed and incubated with HRP conjugated secondary antibody for one hour (ab6721).

Diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA) chromagen was used to identify immunoreactive structures. Whole synchondroses including abutting trabecular bone, as well as individual zones were digitally isolated for direct comparison between control and levothyroxine (low and high) exposed individuals. At least 3 sections 30 μ m apart per individual per treatment for each target were analyzed using Image J Software and the IHC Profiler Open Source Plugin for automated scoring of percent positivity [39]. Measures were compared for effects by dose; p 0.05 was considered significant.

To assess the maturity of the cellular matrix in the cartilaginous synchondrosis, picrosirius red staining was performed per standard protocol. Stained sections were photographed under polarized light using a Motic Inverted Microscope with attached camera. Contribution of green (immature), yellow (intermediate), and red (mature) fibers was assessed using Visopharm software (Visopharm, Hoerscholm Denmark).

Tissue Based Quantitative Polymerase Chain Reaction

A set of randomly selected P20 skulls (n=3 control, n=3 high dose) were not fixed, but were used for RNA isolation from cranial base tissue. The cranial base including the cartilaginous regions of the SOS, and ISS and the intervening bone, were excised. The extirpated tissue was homogenized in a liquid nitrogen cooled mortar and digested in TRIZOL (ThermoFisher). RNA was isolated using the Qiagen RNEasy mini kit (Qiagen, Valenica, CA, USA). Quantity and quality of RNA was assessed using a Synergy H1 Microplate reader and a Take3 Microvolume Plate (BioTek, Winooski, VT, USA). cDNA synthesis was performed using Superscript II Reverse Transcriptase and random hexamer primer (ThermoFisher) and then subjected to quantitative PCR using Applied Biosystems TaqMan Gene Expression Master Mix and the following TaqMan assays for the *Igf1* pathway: *Htra1* (HtrA Serine Peptidase 1 (Mm00479892_m1)), *Igf1* (Insulin Like Growth Factor 1 (Mm00439560_m1)); for proliferation; *Ki67* (Marker of Proliferation Ki67 (Mm01278617_m1)), for apoptosis; *Caspase 3* (Caspase 3 (Mm01195085_m1)), for osteogenesis; *Alp* (Alkaline phosphatase (Mm00475834_m1)), and for matrix composition; *ColIII* (Collagen Type II (Mm01309565_m1)), *ColX* (Collagen Type X (Mm00487041_m1), *Acan* (Aggrecan (Mm00545794_m1). Data were normalized to 18S ribosomal RNA (Mm03928990_g1) expression by CT. Quantitative data were compared for gene expression change by CT. We used statistical analyses for qrt-PCR data as previously

published to determine differences for gene expression after levothyroxine exposure [40]. Differences were considered significant if $p \leq 0.05$.

Western Blots on Cranial Base Tissue

A parallel set of randomly selected P20 skulls ($n=3$ control, $n=3$ high dose) were not fixed, but placed in ice cold PBS. Subsequently, the cranial base was identified and isolated as above. The tissue was then homogenized as above and protein was extracted with cold RIPA buffer (ThermoFisher). Total protein was quantified using a Bradford assay (ThermoFisher). Whole tissue protein extracts were separated by 10% SDS-PAGE. Equal amounts of protein per lane were loaded and transferred onto PVDF membrane (BioRad, Hercules, CA, USA). The blots were probed with the following antibodies diluted in Tris-buffered saline, 0.1% Tween 20 with 5% (wt/vol) bovine serum albumin: Htra1 (AbCam, ab38611, 1:1000), Igf1 (ab63926, 1:500), Alp (ab108337, 1:16,000), active Caspase 3 (ab49822; 1:500), PcnA (ab18197; 1:250), Gapdh (ab181602; 1:10,000) and B-actin (Cell Signaling 4967; 1:10,000). Incubation with horseradish peroxidase-conjugated anti-rabbit IgG (ab6721; 1:3000) followed. The protein was visualized by enhanced chemiluminescence ECL Clarity (BioRad) detection reagents. Band intensity was quantified using NIH Image J software.

Results

In utero Levothyroxine exposure alters the murine synchondrosis

Representative μ CT reconstructions of isolated cranial bases from P15, P20, and P25 mice exposed to control, low, and high dose levothyroxine *in utero* are included in Figure 1. There was approximately equal representation of sex among 226 animals (Male=39.2%; Female=34.3%; Undetermined=26.5%) (Table 1). Animal weight did not differ by sex, but was greater in high dose exposed at P15 and P20 but less at P25 ($p<0.05$) indicating long-term effects of exposure. Assessment of cranial base length indicates an increase in overall length in high dose exposed individuals as compared to control and low dose exposed at P20. That length is maintained to P25 while low dose exposed and control individuals increase in length. This relationship remained in both the anterior and posterior portions of the cranial base. Results suggest increased bone volume in the SOS with high dose exposure compared to control at P15 ($p=0.031$), and a decreased bone volume in the ISS for both low and high dose compared to control ($p<0.001$, $p=0.017$ respectively) and SOS for high dose compared to control ($p=0.025$) at P20. This imbalance resolved by P25 (Figure 1).

Figure 2 demonstrates representative H&E staining of sections of the SOS from P20 mice unexposed (control) or exposed to low or high dose levothyroxine (Figure 2A–F). Histomorphometric analysis was conducted on 3 individuals from each group (control, low, high) at P15, P20, and P25. The area of the SOS including the abutting trabeculae is less in the low dose exposure as compared high does at P15 and as compared to both control and high dose exposures at P20 ($p<0.001$). These differences resolved by P25 (Figure 2G). Similarly, the length of the SOS is less in low dose treatment at P20 compared to control and high dose, and P25 compared to high dose ($p<0.001$) (Figure 2H). Changes to the height of the SOS were seen early with the low dose being shorter than control and high dose at P15 ($p<0.001$). At P20 high dose demonstrated greater height as compared to control ($p<0.05$)

(Figure 2I). Investigation of the lengths of the specific zones of the SOS revealed longer central resting zones in low dose exposed individuals compared to control and high dose at P20 ($p<0.001$) (Figure 2J). For the proliferative zone, control (no exposure) was longer than both doses ($p<0.05$) at P20. At both P20 and P25 high dose exposed proliferative zone was longer than low dose ($p<0.03$) (Figure 2K). The hypertrophic zone abutting the trabeculae was longer in the high dose exposed at all time-points when compared to low dose ($p<0.002$). The control group was greater than both levothyroxine doses at P20 ($p<0.001$) (Figure 2L).

Histological assessment of cellular activity in in utero Levothyroxine exposed Spheno-occipital Synchondroses

Figure 3A–C demonstrates representative images of immunohistochemistry on sections of SOS from P20 mice either unexposed or exposed to levothyroxine. Zone specific quantification of serine protease Htra1 staining indicates a decreased level of activity in the resting zone ($p=0.006$) of the exposed synchondroses (both doses), and in the proliferative and hypertrophic zones of low dose exposed individuals as compared to control ($p=0.005$) (Figure 3A–D). Staining for Igf1 which is partially regulated by Htra1 (Figure 3E–H) demonstrated a significant increase in staining with high dose exposure ($p=0.008$) when the cartilaginous region was combined with the adjacent trabecular bone. Additionally, within the isolated resting zone, low dose exposure demonstrated significantly less Igf1 staining compared to high dose exposure ($p=0.009$). Assessment of gene activity indicated a decrease in Igf1 expression with high dose exposure ($p<0.05$) (Figure 3I). No significant differences in Htra1 or Igf1 protein levels were observed (Figure 3J–K).

Figure 4 demonstrates representative images of PCNA staining. No statistically significant differences in staining intensity were found (Figure 4A–D). Increased Alp activity was observed in the high dose exposed synchondroses ($p=0.004$). (Figure 4E–H). In addition to increased Alp activity in high dose exposed, a significant increase in Caspase3 activity was found. The cartilaginous synchondrosis ($p<0.001$), the proliferative zone ($p=0.002$), the hypertrophic zone ($p<0.001$) and the isolated adjacent trabeculae ($p=0.05$) of levothyroxine exposed pups demonstrate increased activity compared to control. Further, the proliferative zone demonstrated increased caspase activity in the high dose as compared to low (Figure 4I–L). On the level of gene expression however, only *Alp* expression is significantly decreased with exposure to levothyroxine ($p<0.05$) (Figure 4M). Assessment of protein indicates no significant differences (Figure 4N–O).

Histological assessment of cellular matrix formation of in utero Levothyroxine exposed Spheno-occipital Synchondroses

Figure 5 demonstrates representative staining of the extracellular matrix in and around the SOS of P20 mouse pups unexposed or exposed to high or low levels of levothyroxine. Low magnification picrosirius red staining (Figure 5A–C) demonstrates greater density of collagen fibers in the trabeculae of high dose exposed individuals. Polarization and higher magnification of the same samples (Figure 5D–F) reveals a greater density of mature orange/red and less mature blue/green fibers in the exposed samples as compared to control samples. Quantification of the prevalence of those colors indicates significantly more green,

yellow, and red fibers in the exposed samples ($p<0.009$) (Figure 5G). Type II collagen (Figure 5H–K) is abundant in both groups. Zone specific quantification of staining intensity indicates an increase in type II collagen in the hypertrophic zone of exposed cranial bases ($p=0.006$). Type X collagen (Figure 5L–O) is also identified in unexposed and levothyroxine exposed samples. No significant differences in staining intensity were identified. Likewise, Aggrecan (Figure 5P–S) can be observed in both groups. More intense staining was found in the synchondrosis and adjacent trabeculae of the control specimen ($p=0.003$). This decreased aggrecan staining in the exposed samples matched the gene expression assessment ($p<0.05$) (Figure 5T).

Discussion

Here we sought to elucidate the effect of *in utero* levothyroxine exposure on the synchondroses of the mouse. In agreement with previous data revealing a brachicranic phenotype, and a shortened bone length individuals exposed to high (~667ng/day) dosages of levothyroxine presented with decreased cranial base length [41, 42]. Both low (~167ng/day) and high dosages of levothyroxine correlate with decreased bone volume in the cranial base at P20. The cranial base has a primary role in driving postnatal growth and form, and both the inter-sphenoidal and sphenoid-occipital (ISS and SOS) synchondroses remain patent in wild-type mice up to P20 [43]. The decreased bone volume with exposure seen at P20 could indicate a delay in ablation of the cartilaginous regions of the cranial base. Fusion of the pre- and basi-sphenoid and basioccipital bones normally occurs via bony outgrowths extending from the ectocranial margins of the bones of the cranial base. Thus, some areas of the synchondrosis remain patent while others fuse [43]. Cranial dysmorphology may arise from abnormal cranial base, facial bone, or calvarial suture growth. Small but biologically significant changes in any of these regions, may be difficult to observe radiographically, but can result in craniofacial abnormalities [18, 43, 44]. Further, changes in the growth trajectory of the cranial base may contribute to craniofacial growth disturbances [21–23].

Examination of cranial bases from P20 wild-type mice exposed and unexposed (control) indicate differences in cranial base composition as specified by changes in area, length, and height of the low dose exposed individuals as compared to both the control and the higher dose exposed synchondroses. The most dramatic effects of levothyroxine exposure on the morphology of the cranial base are observed at P20 indicating that levothyroxine exposure may interfere with expected synchondrosis fusion [43]. That the low dose exposure was observed to have a greater overall effect on synchondroses morphology may be indicative of a response that is specific to that dosage. Overall, it is interesting that most differences due to exposure were resolved by P25 perhaps indicating some compensatory growth.

Assessment of cellular activity within the SOS revealed an overall increase in Igf1 activity via immunohistochemistry. Similar results were not observed for gene expression or protein levels. These results may indicate specificity of the effect to the cartilaginous cranial base. Our immunohistochemical assessment allowed for localization of Igf1 activity to the cartilaginous cranial base whereas the other assessments may include confounding factors from surrounding tissue. The correlation between increased thyroid hormone and Igf1 enhanced bone formation has been well established and here we observe increased Igf1

activity in the synchondroses of mice exposed to levothyroxine [2, 8]. Such Igf1 activity effects chondrocyte proliferation while Htra1 activity is associated with chondrocyte differentiation. Binding of Htra1 with Igf1 provides a regulatory mechanism slowing the turnover of cartilage matrix for mineralized bone [13, 45]. The increase in Igf1 activity along with the decrease in Htra1 activity and the decrease in bone volume in synchondroses exposed to levothyroxine may be indicative of interference with the pace of craniofacial growth and development at the cranial base [1, 11, 45]. Further, differences in Igf1 and Htra1 activity between dosages may indicate multiple levels of interference. This interference is further validated by the increase in Alp and apoptotic activity observed in the synchondroses exposed to levothyroxine. The increases in Alp and Caspase 3 activity may indicate an acceleration of bone formation and cell turnover precipitating premature cell ablation in the synchondroses. Overall, the synchondroses from exposed individuals appear to be more active (increased differentiation and cell turnover) suggesting an interference with the tight regulation of cell cycling needed to maintain this important region. It is possible that the observed changes to cell metabolism, differentiation, and apoptosis are indicative of accelerated growth associated with thyrotoxicosis. Growth within the synchondroses may be accelerated, and then may resolve to early quiescence disrupting the expected progression of synchondrosis fusion [42]. The differences observed between dosages may indicate different pacing of cranial base fusion.

It is likely that the observed increase in Igf1 activity drives the maturation of the extracellular matrix in the synchondroses exposed to levothyroxine. However, it is also possible that such matrix maturation is a direct effect of the levothyroxine exposure. Thyroid hormone has been found to act directly on the hypertrophic zone of endochondral bone formation causing accelerated maturation of the chondrocytes resulting in changes to the extracellular matrix composition including those noted here [13]. The levothyroxine exposed groups presented with more collagen type II in the hypertrophic zone indicating matrix that is actively processing towards bone. An increase in the presence of type II collagen has been correlated with an increase in Igf1 activity specifically in proliferating cells (proliferative and hypertrophic zones) [8, 11]. Additionally, thyroid hormone promotes hypertrophic chondrocyte differentiation towards mature chondrocytes which produce collagen type X [9, 13]. Increases in Htra1 activity have been shown to enhance removal of cartilage matrix to allow for the formation of mineralized bone matrix [45]. Here we see increases in Igf1 activity without an increase in Htra1 activity which may be responsible for the abundance of matrix observed in the exposed synchondroses.

The synchondroses from mice exposed to levothyroxine are likely displaying two separate effects driving change in cranial base composition which ultimately may drive the observed alterations to craniofacial morphology [41]. Firstly, we observe increased Igf1 signaling which induces a change in cellular activity in the growth center [2, 8]. Secondly, exposure to levothyroxine appears to cause an acceleration of bone growth by affecting the maturation of hypertrophic chondrocytes [6, 10, 13, 15]. Together these factors are likely responsible for the increased metabolic activity found in the synchondrosis of mice exposed to levothyroxine. Disruption via genetic or epigenetic factors to the tightly regulated developmental processes in this anatomical region can have significant and lasting effects on craniofacial development [44]. These data allow for the interpretation that high levels of

thyroid hormone during pregnancy may lead to an increased risk of craniofacial abnormalities in offspring.

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References

1. Pirinen S. Endocrine regulation of craniofacial growth. *Acta odontologica Scandinavica*. 1995; 53:179–185. [PubMed: 7572094]
2. Akita S, Hirano A, Fujii T. Identification of IGF-I in the calvarial suture of young rats: histochemical analysis of the cranial sagittal sutures in a hyperthyroid rat model. *Plastic and reconstructive surgery*. 1996; 97:1–12. [PubMed: 8532765]
3. Akita S, Nakamura T, Hirano A, Fujii T, Yamashita S. Thyroid hormone action on rat calvarial sutures. *Thyroid: official journal of the American Thyroid Association*. 1994; 4:99–106. [PubMed: 8054867]
4. Stevens DA, Harvey CB, Scott AJ, O'Shea PJ, Barnard JC, Williams AJ, Brady G, Samarut J, Chassande O, Williams GR. Thyroid hormone activates fibroblast growth factor receptor-1 in bone. *Molecular endocrinology (Baltimore, Md)*. 2003; 17:1751–1766.
5. Ortner DJ, Hotz G. Skeletal manifestations of hypothyroidism from Switzerland. *American journal of physical anthropology*. 2005; 127:1–6. [PubMed: 15386228]
6. McNabb, FMA. *Thyroid hormones*. Prentice Hall; Englewood Cliffs, N.J.: 1992.
7. Oppenheimer, JH., Samuels, HH., Apriletti, JW. *Molecular basis of thyroid hormone action*. Academic Press; New York: 1983.
8. Bassett JH, Williams GR. The molecular actions of thyroid hormone in bone. *Trends in endocrinology and metabolism: TEM*. 2003; 14:356–364. [PubMed: 14516933]
9. Harvey CB, O'Shea PJ, Scott AJ, Robson H, Siebler T, Shalet SM, Samarut J, Chassande O, Williams GR. Molecular mechanisms of thyroid hormone effects on bone growth and function. *Molecular genetics and metabolism*. 2002; 75:17–30. [PubMed: 11825060]
10. Mosekilde L, Eriksen EF, Charles P. Effects of thyroid hormones on bone and mineral metabolism. *Endocrinology and metabolism clinics of North America*. 1990; 19:35–63. [PubMed: 2192868]
11. Robson H, Siebler T, Shalet SM, Williams GR. Interactions between GH, IGF-I, glucocorticoids, and thyroid hormones during skeletal growth. *Pediatric research*. 2002; 52:137–147. [PubMed: 12149488]
12. Wei X, Hu M, Mishina Y, Liu F. Developmental Regulation of the Growth Plate and Cranial Synchondrosis. *J Dent Res*. 2016
13. Robson H, Siebler T, Stevens DA, Shalet SM, Williams GR. Thyroid hormone acts directly on growth plate chondrocytes to promote hypertrophic differentiation and inhibit clonal expansion and cell proliferation. *Endocrinology*. 2000; 141:3887–3897. [PubMed: 11014246]
14. Rasmussen SA, Yazdy MM, Carmichael SL, Jamieson DJ, Canfield MA, Honein MA. Maternal thyroid disease as a risk factor for craniosynostosis. *Obstetrics and gynecology*. 2007; 110:369–377. [PubMed: 17666613]
15. Radetti G, Zavallone A, Gentili L, Beck-Peccoz P, Bona G. Foetal and neonatal thyroid disorders. *Minerva pediatrica*. 2002; 54:383–400. [PubMed: 12244277]
16. Mauceri L, Ruggieri M, Pavone V, Rizzo R, Sorge G. Craniofacial anomalies, severe cerebellar hypoplasia, psychomotor and growth delay in a child with congenital hypothyroidism. *Clinical dysmorphology*. 1997; 6:375–378. [PubMed: 9354849]

17. Rogers GF, Mulliken JB. Involvement of the basilar coronal ring in unilateral coronal synostosis. *Plast Reconstr Surg*. 2005; 115:1887–1893. [PubMed: 15923833]
18. Burdi AR, Kusnetz AB, Venes JL, Gebarski SS. The natural history and pathogenesis of the cranial coronal ring articulations: implications in understanding the pathogenesis of the Crouzon craniosynostotic defects. *The Cleft palate journal*. 1986; 23:28–39. [PubMed: 3455900]
19. Sperber, GH., Sperber, SM., Guttman, GD., Sperber, GH. *Craniofacial embryogenetics and development*. People's Medical Pub; House USA, Shelton, CT: 2010.
20. Bosma, JF. *Symposium on Development of the Basicranium*. U.S. Dept. of Health, Education, and Welfare, Public Health Service for sale by the Supt. of Docs., U.S. Govt. Print. Off; Bethesda, Md. Washington: 1976.
21. McGrath J, Gerety PA, Derderian CA, Steinbacher DM, Vossough A, Bartlett SP, Nah HD, Taylor JA. Differential closure of the spheno-occipital synchondrosis in syndromic craniosynostosis. *Plast Reconstr Surg*. 2012; 130:681e–689e. [PubMed: 22575855]
22. Nagata M, Nuckolls GH, Wang X, Shum L, Seki Y, Kawase T, Takahashi K, Nonaka K, Takahashi I, Noman AA, Suzuki K, Slavkin HC. The primary site of the acrocephalic feature in Apert Syndrome is a dwarf cranial base with accelerated chondrocytic differentiation due to aberrant activation of the FGFR2 signaling. *Bone*. 2011; 48:847–856. [PubMed: 21129456]
23. Tahir Y, Paliga JT, Vossough A, Bartlett SP, Taylor JA. The spheno-occipital synchondrosis fuses prematurely in patients with Crouzon syndrome and midface hypoplasia compared with age- and gender-matched controls. *Journal of oral and maxillofacial surgery: official journal of the American Association of Oral and Maxillofacial Surgeons*. 2014; 72:1173–1179.
24. Richardson S, Browne ML, Rasmussen SA, Druschel CM, Sun L, Jabs EW, Romitti PA. National Birth Defects Prevention S. Associations between periconceptional alcohol consumption and craniosynostosis, omphalocele, and gastroschisis. *Birth defects research Part A, Clinical and molecular teratology*. 2011; 91:623–630. [PubMed: 21630421]
25. Browne ML, Hoyt AT, Feldkamp ML, Rasmussen SA, Marshall EG, Druschel CM, Romitti PA. Maternal caffeine intake and risk of selected birth defects in the National Birth Defects Prevention Study. *Birth defects research Part A, Clinical and molecular teratology*. 2011; 91:93–101. [PubMed: 21254365]
26. Reefhuis J, Honein MA, Schieve LA, Rasmussen SA. National Birth Defects Prevention S. Use of clomiphene citrate and birth defects, National Birth Defects Prevention Study, 1997–2005. *Human reproduction*. 2011; 26:451–457. [PubMed: 21112952]
27. Carmichael SL, Rasmussen SA, Lammer EJ, Ma C, Shaw GM. National Birth Defects Prevention S. Craniosynostosis and nutrient intake during pregnancy. *Birth defects research Part A, Clinical and molecular teratology*. 2010; 88:1032–1039. [PubMed: 20842649]
28. Alwan S, Reefhuis J, Rasmussen SA, Olney RS, Friedman JM. National Birth Defects Prevention S. Use of selective serotonin-reuptake inhibitors in pregnancy and the risk of birth defects. *The New England journal of medicine*. 2007; 356:2684–2692. [PubMed: 17596602]
29. Rasmussen SA, Yazdy MM, Frias JL, Honein MA. Priorities for public health research on craniosynostosis: summary and recommendations from a Centers for Disease Control and Prevention-sponsored meeting. *American journal of medical genetics Part A*. 2008; 146A:149–158. [PubMed: 18080327]
30. Krause K, Weiner J, Hones S, Kloting N, Rijntjes E, Heiker JT, Gebhardt C, Kohrle J, Fuhrer D, Steinhoff K, Hesse S, Moeller LC, Tonjes A. The Effects of Thyroid Hormones on Gene Expression of Acyl-Coenzyme A Thioesterases in Adipose Tissue and Liver of Mice. *Eur Thyroid J*. 2015; 4:59–66. [PubMed: 26601074]
31. Capuco AV, Kahl S, Jack LJ, Bishop JO, Wallace H. Prolactin and growth hormone stimulation of lactation in mice requires thyroid hormones. *Proc Soc Exp Biol Med*. 1999; 221:345–351. [PubMed: 10460696]
32. Thordarson G, Fielder P, Lee C, Hom YK, Robleto D, Ogren L, Talamantes F. Mammary gland differentiation in hypophysectomized, pregnant mice treated with corticosterone and thyroxine. *Biol Reprod*. 1992; 47:676–682. [PubMed: 1382632]

33. Capelo LP, Beber EH, Huang SA, Zorn TM, Bianco AC, Gouveia CH. Deiodinase-mediated thyroid hormone inactivation minimizes thyroid hormone signaling in the early development of fetal skeleton. *Bone*. 2008; 43:921–930. [PubMed: 18682303]
34. Darnerud PO, Morse D, Klasson-Wehler E, Brouwer A. Binding of a 3,3', 4,4'-tetrachlorobiphenyl (CB-77) metabolite to fetal transthyretin and effects on fetal thyroid hormone levels in mice. *Toxicology*. 1996; 106:105–114. [PubMed: 8571380]
35. Lamb, Jct, Harris, MW., McKinney, JD., Birnbaum, LS. Effects of thyroid hormones on the induction of cleft palate by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in C57BL/6N mice. *Toxicology and applied pharmacology*. 1986; 84:115–124. [PubMed: 3715858]
36. Lamberg BA, Helenius T, Liewendahl K. Assessment of thyroxine suppression in thyroid carcinoma patients with a sensitive immunoradiometric TSH assay. *Clinical endocrinology*. 1986; 25:259–263. [PubMed: 3791667]
37. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol*. 2010; 8:e1000412. [PubMed: 20613859]
38. Parsons TE, Weinberg SM, Khaksarfard K, Howie RN, Elsalanty M, Yu JC, Cray JJ Jr. Craniofacial shape variation in Twist1+/- mutant mice. *Anatomical record*. 2014; 297:826–833.
39. Varghese F, Bukhari AB, Malhotra R, De A. IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. *PLoS One*. 2014; 9:e96801. [PubMed: 24802416]
40. Yuan JS, Reed A, Chen F, Stewart CN Jr. Statistical analysis of real-time PCR data. *BMC bioinformatics*. 2006; 7:85. [PubMed: 16504059]
41. Howie RN, Durham EL, Black L, Bennfors G, Parsons TE, Elsalanty ME, Yu JC, Weinberg SM, Cray JJ Jr. Effects of In Utero Thyroxine Exposure on Murine Cranial Suture Growth. *PloS one*. 2016; 11:e0167805. [PubMed: 27959899]
42. O'Shea PJ, Harvey CB, Suzuki H, Kaneshige M, Kaneshige K, Cheng SY, Williams GR. A thyrotoxic skeletal phenotype of advanced bone formation in mice with resistance to thyroid hormone. *Molecular endocrinology (Baltimore, Md)*. 2003; 17:1410–1424.
43. Vora SR, Camci ED, Cox TC. Postnatal Ontogeny of the Cranial Base and Craniofacial Skeleton in Male C57BL/6J Mice: A Reference Standard for Quantitative Analysis. *Front Physiol*. 2015; 6:417. [PubMed: 26793119]
44. Lieberman DE, Hallgrímsson B, Liu W, Parsons TE, Jamniczky HA. Spatial packing, cranial base angulation, and craniofacial shape variation in the mammalian skull: testing a new model using mice. *J Anat*. 2008; 212:720–735. [PubMed: 18510502]
45. Tsuchiya A, Yano M, Tocharus J, Kojima H, Fukumoto M, Kawaichi M, Oka C. Expression of mouse HtrA1 serine protease in normal bone and cartilage and its upregulation in joint cartilage damaged by experimental arthritis. *Bone*. 2005; 37:323–336. [PubMed: 15993670]

Fig. 1. Effects of *in utero* levothyroxine exposure on murine speno-occipital and inter-sphenoidal synchondrosis morphology

A–C. Endocranial (above) and sagittal (below) view of representative μ CT reconstructions of P15(A), P20(B), and P25(C) mouse skulls exposed to control (no exposure, left), low (~167ng/day, middle), and high (~667ng/day, right) dosages of levothyroxine. Surrounding structures have been digitally cropped and an example area of analysis has been boxed. SOS (arrow). ISS (arrowhead). Whole skull insets (A) indicate orientation. D. μ CT assessment of SOS micro-architecture suggests increased bone volume with high dose exposure compared to control at P15 and the opposite at P20. E–G. Measure of cranial base length indicates an increase in high dose exposed individuals as compared to control and low dose at P20. At P25 low dose exposed is greater than high and control. This relationship is maintained in both the anterior and posterior cranial base. H. μ CT assessment of ISS microarchitecture suggests increased bone volume with both low and high dose exposure at P20 compared to control. All measurements presented as mean $\pm$ SEM. Different than control: * $p=0.05$ – 0.011 ;

** $p=0.01-0.001$; *** $p<0.001$ Different between doses: # $p=0.05-0.011$; ## $p=0.01-0.001$;
$p<0.001$

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Fig. 2. Histomorphometric assessment of speno-occipital synchondrosis morphology after *in utero* levothyroxine exposure

A–F. Representative low (A,C,E) and high (B,D,F) magnification hematoxylin and eosin stained SOS from P20 mice exposed to control (A–B), low (C–D), and high (E–F) doses of levothyroxine. Synchondrosis plus adjacent trabeculae is outlined (A). Resting zone (RZ), proliferative zone (PZ), hypertrophic zone (HZ), and trabecula bone (TB) are noted. G. Assessment of area of the SOS including adjacent trabeculae indicates decreased area for the low dose exposed at P15 (compared to high dose), and at P20 (compared to control and high dose). H. Length of the SOS indicating narrowing with low dose exposure at P20 (compared to control and high dose) and P25 (compared to high dose). I. Height of the SOS demonstrates decreased height with low dose exposure at P15 (compared to control and high dose), and increased height with high dose exposure at P20 (compared to control). J. Resting zone length increased with low dose exposure at P20. K. Proliferative zone length was greater in control at P20 (compared to low and high dose) and low dose was less than high dose exposed at both P20 and P25. L. Length of the hypertrophic zone was greater in high dose exposed as compared to low dose at P15, P20 and P25, however length of the control

was greatest at P20. All measurements presented as mean $\pm$ SEM. * $p=0.05-0.011$;
** $p=0.01-0.001$; *** $p<0.001$

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Fig. 3. Histological markers of cell metabolism in control and levothyroxine exposed pheno-occipital synchondroses

A–D. Immunohistochemistry staining for serine protease *Htra1* on cranial base SOS from P20 pups either unexposed (A) or exposed to low (B) or high (C) doses of levothyroxine. Increased staining in the control (A) was confirmed with quantification of percent staining (D). E–H Immunohistochemistry staining for insulin-like growth factor (*Igf1*) on cranial base SOS from P20 mouse pups either unexposed (E) or exposed to low (F) or high (G) doses of levothyroxine. Increased staining in the resting zone and trabeculae of the exposed sample was confirmed with quantification (H). I–K. Gene expression for *Htra1* and *Igf1* in cranial base derived tissue reveals a decrease in *Igf1* expression in high dose exposed tissue (I), however western blot analysis indicated no change due to exposure (J–K). All measurements presented as mean $\pm$ SEM. Different than control: * $p=0.05$ – 0.011 ; ** $p=0.01$ – 0.001 ; *** $p<0.001$ Different between doses: # $p=0.05$ – 0.011 ; ## $p=0.01$ – 0.001 ; ### $p<0.001$

Fig. 4. Histological markers of proliferation and bone formation in control and levothyroxine exposed speno-occipital synchondroses

A–D. Cranial base SOS from control unexposed (A), low (B), and high (C) dose levothyroxine exposed P20 mice stained for Proliferating Cell Nuclear Antigen (PCNA). Quantification of percent staining revealed no significant differences (D). E–H. SOS from control unexposed (E) low (F), and high (G) dose levothyroxine exposed P20 mice stained for alkaline phosphatase (Alp) activity. Note increased staining in the exposed synchondrosis +trabeculae (H). I–L. SOS from control unexposed (I), low (J) and high (K) dose levothyroxine exposed P20 mice stained for apoptotic activity (active Caspase 3). Quantification of percent staining concurred with the qualitative assessment of more staining in the exposed samples (L). M–O. Gene expression for proliferation (*Ki67*), apoptosis (*Caspase 3*), and differentiation (*Alp*) indicated decreased expression of *Alp* with exposure (M); however, western blot analysis indicated no change due to exposure (N–O). All measurements presented as mean ±SEM. Different than control: * $p=0.05-0.011$; ** $p=0.01-0.001$; *** $p<0.001$ Different between doses: # $p=0.05-0.011$; ## $p=0.01-0.001$; ### $p<0.001$

Fig. 5. Histological markers of matrix composition in control and levothyroxine exposed sphenoccipital synchondroses

A–G: SOS from control unexposed (A,D), low (B,E), and high (C,F) dose levothyroxine exposed P20 mice stained with picrosirius red. Un-polarized images (AC) highlight greater density of collagen fibers emanating from hypertrophic zones of exposed synchondroses. Polarization (D–F), higher magnification (insets), and quantification (G) of the same samples reveal more collagen fibers of all types in exposed samples. H–K. Immunohistochemistry staining for type II collagen (H–K), type X collagen (L–O), and aggrecan (P–S) on SOS from P20 pups unexposed (H, L, P), or exposed to low (I,M,Q) or high (J,N,R) doses of levothyroxine. Quantification of percent staining confirmed Type II collagen is abundant throughout the synchondroses of exposed individuals (K) whereas type X collagen (O) and aggrecan (S) are less abundant. T. Gene expression for Type II collagen (*Col2a1*), Type X collagen (*Col10a1*), and Aggrecan (*Acan*) in cranial base tissue reveals a

decrease in *Acan* expression in exposed tissue. All measurements presented as mean $\pm$ SEM. Different than control: * $p=0.05-0.011$; ** $p=0.01-0.001$; *** $p<0.001$ Different between doses: # $p=0.05-0.011$; ## $p=0.01-0.001$; ### $p<0.001$

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Table 1

Group sample size of C57BL6 pups

	15 Day Pups	20 Day Pups	25 Day Pups
Control	42 (42U)	19 (10M, 9F)	23 (13M, 10F)
Low (~167 ng per day)	20 (9M, 11F)	18 (9M, 9F)	32 (13M, 19F)
High (~ 667 ng per day)	19 (5U, 6M, 8F)	22 (14M, 8F)	31 (19M, 12F)

(U) – undetermined, (M) – male, (F) – female