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Estrogen receptor alpha signaling in extrahypothalamic neurons during late puberty decreases bone size and strength in female but not in male mice

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Abstract

Sexually dimorphic bone structure emerges largely during puberty. Sex steroids are critical for peak bone mass acquisition in both genders. In particular, the biphasic effects of estrogens mediate the skeletal sexual dimorphism. However, so far the stimulatory vs inhibitory actions of estrogens on bone mass are not fully explained by direct effects on bone cells. Recently, it has become evident that there is possible neuroendocrine action of estrogen receptor alpha (ERα) on the skeleton. Based on these considerations, we hypothesized that neuronal ERα-signaling may contribute to the skeletal growth during puberty. Here, we generated mice with tamoxifen-inducible Thy1-Cre mediated ERα inactivation during late puberty specifically in extrahypothalamic neurons (N-ERαKO). Inactivation of neuronal ERα did not alter the body weight in males, whereas N-ERαKO females exhibited a higher body weight and increased body and bone length compared to their control littermates at 16 weeks of age. Ex vivo microCT analysis showed increased radial bone expansion of the midshaft femur in female N-ERαKO along with higher serum levels of insulin-like growth factor (IGF)-1 as well as IGF-binding protein (IGFBP)-3. Furthermore, the 3-point bending test revealed increased bone strength in female N-ERαKO. In contrast, inactivation of neuronal ERα had no major effect on bone growth in males. In conclusion, we demonstrate that central ERα-signaling limits longitudinal bone

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growth and radial bone expansion specifically in females potentially by interacting with the GH/IGF-1 axis.

1 | INTRODUCTION

Gender skeletal dimorphism, characterized by larger and longer bones in males compared to females, is largely established during puberty.[1-4] Sex steroids are generally considered as the primary mediators of skeletal sexual dimorphism in cortical bone size and thereby strength.[2-4] Indeed, sex steroid deficiency, induced by ovariectomy and orchidectomy in animal models, increases vs decreases radial bone growth in female and male rodents, respectively.[5,6] Therefore, androgens were traditionally considered to promote and estrogens to inhibit the cortical bone size. However, the more recent consensus is that during early puberty estrogens promote peak bone mass acquisition in females, while both estrogens and androgens are required for optimal periosteal cortical bone expansion in males. The stimulatory action of estradiol (E2) is mediated mainly indirectly via growth hormone (GH) and insulin-like growth factor (IGF)-1 secretion.[2,7] At the end of puberty, however, E2 exerts inhibitory actions on bone size in females more than males.[8-10] As a result of these combined actions, males are on average taller and have a greater bone cross-sectional area than females.[11] Previous studies focused mainly on the stimulatory E2 action on bone. Therefore, the mechanisms underlying inhibitory E2 action, which potentially is the major determinant of the difference in bone mineral acquisition between males and females,[11] remain poorly understood.

Thus far, estrogen receptor beta (ERβ), not alpha (ERα), is considered to mediate suppressive E2 action on the cortical bone size in females.[12,13] Shortly after the pubertal maturation, female global ERβKO mice showed increased longitudinal and transverse skeletal growth, whereas male global ERβKO mice displayed normal bone phenotype.[12] In contrast, global ERαKO mice have reduced the cortical bone size (lower bone length and width) in both sexes.[13-15] However, the bone phenotype of these transgenic mice is confounded by elevated serum testosterone (T) and E2 as well as low IGF-1 levels as a result of altered negative feedback regulation of the hypothalamic-pituitary-gonadal (HPG) axis. Therefore, a potential inhibitory effect of E2 via ERα on bone growth is difficult to assess in a model with ubiquitous disruption of the receptor. Moreover, it seems unlikely that inhibitory effects of E2 on bone are directly mediated via ERα in bone cells. Indeed, most of the bone-cell specific ERαKO mouse models, which lack ERα in mesenchymal progenitors or mature osteoblasts, show reduced cortical bone mass in both genders and hence support a stimulatory role for ERα on bone.[16-18]

Alternatively, potential inhibitory effects of ERα on bone via the neuronal system are plausible since bone is highly innervated[19,20] and ERα is enriched throughout the central nervous system.[21] In line with this notion, different mouse models of neuronal ERα deletion showed increased cortical bone mass in female mice, suggesting inhibitory ERα-mediated action in the brain.[22-24] However, in these models, ERα was also deleted in hypothalamic neurons which may lead to potential confounding effects on bone due to altered circulating sex steroids levels. Moreover, these non-inducible models did not enable to distinguish the relative contribution of stimulatory vs inhibitory actions of E2 via ERα. Furthermore, potential impact of ERα deletion on the GH/IGF-1 axis was not documented. Therefore, there is a need for an inducible knockout mouse model to investigate neuronal ERα effects on growth and sexual dimorphism in bone structure while avoiding potential confounding effects of altered sex steroid or GH/IGF-1 concentrations.

To this aim, we generated mice with tamoxifen-inducible Thy1-Cre mediated ERα inactivation during late puberty specifically in extrahypothalamic neurons. We hypothesized that disruption of extrahypothalamic neuronal ERα signaling during late puberty would increase cortical bone size.

2 | MATERIALS AND METHODS

2.1 | Animal experiments

All animal experiments were approved by the KU Leuven ethical committee (P192/2016). To generate a neuron-specific ERα knockout mouse model, a tamoxifen inducible Cre-LoxP system was used. ERα flox mice were provided by prof. Jan-Åke Gustafsson (Karolinska Institute, Stockholm, Sweden).[25] Heterozygous female mice carrying ERα with a floxed exon 3 were mated with male mice expressing CreERT2 under the control of a modified Thy1 promoter, which shows robust expression in both central and peripheral nervous system except the hypothalamus, caudate putamen, ventral striatum, and basal forebrain.[26] All transgenic mice were backcrossed to the C57BL6/J background and genotyped by PCR-based analysis of genomic DNA samples. Our group previously established a tamoxifen regimen to induce neuron-specific deletion of androgen receptor using the same

CreERT2.[27] To minimize avoid off-target effects of tamoxifen on sex steroid-sensitive organs such as reproductive tissues and bones while having enough recombinase efficiency, 6-week-old ERαfl/fl; Thy-CreERT2+/− mice and their control littermates were orally gavaged with tamoxifen (190 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) once daily for 2 (males) or 4 (females) consecutive days. A subgroup of female N-ERαKO and control littermates were subjected to chemical castration by administration of the gonadotropin releasing hormone antagonist, Degarelix (Ferring Pharmaceuticals, Saint-Prex, Switzerland; subcutaneous injection of 25 mg/kg body weight every 4 weeks starting from the last day of tamoxifen administration). Mice were group-housed (3-5 animals/cage) in conventional facilities at 20°C with 12-hour light/dark cycle and ad libitum access to water and standard chow. At 16 weeks of age, all animals were euthanized with pentobarbital anesthesia followed by cardiac puncture. No differences were observed between the three control genotypes (ERαfl/fl; Thy-CreERT2−/−, ERαwt/wt; Thy-CreERT2+/− and ERαwt/wt; Thy-CreERT2−/−) in any of the parameters. Hence, the data from the three genotypes were pooled as control condition (CON).

2.2 | MicroCT

Both axial and appendicular bones were scanned using Skyscan 1172 (Bruker, Kontich, Belgium) with 5 μm pixel size, 0.5 mm Al filter, 50 kV, 200 μA, 180° angular rotation at 0.4° steps and 590 ms integration time. All images were reconstructed using the NRecon program and analyzed by CTAn software as described.[28] For cortical bone, a 0.5 mm region of interest in the distal femur was selected starting at 4.5 mm from the distal edge of the growth plate. For femoral trabecular bone, a 2 mm segment starting at 0.25 mm from the distal growth plate was analyzed. For trabecular bone of vertebra, whole vertebral body of lumbar 5 (L5) was analyzed. Parameters included trabecular bone volume fraction (BV/TV, %), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, μm), trabecular separation (Tb.Sp, μm), total cross-sectional tissue area (Tt.Ar, mm^2), cortical bone area (Ct.Ar, mm^2), medullary area (Ma.Ar, mm^2), cortical thickness (Ct.Th, mm), periosteal circumference (Ct.PC, mm), and endosteal circumference (Ct.EC, mm).

2.3 | Serum analysis

Serum levels of E2 (sensitivity 0.3 pg/mL) and T (sensitivity 4 pg/mL) were measured in a single run by gas chromatography tandem mass spectrometry. After the addition of isotope-labeled standards, steroids were extracted to chlorobutane, purified on a silica column and derivatized using pentafluorobenzylhydroxylamine hydrochloride followed by pentafluorobenzyl chlorid. Steroids were analyzed in multiple reactions monitoring mode with ammonia as reagent gas using an Agilent 7000 triple quadrupole mass spectrometer equipped with a chemical ionization source.[29] Serum LH was measured by enzyme-linked immunosorbent assay at the University of Virginia Center of Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA, USA) as described before.[30] Serum IGFBP-3 and TRAcP 5b levels were measured using a commercial enzyme-linked immunosorbent assay kit (RAB0236, Sigma-Aldrich; SB-TR103, Immunodiagnostic systems) according to the manufacturers' instructions. Serum IGF-1 and osteocalcin levels were measured using an in-house radioimmunoassay as previously described.[31]

2.4 | Biomechanical testing

At termination point, femurs were collected and kept in 1x PBS at −20°C. A destructive 3-point bending test was performed at the midshaft region with a displacement rate of 0.1 mm/s and a span length of 7 mm (TestBench LM1, EnduraTEC Systems Group, Bose Corp., Minnetonka, MN, USA). The load-displacement curve was used to calculate ultimate bone strength (N), stiffness (N/mm), and work-to-failure (mJ).

2.5 | Quantitative real-time PCR

Total RNA was extracted from the tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. CDNA was synthesized from 1 μg RNA using FastGene Scriptase II kit (NIPPON GENETICS Europe, Dueren, Germany) and random hexamer primers. ERα (Mm00433147_m1) and ERβ (Mm00599819_m1) expressions levels were assessed by the Applied Biosystems StepOnePlus Real-Time PCR system (Applied Biosystems), using TaqMan Fast Advanced Master Mix (Applied Biosystems) according to the manufacturer's instructions. The relative expression levels of the target genes were calculated as a ratio to the Hypoxanthine guanine phosphoribosyl transferase (Hprt) gene (Table S1).

2.6 | Statistics

Statistical analysis was performed using GraphPad Prism v7.04 (GraphPad, La Jolla, CA, USA). Unpaired two-tailed Student's t test was used to analyze differences between two groups. Two-way ANOVA with Bonferroni post hoc test was used in experiments with more than one independent

variable. Data are represented as mean ± SEM, and $P < .05$ was considered as statistically significant.

3 | RESULTS

3.1 | Validation of neuron-specific deletion of ERα in male and female mice

Ten days after tamoxifen administration, PCR analysis of tissue-extracted DNA from male and female N-ERαKO mice revealed that excision of the floxed ERα exon 3 was limited to neuronal tissues in both sexes. Reproductive tissues and long bones were not affected (Figure 1A,B). Moreover, qPCR analysis revealed that male and female N-ERαKO mice showed a significant decrease in ERα transcript levels in brain stem (−44.6%, $P < .0001$; −51.2%, $P < .0001$), cerebral cortex (−45.5%, $P < .01$; −59.3%, $P < .01$), and spinal cord (−46.7%, $P < .0001$; −50.4%, $P < .0001$) but not in hypothalamus, tibia, or gonads (Figure 1C,D).

## 3.2 | Hypothalamic-pituitary-gonadal axis is not affected in neuronal ERαKO mice

To determine whether the negative feedback of HPG axis was disturbed, serum sex steroid levels and reproductive organs weights were determined at 16 weeks of age, 10 weeks after tamoxifen treatment. Analysis of serum T and E2 (Figure 2A) along with serum LH (Figure 2B) revealed unchanged levels in male and female N-ERαKO mice compared to control littermates. Also, seminal vesicles and uterine weights

were not significantly altered in male and female N-ERαKO mice (Figure 2C) nor was the weight of liver and thymus (Table S2), suggesting normal negative feedback regulation in mutant mice.

3.3 | Deletion of ERα in extrahypothalamic neurons in late puberty induces greater bone size and strength in female mice

Unlike males, female N-ERαKO mice showed an increase in body weight (+6.3%, $P < .01$) (Figure 3A), which was not due to changes in body composition as indicated by similar skeletal muscle and fat mass compared to the control group (Table S2). In line with the increase in body weight, female N-ERαKO mice had increased length of nose-to-tail (+2%, $P < .05$), femur (+2.4%, $P < .01$) and vertebral column (+4.8%, $P < .01$) compared to control littermates (Figure 3B-D). MicroCT analysis of the femoral midshaft region revealed significantly increased total cross-sectional tissue area (+7.9%, $P < .01$) and cortical bone area (+6.8%, $P < .05$) which resulted in increased medullary area (+9.4%, $P < .05$) in female N-ERαKO compared with control genotypes, while cortical thickness was unaffected (Figure 3E-H). These findings were confirmed in the tibia (Figure S1). This effect of inactivation of neuronal ERα on cortical bone growth was absent in chemically castrated female mice (Figure S2), supportive of a role for E2 in mediating the suppressive effect of neuronal ERα on bone. Deletion of ERα in extrahypothalamic neurons did not affect trabecular bone parameters in the metaphyseal region of femur or L5 vertebra in both sexes (Table S3). Serum analysis showed reduced TRAcP 5b levels in female N-ERαKO compared to controls, while
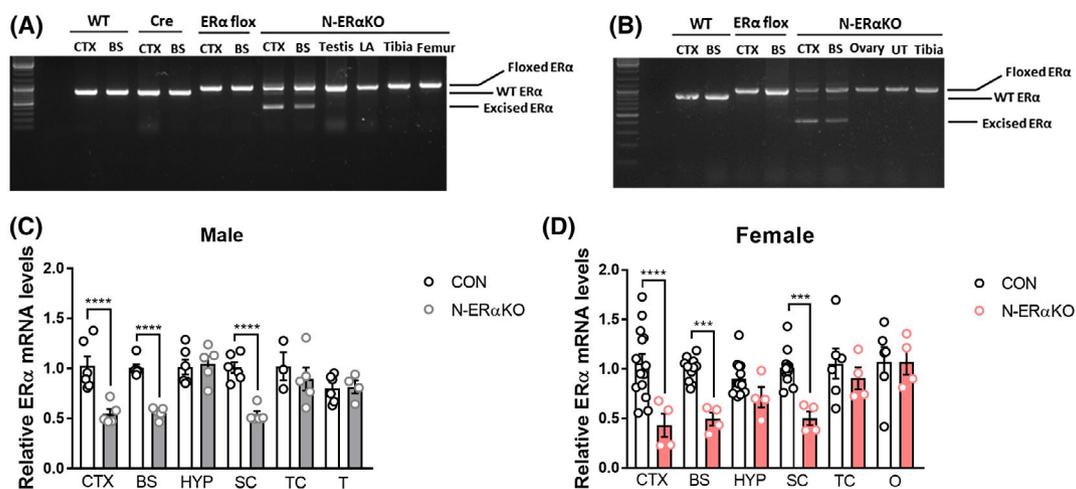
FIGURE 1 Validation of the N-ERαKO mouse model. A and B, Representative images of PCR analysis of DNA extracted from brain and non-neuronal tissues of 8-week-old male (A) and female (B) mice of the indicated genotypes. C and D, Quantitative real-time RT-PCR analysis of ERα mRNA in brain, spinal cord and tibia of 8-week-old male (C) and female (D) N-ERαKO and CON mice. Data are expressed as mean ± SEM. Number of animals: male CON = 3-6, male N-ERαKO = 5; female CON = 6-14, female N-ERαKO = 4. Two-way ANOVA with Bonferroni post hoc test was used, ***$P < .001$, ****$P < .0001$ versus CON. BS = brainstem; CTX = cerebral cortex; HYP = hypothalamus; LA = levator ani; O = ovary; SC = spinal cord; T = testis; TC = tibia cortex; UT = uterus
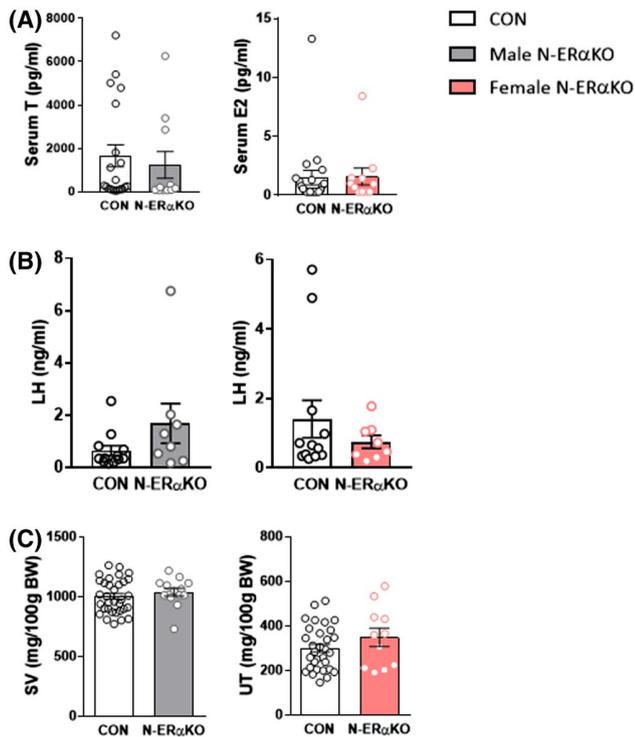
FIGURE 2 Serum sex steroid levels, sex steroid-sensitive tissue weights, and serum LH levels in N-ERαKO mice. A, Serum levels of T in male (left panel) and E2 in female (right panel) 16-week-old mice. B, Serum LH levels in male (left panel) and female (right panel) 16-week-old mice. C, Seminal vesicle weight in male (left panel) and uterus weight in female (right panel) 16-week-old mice. Data represent mean ± SEM. Number of animals: male CON = 12-34, male N-ERαKO = 8-14; female CON = 12-31, female N-ERαKO = 8-11. The unpaired two-tailed *t* test revealed no differences between groups. BW = body weight; E2 = estradiol; LH = luteinizing hormone; SV = seminal vesicle; T = testosterone; UT = uterus

osteocalcin levels were comparable between the two groups (Figure S3). No changes in serum osteocalcin or TRAcP 5b were detected in male N-ERαKO mice (Figure S3). Since larger bones (both longer and wider) are related to bone strength, mechanical properties of the femurs were analyzed by a 3-point bending test. Female N-ERαKO mice displayed increased bone strength (+8.7%, $P < .05$) (Figure 3I), whereas stiffness and work-to-failure were unaffected (Figure S4). Overall, there was no detectable bone phenotype observed in male N-ERαKO at 16 weeks of age.

3.4 | Longitudinal and radial bone expansion in female N-ERαKO is associated with increased IGF-1 and IGFBP 3 levels

To investigate whether the increased cortical bone size in female N-ERαKO was related to the GH-IGF-1 axis, IGF-1, and IGFBP 3, which are both strong indicators for GH regulation, were determined. Male N-ERαKO did not show

significant differences compared to control mice, while female mutant mice displayed increased circulating levels of IGF-1 (16.3%, $P < .05$) and IGFBP-3 (21.1%, $P < .05$) at 16 weeks of age (Figure 4A,B).

4 | DISCUSSION

To uncover the importance of central ERα signaling for bone mass acquisition during puberty in both sexes, we generated mice with inactivation of ERα in extrahypothalamic neurons. We demonstrate that neuronal ERα is a major determinant of sexual skeletal dimorphism. Indeed, neuronal ERα inactivation increased linear growth, radial bone expansion and mechanical strength in female mice, whereas no effects on bone were observed in male mice. The male-like skeletal phenotype of female N-ERαKO was potentially mediated by GH/IGF-1 signaling (Figure 5).

Thus far, ERβ has been considered as the main determinant of the difference in bone size between sexes. Female ERβKO mice showed more pronounced bone growth in both appendicular and axial length as well as radial cortical expansion.[12,13,32] Moreover, no effects were observed in male ERβKO mice indicating that ERβ-mediated inhibitory action is sex-specific. Interestingly, the increased femur length observed in female ERβKO was accompanied by increased IGF-1 levels, supporting involvement of the GH/IGF-1 axis as underlying mechanism. Since there is possible interaction between ERα and ERβ as proposed by Lindberg et al,[33] we examined ERβ mRNA expression levels in the different brain regions where Thy1-Cre is expressed (Figure S5). No changes in ERβ expression were detected in female N-ERαKO mice, excluding involvement of ERβ in the phenotype. Therefore, on top of previous observations of inhibitory E2 action via ERβ, our study suggests another inhibitory mechanism of E2 on sexually dimorphic bone development via neuronal ERα during late puberty.

ERα has been traditionally viewed as the dominant receptor mediating stimulatory E2 actions on bone by promoting the cortical bone development. Global deletion of ERα reduced both appendicular and axial growth as well as radial cortical bone expansion in female mice.[13] However, the bone phenotype of global ERαKO mice was confounded by a disrupted HPG axis as evidenced by strongly increased serum T and E2 as well as low IGF-1 levels. Indeed, the decrease in cortical bone observed in global ERαKO female mice was accompanied by an increase in trabecular bone mass, a parameter which is highly sensitive to sex steroids levels.[34] Therefore, it is difficult to assess the relative contribution of stimulatory vs inhibitory E2 actions via ERα on bone growth using these transgenic mouse models with a disrupted HPG axis. Moreover, it seems unlikely that inhibitory E2 effects on bone are directly mediated by ERα in bone cells. Indeed,
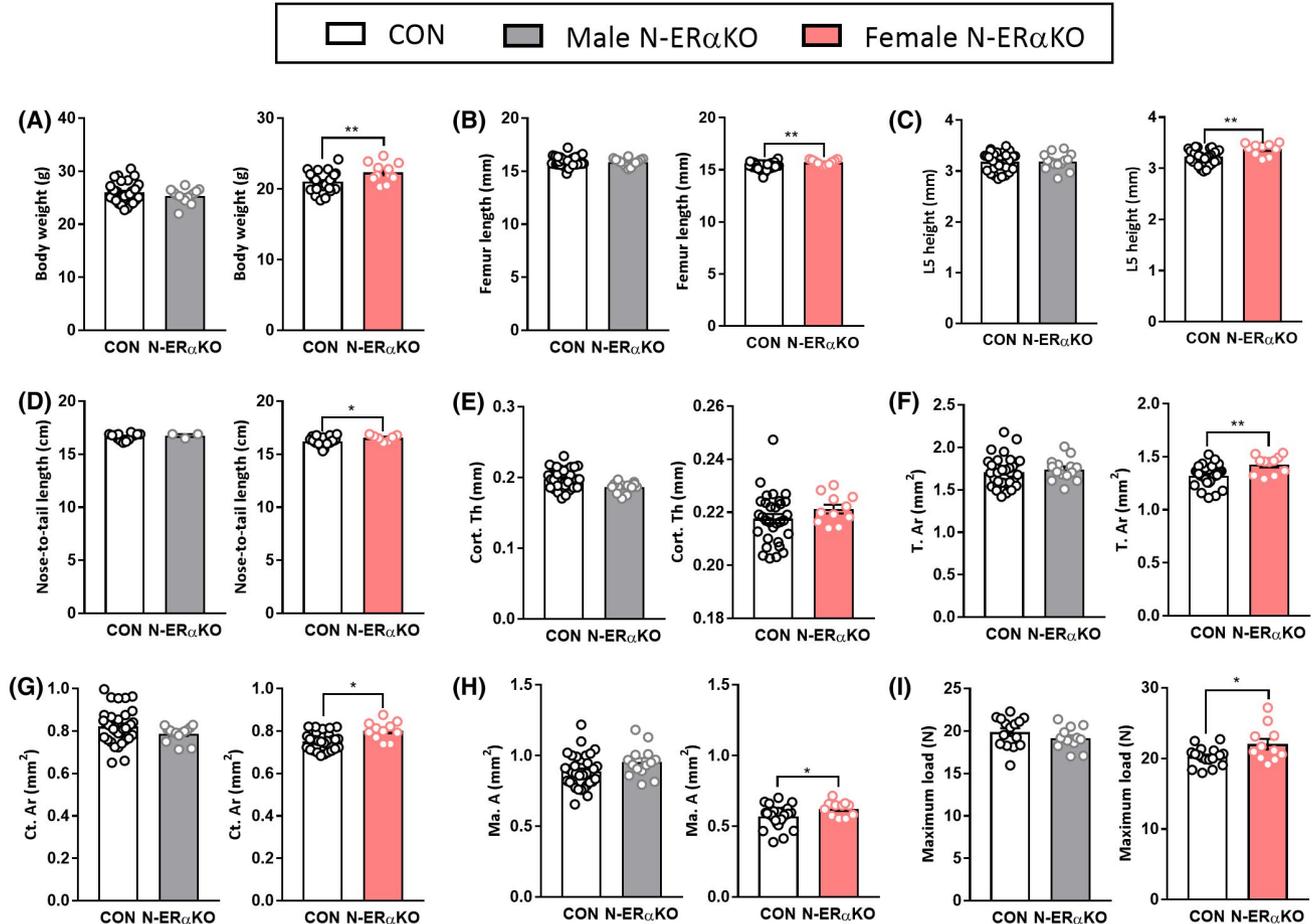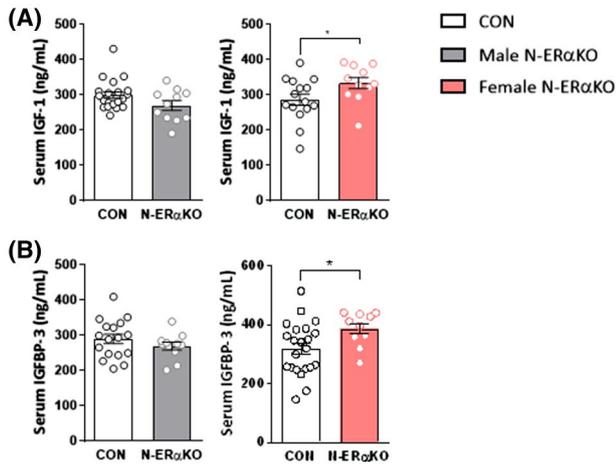
FIGURE 3 Effect of neuronal ERα inactivation on body weight and bone. All parameters were determined in 16-week-old male (left panels) and female (right panels) mice. A-D, Body weight (A), femur length (B), L5 height (C) and nose-to-tail length (D) of N-ERαKO and CON mice. E-H, Cortical bone measurements of midshaft region of femur. I, Maximum load measured at midshaft region of femur by 3-point bending test. Ct.Ar = cortical area; Ct.Th = cortical thickness; Ma.Ar = medullary area; Tt.Ar = total cross-sectional tissue area. Data are expressed as mean ± SEM. Number of animals: male CON = 29-34, male N-ERαKO = 12-14; female CON = 28-31, female N-ERαKO = 10-11. The unpaired two-tailed *t* test was used, *$P < .05$, **$P < .01$ versus CON

bone cell-specific ERαKO mouse models rather support stimulatory effects of ERα on periosteal expansion through early and mature osteoblast lineage cells.[17,35,36] An indirect mediator of suppressive E2 effects via ERα on skeletal growth is the chondrocyte. During late puberty, high estrogen concentrations directly act on the epiphyseal growth plate by reducing its width and proliferative capacity.[7,37] Patients with a disruptive ERα or CYP19A1 gene mutation experience absence of pubertal growth spurt and continue to grow due to lack of epiphyseal closure.[38,39] Similarly, mice with chondrocyte-specific deletion of ERα had longer bones than their wild type controls at 1 year of age. Collectively, these data suggest inhibitory E2 action on the skeleton through ERα in cartilage cells.[7]

In contrast to the global ERαKO model, the bone phenotype of our mice with extrahypothalamic neuron-specific ERαKO deletion is independent from disturbed negative feedback on HPG axis since sex steroid levels and gonadotropins were unaffected. Hence, our findings demonstrate

that cortical bone size is not mainly explained by circulating sex steroids. In line with the normal sex steroid levels, there were no changes in trabecular bone mass in female N-ERαKO mice.

In contrast to our findings, previous investigations on the effect of neuronal ERα signaling on bone showed higher trabecular bone mass in female mice with central ERα deletion. When ERα was deleted in neuronal and glial precursor cells using Nestin-Cre, young adult female mice showed higher trabecular bone mass in both the appendicular and axial skeleton as well as higher cortical bone mass.[24] Increased bone mass acquisition in female Nestin-ERαKO mice was proposed to be mediated by decreased leptin sensitivity in the hypothalamus leading to elevated secretion of leptin from white adipose tissue.[24] Similarly, when ERα was specifically deleted in ERα-expressing neurons in the arcuate nucleus of the hypothalamus using POMC-Cre and NKX2-1-Cre, female mutant mice displayed higher trabecular bone mass along with increased cortical bone mineral content which led

FIGURE 4 Effect of neuronal ERα inactivation on GH/IGF-1 axis. Serum levels of IGF-1 (A) and IGFBP-3 (B) in male (left panels) and female (right panels) 16-week-old N-ERαKO and CON mice. Data are expressed as mean ± SEM. Number of animals: male CON = 17-21 male N-ERαKO = 11; female CON = 15-22, female N-ERαKO = 10-11. The unpaired two-tailed *t* test was used, *$P < .05$ versus CON. GH = growth hormone; IGF-1 = insulin like growth factor-1; IGFBP-3 = insulin like growth factor binding protein-3
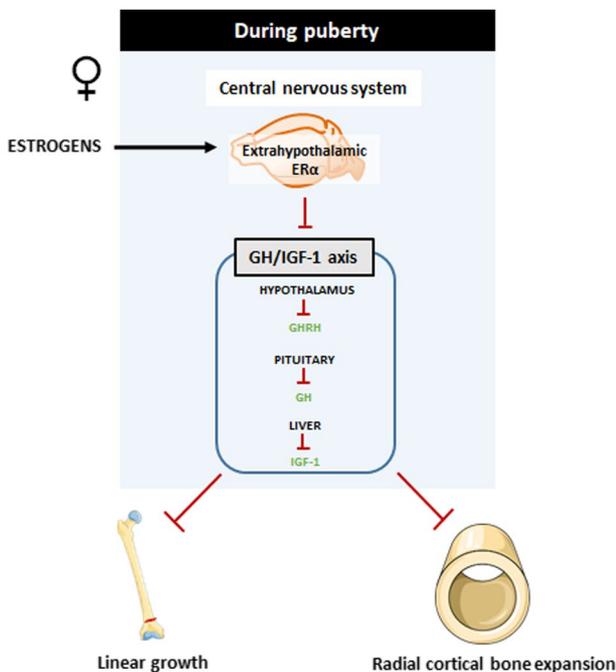
FIGURE 5 Proposed central ERα action on bone growth. Central estrogen signaling limits both linear growth and radial bone expansion during late puberty indirectly via inhibition of the GH/IGF-1 axis in female mice

to increased mechanical bone strength compare to control mice.[22,23] However, the elevated serum E2 levels or increased uterine weight in these hypothalamic transgenic mouse models indicate disturbance in feedback regulation which may have confounded their bone phenotype. In this view, our study extends current understanding of brain-bone axis since deletion of hypothalamic ERα was avoided in our animal model resulting in normal HPG function.

The sex-specific cortical bone phenotype of our N-ERαKO model appears to be dependent on GH/IGF-1, since both serum IGF-1 and IGFBP-3, as a proxy of GH secretion, were upregulated in female mutant mice. Therefore, it is plausible that the inhibitory central ERα action on bone size during late puberty is indirectly mediated via GH/IGF-1. During puberty, the GH/IGF-1 axis is the major determinant of longitudinal and transversal bone growth. Mice with deletion of the GH receptor showed no skeletal sexual dimorphism with very low circulating IGF-1.[6] Accordingly, the pituitary GH secretion pattern is sex dependent and related to neonatal imprinting.[40,41] In addition, GH production and release are positively correlated with E2 in both humans and rodents.[42-44] In fact, GH gene expression is sexually dimorphic and, in particular, GH is co-expressed with ERα not only in the hypothalamus but also in extrahypothalamic brain regions including cerebral cortex.[44] Collectively, while these previous observations demonstrate that the GH/IGF-1 axis mediates sexual skeletal dimorphism, our study for the first time provides a possible link between the somatotropic axis and neuronal ERα signaling in the establishment of skeletal sexual dimorphism in mice.

It could be tempting to speculate that ERα action in the brain might also be involved in periosteal bone expansion following menopause.[45] However, postmenopausal periosteal bone expansion is much slower than during growth and—in contrast to our observations in female N-ERαKO mice—accompanied by cortical bone loss.[45]

The main strength of our study is that we were able to shut down ERα signaling in a specific location and time (ie, in extrahypothalamic neurons during late puberty). However, our N-ERαKO mouse model also has some limitations. First, while the 50% reduction in ERα mRNA levels in extrahypothalamic brain regions may be due to contaminating ERα-expressing glial cells and pericytes, incomplete neuronal ERα deletion cannot be excluded. Moreover, although our aim was to specifically target the nervous system while avoiding disruption of hypothalamic ERα, changes in GH/IGF-1 signaling do occur in our model, indicating interaction between extrahypothalamic neuronal ERα signaling and hypothalamic regions implicated in GH signaling.

In summary, we here report that neuronal ERα signaling is necessary to limit bone size and strength in female mice at the end of puberty. This inhibitory E2 action appears to be mediated by the GH/IGF-1 axis.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Authors' roles: Study design: F. Jardí, B. Decallonne, G. Carmeliet, F. Claessens, V. Dubois, and D. Vanderschueren. Study conduct: N.R. Kim, F. Jardí, D. Schollaert, L. Deboel. Data collection: N.R. Kim. Data analysis: N.R. Kim. Data interpretation: N.R. Kim, L. Antonio, B. Decallonne, G. Carmeliet, F. Claessens, C. Ohlsson, M.K. Lagerquist, V. Dubois, and D. Vanderschueren. Drafting manuscript: N.R. Kim and V. Dubois. Revising manuscript content: all authors. Approving final version of manuscript: all authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.