Inactivation of AR or ERα in extrahypothalamic neurons does not affect osteogenic response to loading in male mice

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ABSTRACT

Failure of bone mass maintenance in spite of functional loading is an important contributor to osteoporosis and related fractures. While the link between sex steroids and the osteogenic response to loading is well-established, the underlying mechanisms are unknown, hampering clinical relevance. Androgens inhibit mechanoresponsiveness in male mice, but the cell type mediating this effect remains unidentified. To evaluate the role of neuronal sex steroid receptor signaling in the male bone's adaptive capacity, we subjected adult male mice with an extrahypothalamic neuron-specific knockout of the androgen receptor (N-ARKO) or the estrogen receptor alpha (N-ERαKO) to in vivo mechanical stimulation of the tibia. Loading increased cortical thickness in the control animals mainly through periosteal expansion, as total cross-sectional tissue area and cortical bone area but not medullary area were higher in the loaded compared to the unloaded tibia. Trabecular bone volume fraction also increased upon loading in the control group, mostly due to trabecular thickening. N-ARKO and N-ERαKO males displayed a loading response at both the cortical and trabecular bone compartments which was not different from their control littermates. In conclusion, we show that the presence of AR or ERα in extrahypothalamic neurons is dispensable for the osteogenic response to mechanical loading in male mice.

Keywords: Testosterone, neurons, bone, mechanical loading

INTRODUCTION

Functional adaptation of the skeleton is characterized by its ability to adjust bone mass and architecture to withstand reasonable loads (1). The most widespread failure of this adaptation mechanism is the decline in bone mass occurring despite functional loading in postmenopausal and age-related osteoporosis (2,3). This bone loss results in an increased incidence of osteoporotic fractures, which are more prevalent in women but associated with greater morbidity and mortality in men, principally because they occur later in life (4). Several studies have suggested that changes in sex steroid levels such as observed during puberty, after menopause or in aging affect the adaptive response of bone (5,6). In humans, however, studying the effects of sex steroids on the bone's osteogenic response to loading independent of muscle-bone interactions is not feasible.

Mouse models with direct in vivo skeletal loading allow mechanistic elucidation of these effects, independent of muscle (7). In females, a landmark study from the group of Lanyon and colleagues (8) demonstrated that the estrogen receptor alpha (ERα) is crucial for the osteogenic response to loading. The finding of diminished mechanoresponsiveness in female mice with global ERα knockout (KO) was confirmed by subsequent studies (9,10). This effect occurs in a ligand-independent manner involving activation function 1 in the N-terminal domain of ERα (10). In males, however, the relationship between sex steroids, their receptors, and the skeletal response to loading is less clear. Indeed, while orchidectomy consistently enhances the osteogenic response indicating that circulating sex steroids suppress the bone's adaptive capacity (11,12), the underlying mechanisms remain to be elucidated. Testosterone (T), the main circulating sex steroid in males, exerts its effects either directly via the androgen receptor (AR) or indirectly through the estrogen receptors (ERα/β) as the substrate for aromatization into estrogens (13). Evidence supporting a role for the AR in mediating the suppressive effect of androgens on the male bone's osteogenic response to loading includes the enhanced mechanoresponsiveness of global ARKO males (9) and the finding that the nonaromatizable androgen dihydrotestosterone produces equal or even stronger inhibition of

loading-induced bone formation compared with T (12). The involvement of the ERα in the male bone's adaptive capacity, on the other hand, continues to be debated, as conflicting results showing enhanced or unaltered osteogenic responses were obtained in male global ERαKO mice (9,14).

Another unresolved issue in the field relates to the cell type mediating the suppressive effect of androgens on the osteogenic response to loading in males. A first obvious candidate is the osteocyte, as this predominant cell type in bone harbors mechanosensing properties enabling it to translate mechanical loading into biochemical signals that mediate bone formation and resorption (15). Osteocyte-ablated mice show resistance to unloading-induced bone loss (16), providing further evidence for the role of osteocytes in mechanotransduction. Surprisingly, however, we and others have demonstrated that the osteocyte is not the main mediator of androgen effects on the male bone's adaptive response to loading. Indeed, male mice with osteocyte-specific ablation of AR or ERα show unaltered osteogenic response (17-19). Another plausible candidate is the neuronal cell. Emerging evidence describes a physical and functional association between the neuronal and skeletal systems that form a neuro-osteogenic network (20). Furthermore, neuronal blocking during loading abrogates bone formation (21), and both AR and ERα are expressed throughout the nervous system (22). Finally, we and others have shown that sex steroid action on the skeleton is in part dependent on a bone-brain crosstalk, as neuronal AR signaling slows the age-related cortical thinning in male mice (23) while neuronal ERα signaling participates to the development of skeletal sexual dimorphism (24-27). Whether these signaling pathways are also involved in the suppressive effect of androgens on the male bone's adaptive capacity is however unknown. The aim of the present study was therefore to examine the role of neuronal AR and ERα signaling in the osteogenic response to loading in male mice.

MATERIALS AND METHODS

Animals

Male mice with an extrahypothalamic neuron-specific knockout of the androgen receptor (N-ARKO) or the estrogen receptor alpha (N-ERαKO) were generated using a tamoxifen inducible Cre-LoxP system as previously described (23,24) and maintained on a C57BL/6J genetic background. Briefly, female mice carrying a heterozygous floxed exon 2 of the AR (28) or a heterozygous floxed exon 3 of the ERα (29) were mated in successive generations with male mice expressing CreERT2 under the control of a modified Thy1 promoter, which shows robust expression in both the central and peripheral nervous system except for the hypothalamus, caudate putamen, ventral striatum, and basal forebrain (30). At 6 weeks of age, male N-ARKO mice (ARfl/Y;Thy1-CreERT2+/-) and control littermates (ARfl/Y; Thy1-CreERT2-/-, ARwt/Y; Thy1-CreERT2+/-, and ARwt/Y; Thy1-CreERT2-/-) as well as male N-ERαKO mice (ERαfl/fl; Thy1-CreERT2+/-) and control littermates (ERαfl/fl; Thy1-CreERT2-/-, ERαwt/wt; Thy1-CreERT2+/-, and ERαwt/wt; Thy1-CreERT2-/-) were orally gavaged with tamoxifen (190 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) once daily for 2 consecutive days. Our group previously showed that this tamoxifen regimen results in enough recombinase efficiency to induce neuron-specific deletion of the sex steroid receptors while minimizing off-target effects of tamoxifen on sex steroid-sensitive organs such as reproductive tissues and bones (31). At 16 weeks of age, N-ARKO and control littermates as well as N-ERαKO and control littermates (n=8-10 mice/group) were subjected to the mechanical loading protocol described below. At 18 weeks of age, all animals were euthanized with pentobarbital anesthesia followed by cardiac puncture, and tibiae were harvested for micro-computed tomography analysis. An additional cohort of N-ARKO and control littermates as well as N-ERαKO and control littermates (n=6-10 mice/group) were sacrificed at 8 weeks of age for collection of various neural and non-neural tissues.

To determine the effects of our tamoxifen induction scheme on bone morphology and the osteogenic response to loading, 32 male wildtype mice (C57BL/6J) obtained from the KU Leuven

animal facility were orally gavaged with tamoxifen (190 mg/kg body weight) (n=16) or vehicle (1 in 10 dilution of ethanol in sunflower seed oil) (n=16) once daily for 2 consecutive days at 6 weeks of age. A subgroup was sacrificed 24h after the second administration to monitor acute tamoxifen effects on hepatic gene expression (n=6 mice/group). The remaining animals (n=10/group) were subjected to the mechanical loading protocol at 16 weeks of age and sacrificed at 18 weeks of age for collection of liver and tibiae.

An additional group of 6 male wildtype mice (C57BL/6J) obtained from the KU Leuven animal facility were used to measure sex steroid receptor expression in different regions of the brain at 8 weeks of age.

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 food and water. The animal experiments were conducted in accordance with the KU Leuven guidelines for animal experimentation and approved by the KU Leuven ethical committee (P041/2014 and P192/2016).

Real-time quantitative PCR

Murine tissues (various brain regions and liver) collected at sacrifice were snap-frozen in liquid nitrogen and stored at -80°C until processing. The bone marrow fraction was removed from the tibia by centrifugation of the bones for 2.5 minutes at full speed after removal of the proximal ends, yielding tibial cortical bone which was stored at -80°C. Total RNA was extracted from the tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. CDNA was synthesized from 1 µg RNA using FastGene Scriptase II kit (NIPPON Genetics Europe, Dueren, Germany) and random hexamer primers. The PCR reactions were performed using Fast SYBR Green Master Mix or TaqMan Fast Advanced Master Mix and the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). For assessment of *Esr1* expression levels, primers from the Mm00433147_m1 assay (Thermo Fisher Scientific, Waltham, MA, USA) were used. The

other primer sequences are described in **Supplemental Table 1** (32). The relative expression levels of the target genes *Ar* and *Esr1* were calculated as a ratio to the expression levels of the *Actb*, *Gapdh* and *Hprt* housekeeping genes.

In vivo mechanical loading

Mice were anesthetized with isoflurane while the flexed knee and ankle joints of the right leg were positioned in concave cups. The upper cup, into which the knee was positioned, was attached to the actuator arm of a servohydraulic loading machine (ElectroForce 3100 Test Instrument, Bose Corporation) and the lower cup to a dynamic load cell. The right tibia was held in place by a low level of a continuous static preload, onto which higher levels of intermittent dynamic load were superimposed. In the current study, 16.5 N dynamic load and 2.0 N static preload were applied in a series of 40 trapezoidal-shaped pulses (0.025 sec loading, 0.050 sec hold at 16.5 N, and 0.025 sec unloading) with a 10-second rest interval between each pulse. Left tibiae served as internal controls (33). All animals underwent the mechanical loading protocol three times a week on alternate days for two weeks.

Micro-computed tomography

Tibiae 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. Images were reconstructed with the NRecon software (Bruker) and morphometric parameters were calculated using CTAn (Bruker) as previously described (34). For cortical bone analysis, a 0.5 mm region of interest in the tibial shaft was selected starting at 3 mm from the proximal growth plate and moving towards the diaphysis. For trabecular bone analysis, a 1.5 mm segment was selected starting at 0.75 mm from the proximal growth plate and moving towards the diaphysis. Parameters are reported according to the ASBMR guidelines (35) and include cortical thickness (Ct.Th, mm), total cross-sectional tissue area (Tt.Ar, mm^2), cortical bone area (Ct.Ar, mm^2), medullary area (Ma.Ar, mm^2),

trabecular bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, µm), trabecular separation (Tb.Sp, µm), and trabecular number (Tb.N, 1/mm). Loading-related changes (%) were calculated as (loaded bone – unloaded bone) / unloaded bone x 100. Three-dimensional image rendering was performed using CTVol (Bruker).

Statistical analysis

Statistical analysis was performed using GraphPad Prism v9.3.0 (GraphPad, La Jolla, CA, USA). Repeated-measures ANOVA was performed to assess the interaction between genotype and loading followed by Sidak's multiple comparisons test. Data are presented as mean ± SD and two-tailed $P<0.05$ was considered as statistically significant.

RESULTS

Validation of neuron-specific sex steroid receptor deletion in the N-ARKO and N-ERαKO models

We first mapped sex steroid receptor expression in the male mouse brain. To this end, AR and ERα levels were quantified in various brain regions of 8-week-old wildtype male mice. Not only the hypothalamus, but also extrahypothalamic regions such as cerebral cortex, striatum, hippocampus, and amygdala displayed considerable AR expression (**Supplemental Figure S1A**) (32). With regards to ERα, we found the highest expression in the hypothalamus, although it was also detected in the different extrahypothalamic regions we analyzed (**Supplemental Figure S1B**) (32).

We previously showed that tamoxifen administration induces the excision of floxed AR exon 2 or floxed ERα exon 3 in neural tissues of N-ARKO and N-ERαKO mice, respectively, whereas non-neural tissues do not show CreERT2-mediated recombination (23,24). Here, we confirm that AR expression is reduced in extrahypothalamic brain regions of N-ARKO animals including cerebral cortex (-84%, $P<0.0001$), brain stem (-68%, $P<0.0001$), and spinal cord (-73%, $P<0.0001$), while unaffected in hypothalamus and tibia (**Figure 1A left panel**). In contrast, no changes were observed in ERα

expression (**Figure 1A right panel**). In N-ERαKO mice, AR levels were stable (**Figure 1B left panel**), whereas ERα expression was decreased in cerebral cortex (-38%, *P*=0.0036), brain stem (-44%, *P*=0.0006), and spinal cord (-52%, *P*<0.0001), but comparable to control levels in hypothalamus and tibia (**Figure 1B right panel**).

To evaluate the role of neuronal AR and ERα signaling in the male bone's adaptive capacity, we next subjected 16-week-old male N-ARKO and N-ERαKO mice as well as their control littermates to in vivo mechanical loading of the right tibia, while the left tibia was used as an internal control. After two weeks of loading, both tibiae were collected and the osteogenic response was assessed by microCT analysis.

Loading-induced osteogenic response in N-ARKO male mice

Loading increased cortical thickness in the control animals (+19%, *P*<0.0001) (**Figure 2A** and **2B left panel**). This effect was due to periosteal expansion, as total cross-sectional tissue area and cortical bone area but not medullary area were higher in the loaded compared to the unloaded tibia (**Figure 2C-2E**). In N-ARKO males, cortical thickness also increased (+15%, *P*<0.0001) (**Figure 2A** and **2B left panel**) due to periosteal expansion (**Figure 2C-2E**). The loading-induced increase in cortical thickness was similar in both genotypes (P=0.34) (**Figure 2B right panel**).

Cancellous bone was also responsive to loading, as evidenced by the increased trabecular bone volume fraction in both control and N-ARKO males (+33%, P<0.0001; +26%, P=0.0002) (**Figure 3A** and **3B left panel**). There was no significant difference in the loading-induced increase in trabecular volume fraction between both genotypes (P=0.17) (**Figure 3B right panel**). The increase in cancellous bone upon loading was mostly due to an increase in trabecular thickness (**Figure 3C**), as loading-induced changes in trabecular separation and number were marginal, except for a small but significant increase in trabecular number in the control animals (**Figure 3D** and **3E**).

Loading-induced osteogenic response in N-ERαKO male mice

Cortical thickness increased upon loading in the control animals (+18%, *P*<0.0001) (**Figure 4A** and **4B left panel**), as did total cross-sectional tissue area and cortical bone area but not medullary area (**Figure 4C-4E**). N-ERαKO males also showed an osteogenic response, with cortical thickness increasing (+15%, *P*=0.0027) (**Figure 4A** and **4B left panel**) due to periosteal expansion (**Figure 4C-4E**). There was no significant difference in the loading-induced increase in cortical thickness between both genotypes (P=0.59) (**Figure 4B right panel**).

Loading increased the trabecular bone volume fraction in both control and N-ERαKO animals (+29%, P=0.0004; +31%, P=0.0017) (**Figure 5A** and **5B left panel**). This increase was similar in both genotypes (P=0.86) (**Figure 5B right panel**) and mostly attributable to an increase in trabecular thickness (**Figure 5C**). Indeed, trabecular separation was unaffected by loading (**Figure 5D**), while effects on trabecular number were marginal except for a small but significant increase in the control animals (**Figure 5E**).

Tamoxifen effects on loading-induced osteogenic response

As in aforementioned experiments, all groups including the control littermates were treated with tamoxifen, we aimed to verify the effects of our tamoxifen regimen on bone morphology and the osteogenic response to loading. To this end, male wildtype mice were treated with 190 mg/kg tamoxifen (TAM) or vehicle (VEH) once daily for 2 consecutive days at 6 weeks of age (similar to the administration scheme used to generate the N-ARKO and N-ERαKO models), subjected to the mechanical loading protocol at 16 weeks of age and sacrificed at 18 weeks of age. Tamoxifen administration at 6 weeks of age was effective, as evidenced by the increased expression of several tamoxifen-responsive hepatic genes 24h after the second administration (**Supplemental Figure S2A upper panel**) (32,36). This hepatic induction was transient, since gene expression had returned to base levels at 18 weeks of age (**Supplemental Figure S2A lower panel**) (32). In both VEH and TAM

groups, mechanical loading increased cortical thickness by periosteal expansion, as well as trabecular bone volume fraction by trabecular thickening. (**Supplemental Figure S2B-S2I**) (32). While the loading-induced increase in cortical thickness was similar between VEH and TAM groups, the increase in trabecular bone volume fraction was more pronounced in the TAM-treated animals, likely due to a lower baseline trabecular thickness (**Supplemental Figure S2B-S2I**) (32).

DISCUSSION

Male osteoporosis and related fractures represent an important public health issue, with considerable economic impact as well as high personal burden (37). Failure of the skeleton to maintain adequate bone mass in spite of functional loading in elderly men is an important contributor to age-related osteoporosis (3). While the link between sex steroids and the osteogenic response to loading is well-established (2), lack of knowledge of the underlying mechanisms hampers clinical relevance. Therefore, there is an unmet need to investigate the effects of sex steroids on male bone's adaptive capacity.

Androgens inhibit the osteogenic response to mechanical loading in adult male mice (11,12). Previous findings indicate that both AR and ERα might be implicated in this suppressive effect (9,14) (**Figure 6**). A key question, however, remains unresolved: which cell type mediates the interaction between androgens and the adaptive response of the male skeleton? Neurons innervating the bone arose as potential candidates, as both sex steroid receptors are expressed throughout the nervous system (22) and functional adaptation of the skeleton to loading is neuronally regulated (21). In this study, we demonstrate that the presence of AR or ERα in extrahypothalamic neurons is dispensable for the male osteogenic response to loading at both the cortical and trabecular bone compartments. Indeed, tibiae from both N-ARKO and N-ERαKO male mice showed periosteal expansion and trabecular thickening upon in vivo mechanical loading to a similar extent as observed in their control

littermates. Hence, we conclude that extrahypothalamic neurons are not the main mediators of sex steroid effects on the male bone's adaptive capacity (**Figure 6**).

Tamoxifen affects bone homeostasis, which might confound the bone phenotype of knockout models when using this drug to induce Cre recombinase-mediated gene deletion (38). Therefore, we previously tested several induction schemes and showed that a shortened tamoxifen regimen of two doses effectively induces Cre recombinase while having only marginal effects on androgen bioactivity and bone morphology (31). Based on those findings, we applied this scheme to generate our neuronal sex steroid receptor knockouts. Given that the skeletal response to mechanical loading is highly sensitive to even subtle differences in bone morphology at baseline, we here subjected all experimental groups, including controls, to the tamoxifen regimen, to avoid differences in osteogenic response between controls and knockout animals arising from modest tamoxifen-induced alterations in baseline bone morphology which would be independent from our pathway of interest i.e. neuronal sex steroid receptor signaling. In a separate experiment, we compared the tamoxifen scheme with vehicle to pinpoint these potential tamoxifen-related differences in bone morphology in the unloaded bones and their effect on the loading response. While the loading-induced increase in cortical bone was unaffected, the increase in trabecular bone volume fraction was enhanced by tamoxifen, likely due to a lower baseline trabecular thickness.

Previously developed models of neuronal sex steroid receptor deletion (25-27), in which ablation also occurs in the hypothalamus, display elevated circulating sex steroid levels and/or increased reproductive organ weight. These observations indicate disturbances in the hypothalamic-pituitary-gonadal feedback, which might confound the bone phenotype including the osteogenic response to mechanical loading. To avoid this bias, we chose to develop neuronal knockout lines in which hypothalamic AR and ERα expression is intact. The main strength of this study hence resides in the unaffected levels of sex steroids and gonadotropins in our mouse models (23,24), enabling to study the role of neuronal sex steroid receptor signaling independent of the negative feedback regulation

via the hypothalamic-pituitary-gonadal axis. A limitation of this study is that - as for all murine KO models – only a partial reduction of the gene of interest is observed (80% and 50% decrease in AR and ERα mRNA, respectively). While the remaining levels in extrahypothalamic brain regions may be due to contaminating sex steroid receptor-expressing glial cells and pericytes, incomplete neuronal deletion cannot be excluded. However, it should be noted that these reductions were sufficient to generate a bone phenotype. Indeed, decrease in extrahypothalamic AR expression accelerates age-related cortical thinning in male mice (23), while diminished extrahypothalamic ERα expression results in increased linear growth and radial bone expansion during late puberty in female mice (24). Thus, while being essential for acquisition (ERα in females) or maintenance (AR in males) of the skeleton, extrahypothalamic sex steroid receptor signaling is dispensable for its response to mechanical loading. Moreover, as POMC neurons in the hypothalamus have been shown to be involved in estrogenic effects on bone (26), we cannot rule out the possibility that sex steroid signaling in hypothalamic neurons might participate to the male bone's adaptive capacity (**Figure 6**). Finally, our study was restricted to male mice. Whether neuronal sex steroid signaling is implicated in the female bone's adaptive capacity remains to be investigated.

Previous mechanistic studies aiming to unravel sex steroid effects on the male skeletal response to loading have focused on the osteocyte, given the key role of this predominant skeletal cell type in mechanoresponsiveness (15). However, targeted deletion of AR or ERα in osteocytes of male mice did not impair periosteal expansion of the bones upon mechanical stimulation (17-19), indicating sex steroid receptor signaling in the osteocyte is not mediating the suppression of the loading response by androgens (**Figure 6**). In females also, the osteocyte has been excluded as the main mediator of sex steroid effects on skeletal adaptation (39). Recent studies nevertheless suggest that estrogen withdrawal results in intrinsic changes in the ability of the osteocyte to sense and respond to mechanical loading (40,41). Of note, Cre recombinase activity in abovementioned conditional knockouts was driven by the Dmp1 or osteocalcin promoter, resulting in sex steroid receptor ablation in mature osteoblasts and/or terminally differentiated osteocytes. Previous research,

however, has indicated that other cell types in bone are also important players in the skeletal response to loading. Indeed, biasing of the cell fate of bone marrow-resident mesenchymal stem cells away from adipogenesis and toward osteoblastogenesis by mechanical strain contributes to the bone's adaptive response (42,43). In addition, mechanical stimulation results in rapid changes in periosteal gene expression (44), and osteoprogenitor cells in the periosteum contribute to the increase in osteoblast numbers in response to loading (45). Sex steroid receptors have been detected in several cell types along the differentiation of mesenchymal precursors to osteoblasts, as well as in other cell types residing in the bone marrow (2). Altogether, it seems plausible that the inhibition of mechanoresponsiveness by androgens in males may rely on sex steroid receptor actions in mesenchymal stem cells, osteoprogenitor cells or other periosteal cell populations (**Figure 6**). Additional studies investigating the effect of targeted AR or ERα deletion in these cell types on the loading response will increase our mechanistic insights into the interplay between sex steroids and mechanoresponsiveness, and might in the long run impact clinical strategies for the management of osteoporosis.

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Author Contributions: V.D. and D.V. conceptualized and designed the study. N.R.K., K.D., V.D., V.S., D.S., and L.D. performed the experiments. N.R.K. and V.D. collected and analyzed most of the data. N.R.K., K.D., V.S. L.A., B.D., F.C., D.V., and V.D. contributed to data interpretation. V.D. wrote and drafted the manuscript. All authors participated to critical revisions of the manuscript and approved the final version of the manuscript.

ADDITIONAL INFORMATION

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FIGURE LEGENDS

Figure 1. Sex steroid receptor expression levels in the N-ARKO and N-ERaKO mouse models. (A-B) Relative expression levels of the *Ar* (A) and *Esr1* (B) genes, encoding the sex steroid receptors AR and ERα, respectively, assessed by RT-qPCR analysis of various tissues from 8-week-old male mice with extrahypothalamic neuron-specific deletion of the AR (N-ARKO) and control littermates. **(C-D)** Relative expression levels of the *Ar* (C) and *Esr1* (D) genes, assessed by RT-qPCR analysis of various tissues from 18-week-old male mice with extrahypothalamic neuron-specific deletion of the ERα (N-ERαKO) and control littermates. Data are normalized to control male levels in each panel and expressed as mean ± SD (n=4-10 mice/group), and were analyzed by two-way ANOVA followed by Sidak's multiple comparisons test. CTX: brain cortex; BS: brain stem; SC: spinal cord; HYP: hypothalamus; TIB: tibial cortex.

Figure 2. N-ARKO male cortical bone shows unaltered osteogenic response to mechanical loading. (A) Three-dimensional images of cortical bone of tibiae from 18-week-old male mice with extrahypothalamic neuron-specific deletion of the AR (N-ARKO) and control littermates subjected to in vivo mechanical loading for two weeks. **(B)** Cortical thickness (Ct.Th) measured by ex vivo microCT analysis of tibiae from 18-week-old male N-ARKO mice and control littermates subjected to in vivo mechanical loading for two weeks. Absolute values (left panel) and relative loading-related changes [(loaded bone – unloaded bone) / unloaded bone x 100] (right panel) of Ct.Th are shown. **(C-E)** Total cross-sectional tissue area (Tt.Ar) (C), cortical bone area (Ct.Ar) (D) and medullary area (Ma.Ar) (E) in tibiae from the same animals. Data are expressed as mean ± SD (n=8-10 mice/group) and were analyzed by repeated-measures ANOVA to assess the interaction between genotype and loading followed by Sidak's multiple comparisons test.

Figure 3. N-ARKO male trabecular bone shows unaltered osteogenic response to mechanical loading. (A) Three-dimensional images of trabecular bone of tibiae from 18-week-old male mice

with extrahypothalamic neuron-specific deletion of the AR (N-ARKO) and control littermates subjected to in vivo mechanical loading for two weeks. **(B)** Trabecular bone volume fraction (BV/TV) measured by ex vivo microCT analysis of tibiae from 18-week-old male N-ARKO mice and control littermates subjected to in vivo mechanical loading for two weeks. Absolute values (left panel) and relative loading-related changes [(loaded bone – unloaded bone) / unloaded bone x 100] (right panel) of BV/TV are shown. **(C-E)** Trabecular thickness (Tb.Th) (C), trabecular separation (Tb.Sp) (D) and trabecular number (Tb.N) (E) in tibiae from the same animals. Data are expressed as mean ± SD (n=8-10 mice/group) and were analyzed by repeated-measures ANOVA to assess the interaction between genotype and loading followed by Sidak's multiple comparisons test.

Figure 4. N-ERαKO male cortical bone shows unaltered osteogenic response to mechanical loading. **(A)** Three-dimensional images of cortical bone of tibiae from 18-week-old male mice with extrahypothalamic neuron-specific deletion of the ERα (N-ERαKO) and control littermates subjected to in vivo mechanical loading for two weeks. **(B)** Cortical thickness (Ct.Th) measured by ex vivo microCT analysis of tibiae from 18-week-old male N-ERαKO mice and control littermates subjected to in vivo mechanical loading for two weeks. Absolute values (left panel) and relative loading-related changes [(loaded bone – unloaded bone) / unloaded bone x 100] (right panel) of Ct.Th are shown. **(C-E)** Total cross-sectional tissue area (Tt.Ar) (C), cortical bone area (Ct.Ar) (D) and medullary area (Ma.Ar) (E) in tibiae from the same animals. Data are expressed as mean ± SD (n=8-10 mice/group) and were analyzed by repeated-measures ANOVA to assess the interaction between genotype and loading followed by Sidak's multiple comparisons test.

Figure 5. N-ERαKO male trabecular bone shows unaltered osteogenic response to mechanical loading. (A) Three-dimensional images of trabecular bone of tibiae from 18-week-old male mice with extrahypothalamic neuron-specific deletion of the ERα (N-ERαKO) and control littermates subjected to in vivo mechanical loading for two weeks. **(B)** Trabecular bone volume fraction (BV/TV) measured by ex vivo microCT analysis of tibiae from 18-week-old male N-ERαKO mice and control

littermates subjected to in vivo mechanical loading for two weeks. Absolute values (left panel) and relative loading-related changes [(loaded bone – unloaded bone) / unloaded bone x 100] (right panel) of BV/TV are shown. **(C-E)** Trabecular thickness (Tb.Th) (C), trabecular separation (Tb.Sp) (D) and trabecular number (Tb.N) (E) in tibiae from the same animals. Data are expressed as mean ± SD (n=8-10 mice/group) and were analyzed by repeated-measures ANOVA to assess the interaction between genotype and loading followed by Sidak's multiple comparisons test.

Figure 6. Candidate mediators of the suppressive effect of androgens on the osteogenic response to mechanical loading in male mice. Androgens inhibit the osteogenic response to mechanical loading in adult male mice through both AR and ERα. Osteocytes, osteoblasts and extrahypothalamic neurons are not the main cellular mediators of this suppressive effect. The inhibition of mechanoresponsiveness by androgens may rely on sex steroid receptor actions in hypothalamic neurons, mesenchymal stem cells, osteoprogenitor cells or other periosteal cell populations.

Figure 1

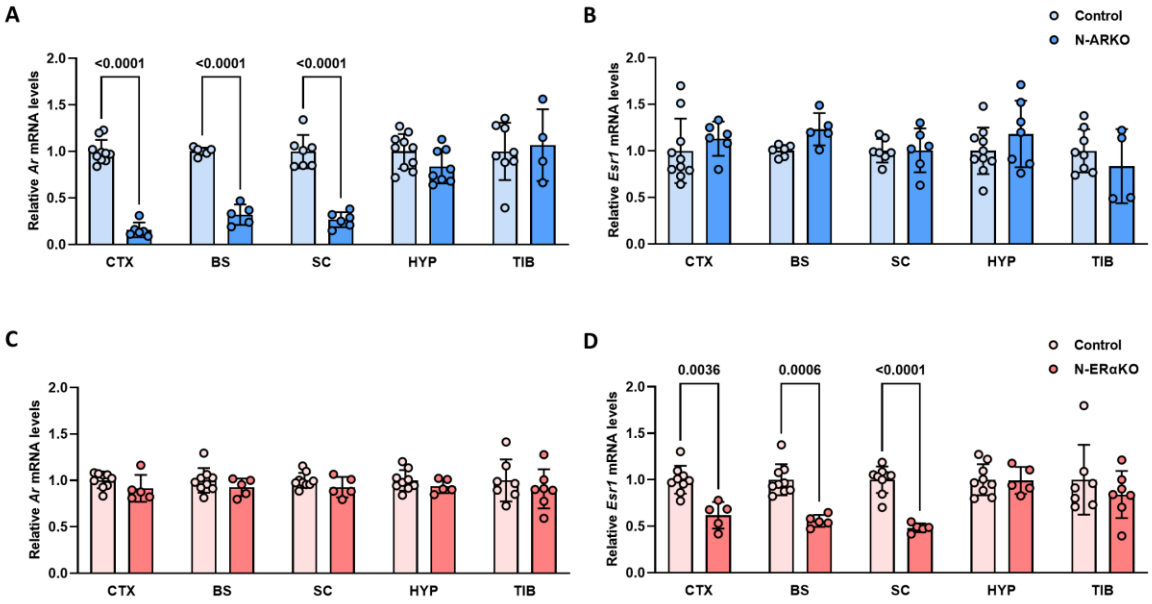
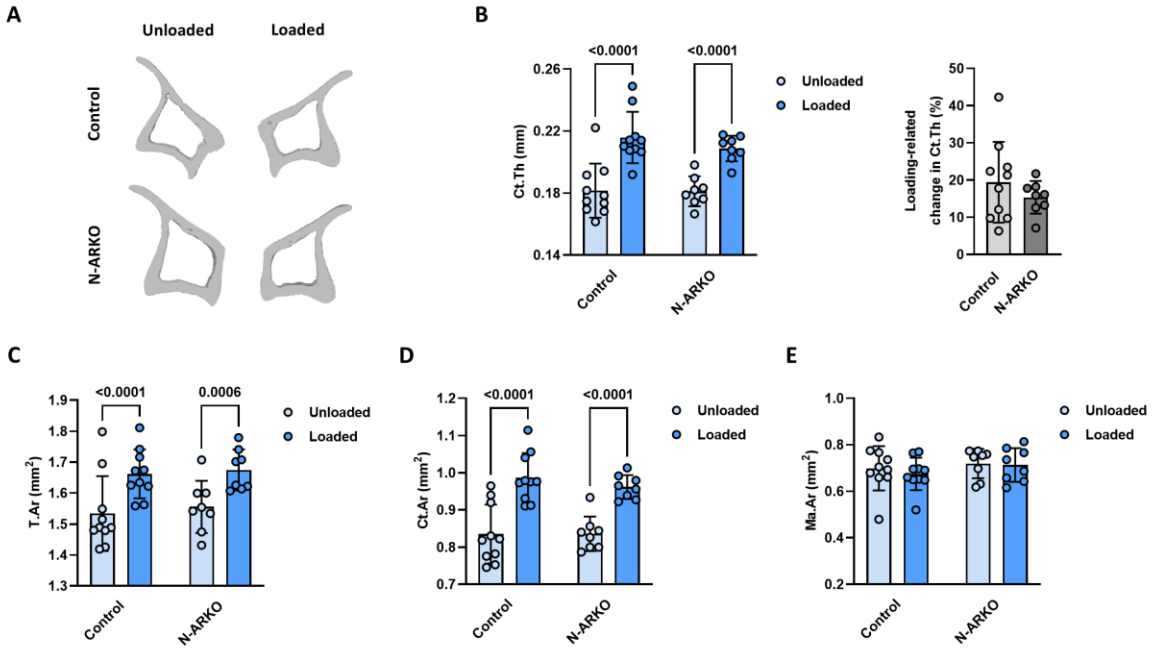


Figure 2



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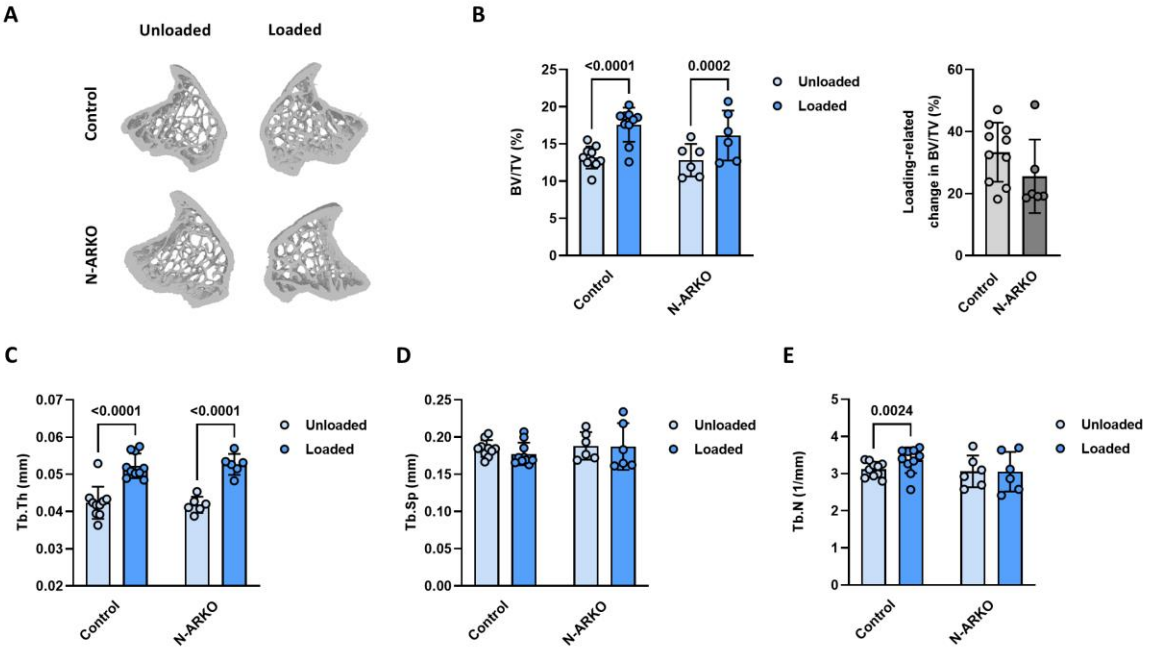

Figure 4

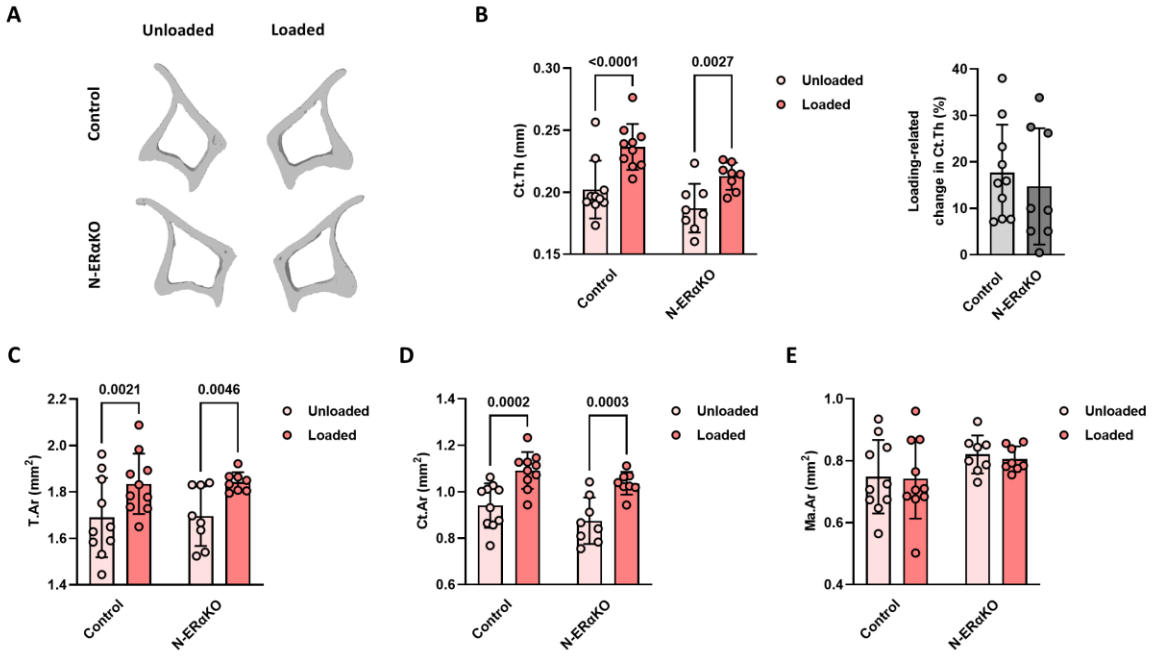

Figure 5

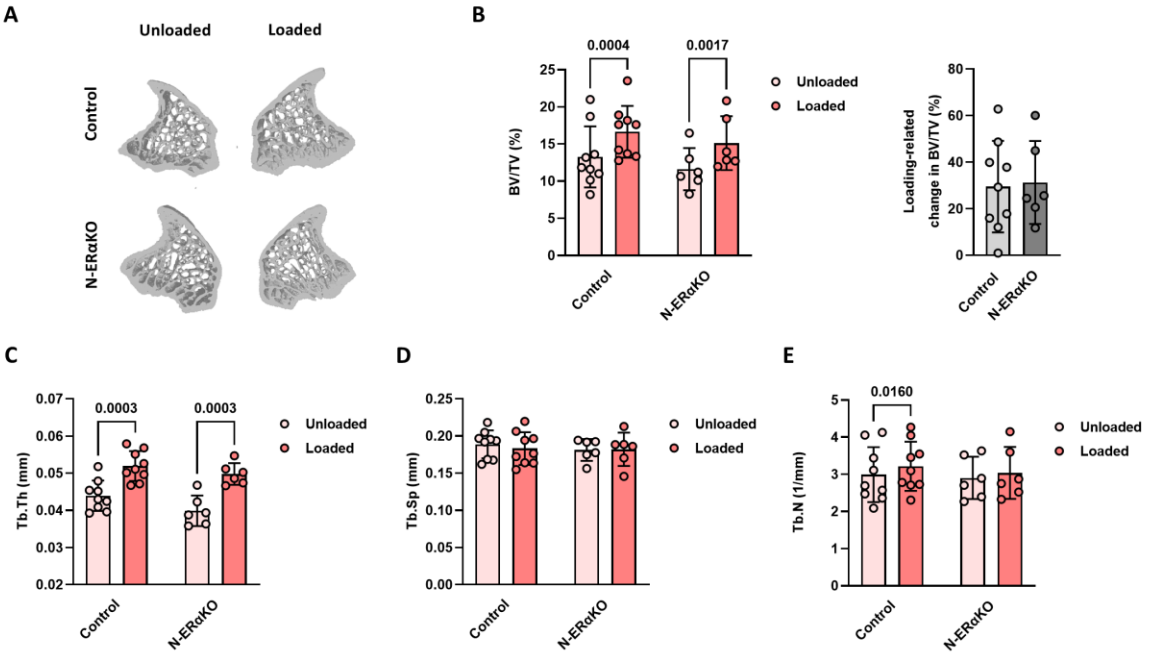

Figure 6