



# Global Disruption of $\alpha$ 2A Adrenoceptor Barely Affects Bone Tissue but Minimizes the Detrimental Effects of Thyrotoxicosis on Cortical Bone

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Evidence shows that sympathetic nervous system (SNS) activation inhibits bone formation and activates bone resorption leading to bone loss. Because thyroid hormone (TH) interacts with the SNS to control several physiological processes, we raised the hypothesis that this interaction also controls bone remodeling. We have previously shown that mice with double-gene inactivation of  $\alpha$ 2A- and -adrenoceptors ( $\alpha$ 2A/2C-AR<sup>-/-</sup>) present high bone mass (HBM) phenotype and resistance to thyrotoxicosis-induced osteopenia, which supports a TH-SNS interaction to control bone mass and suggests that it involves  $\alpha$ 2-AR signaling. Accordingly, we detected expression of  $\alpha$ 2A-AR,  $\alpha$ 2B-AR and  $\alpha$ 2C-AR in the skeleton, and that triiodothyronine (T3) modulates  $\alpha$ 2C-AR mRNA expression in the bone. Later, we found that mice with single-gene inactivation of  $\alpha$ 2C-AR ( $\alpha$ 2C-AR<sup>-/-</sup>) present low bone mass in the femur and HBM in the vertebra, but that both skeletal sites are resistant to TH-induced osteopenia, showing that the SNS actions occur in a skeletal site-dependent manner, and that thyrotoxicosis depends on  $\alpha$ 2C-AR signaling to promote bone loss. To further dissect the specific roles of  $\alpha$ 2-AR subtypes, in this study, we evaluated the skeletal phenotype of mice with single-gene inactivation of  $\alpha$ 2A-AR ( $\alpha$ 2A-AR<sup>-/-</sup>), and the effect of daily treatment with a supraphysiological dose of T3, for 4 or 12 weeks, on bone microarchitecture and bone resistance to fracture. Micro-computed tomographic ( $\mu$ CT) analysis revealed normal trabecular and cortical bone structure in the femur and vertebra of euthyroid  $\alpha$ 2A-AR<sup>-/-</sup> mice. Thyrotoxicosis was more detrimental to femoral trabecular bone in  $\alpha$ 2A-AR<sup>-/-</sup> than in WT mice, whereas this bone compartment had been previously shown to present resistance to thyrotoxicosis in  $\alpha$ 2C-AR<sup>-/-</sup> mice. Altogether these findings reveal that TH excess depends on  $\alpha$ 2C-AR signaling to negatively affect femoral trabecular bone. In contrast, thyrotoxicosis was more deleterious to femoral and vertebral cortical bone in WT than in  $\alpha$ 2A-AR<sup>-/-</sup> mice, suggesting that  $\alpha$ 2A-AR signaling contributes to TH actions on

cortical bone. These findings further support a TH-SNS interaction to control bone physiology, and suggest that  $\alpha$ 2A-AR and  $\alpha$ 2C-AR signaling pathways have key roles in the mechanisms through which thyrotoxicosis promotes its detrimental effects on bone remodeling, structure and resistance to fracture.

**Keywords:** thyroid hormone, thyrotoxicosis, sympathetic nervous system,  $\alpha$ 2A-adrenoceptor, cortical bone, trabecular bone, bone remodeling

## INTRODUCTION

Thyroid hormone (TH) is recognized as an important regulator of bone remodeling, having a key role in the maintenance of bone mass and bone integrity (1). TH affects the skeleton indirectly, modulating the expression or action of other hormones and factors, such as GH and IGF-1 (2, 3), but also acts directly in the skeleton, controlling the proliferation, differentiation and/or activity of the main skeletal cells (4–7). Thyroid hormone receptors (TR) were detected in osteoblasts (8–11), osteocytes (9), osteoclasts (9, 12) and chondrocytes (13). In conditions of TH deficiency, both bone formation and resorption are decreased, leading to a state of low bone turnover. In this condition bone mass may be slightly increased or unchanged (14–16). In contrast, TH excess increases both osteoblastic and osteoclastic activities, but the latter is favored, leading to negative balance of calcium and bone loss (17–20). Therefore, thyrotoxicosis is an established cause of secondary osteoporosis, due to high bone turnover, with accelerated bone loss (16, 21, 22).

Over the last two decades, data has uncovered the sympathetic nervous system (SNS) as another potent regulator of bone remodeling (23). Evidence shows that SNS activation increases bone resorption and decreases bone formation leading to bone loss (24, 25), in a process that involves  $\beta$ 2 adrenoceptors ( $\beta$ 2-AR), expressed in osteoblasts (26, 27). However, studies by our group showed that female mice with global double inactivation of  $\alpha$ 2A and  $\alpha$ 2C adrenoceptors ( $\alpha$ 2A/C-AR<sup>-/-</sup> mice) present a striking high bone mass (HBM) phenotype along with improved resistance to fracture, increased bone formation rate and decreased bone resorption, regardless of presenting increased sympathetic outflow and, therefore, a chronic increase in serum levels of catecholamines (28). The  $\alpha$ 2-adrenoceptor ( $\alpha$ 2-AR) family comprises  $\alpha$ 2A-,  $\alpha$ 2B- and  $\alpha$ 2C-AR subtypes (29–31), which are expressed in presynaptic membranes of adrenergic neurons, where they act as autoreceptors (receptors stimulated by the neurotransmitter released by the neuron where they are located) inhibiting the release of catecholamines (32, 33), especially norepinephrine (NE). Therefore,  $\alpha$ 2A/C-AR<sup>-/-</sup> mice represent a mouse model of chronic sympathetic hyperactivity (34). Thus, the HBM phenotype in these animals revealed that  $\beta$ 2-AR is not the only adrenoceptor involved in the control of bone turnover and raised the hypothesis that  $\alpha$ 2-AR subtypes also mediate SNS signaling in the skeleton (28). Accordingly, we and others found expression of  $\alpha$ 2A-,  $\alpha$ 2B- and  $\alpha$ 2C-AR in osteoblasts (28, 35, 36), osteoclasts, osteocytes and chondrocytes (28). In addition, *in vitro* findings reveal direct  $\alpha$ 2-AR signaling-mediated actions of the SNS in the skeleton (28, 37).

An important characteristic of TH is its interaction with the SNS to regulate several physiological processes (38). It is well known that TH-SNS interactions are necessary for maximum thermogenesis, lipolysis and lipogenesis (39). Interestingly, several clinical manifestations of thyrotoxicosis are indicative of increased adrenergic activity, such as tachycardia, increased cardiac output, increased glycogen and lipid mobilization, enhanced thermogenesis, tremor, hyperkinetic behavior, and sweating (40). In contrast, responses to adrenergic stimulation are low or blunted during hypothyroidism, leading, for example, to cold intolerance and limited metabolic responses to exercise (38, 41). Considering that both SNS hyperactivity and thyrotoxicosis have osteopenic effects, we raised the hypothesis that a TH-SNS interaction also occurs to regulate bone remodeling. The fact that treatment of hyperthyroid patients with propranolol, a  $\beta$  adrenoceptor antagonist, corrects the thyrotoxicosis-induced hypercalcemia (42) and decreases the urinary excretion of hydroxyproline, a biochemical marker of bone resorption (43), support a possible TH-SNS interaction to control bone remodeling, in addition to suggest that this interaction depends on the  $\beta$ -AR signaling pathway.

Recent studies by our group further support the hypothesis of a TH-SNS interaction to control bone remodeling, and reveals that  $\alpha$ 2-AR signaling has an important role in this interaction (28, 37, 44). We found that young adult female  $\alpha$ 2A/2C-AR<sup>-/-</sup> mice are resistant to the deleterious effects of thyrotoxicosis on bone mass, on cortical and trabecular bone microarchitecture, and on bone resistance to fracture (37). In addition, *in vitro* studies bring evidence that TH-SNS interactions are likely to occur locally in the skeleton, via  $\alpha$ 2A-AR and/or  $\alpha$ 2C-AR signaling. Later, with the aim of discriminating the roles of the different  $\alpha$ 2-AR isoforms, we evaluated the bone phenotype of mice with the single gene inactivation of  $\alpha$ 2C-AR subtype ( $\alpha$ 2C-AR<sup>-/-</sup>) (44), which mRNA expression had been previously shown to be downregulated by TH in the femur (37). While  $\alpha$ 2A/2C-AR<sup>-/-</sup> mice present a generalized phenotype of HBM (28), we found that  $\alpha$ 2C-AR<sup>-/-</sup> animals present higher trabecular bone mass in the vertebra and lower trabecular bone mass in the femur (when compared with WT mice), which was accompanied by decreased bone strength in the femur and tibia (44). This heterogeneous bone phenotype of  $\alpha$ 2CAR<sup>-/-</sup> mice reinforces the hypothesis that the SNS regulates bone remodeling and structure, via  $\alpha$ 2C-AR signaling, and suggests that this regulation is not the same across the skeleton, but rather may vary depending on the skeletal site. In spite of this heterogeneous bone phenotype,  $\alpha$ 2C-AR<sup>-/-</sup> mice present resistance to the thyrotoxicosis-induced bone deterioration in both skeletal sites (femur and vertebra),

likewise  $\alpha$ 2A/2C-AR<sup>-/-</sup> mice (37, 44). These findings strongly suggest that the mechanism of action of TH to promote bone loss depends on  $\alpha$ 2C-AR subtype signaling. To confirm this hypothesis, in the present study, we characterized the bone phenotype of mice with global gene inactivation of only  $\alpha$ 2A-AR subtype, and the skeletal responses of these single KO animals ( $\alpha$ 2A-AR<sup>-/-</sup> mice) to a 4- or 12-week-long-chronic condition of thyrotoxicosis.

## MATERIALS AND METHODS

### Animals and Treatment

All the experimental procedures were carried out in accordance with the ethical principles and guidelines for animal research set by the Brazilian Society of Animal Experimentation, and were approved by the Ethics Committee on Animal Use (ECAU) of the Institute of Biomedical Sciences, University of São Paulo (protocol number 35/page 85/book 02). A cohort of female congenic C57BL/6J (B6) mice with gene inactivation of  $\alpha$ 2A-adrenoceptors ( $\alpha$ 2A-AR<sup>-/-</sup>) (34) and their wild-type (WT) controls (B6) were studied. All animals were 30 days old at baseline. The animals were kept under light- and temperature-controlled conditions (alternating cycles of light/dark for 12 h at a temperature of approximately 25°C), with *ad libitum* access to food and water. Thyrotoxicosis was induced by daily and i.p. administration of T3 (Sigma, St Louis MO, USA), at a dose of 7  $\mu$ g T3/100g•body weight (BW)/day, which is equivalent to 20 times the physiological dose of T3 per day. T3-untreated (-) animals received daily i.p. injections of saline. Animals were treated with T3 or saline for 4 or 12 weeks, at the same time of the day each day. Animals were weighed once a week to follow changes in BW over the experimental period, in order to indirectly monitor a thyrotoxic state and with the purpose of adjusting the amount of T3 to be administered, to maintain the supraphysiological dose (20xT3), during the whole treatment period. Female mice were grouped (7 animals per group) as follows: WT and  $\alpha$ 2A-AR<sup>-/-</sup> (T3-untreated animals, receiving saline), WT+T3 and  $\alpha$ 2A-AR<sup>-/-</sup> + T3. At the end of the treatment period, animals were euthanized by exposure to CO<sub>2</sub>.

### Serum T3 and T4 Assay

In order to confirm a thyrotoxic state, serum levels of T3 and thyroxine (T4) were measured at the end of the experimental period. Just after euthanasia, blood was collected by cardiac puncture. Serum was isolated by centrifugation and serum levels of total T4 and T3 were measured using radioimmunoassay commercial kits (RIA-gnost T4 and RIA-gnost T3; CIS Bio International, Gif-sur-Yvette, France). For the T4 and T3 assays, standard curves were built in our laboratory with a pool of charcoal-stripped mouse serum. Blood samples were always collected 2 h after the last T3 administration.

### Fat, Skeletal Muscles and Heart Mass

Considering the characteristic effects of thyrotoxicosis on fat mass, skeletal muscle mass and heart mass (45, 46), we measured these parameters at the end of the treatment period to also confirm a thyrotoxic state. Immediately after euthanasia, the

axillar and retroperitoneal fat pads; the extensor digitorum longus (EDL), gastrocnemius and rectus femoris muscles; and the heart were dissected out and weighed for wet-mass determination. The skeletal muscles and heart samples were dehydrated at 60°C for 48 h and weighed again for dry-mass determination. Fat and heart masses were expressed in milligrams per gram of BW, whereas muscle masses were expressed in milligrams per tibial or femoral length.

### Micro-Computed ( $\mu$ CT) Analysis of the Femur and L5

Bone structural parameters of the right femur and the fifth lumbar vertebra (L5) were obtained using the  $\mu$ CT unit Skyscan 1174 (Bruker MicroCT, Kontich, Belgium), and the CtAn Software, version 1.5 (Bruker MicroCT). The X-ray settings were standardized to 100 kV for the baseline specimens, with an exposure time of 590 ms. A 0.05-mm-thick aluminum filter and a beam-hardening algorithm were used to minimize beam-hardening artifacts. The vertebral body of L5 and the distal metaphysis of the femur were the selected regions of interest (ROI). The trabecular bone parameters analyzed are trabecular volume (BV/TV); trabecular number (Tb.N); trabecular thickness (Tb.Th); trabecular separation (Tb.Sp); structure model index (SMI), which indicates the prevalence of rod-like (cylinder) and plate-like trabeculae (an ideal plate and cylinder have SMI values of 0 and 3, respectively) (47); trabecular pattern factor (Tb.Pf), a trabecular connectivity index (lower indexes indicate better connectivity of the trabecular structure) (48); trabecular porosity (Tb. Po); and trabecular bone mineral density (BMD). The cortical bone parameters analyzed are the total tissue area (T.Ar), which is the total area delimited by the periosteum, including the medullary and cortical area; cortical bone area (Ct.Ar); medullary area (Ma.Ar), cortical bone thickness (Ct.Th); periosteal perimeter (Ps.Pm); endocortical or endosteal perimeter (Ec.Pm); cortical porosity (Ct.Po), and cortical BMD.

### Three-Point Bending Test of the Femur

The right femurs were dissected and submitted to the three-point bending test, using the Instron testing system Model 3344 (Instron Corporation, MA, USA). During the test, the anterior cortex of the femur was placed in compression and the posterior cortex in tension. The distal and proximal extremities of the femur were supported on two anvils, spaced by a distance that equals the half of the femoral length (femoral length/2). A force was applied perpendicularly to the longitudinal axis of the bone, at the midpoint between the two supports, in the anteroposterior direction of the femur, by a crosshead, at a constant velocity of 5 mm/min, until the bone completely ruptured. To stabilize the specimen, a small preload (5% of the average maximum load) was applied before real testing. The applied force and the displacement of the crosshead were monitored and registered, at a sampling rate of 80 Hz, by the Bluehill software (version 3, Instron Corporation). The following biomechanical parameters were obtained and evaluated: Maximum load (N), which corresponds to the highest force applied during the test (maximum force at which the bone

is able to withstand); tenacity (mJ), which is the bone's ability to withstand a fracture; and stiffness (N/mm), a measure of the rigidity of the bone tissue.

## Real-Time PCR

Expression of  $\alpha 2A$ -AR,  $\alpha 2B$ -AR,  $\alpha 2C$ -AR,  $\beta 1$ -AR, and  $\beta 2$ -AR were determined by real-time PCR in the whole femur. The femurs were dissected and then crushed in a steel mortar and pestle set (Fisher Scientific International, Inc., Hampton, NH) precooled in dry ice. The crushed bones were transferred to microfuge tubes precooled in ice and total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Total RNA was reverse transcribed using RevertAid-H-Minus M-MuLV Reverse Transcriptase (Fermentas, Hanover, MD) to synthesize the first-strand cDNA, which was used as a template. SYBR Green Super Mix (Applied Biosystems, Warrington, UK) was used for the real-time PCR using the ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA). All primers used in this study [ $\alpha 2A$ -AR, forward (F): CGC AGG CCA TCG AGT ACA A and reverse (R): GAT GAC CCA CAC GGT GAC AA (NM\_007417.3);  $\alpha 2B$ -AR, forward (F): TCC CTC TGG GAG GCA AGT G and reverse (R): GGC CAG GAT TCC AGA CCA TT (NM\_009633.3);  $\alpha 2C$ -AR, F: CAT GGG CGT GTT CGT ACT GT and R: CAG GCC TCA CGG CAG ATG (NM\_007418.3);  $\beta 1$ -AR, forward (F): TCG TCC GTC GTC TCC TTC TAC and reverse (R): ACA CCC GCA GGT ACA CGA A (NM\_007419);  $\beta 2$ -AR, forward (F): GCC ACG ACA TCA CTC AGG AAC and reverse (R): CGA TAA CCG CAC TGA GGA TGG (NM\_007420); 18S, F: GTA ACC CGT TGA ACC CCA TT and R: CCA TCC AAT CGG TAG TAG CG (NM\_11188)] were designed using the Primer Express software (Applied Biosystems<sup>TM</sup>) and were synthesized (Integrated DNA Technologies, Coralville, IA) specifically for real-time PCR. All CT values were normalized using 18S as the internal control, which was validated for this study, showing to be stable (its expression did not vary due to mice lineage or T3 treatment). Relative gene expression quantification was assessed by the CT method, as described previously by Livak and Schmittgen (49). The final values for samples are reported as fold induction relative to the expression of the control, with the mean control value being arbitrarily set to 1.

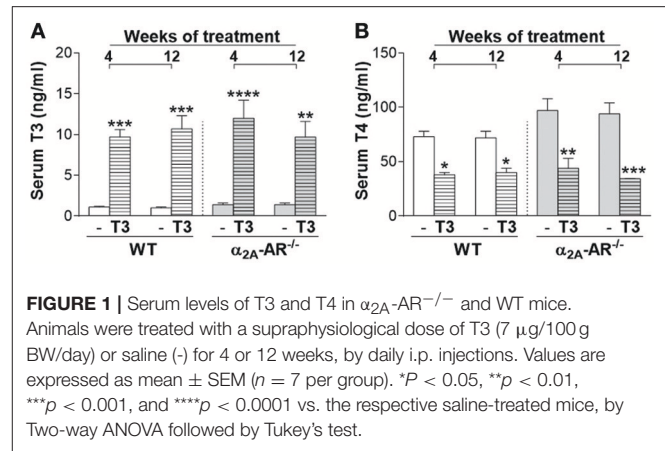
## Statistical Analysis

The statistical significance was determined by the Two-way analysis of variance (ANOVA), followed by the Tukey's test. Results were expressed as mean  $\pm$  standard error of the mean (SEM). For all tests,  $p < 0.05$  was considered statistically significant. For the statistical tests, we used the GraphPad InStat Software (GraphPad Software, Inc., San Diego, CA, USA).

## RESULTS

### Effect of T3 Treatment on Serum Levels of T3 and T4

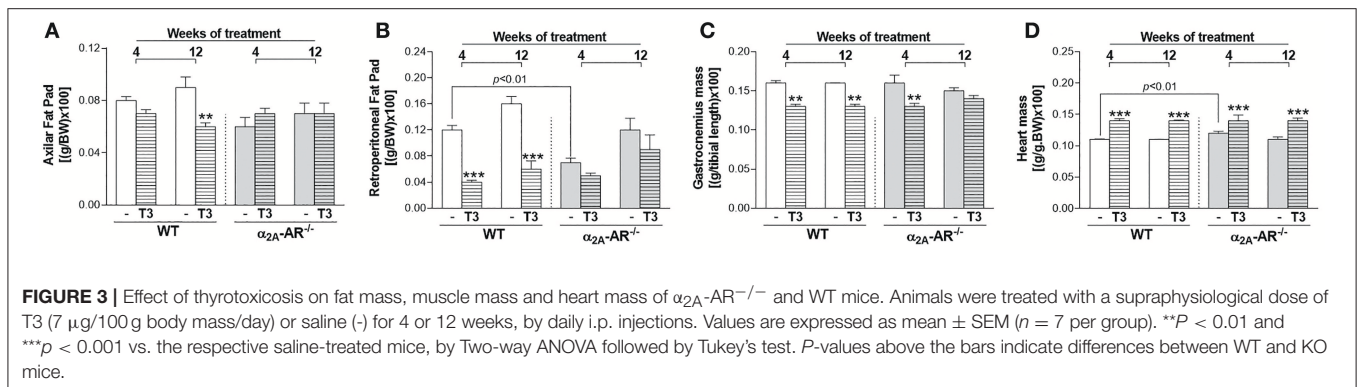
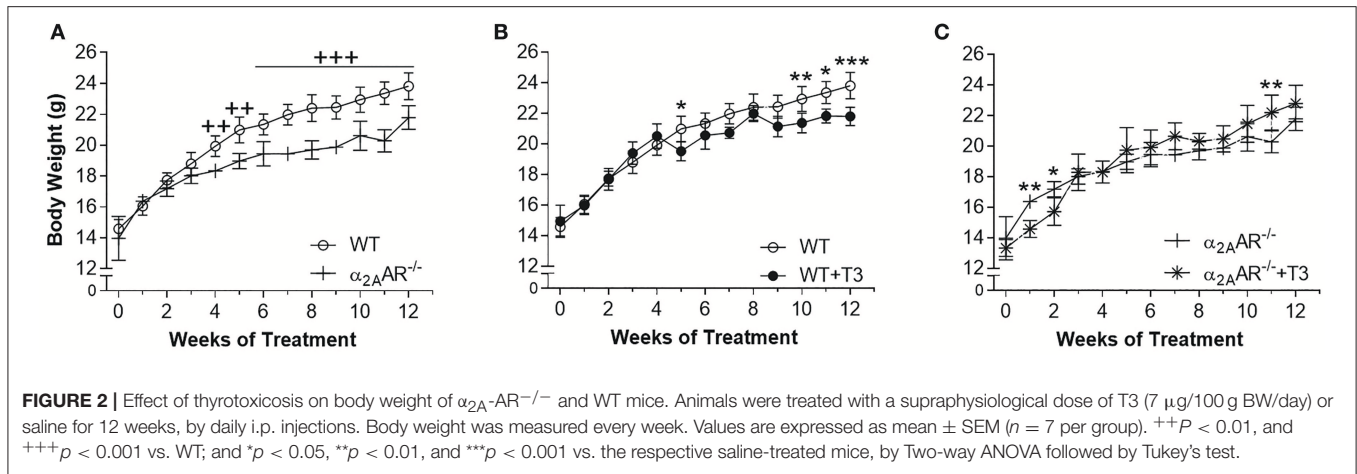
Serum levels of T3 were significantly higher (7- to 11-fold) in WT and  $\alpha 2A$ -AR<sup>-/-</sup> animals treated with T3 for 4 or 12 weeks, when compared with their respective saline-treated



controls (**Figure 1A**). On the other hand, T4 concentrations (**Figure 1B**) were significantly lower (44–64%) in WT and  $\alpha 2A$ -AR<sup>-/-</sup> animals treated with T3 (vs. saline-treated animals), which reflects the suppression of the hypothalamic-pituitary-thyroid (HPT) axis promoted by T3 excess (50), and confirms a thyrotoxic state in both WT and KO animals. Serum levels of T3 and T4 were not different between WT and  $\alpha 2A$ -AR<sup>-/-</sup> animals.

### Effect of T3 Treatment on Body Composition and Heart Mass

To indirectly confirm a thyrotoxic state, we evaluated the effect of T3 treatment on body composition and heart mass. **Figure 2A** shows that  $\alpha 2A$ -AR<sup>-/-</sup> mice presented 8–13% lower BW than WT mice from weeks 4 to 12 of this study, when animals were in the age range of 58–114 days. T3 treatment lead to lower BW in WT animals (**Figure 2B**), on weeks 5, 10, 11, and 12 of treatment (7–8%). In  $\alpha 2A$ -AR<sup>-/-</sup> animals (**Figure 2C**), BW decreased 11 and 9% after 1 and 2 weeks of T3 treatment, respectively; but then tended to be higher in T3 treated KO animals as compared with saline-treated KO mice. In fact, on week 11, BW was 9% higher in KO mice treated with T3. In WT animals, T3 treatment significantly decreased the axillar fat mass, after 12 weeks of treatment (33%); and the retroperitoneal fat mass, after 4 and 12 weeks of treatment (66 and 62%, respectively), which was not observed in KO animals (**Figures 3A,B**). The retroperitoneal fat mass showed to be 42% lower in euthyroid KO animals vs. euthyroid WT animals by the end of the 4th week of this study, when animals were 58 days old (**Figure 3B**), but later these differences were not present anymore. Four weeks of treatment with T3 did not significantly affect the mass of EDL and rectos femoris muscles both in WT and KO animals (not shown), but significantly decreased by 19% the gastrocnemius mass in both WT and KO animals, whereas 12 weeks of treatment with T3 only decreased the gastrocnemius mass in WT animals (19%), but not in KO animals (**Figure 3C**). Treatment with the supraphysiological dose of T3 (20xT3) promoted cardiac hypertrophy, evidenced by the increased dry mass of the heart (between 17 and 27%), in both WT and KO animals after 4 and 12 weeks of treatment (**Figure 3D**).



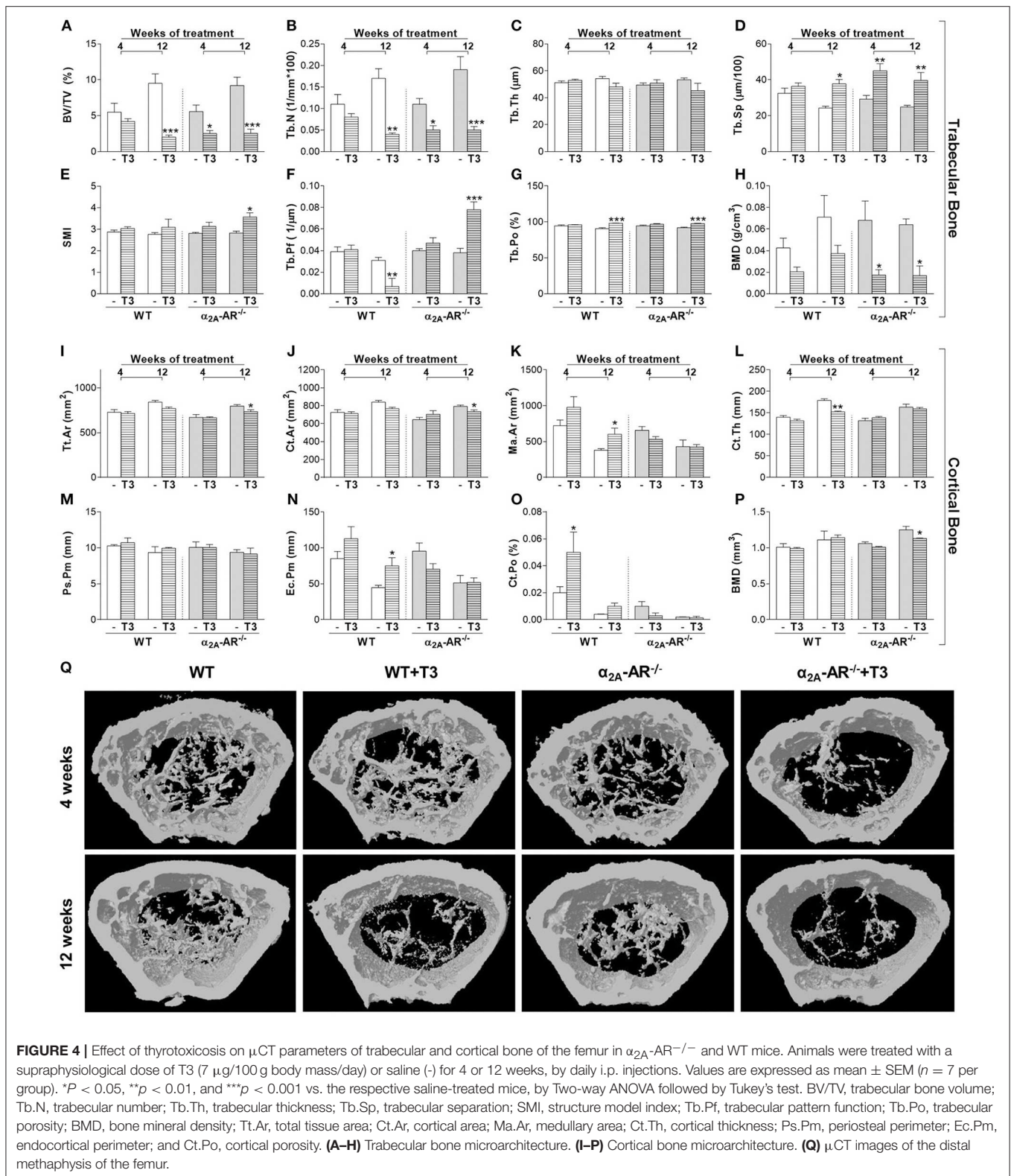
## Effect of T3 Treatment on Trabecular and Cortical Bone of the Femur

The  $\mu$ CT analysis of the femur showed that trabecular and cortical bone parameters were not different between WT and  $\alpha 2A$ -AR<sup>-/-</sup> mice treated with saline (Figure 4). As expected, T3 treatment caused deleterious effects on both trabecular and cortical compartments of the femur, but trabecular bone of KO animals showed to be more sensitive to T3 effects than that of WT animals (Figures 4A–H). The 4-week treatment with T3 significantly decreased BV/TV (54%; Figure 4A), Tb.N (55%; Figure 4B), and trabecular BMD (74%; Figure 4H); and increased Tb.Sp (54%; Figure 4D) only in KO, but not in WT animals. The 12-week treatment with T3 significantly decreased BV/TV (Figure 4A) and Tb.N (Figure 4B), and increased Tb.Sp (Figure 4D) and Tb.Po (4G) in both WT and KO animals (78 and 72%, 76 and 73%, 55 and 59%, and 8 and 6%, respectively). However, this longer treatment increased SMI (27%; Figure 4E), which indicates an increase in rod-like trabeculae (vs. plate-like trabeculae); enhanced Tb.Pf (2.1-fold; Figure 4F), which indicates a lower trabecular connectivity; and decreased trabecular BMD (74%; Figure 4H) only in KO animals, and not in WT animals. Interestingly, some cortical bone parameters of the femur were more affected by thyrotoxicosis in WT animals, while others were more affected by T3 in  $\alpha 2A$ -AR<sup>-/-</sup> animals (Figures 4I–Q). T3

treatment for 4 weeks practically had no effect on cortical bone of both WT and KO animals, except for Ct.Po, which was increased 2.5 times in WT mice, but not in KO animals. The longer T3 treatment (12 weeks) decreased Tt.Ar (8%), Ct.Ar (7%) and cortical BMD (10%) only in KO mice. On the other hand, the 12-week T3-treatment decreased Ct.Th (15%) and increased Ec.Pm (68%) and Ma.Ar (58%) only in the femur of WT animals, which suggests that TH-induced endocortical bone resorption depends on  $\alpha 2A$ -AR signaling.

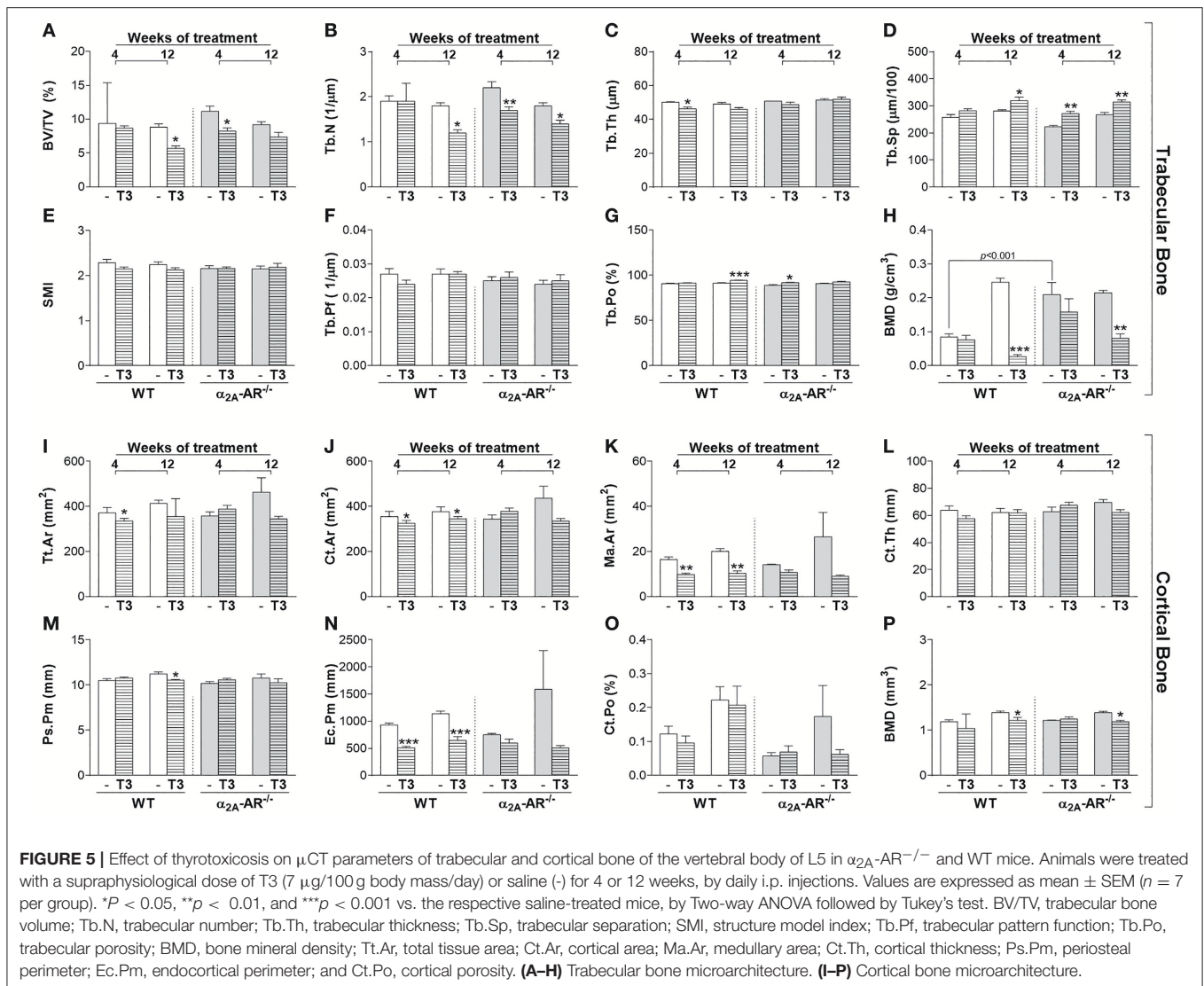
## Effect of T3 Treatment on Trabecular and Cortical Bone of the Vertebra (L5)

Trabecular and cortical bone parameters of the vertebral body of L5 were not different between euthyroid WT and  $\alpha 2A$ -AR<sup>-/-</sup> mice as well (Figure 5), except for trabecular BMD that was 2.5-fold higher in KO mice on the fourth week of this study, when animals were 58-day old, but this difference was no longer observed by the end of the study, when animals were 114 days old (Figure 5H). Trabecular bone of L5 also showed to be more sensitive to thyrotoxicosis in  $\alpha 2A$ -AR<sup>-/-</sup> mice than in WT animals, but this difference was only observed after the shorter period of T3 treatment (4 weeks). T3 treatment for 4 weeks practically had no effect on the trabecular bone of L5 in WT mice, it only decreased Tb.Th (7%) in these animals



(Figure 5C). In contrast, in  $\alpha_{2A}\text{-AR}^{-/-}$  mice, 4 weeks of T3 treatment decreased BV/TV (27%; Figure 5A) and Tb.N (22%; Figure 5B), and increased Tb.Sp (13%; Figure 5D) and Tb.Po

(3%; Figure 5G). Twelve-weeks of T3 treatment was slightly more detrimental to WT mice. T3 increased Tb.Sp and decreased Tb.N and trabecular BMD in both WT and KO animals (13 and



17%, 33 and 23%, and 89 and 62%, respectively); but decreased BV/TV (35%) and increased Tb.Po (3%) only in WT animals (Figures 5A,G). In contrast to trabecular bone, cortical bone of L5 showed to be more sensitive to thyrotoxicosis in WT than in  $\alpha 2A$ -AR $^{-/-}$  mice. T3 treatment decreased Tt.Ar (10% after 4 weeks), Ct.Ar (8% after 4 and 12 weeks), Ma.Ar (41 and 49% after 4 and 12 weeks, respectively), Ps.Pm (6% after 12 weeks) and Ec.Pm (45 and 43% after 4 and 12 weeks, respectively) only in WT animals (Figures 5I–K,N, respectively), but not in KO animals. BMD was decreased after 12 weeks of T3 treatment in both WT and KO animals in about 12% (Figure 5P). These data suggest that TH interacts with  $\alpha 2A$ -AR signaling to promote its detrimental effects on cortical bone of the vertebra.

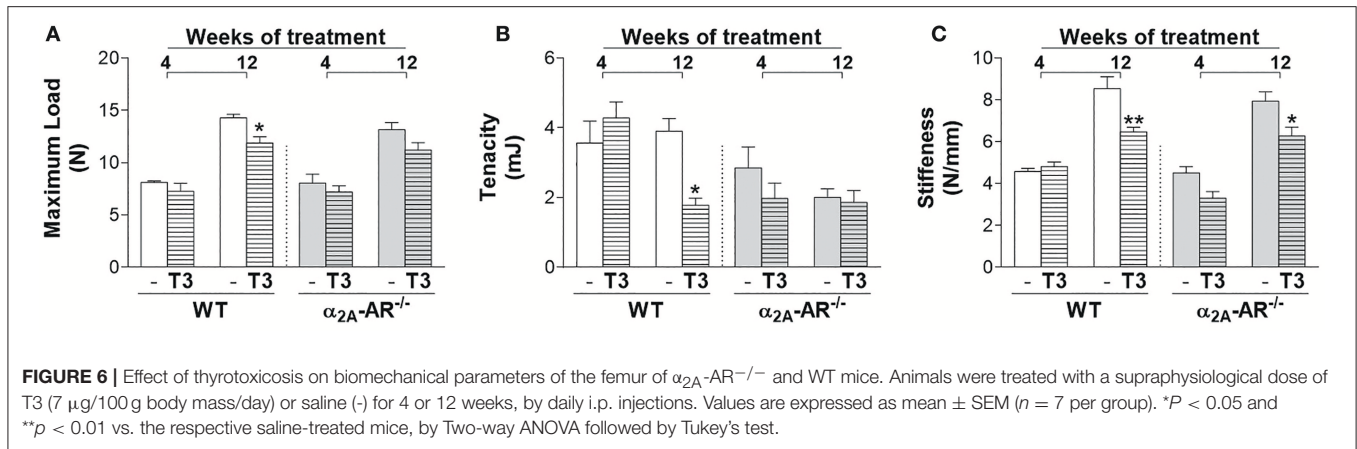
### Effect of T3 Treatment on Biomechanical Parameters of the Femur

Through the three-point bending test, we analyzed the effect of thyrotoxicosis on the femoral resistance to fracture. According

to the  $\mu$ CT data, all the biomechanical parameters analyzed were not different between WT and  $\alpha 2A$ -AR $^{-/-}$  animals. T3 treatment for 4 weeks had no effect on any parameter. On the other hand, when T3 treatment was extended to 12 weeks, it promoted a significant decrease in maximum load (17%) and tenacity (54%), only in WT animals and not in  $\alpha 2A$ -AR $^{-/-}$  animals (Figures 6A,B); but significantly decreased stiffness (Figure 6C) in both WT and KO animals (24 and 21%, respectively). These findings suggested that  $\alpha 2A$ -AR signaling mediates the detrimental effects of TH on the femoral ability to resist fracture.

### Effect of T3 on mRNA Expression of $\alpha 2$ and $\beta$ Adrenoceptors

T3 treatment had no effect on the mRNA expression of  $\alpha 2A$ -AR and  $\alpha 2B$ -AR (Figure 7A) in the whole femur of WT and/or KO mice (Figures 7A,B). On the other hand, T3 significantly decreased  $\alpha 2C$ -AR and  $\beta 1$ -AR mRNA expression (44 and 50%,



respectively) in the femur of WT mice, but not of  $\alpha$ 2A-AR<sup>-/-</sup> mice (Figures 7C,D). On the other hand,  $\beta$ 2-AR mRNA expression (Figure 7E) was decreased by T3 treatment in both WT and KO femurs (53 and 52%, respectively). Figure 7 also shows that the femoral mRNA expression of  $\alpha$ 2B-AR,  $\alpha$ 2C-AR,  $\beta$ 1-AR and  $\beta$ 2-AR is not different between WT and  $\alpha$ 2A-AR<sup>-/-</sup> mice.

## DISCUSSION

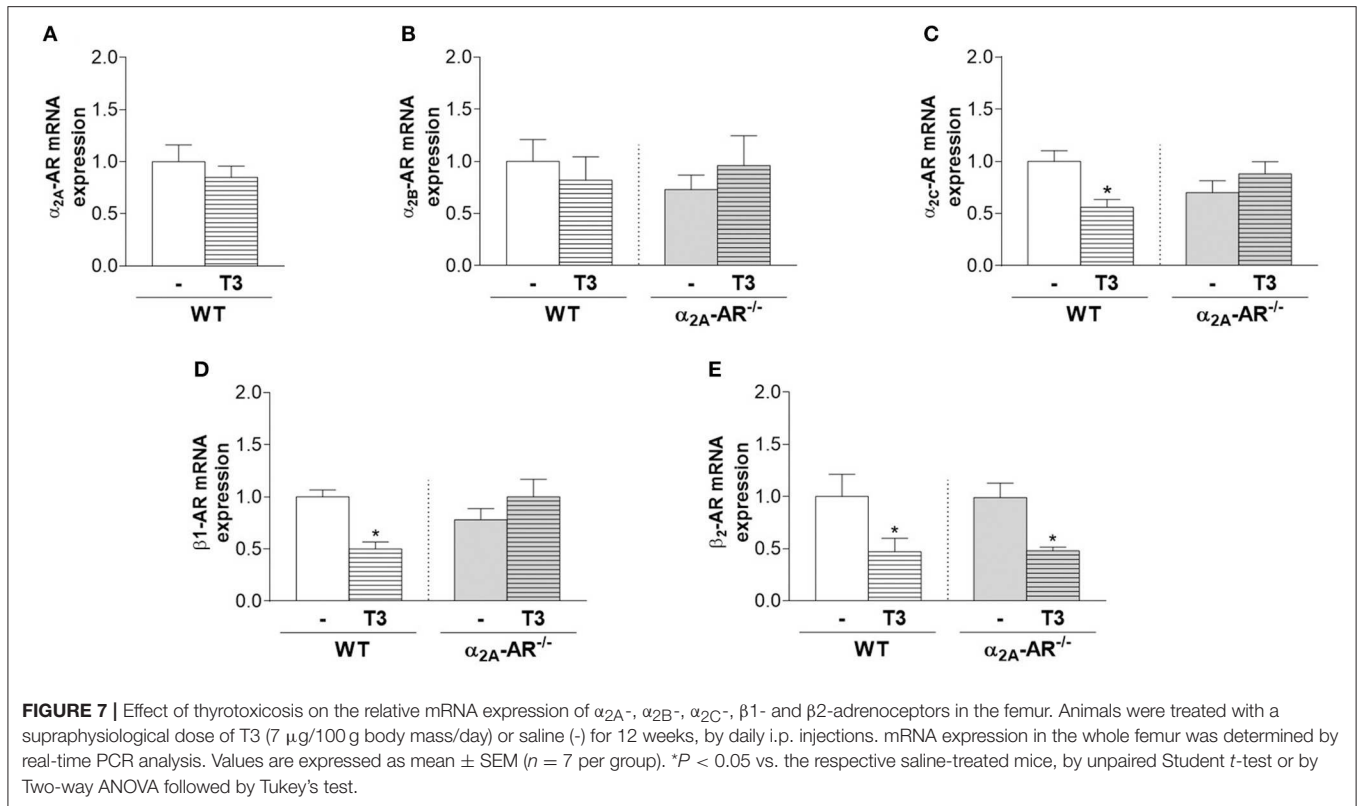
In the present study, we first characterized the skeletal phenotype of mice with single-gene inactivation of  $\alpha$ 2A-AR ( $\alpha$ 2A-AR<sup>-/-</sup> mice), in an attempt to dissect the specific roles of  $\alpha$ 2-AR subtypes in bone physiology. Surprisingly, the  $\mu$ CT analysis of the trabecular and cortical bone parameters of the femur and vertebra (vertebral body of L5) showed no differences between  $\alpha$ 2A-AR<sup>-/-</sup> mice and their WT controls in euthyroidism. These observations contrast with mice with global double-gene inactivation of  $\alpha$ 2A- and  $\alpha$ 2C-AR ( $\alpha$ 2A/C-AR<sup>-/-</sup> mice), which exhibit a generalized HBM phenotype (28). These double KO mice present increased trabecular and cortical bone in the femur and vertebra, but mainly in the vertebra (vs. femur) and in the trabecular bone (vs. cortical bone) (Table 1), which initially suggested an osteopenic role of  $\alpha$ 2A- and/or  $\alpha$ 2C-AR signaling in the bone tissue. Accordingly, histomorphometric analysis showed that these animals present increased bone formation and decreased bone resorption, with higher BMD mainly in the vertebra (28). We later studied mice with global single-gene KO of  $\alpha$ 2C-AR ( $\alpha$ 2C-AR<sup>-/-</sup> mice) (44). We found that  $\alpha$ 2C-AR<sup>-/-</sup> mice present a HBM phenotype in the vertebra, with increased trabecular bone, but normal cortical bone (Table 1). The collective analysis of the vertebral phenotype of  $\alpha$ 2A/C-AR<sup>-/-</sup> mice (increased trabecular and cortical bone) and  $\alpha$ 2C-AR<sup>-/-</sup> mice (increased trabecular bone) indicated that  $\alpha$ 2C-AR signaling mediates osteopenic actions of the SNS on vertebral trabecular bone and suggested a negative action of  $\alpha$ 2A-AR signaling on vertebral cortical bone (28, 44). The present finding that euthyroid  $\alpha$ 2A-AR<sup>-/-</sup> mice present a normal skeletal phenotype strengthens the role of  $\alpha$ 2C-AR signaling in mediating

detrimental actions of the SNS on trabecular bone of the vertebra; on the other hand, it does not sustain, but does not exclude, a detrimental action of  $\alpha$ 2A-AR signaling on vertebral cortical bone.

In contrast to the HBM phenotype in the vertebra,  $\alpha$ 2C-AR<sup>-/-</sup> mice showed a low bone mass (LBM) phenotype in the femur, with lower trabecular bone and nearly normal cortical bone (Table 1) (44). The lower trabecular content in the femur of  $\alpha$ 2C-AR<sup>-/-</sup> mice raised two hypotheses: (i)  $\alpha$ 2C-AR signaling has anabolic actions on trabecular bone of the femur; and/or (ii)  $\alpha$ 2A-AR signaling has a predominant role in mediating osteopenic actions of the SNS on trabecular bone of the femur. Considering this latter hypothesis, one could expect increased trabecular bone in the femur of  $\alpha$ 2A-AR<sup>-/-</sup> mice. However, the current study showed normal trabecular bone phenotype in the femur of euthyroid  $\alpha$ 2A-AR<sup>-/-</sup> mice, which does not sustain a predominant catabolic role of  $\alpha$ 2A-AR signaling in euthyroid conditions, but supports anabolic actions of  $\alpha$ 2C-AR signaling on the trabecular compartment of the femur. On the other hand, negative actions of  $\alpha$ 2A-AR signaling on femoral trabecular bone cannot be excluded, as it will be discussed next.

It is important to consider that all these mouse models present increased SNS outflow, since  $\alpha$ 2A-AR and  $\alpha$ 2C-AR are autoreceptors that inhibit the secretion of catecholamines (32, 33). The first one is the major presynaptic regulator of sympathetic NE release (32, 33), whereas  $\alpha$ 2C-AR is the main feedback receptor of adrenaline secretion from the chromaffin cells in the adrenal medulla (51), in addition to contribute to inhibition of NE release from sympathetic nerves (34). Therefore, it is important to consider that other adrenoceptors may be activated in these  $\alpha$ 2-AR KO mouse models. Evidence suggests that  $\beta$ 2-AR is the main adrenoceptor to directly mediated SNS actions in the skeleton. Pharmacological blockade and activation of  $\beta$ 2-AR signaling, respectively, increases and decreases bone mass (52–54), whereas global or osteoblast-specific genetic ablation of  $\beta$ 2-AR in mice results in a HBM phenotype by 6 months of age (27, 55). Activation of  $\beta$ 2-AR signaling inhibits osteoblast proliferation and activity and induces the expression of RANKL (receptor activator of nuclear factor kappa-B ligand), which binds to its receptor, RANK, in osteoclast precursor





cells or in mature osteoclasts to activate osteoclastogenesis and to increase osteoclastic activity, respectively (27). On the other hand,  $\beta$ <sub>1</sub>-AR signaling seems to exert anabolic actions in the skeleton and, therefore, opposite effects in relation to  $\beta$ <sub>2</sub>-AR signaling. Nevertheless, euthyroid  $\beta$ <sub>1</sub>-AR<sup>-/-</sup> mice show decreased trabecular bone in the vertebra but normal trabecular bone in the femur (56). There is also evidence that  $\alpha$ <sub>1</sub> adrenoceptors have a role in regulating osteoblast function (57, 58). Recently,  $\alpha$ <sub>1B</sub>-AR signaling was shown to be required for bone formation and  $\alpha$ <sub>1B</sub>-AR<sup>-/-</sup> mice was shown to display reduced trabecular bone in the femur (59). Thus, the trabecular phenotypes in the femur of  $\alpha$ <sub>2A</sub>/ $\alpha$ <sub>2C</sub>-AR<sup>-/-</sup>,  $\alpha$ <sub>2C</sub>-AR<sup>-/-</sup>, and  $\alpha$ <sub>2A</sub>-AR<sup>-/-</sup> mice are expected to rely on a balance/unbalance among the actions of  $\alpha$ <sub>2A</sub>-AR,  $\alpha$ <sub>2C</sub>-AR and the other adrenoceptors, which adds great complexity to the actions of the SNS in the skeleton. Considering the well characterized actions of  $\beta$ <sub>2</sub>-AR signaling in the bone tissue, the increased trabecular bone in the femur of  $\alpha$ <sub>2A</sub>/ $\alpha$ <sub>2C</sub>-AR<sup>-/-</sup> mice suggests that lack of  $\alpha$ <sub>2A</sub>-AR results in anabolic effects that could overcome the negative effects of  $\beta$ <sub>2</sub>-AR activation and the lack of  $\alpha$ <sub>2C</sub>-AR-mediated anabolic actions (Figure 8A). On the other hand, in  $\alpha$ <sub>2C</sub>-AR<sup>-/-</sup> mice, the osteopenic actions of  $\alpha$ <sub>2A</sub>-AR and  $\beta$ <sub>2</sub>-AR and the lack of  $\alpha$ <sub>2C</sub>-AR-mediated anabolism result in lower trabecular bone content (Figure 8B). Finally, in  $\alpha$ <sub>2A</sub>-AR<sup>-/-</sup> mice (Figure 8C), a balance between  $\alpha$ <sub>2C</sub>-AR-mediated anabolism and  $\beta$ <sub>2</sub>-AR-mediated catabolism could result in normal trabecular bone mass (as in WT controls). It is noteworthy that in the present study, the mRNA expression of

$\alpha$ <sub>2B</sub>-,  $\alpha$ <sub>2C</sub>-,  $\beta$ <sub>1</sub> and  $\beta$ <sub>2</sub>-adrenoceptors was not different between WT and KO animals, but, as discussed above, it is expected that the activation of these receptors differs between WT and KO mice. Further studies are necessary to investigate the actions and interactions among all these adrenoceptors to control bone remodeling.

Regarding the femoral cortical bone,  $\alpha$ <sub>2A</sub>-AR<sup>-/-</sup> mice present no alterations;  $\alpha$ <sub>2A</sub>/ $\alpha$ <sub>2C</sub>-AR<sup>-/-</sup> mice present an increase only in volumetric BMD (28), suggesting negative actions of  $\alpha$ <sub>2</sub>-AR signaling in this parameter; whereas  $\alpha$ <sub>2C</sub>-AR<sup>-/-</sup> mice present an increase in Ma.AR (44), which is suggestive of increased endosteal resorption. This finding suggests anabolic actions of  $\alpha$ <sub>2C</sub>-AR signaling also in the cortical bone of the femur. Accordingly,  $\alpha$ <sub>2A</sub>-AR<sup>-/-</sup> mice show normal (same as WT mice) resistance to fracture whereas  $\alpha$ <sub>2C</sub>-AR<sup>-/-</sup> mice display reduced resistance to fracture (Table 1), determined by the three-point bending test (decreased maximum load and resilience). It is noteworthy that this test measures mostly the resistance of the femoral diaphysis to fracture, which is a skeletal site mainly composed by cortical bone. These findings, therefore, support a positive role of  $\alpha$ <sub>2C</sub>-AR signaling in the cortical bone and in its ability to resist fracture.

To add insights to our understanding about the interaction of TH with the SNS to control bone physiology, and to clarify the specific roles of  $\alpha$ <sub>2</sub>-AR subtypes in this process, we evaluated the bone responses of  $\alpha$ <sub>2A</sub>-AR<sup>-/-</sup> mice to 4 and 12 weeks of daily treatment with 20xT3. This daily treatment increased serum levels of T3 and decreased serum levels of T4 in both WT and

**TABLE 1** | Trabecular and cortical bone microarchitecture and femoral resistance to fracture in  $\alpha 2$ -AR KO mouse models.

	$\alpha 2A/2C$ -AR <sup>-/-</sup> (28)	$\alpha 2C$ -AR <sup>-/-</sup> (44)	$\alpha 2A$ -AR <sup>-/-</sup>
<b>VERTEBRA</b>			
Trabecular	↑BV/TV, ↑Tb.N ↓Tb.Pf and ↓SMI	↑BV/TV, ↑Tb.N, ↑Tb.Th ↓Tb.Sp	n.d.
Cortical	↑Ct.Th, ↑BMD, ↑Ct.BV ↓Ma.Ar, ↓Ec.Pm	n.d.	n.d.
<b>FEMUR</b>			
Trabecular	↑BV/TV ↓Tb.Pf and ↓SMI	↓BV/TV, ↓Tb.N ↑Tb.Sp	n.d.
Cortical	↑BMD	↑Ma.Ar	n.d.
Fracture resistance	↑Maximum load	↓Maximum load	n.d.

Bone microarchitecture was determined by  $\mu$ CT analysis and bone resistance to fracture by the three-point bending test. Arrows indicate if each parameter decreased (↓) or increased (↑) in KO mice vs. WT mice. n.d. indicates no difference vs. WT. Animals were 114 day-old when the tests were performed.  $\mu$ CT parameters: BV/TV, trabecular bone volume; Tb.N; trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.Pf, trabecular pattern factor; SMI, structure model index; BMD, bone mineral density; Ct.Th, cortical thickness; Ct.BV, cortical bone volume; Ma.Ar, medullary area; Ec.Pm, endocortical perimeter. Biomechanical parameter: maximum load and resilience.

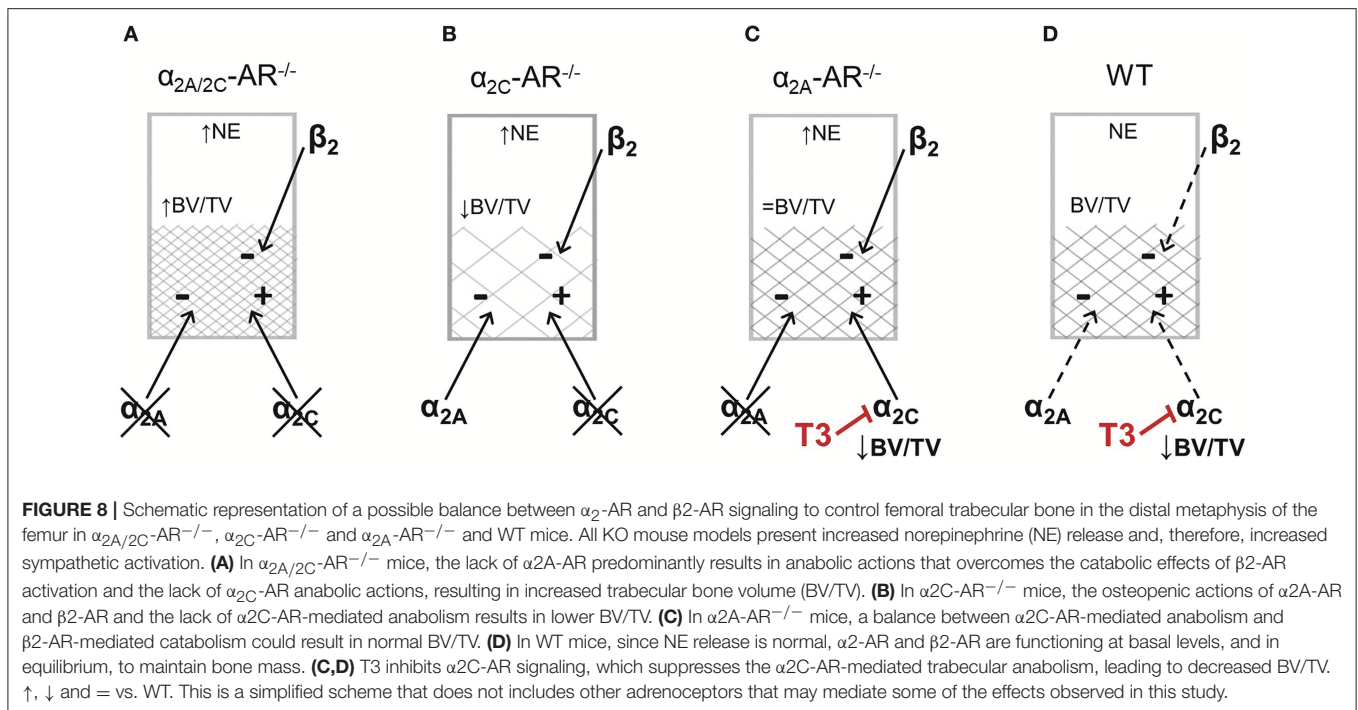
KO mice, which reflects the suppression of the HPT axis by TH excess (50), and confirms a thyrotoxic state in these animals. To further confirm a thyrotoxic state, we investigated the effect of T3 treatment on body composition. TH excess for 4 and 12 weeks promoted the characteristic effects of thyrotoxicosis in WT mice, including some decrease in BW, fat mass (axillar and retroperitoneal fat pads) and muscle mass (gastrocnemius mass). Besides, this T3 treatment increased heart mass, which reflects cardiac hypertrophy, a known consequence of toxic levels of TH (60, 61). Responses of  $\alpha 2A$ -AR<sup>-/-</sup> mice to thyrotoxicosis were different from those of WT mice in several parameters, including bone parameters, as it will be discussed below. KO animals showed an 8–13% lower BW than WT mice from 58 days of age until the end of the study (8 weeks later), which is probably explained by increased lipolysis. There is evidence that  $\alpha 2$ -AR signaling has antilipolytic actions (62, 63). Thus, the increased NE release and the impaired  $\alpha 2$ -AR-dependent antilipolysis are likely to facilitate the lipolytic action of  $\beta$ -AR (64). Accordingly,  $\alpha 2A$ -AR<sup>-/-</sup> animals presented lower retroperitoneal fat pad than WT mice. Surprisingly, TH failed to promote reductions in the retroperitoneal and axillar fat pads in KO animals, suggesting a complex role of  $\alpha 2A$ -AR in lipolysis, which remains to be investigated. Heart mass was increased in 58-day-old saline-treated  $\alpha 2A$ -AR<sup>-/-</sup> mice (vs. WT mice), which reflects cardiac hypertrophy because of the enhanced sympathetic activity (34). Nevertheless, TH promoted cardiac hypertrophy in  $\alpha 2A$ -AR<sup>-/-</sup> mice as much as in WT mice, further confirming a thyrotoxic state in both mouse lineages.

Regardless of the normal bone phenotype in euthyroid  $\alpha 2A$ -AR<sup>-/-</sup> mice, in general, trabecular bone showed to be more sensitive to thyrotoxicosis, whereas cortical bone showed to be resistant to the osteopenic effects of thyrotoxicosis in these KO animals, when compared with their WT controls. In order to gain

insights into the role of each  $\alpha 2$ -AR subtypes on the TH-SNS interaction to control bone physiology, we compared these new findings with the already published data of  $\alpha 2A/C$ -AR<sup>-/-</sup> and  $\alpha 2C$ -AR<sup>-/-</sup> mice (28, 37, 44). The higher sensitivity of trabecular bone to TH in  $\alpha 2A$ -AR<sup>-/-</sup> mice contrasts the lower sensitivity of trabecular bone to TH in  $\alpha 2A/C$ -AR<sup>-/-</sup> and  $\alpha 2C$ -AR<sup>-/-</sup> mice (37, 44), which suggests that  $\alpha 2C$ -AR signaling has a key role in the mechanism by which TH excess promotes its detrimental effects on trabecular bone. On the other hand, cortical bone of all these KO models showed some degree of resistance to thyrotoxicosis (37, 44), suggesting that  $\alpha 2A$ -AR and  $\alpha 2C$ -AR signaling contributes to TH actions on cortical bone.

The current study showed that TH excess for 4 weeks decreased BV/TV, Tb.N and increased Tb.Sp and Tb.Po only in the vertebra of  $\alpha 2A$ -AR<sup>-/-</sup> mice, whereas WT mice showed only a reduction in Tb.Th. However, after 12 weeks of TH treatment, vertebral trabecular bone of WT mice showed to be slightly more sensitive to the detrimental effects of thyrotoxicosis than  $\alpha 2A$ -AR<sup>-/-</sup> mice, since 20xT3 decreased BV/TV and increased Tb.Po only in WT animals. Conciliating these new findings with the observation that vertebral trabecular bone of  $\alpha 2C$ -AR<sup>-/-</sup> mice shows resistance to thyrotoxicosis after 4 and 12 weeks of T3 treatment (44), we could suppose that  $\alpha 2C$ -AR signaling is the main  $\alpha 2$ -AR subtype to mediate TH actions on trabecular bone of the vertebra until 4 weeks of thyrotoxicosis, but that, in longer situations of thyrotoxicosis (12 weeks),  $\alpha 2A$ -AR signaling could also contribute to the detrimental effects of TH excess. Interestingly, cortical bone of the vertebra showed to be more sensitive to thyrotoxicosis in WT than in  $\alpha 2A$ -AR<sup>-/-</sup> mice. Thyrotoxicosis negatively affected Ct.Ar, Ma.Ar, Ps.Pm, and Ec.Pm in WT mice and not in  $\alpha 2A$ -AR<sup>-/-</sup> mice. Similar effects of toxic levels of T3 were also observed in  $\alpha 2A/2C$ -AR<sup>-/-</sup> mice (37), which were resistant to TH-induced decreases in Ct.Th, T.Ar, Ct.Ar, Ct.BV, and Ma.Ar, and to TH-induced increases in Ct.Po. and in  $\alpha 2C$ -AR<sup>-/-</sup> mice (44), which were resistant to TH-induced decreases in T.Ar and increases in Ec.Pm. Altogether, these findings suggest that both  $\alpha 2A$ -AR and  $\alpha 2C$ -AR signaling pathways mediate TH actions on the cortical compartment of the vertebra.

The mechanisms by which TH interacts with the SNS to control bone morphophysiology in the femur seems to be different than that in the vertebra. As discussed before,  $\alpha 2A/2C$ -AR<sup>-/-</sup> mice present increased trabecular (mainly) and cortical bone in the femur (37);  $\alpha 2C$ -AR<sup>-/-</sup> mice present lower trabecular bone and nearly normal femoral cortical bone (44); whereas  $\alpha 2A$ -AR<sup>-/-</sup> mice present normal trabecular and cortical bone in the femur (Table 1). Considering these findings, anabolic roles of  $\alpha 2C$ -AR signaling in the trabecular compartment of the femur have emerged. The study of  $\alpha 2A/2C$ -AR<sup>-/-</sup> and  $\alpha 2C$ -AR<sup>-/-</sup> mice showed that trabecular and cortical compartments of the femur are resistant to detrimental effects of thyrotoxicosis (37, 44). In contrast, the present study shows that thyrotoxicosis was clearly more deleterious to the trabecular bone of the femur in  $\alpha 2A$ -AR<sup>-/-</sup> mice than in WT mice, whereas cortical bone of the femur was less sensitive to thyrotoxicosis in  $\alpha 2A$ -AR<sup>-/-</sup> mice. Altogether, these studies suggest that  $\alpha 2C$ -AR signaling, but not  $\alpha 2A$ -AR, is necessary for TH to promote its detrimental



effects in the trabecular bone of the femur, whereas both  $\alpha$ <sub>2A</sub>-AR and  $\alpha$ <sub>2C</sub>-AR mediate TH effects in the femoral cortical bone.

The three-point bending test showed that thyrotoxicosis decreased femoral resistance to fracture (lower maximum load and tenacity) in WT mice, but not in  $\alpha$ <sub>2A</sub>-AR<sup>-/-</sup> animals. This finding is consistent with the more deleterious effects of thyrotoxicosis in the cortical bone of WT mice. Similar results were observed in  $\alpha$ <sub>2A/2C</sub>-AR<sup>-/-</sup> and  $\alpha$ <sub>2C</sub>-AR<sup>-/-</sup>, which also showed resistance to the detrimental effects of TH on bone ability to resist fracture. These findings, therefore, support a role of both  $\alpha$ <sub>2A</sub>-AR and  $\alpha$ <sub>2C</sub>-AR signaling pathways in mediating detrimental actions of TH on cortical bone of the femur.

The fact that  $\alpha$ <sub>2C</sub>-AR signaling arises as having anabolic actions on trabecular and cortical femoral bone while seems to be necessary for TH to promote its deleterious effects in the femur is intriguing. We, therefore, suggest that one mechanism by which TH promote its deleterious effects in the femur is decreasing  $\alpha$ <sub>2C</sub>-AR signaling (Figures 8C,D), which would decrease bone anabolism and favor catabolism. Accordingly, we found that T3 decreases  $\alpha$ <sub>2C</sub>-AR mRNA expression in the femur of WT mice. This effect, however, was not observed in  $\alpha$ <sub>2A</sub>-AR<sup>-/-</sup> mice. It is important to consider that TH actions on the expression of adrenoceptors are normally modest or nonexistent, and usually cannot explain the T3-SNS interactions (38). Evidence shows that TH usually modulates more distal cellular effectors in the adrenoceptors signaling pathways (38). Further studies will be necessary to confirm if T3 really suppresses  $\alpha$ <sub>2C</sub>-AR signaling in bone cells and in which level it occurs. We also found that T3 had no effect on  $\alpha$ <sub>2A</sub>- and  $\alpha$ <sub>2B</sub>-AR mRNA expression. On the other hand, T3 decreased femoral mRNA expression of  $\beta$ <sub>1</sub>-AR in WT but not in  $\alpha$ <sub>2A</sub>-AR<sup>-/-</sup> mice, whereas decreased  $\beta$ <sub>2</sub>-AR mRNA expression in both WT and  $\alpha$ <sub>2A</sub>-AR<sup>-/-</sup> mice. The relevance

of these modulations cannot be extrapolated at this moment, but the effects of T3 on the expression of these adrenoceptors suggest that the modulation of adrenoceptor signaling may be a point of interaction between TH and the SNS to regulate bone mass.

It is important to consider that besides their function as autoreceptors,  $\alpha$ <sub>2</sub>-AR subtypes are also expressed in non-adrenergic neurons, where they can operate as heteroreceptors to regulate the release of several neurotransmitters in the central and peripheral nervous system, including serotonin, GABA and dopamine, among other neurotransmitters (65). In addition to neuronal locations,  $\alpha$ <sub>2</sub>-AR subtypes are also present in several non-neuronal cells/tissues (vascular vessels, pancreatic islets, etc.), which actions have emerged as relevant to the body (65). The widespread expression of  $\alpha$ -AR subtypes in the central and peripheral nervous system, and in non-neuronal cells adds further complexity to the understanding of the roles of  $\alpha$ <sub>2</sub>-AR signaling in several physiological processes, including bone metabolism. An important limitation of the present study is that it is based on mouse models of global gene inactivation of  $\alpha$ <sub>2</sub>-AR subtypes. Thus, the interference of factors from the central and peripheral nervous system, as well as interferences from systemic and local factors may have occurred in the bone effects of TH observed in the present study. In addition, these global mouse models do not allow the discrimination of the contribution of central and local TH-SNS interactions. Nevertheless, we have previously shown that clonidine (CLO), an  $\alpha$ <sub>2</sub>-AR agonist, increases osteoclastogenesis, *in vitro*, in mouse marrow cells derived from WT animals, but not from  $\alpha$ <sub>2A/2C</sub>-AR<sup>-/-</sup> mice. In contrast, phentolamine, a nonspecific  $\alpha$ -AR antagonist, decreases osteoclast formation in cultures of marrow cells derived from WT but not in the same cells derived from  $\alpha$ <sub>2A/2C</sub>-AR<sup>-/-</sup> animals

(28). In addition, further *in vitro* studies showed that CLO or T3 alone decreased proliferation of calvaria-derived osteoblasts isolated from WT mice, which was further decreased when cells were treated with CLO combined with T3 (CLO+T3). These effects, however, were completely blocked or reversed in  $\alpha$ 2A/2C-AR<sup>-/-</sup> mice-derived cells (37). These *in vitro* studies and the expression of all  $\alpha$ 2-AR isotypes in the main skeletal cells (28) support direct actions of the SNS on the skeleton and show that a TH-SNS interaction is likely to occur locally in the skeleton, via  $\alpha$ 2A-AR and/or  $\alpha$ 2C-AR signaling. Thus, these studies suggest that one mechanism by which TH regulates bone physiology directly in the skeleton involves a local interaction with  $\alpha$ 2-AR signaling.

In summary, euthyroid  $\alpha$ 2A-AR<sup>-/-</sup> mice present normal trabecular and cortical bone in the femur and vertebra, in addition to present normal resistance to fracture. The combined analysis of the bone phenotypes of  $\alpha$ 2A/C-AR<sup>-/-</sup>,  $\alpha$ C-AR<sup>-/-</sup>, and  $\alpha$ 2A-AR<sup>-/-</sup> mice suggests that: (i)  $\alpha$ 2-AR signaling mediates SNS-actions mainly in trabecular bone; (ii)  $\alpha$ 2C-AR signaling predominantly mediates osteopenic actions of the SNS on trabecular bone of the vertebra; (iii)  $\alpha$ 2C-AR signaling has anabolic actions mainly on trabecular bone but also on cortical bone of the femur. Regardless of the normal bone phenotype in euthyroidism,  $\alpha$ 2A-AR<sup>-/-</sup> mice responds differently than WT mice to thyrotoxicosis. The higher sensitivity of trabecular bone to TH in  $\alpha$ 2A-AR<sup>-/-</sup> mice and the lower sensitivity of trabecular bone to TH in  $\alpha$ 2A/C-AR<sup>-/-</sup> and  $\alpha$ 2C-AR<sup>-/-</sup> mice (37, 44) points out  $\alpha$ 2C-AR signaling as a key factor to mediate the detrimental actions of TH on trabecular bone, particularly in the femur. On the other hand, the lower sensitivity of cortical bone to thyrotoxicosis in  $\alpha$ 2A/C-AR<sup>-/-</sup>,  $\alpha$ 2C-AR<sup>-/-</sup>, and  $\alpha$ 2A-AR<sup>-/-</sup> mice (37, 44) suggests that both  $\alpha$ 2A-AR and  $\alpha$ 2C-AR signaling pathways contribute to detrimental actions of TH on cortical

bone of both femur and vertebra. Altogether, these novel findings further sustain a TH-SNS interaction, involving  $\alpha$ 2-AR signaling, to regulate bone remodeling, and, therefore, bone mass, bone integrity and, ultimately, bone resistance to fracture.

## AUTHOR CONTRIBUTIONS

GM: Conceived and performed experiments, carried out data collection and analysis, and wrote the manuscript; MT: performed experiments, carried out data collection and analysis, and reviewed the manuscript; MS, BN-P, and MM-R: performed experiments, carried out data collection, and reviewed the manuscript; PB: Conceived the study and experiments, carried out data analysis and reviewed the manuscript; CG: Conceived the study and experiments, carried out data analysis and wrote the manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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