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Signaling through the M₃ muscarinic receptor favors bone mass accrual by decreasing the sympathetic activity

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Summary

Bone remodeling is regulated by various neuronal inputs, one of the best understood being the sympathetic tone which inhibits bone mass accrual. This function of the sympathetic nervous system raises the prospect that the other arm of the autonomic nervous system, the parasympathetic nervous system, may also affect bone remodeling. Using various mutant mouse strains, each lacking one of the muscarinic receptors that mediate parasympathetic activity, we show here that the parasympathetic nervous system acting through the M₃ muscarinic receptor is a positive regulator of bone mass accrual by increasing bone formation and decreasing bone resorption. Expression studies, cell-specific gene deletion experiments and analysis of compound mutant mice showed that the parasympathetic nervous system favors bone mass accrual by acting centrally and by decreasing the sympathetic tone. By showing that both arms of the autonomic nervous system affect bone remodeling, this study further underscores the importance of its neuronal regulation.

Introduction

Bone remodeling, the physiological function assuring renewal of bone tissue, consists of a perpetual alternance of a resorption phase followed by *de novo* bone formation (Rodan and Martin, 2000). One of the many significant advances that took place in recent years in our understanding of the regulation of bone remodeling has been the identification of its neuronal control which was first revealed when deciphering the mechanisms whereby the adipocyte-derived hormone leptin regulates bone mass (Ducy et al., 2000).

Intracerebroventricular infusion of leptin in wildtype (WT) and leptin-deficient (*ob/ob*) mice before or after chemical lesioning of hypothalamic neuronal populations, together with the

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analysis of multiple cell-specific gene deletion mouse models, have established that leptin inhibits bone mass accrual by acting solely through neuronal means (Shi et al., 2008). First, after binding to its receptor on neurons of the brainstem, leptin inhibits synthesis and release of serotonin (Yadav et al., 2009). Brain-derived serotonin then signals through the Htr2c receptor present on ventromedial hypothalamic (VMH) neurons. This signaling event triggers a decrease of the activity of the sympathetic nervous system (SNS), a known negative regulator of bone mass accrual acting through the β_2 adrenergic receptor (Adrb2) present on osteoblasts (Elefteriou et al., 2005; Fu et al., 2005; Takeda et al., 2002). The crucial role of the SNS in the regulation of bone remodeling begs the question of the possible role that the other arm of the autonomic nervous system, the parasympathetic nervous system (PNS), may have in this context.

The PNS originates from medial medullary sites. Vagal efferent innervations extend from the medulla to postganglionic nerves in various locations in the body (Agostoni et al., 1957; Brodal, 1981). The main neurotransmitter used by PNS endings is acetylcholine, which binds to a small family of G-protein coupled receptors, the muscarinic acetylcholine receptors (Caulfield, 1993; Wess, 1996). Molecular cloning identified five different muscarinic receptors (MRs), which can be subdivided into two major classes. The M_1R , M_3R , and M_5R selectively couple to G-proteins of the G_q family, while the M_2R and M_4R preferentially activate G_i/G_o -type of G-proteins (Caulfield, 1993; Caulfield and Birdsall, 1998; Wess, 1996).

In an effort to determine whether or not the PNS regulates bone remodeling, we studied the bone phenotype of mice lacking muscarinic receptors. Here we show that among the four receptors tested, M_3 receptor (M_3R) is the only muscarinic receptor subtype influencing bone remodeling. Furthermore, studies of mutant mouse strains harboring cell-specific gene deletion establish that the M_3R fulfills this function through its neuronal expression and by decreasing sympathetic activity. Thus, these experiments reveal that the PNS is a positive regulator of bone mass accrual and that, unlike the SNS, it acts through a neuronal, not an osteoblast relay, to fulfill this function.

Results and Discussion

Signaling through M_3R favors bone mass accrual

To determine whether signaling through the PNS affects bone remodeling *in vivo*, we studied various strains of mutant mice, each of them lacking, in all cells, one of the muscarinic receptors that mediate acetylcholine signaling (Wess, 2004; Wess et al., 2007).

For that purpose, histological and microcomputed tomography (μ CT) analyses of vertebrae and long bones were performed in 6-, 12-, and 24-week-old WT and mutant mice. As shown in Figure 1C-E, even when studied in relatively old mice (24 weeks of age), the absence of either M_1R , M_2R or M_4R did not affect significantly bone mass as measured histologically by the ratio of bone volume over tissue volume (BV/TV). None of these three mutations affected either bone formation or bone resorption parameters (Figure 1C-1E). In contrast, female $M_3R^{-/-}$ mice, that are slightly shorter than WT littermates (Figure S1A-S1C) as previously reported (Matsui et al., 2000), displayed at 6-, 12-, and 24-week of age a low bone mass phenotype affecting both the axial (vertebrae) and appendicular (long bones) skeleton (Figure 1A, 1B and S1F), while cortical thickness was unaffected (Figure S1G). The same low bone mass phenotype was observed in male $M_3R^{-/-}$ mice (data not shown). The rest of the analysis was done in female WT and mutant mice.

To better understand the pathogenesis of this low bone mass phenotype, we first asked whether it could be explained by abnormalities in the levels of circulating hormones or growth factors. As shown in Figure 1K, leptin serum levels were significantly lower in $M_3R^{-/-}$ than in WT

littermates, an abnormality that should increase bone mass and therefore could not explain the low bone mass phenotype of the $M_3R^{-/-}$ mice (Karsenty, 2006). In our hands, corticosterone serum levels were within the normal range in $M_3R^{-/-}$ mice (Figure 1K) and therefore could not account for their bone phenotype either. In contrast, serum levels of growth hormone and of insulin-like growth factor 1 (IGF-1), a cytokine that may promote bone formation (Kawai and Rosen, 2008), were decreased in $M_3R^{-/-}$ mice (Figure 1K). One experimental evidence indicated, however, that the low bone mass phenotype observed in $M_3R^{-/-}$ mice was not due primarily to this decrease in circulating levels of growth hormone and IGF-1. Indeed, when $M_3R^{-/-}$ mice were treated (once daily s.c. injection of 2 μ g for 8 weeks) with CJC1295, a synthetic long-acting growth hormone-releasing hormone analog that enhances growth hormone secretion and as a result increases circulating levels of IGF-1 (Jette et al., 2005), we observed, as expected, a normalization of their growth hormone and IGF-1 serum levels (Figure 1L and 1M). However, these CJC1295-treated $M_3R^{-/-}$ mice remained osteopenic (Figure 1N). These observations indicate that parasympathetic signaling through the M_3R affects bone mass accrual and does it, for the most part, in a growth hormone and IGF-1-independent manner.

Decreased bone formation and increased bone resorption in $M_3R^{-/-}$ mice

To define the cellular bases of the low bone mass phenotype observed in $M_3R^{-/-}$ mice, we relied on histological and biochemical studies. Histomorphometry analyses of vertebrae showed that the low bone mass phenotype of $M_3R^{-/-}$ mice was secondary to a decrease in bone formation parameters, such as bone formation rate and osteoblast numbers (Figure 1F and 1G). Importantly, we did not observe any increase in osteoblast apoptosis in $M_3R^{-/-}$ bones (Figure 1H), a feature usually observed when bone formation is hampered by corticosterone (Weinstein et al., 1998). In addition, there was also an increase in bone resorption parameters, such as the surface covered by osteoclasts and an increase in the circulating levels of total deoxypyridinoline, a degradation product of collagen and a biomarker of bone resorption (Eyre et al., 1988), in $M_3R^{-/-}$ mice (Figure 1I and 1J). Taken together, results of these analyses showed that the PNS is a positive regulator of bone mass accrual affecting both the formation and resorption arms of bone remodeling and identified M_3R as a muscarinic receptor involved in this process.

M_3R is expressed in neurons involved in the control of bone mass

Given the direct effect of sympathetic signaling on osteoblasts, we next asked whether the PNS regulates bone mass accrual by acting directly on bone cells or through a different mechanism. As a first approach, we studied M_3R expression.

As shown in Figure 2A and B, M_3R expression in osteoblasts was two to three orders of magnitude lower than in various regions of the brain, or than in other peripheral tissues where it is known to be expressed and functional (Matsui et al., 2000; Yamada et al., 2001). Moreover, M_3R expression in osteoblasts was also three orders of magnitude lower than the expression of *Adrb2*, the adrenergic receptor mediating sympathetic signaling in osteoblasts (Takeda et al., 2002) (Figure 2C). In the face of this rather weak expression of M_3R in osteoblasts and in an effort to explain the bone phenotype of the $M_3R^{-/-}$ mice, we then asked where in the nervous system M_3R was expressed.

In situ hybridization studies revealed that, in addition to its previously described expression in the hippocampus, visual cortex and lateral hypothalamus (data not shown) (Buckley et al., 1988), M_3R is also expressed in neurons of the Dorsal Raphe (DR) and Locus Coeruleus (LC) nuclei in brainstem (Figure 2D and 2E). The DR and LC cluster serotonergic neurons and noradrenergic neurons respectively, two pathways affecting bone mass accrual (Takeda et al., 2002; Yadav et al., 2009). In contrast, we failed to detect any M_3R expression in neurons of VMH, a hypothalamic structure whose integrity is required for the regulation of bone mass

accrual (Takeda et al., 2002) (Figure S2A). We also failed to detect M_3R expression in sympathetic chain ganglia (Figure S2B). In summary, these various studies of M_3R expression indicated that M_3R expression in osteoblasts is barely above the limit of detection of quantitative PCR, while it is expressed in the brain structures regulating bone mass accrual. As a result, they suggested that the PNS favors bone mass accrual through an indirect mechanism, i.e. its expression in neurons, rather than by acting directly on osteoblasts.

Neuronal-specific but not osteoblast-specific inactivation of M_3R results in a low bone mass

To determine formally the cell type in which signaling through M_3R must occur to favor bone mass accrual, we relied on cell-specific gene inactivation experiments in the mouse.

Crossing mice harboring a floxed allele of M_3R (Gautam et al., 2006) with $\alpha_1(I)Collagen$ -Cre mice, a transgenic mouse line expressing the Cre recombinase specifically in osteoblasts (Dacquin et al., 2002), allowed us to generate $M_3R_{osb}^{-/-}$ mice. Southern blot analysis demonstrated that we had successfully deleted M_3R in osteoblasts of $M_3R_{osb}^{-/-}$ mice but not in other tissues (Figure 3A). Study of $M_3R_{osb}^{-/-}$ mice at 12 weeks of age, a time point when $M_3R^{-/-}$ mice are profoundly osteopenic, failed to detect any change in bone mass, bone formation or bone resorption parameters (Figure 3B), indicating that, in the conditions of this experiment, M_3R does not regulate bone mass through its expression in osteoblasts. This result is consistent with the very weak expression of M_3R in these cells, and also consistent with the fact that muscarinic receptor agonist treatment of WT or $M_3R^{-/-}$ osteoblasts does not affect their ability to form nodules and to produce alkaline phosphatase (Figure S3A and S3B).

Next, we asked whether a deletion of M_3R in all neurons would affect bone mass by crossing *Nestin*-Cre transgenic mice (Tronche et al., 1999) with floxed M_3R mice. Southern blot analysis verified that we had effectively deleted M_3R in neurons but not in other cell types (Figure 3C). $M_3R_{neuron}^{-/-}$ mice of either sex showed, at 6 and 12 weeks of age, a low bone mass phenotype of similar severity as the one observed in mice lacking M_3R in all cells (Figure 3D and 3E). These histological findings were confirmed by μ CT analysis (Figure 3F). That the phenotype of $M_3R^{-/-}$ and $M_3R_{neuron}^{-/-}$ mice was of equal severity indicates that the bulk of the influence of the PNS on bone remodeling occurs through a neuronal relay. As it was the case in $M_3R^{-/-}$ mice, this osteopenia was due to a decrease in bone formation and an increase in bone resorption parameters. These results established that it is through its neuronal not through its osteoblastic expression that the M_3R regulates bone mass. Remarkably, appetite, which is low in $M_3R^{-/-}$ mice [Figure S1D and S1E and (Yamada et al., 2001)], was unaffected in $M_3R_{neuron}^{-/-}$ mice (Figure 3G and 3H). This may have at least two explanations. The first one is that the parasympathetic regulation of bone mass accrual occurs independently of its regulation of energy metabolism. A second one is that the deletion of M_3R in neurons is not complete but large enough to affect bone mass but not appetite. Consistent with this latter hypothesis, we should point out that we observed the same discrepancy between the effect on bone mass and appetite in a mouse model of gain of function of leptin signaling (Shi et al., 2008).

$M_3R_{neuron}^{-/-}$ mice displayed, like the $M_3R^{-/-}$ mice, a decrease in growth hormone and IGF-1 circulating levels as previously reported (Gautam et al., 2009). To determine whether this abnormality explained their low bone mass phenotype, we treated them with CJC-1295. Although this treatment normalized the serum levels of growth hormone and IGF-1, the low bone mass phenotype of $M_3R_{neuron}^{-/-}$ mice was not rescued, indicating that this phenotype develops independently of the growth hormone and IGF-1 abnormalities (Figure 3I-3K).

Parasympathetic signaling favors bone mass accrual by inhibiting sympathetic activity

The neuronal mediation of the PNS regulation of bone mass accrual along with the expression pattern of M_3R in the DR nuclei were two reasons that led us to ask whether the PNS regulates

bone mass by affecting synthesis and release of serotonin by brainstem neurons. However, *Tph2* expression was normal in $M_3R^{-/-}$ mice as was the serotonin brainstem content (Figure 4C and 4D). These two lines of experimental evidence argue strongly against a model whereby the PNS would regulate bone mass by affecting serotonergic output.

M_3R is also expressed in the noradrenergic neurons of the LC nuclei in brainstem (Figure 2E) and activation of these neurons is correlated with an increase of sympathetic activity, as measured by an increase in plasma levels of epinephrine (Crawley et al., 1980). Thus, we tested whether signaling through M_3R regulates bone mass accrual by affecting the sympathetic tone. A first experimental argument supporting this mode of action was that urinary levels of epinephrine were elevated in both $M_3R^{-/-}$ and $M_3R_{neuron}^{-/-}$ mice (Figure 4A and 4B). A second experimental evidence relies on genetic epistasis (Cordell, 2002). Indeed, if parasympathetic signaling through M_3R and sympathetic signaling through *Adrb2* lie in the same genetic pathway, one would expect that removing one allele of *Adrb2* from $M_3R^{-/-}$ background should rescue the bone phenotype of the latter mutant mice. Consistent with this hypothesis, $M_3R^{-/-};Adrb2^{+/-}$ mice had normal bone mass, bone formation and bone resorption parameters (Figure 4E). That the low serum levels of growth hormone and IGF-1 characterizing $M_3R^{-/-}$ mice were not corrected in $M_3R^{-/-};Adrb2^{+/-}$ mice (Figure 4F) added further support to the notion that the low bone mass phenotype of the $M_3R^{-/-}$ mice is not secondary to their low serum levels of growth hormone and IGF-1. Thus, our results suggest that the PNS favors bone mass accrual by inhibiting the activity of the sympathetic nervous system.

In summary, we present here *in vivo* evidence that the PNS, signaling through the M_3R is, unlike the SNS, a positive regulator of bone mass. Although they highlight the importance of M_3R in the context of the PNS regulation of bone mass, our results do not rule out that M_5R , which was not tested here, may contribute to the regulation of bone mass by the PNS. An unexpected feature of the PNS regulation of bone mass accrual is that it does not occur on osteoblasts but rather on neurons, where it decreases sympathetic activity. The expression of M_3R in the LC, a brain structure necessary for sympathetic output, and the decrease of epinephrine urinary contents in both $M_3R^{-/-}$ and $M_3R_{neuron}^{-/-}$ mice all argue for a model whereby the PNS would favor bone mass accrual by inhibiting sympathetic activity.

More generally, these observations broaden the importance of the autonomic regulation of bone mass and add further credence to the existence of the neuronal regulation of bone remodeling. The PNS is here to dampen the negative influence of the sympathetic tone on bone mass; such a function is also observed in other physiological systems, such as cardiac and digestive activity among others (Goldstein, 2001). Moreover and although it is difficult to compare their influence, it is striking that both the LRP5/serotonin-dependent and the leptin/sympathetic regulation of bone mass accrual inhibits bone formation (Ducy et al., 2000; Yadav et al., 2008). This observation raises the prospect that other regulators whose role would be to enhance bone formation in physiological situations must exist.

Experimental Procedures

Animals

$M_1R^{-/-}$, $M_2R^{-/-}$, $M_3R^{-/-}$, $M_4R^{-/-}$, $M_3R^{fl/fl}$, $\alpha_1(I)Collagen-Cre$ and *Adrb2*^{+/-} mice used in these studies have been reported previously (Chruscinski et al., 1999; Dacquin et al., 2002; Gautam et al., 2006; Gomez et al., 1999a; Gomez et al., 1999b; Miyakawa et al., 2001; Yamada et al., 2001) and all are on C57BL/6J background. *Nestin-Cre* transgenic mice (C57BL/6J background) were from the Jackson Laboratory (Bar Harbor, MA).

Bone Histology, μ CT Analysis and TUNEL Assays

Static and dynamic histomorphometric analyses were performed on undecalcified vertebral column specimens (See Supplemental Experimental Procedures). μ CT analyses were performed on proximal tibiae using a micro computed tomography system (See Supplemental Experimental Procedures). TUNEL assay was performed on 3-week-old mice to evaluate *in vivo* osteoblast apoptosis as per manufacturer's instructions (In Situ Cell Death Detection Kit, Roche Applied Science, Indianapolis, IN). Four pups per genotype were analyzed and ten calvarial sections were counted per animal.

Food intake, energy expenditure, and CJC-1295 treatment

24-hr food intake was measured using metabolic cages (Nalge) and energy expenditure was assessed by an indirect calorimetry method using a 6-chamber Oxymax system (Columbus Instruments) as previously described (Shi et al., 2008). One-week-old $M_3R^{-/-}$, $M_3R_{neuron}^{-/-}$ and their WT littermates were treated with once daily s.c. injection of 2 μ g of CJC-1295 for 8 weeks.

Bioassays

Serum levels of total DPD (Quidel), leptin (R&D System), growth hormone (DSL), IGF-1 (DSL), corticosterone (MP Biomedicals) were measured using commercially available kits. Urinary epinephrine contents were measured in acidified spot urine samples by EIA (Bi-CAT kit, Alpcos) and creatinine (Quidel) was used to standardize between urine samples.

Molecular studies

RNA isolation (Trizol, Invitrogen) and cDNA synthesis (Superscript III, Invitrogen) were performed as per manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed on an MX3000 instrument (Stratagene, La Jolla, CA) using primers from SABiosciences (Frederick, MD) and the Taq SYBR Green supermix with ROX (Biorad, Hercules, CA). Expression levels of the studied genes were normalized by β -actin levels. Southern blot were performed using probe 3 as previously described (Gautam et al., 2006). In situ mRNA hybridization was performed on 20 μ m cryosections using DIG-labeled riboprobe hybridizing at 68°C (See Supplemental Experimental Procedures).

Statistical analyses

Statistical significance was assessed by unpaired Student's two-tailed t test. Values were considered statistically significant at $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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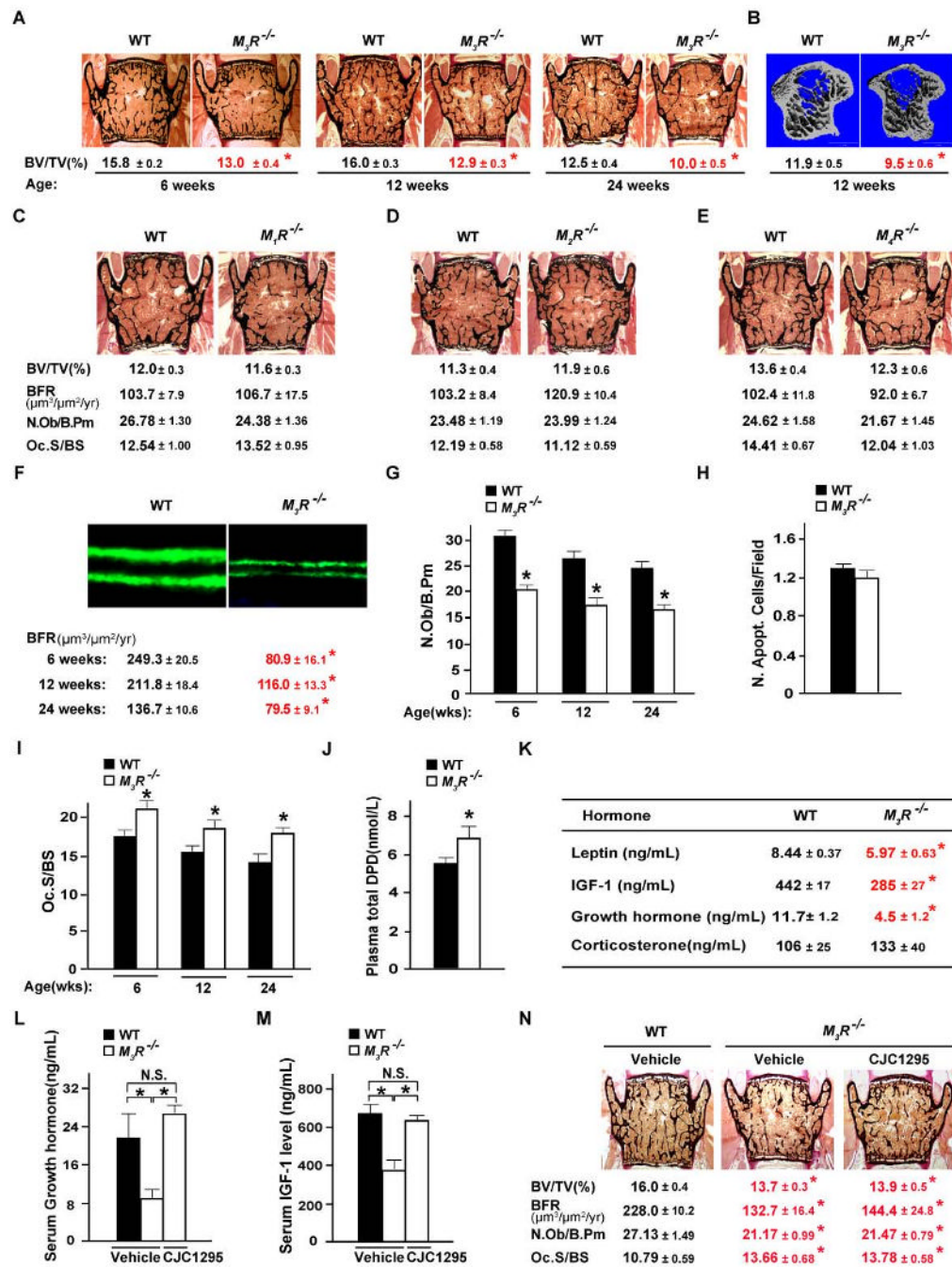


Figure 1. The parasympathetic nervous system, acting through M_3 receptor, favors bone mass accrual by increasing bone formation and decreasing bone resorption

(A) Histological analyses of WT and $M_3R^{-/-}$ vertebrae. Bone mass is measured as the ratio of mineralized trabecular bone volume (black) over total tissue volume (BV/TV, %) after Von Kossa/Van Gieson staining. (B) μ CT analyses of WT and $M_3R^{-/-}$ proximal tibiae. (C-E) Histomorphometric analyses of vertebrae of 24-week-old $M_1R^{-/-}$ (C), $M_2R^{-/-}$ (D), $M_4R^{-/-}$ (E) and corresponding WT littermates. Histomorphometric parameters: BFR, bone formation rate; N.Ob/B.Pm: number of osteoblasts per bone perimeter; Oc.S/BS: osteoclast surface per bone surface. (F, G and I) Histomorphometric analyses of WT and $M_3R^{-/-}$ vertebrae. (H) TUNEL assay of calvarial sections. The number of apoptotic cells per 0.16 mm² field was comparable

between 3-week-old WT and $M_3R^{-/-}$ mice. (J) ELISA analysis of plasma levels of total deoxypyridinoline (DPD), a biomarker of bone resorption, in $M_3R^{-/-}$ mice. (K) Serum levels of hormones or growth factors in WT and $M_3R^{-/-}$ mice. (L-N) ELISA analysis of serum growth hormone (L) and IGF-1 (M) levels and histomorphometric analysis of vertebrae (N) of CJC1295- and vehicle-treated $M_3R^{-/-}$ and WT mice. N.S., statistically non-significant. n = 6-10 for all other experiments; * $P < 0.05$ comparing to WT group; results are represented as mean \pm SEM; error bars represent SEM.

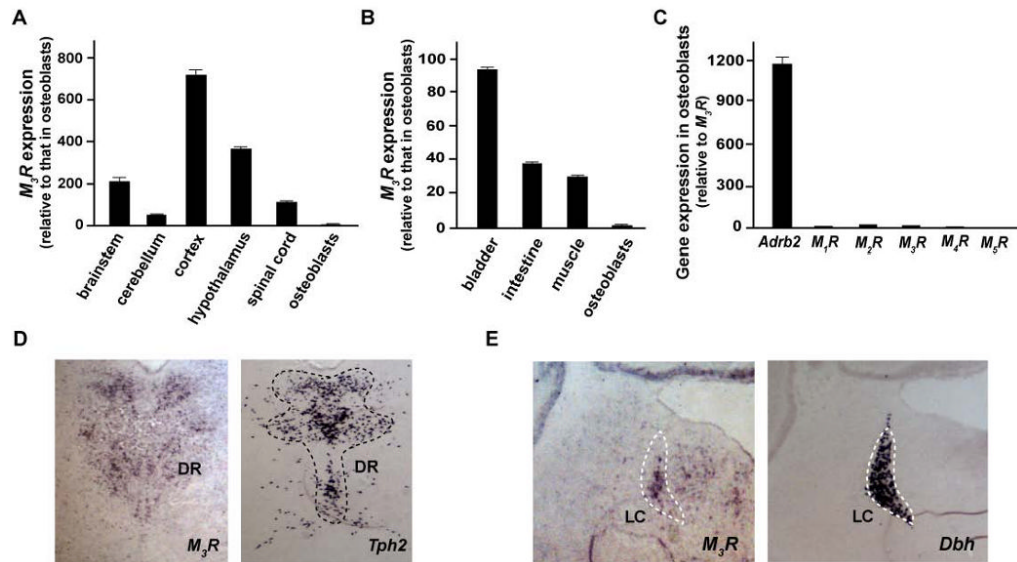


Figure 2. Expression pattern of M_3R

(A-C) Gene expression by real-time quantitative PCR (qPCR) analysis. Comparison of M_3R expression between osteoblasts, various regions of the brain (A) and several peripheral tissues (B). (C) Gene expression of M_1R , M_2R , M_3R , M_4R and M_5R in osteoblasts compared to that of *Adrb2*. The osteoblasts used were primary osteoblast cultures with 5-day induction for differentiation. (D and E) In situ mRNA hybridization analysis of M_3R expression in brain. *Tph2* and *Dbh* are the molecular markers of the Dorsal Raphe (DR) and Locus Coeruleus (LC) nuclei respectively.

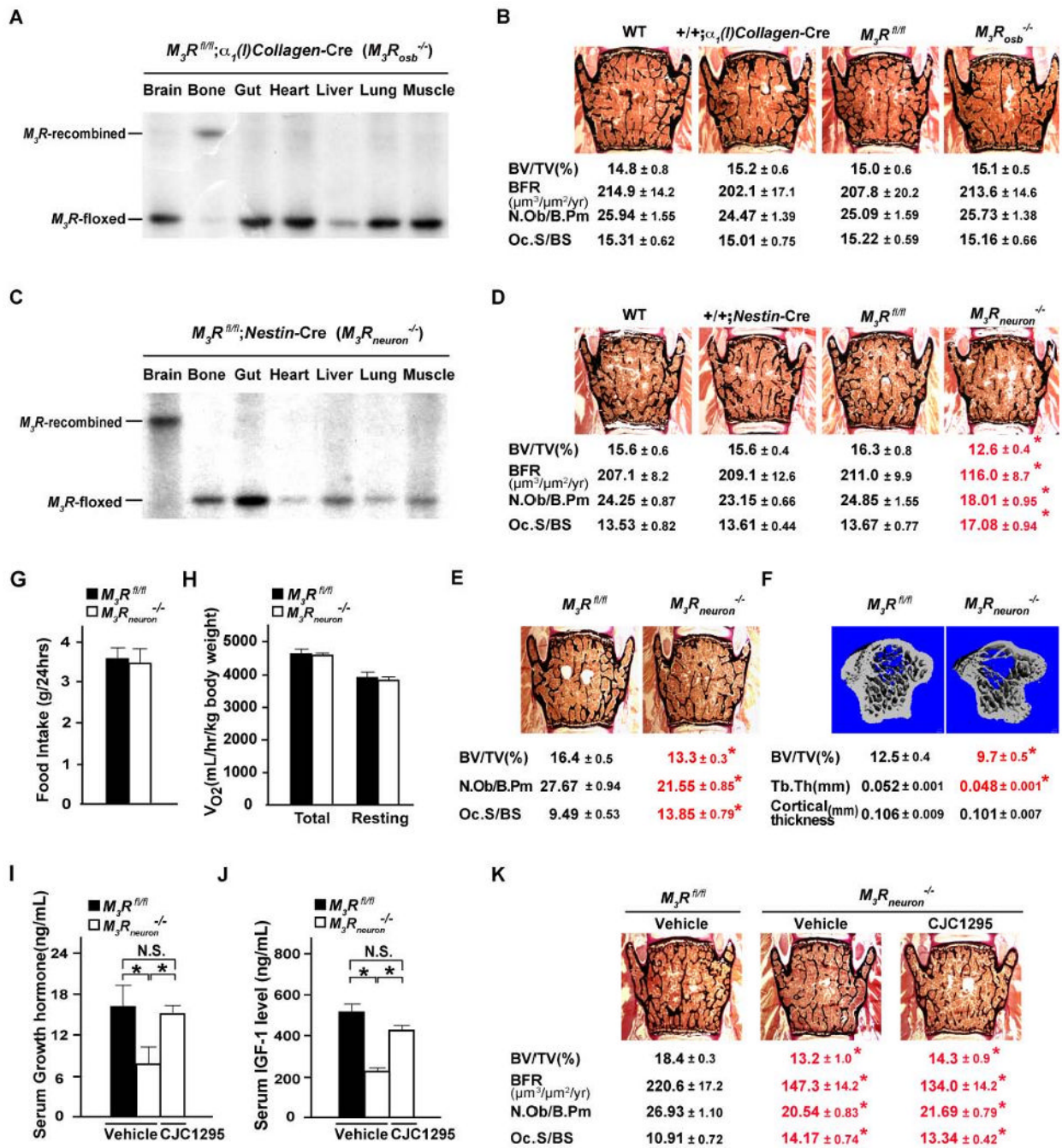


Figure 3. Neuronal mediation of the parasympathetic regulation of bone mass accrual by M₃R (A and C) Recombination efficiency of the *M₃R* locus in various tissues/organs of osteoblast-specific (A) and neuron-specific *M₃R*-deficient mice (C) by southern blot. (B, D and E) Histomorphometric analysis of female 12-week-old *M₃R^{osb}^{-/-}* (B), 12-week-old *M₃R^{neuron}^{-/-}* (D), 6-week-old *M₃R^{neuron}^{-/-}* (E) and corresponding control vertebrae. (F) μ CT analyses of 12-week-old female *M₃R^{fl/fl}* and *M₃R^{neuron}^{-/-}* proximal tibiae. Tb.Th, trabecular thickness. (G and H) 24-hour food intake and energy expenditure assays of *M₃R^{neuron}^{-/-}* and *M₃R^{fl/fl}* control mice. (I-K) ELISA analysis of serum growth hormone (I) and IGF-1 (J) levels and histomorphometric analysis of vertebrae (K) of CJC1295- and vehicle-treated *M₃R^{neuron}^{-/-}* and *M₃R^{fl/fl}* control

mice. N.S., statistically non-significant. n = 6-10 per group; * $P < 0.05$ comparing to WT group; results are represented as mean \pm SEM.

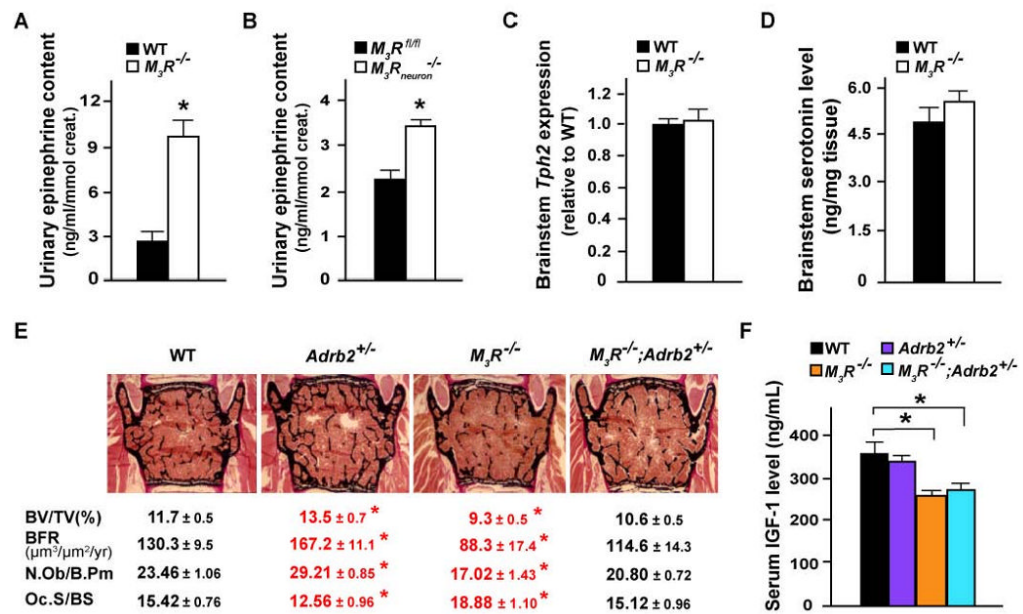


Figure 4. Increased sympathetic activity in $M_3R^{-/-}$ mice leads to low bone mass
 (A and B) ELISA assays of epinephrine content in urine of $M_3R^{-/-}$ (A) and $M_3R_{neuron}^{-/-}$ (B) as well as WT control mice. (C and D) qPCR analysis of *Tph2* mRNA expression (C) and HPLC measurement of serotonin level in brainstem of WT and $M_3R^{-/-}$ mice. (E and F) Histomorphometric analysis of vertebrae (E) and ELISA analysis of serum IGF-1 levels (F) of 24-week-old WT, $Adrb2^{+/-}$, $M_3R^{-/-}$, and $Adrb2^{+/-}; M_3R^{-/-}$ mice. n = 6-10 per group; * $P < 0.05$ comparing to WT group; results are represented as mean \pm SEM.