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# Leptin regulation of bone mass, appetite and energy expenditure relies on its ability to inhibit serotonin synthesis in the brainstem

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## Abstract

Leptin inhibition of bone mass accrual requires the integrity of specific hypothalamic neurons but not expression of its receptor on these neurons. The same is true for its regulation of appetite and energy expenditure. This suggests that leptin acts elsewhere in the brain to achieve these three functions. We show here that brainstem-derived serotonin (BDS) favors bone mass accrual following its binding to Htr2c receptors on ventromedial hypothalamic neurons and appetite via Htr1a and 2b receptors on arcuate neurons. Leptin inhibits these functions and increases energy expenditure because it reduces serotonin synthesis and firing of serotonergic neurons. Accordingly, while abrogating BDS synthesis corrects the bone, appetite and energy expenditure phenotypes caused by leptin deficiency, inactivation of the leptin receptor in serotonergic neurons recapitulates them fully. This study modifies the map of leptin signaling in the brain and identifies a molecular basis for the common regulation of bone and energy metabolisms.



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## Introduction

Leptin is an adipocyte-derived hormone that regulates a broad spectrum of homeostatic functions following its binding to the signaling form of its receptor, ObRb, present on neurons of the central nervous system (Friedman and Halaas, 1998; Spiegelman and Flier, 2001). It is widely assumed that the hypothalamus, where *ObRb* is expressed in several nuclei, is the main site where leptin acts in the brain (Elmquist, 2000).

One homeostatic function regulated by leptin in rodents, sheep and humans is bone remodeling, the mechanism whereby vertebrates renew their bones during adulthood (Karsenty, 2006; Pogoda et al., 2006). Leptin regulates, only through a central relay, both phases of this process, resorption and formation (Ducy et al., 2000; Shi et al., 2008). One mediator linking leptin signaling in the brain to bone remodeling is the sympathetic tone which inhibits bone formation and favors bone resorption through the β2 adrenergic receptor (Adrβ2) expressed in osteoblasts (Elefteriou et al., 2005; Takeda et al., 2002). Hence, sympathetic activity can be used as a readout of leptin regulation of bone mass.

The leptin-dependent central control of bone mass raises the question of the identity of the neurons mediating it. Chemical lesioning experiments performed in both WT and leptin-deficient (*ob/ob*) mice followed by leptin intracerebroventricular (ICV) infusion provided compelling evidence that, to regulate bone mass, leptin requires the integrity of neurons of the ventromedial hypothalamic (VMH) nuclei which in turn influence sympathetic activity (Takeda et al., 2002). Surprisingly however, VMH-specific deletion of *ObRb* does not affect bone mass (Balthasar et al., 2004). At least two interpretations of these experiments can be proposed. The first one is that they are contradicting each other and that, since chemical lesioning is less precise than cell-specific gene deletion, results obtained using the former technique are not reliable (Waddington et al., 2007). A second, more literal, interpretation views these two experiments as complementary and simply states that VMH neurons are necessary for leptin to regulate bone mass but signaling through ObRb on these neurons is not.

This latter interpretation gains further support if one looks at another function regulated centrally by leptin: appetite. Genetic inactivation of *ObRb* in all neurons and chemical destruction of the arcuate nuclei of hypothalamus increases appetite (Cohen et al., 2001), yet inactivation of *ObRb* selectively in arcuate, VMH or in both nuclei does not when mice are fed a normal diet (Balthasar et al., 2004). This inconsistency echoes the one noted above for the regulation of bone mass. Together they raise the prospect that leptin may first act elsewhere in the brain to affect synthesis of neuromediator(s) that in turn influences bone mass and energy metabolism by signaling to hypothalamic neurons.

Serotonin is an indoleamine produced in enterochromaffin cells of the duodenum and in serotonergic neurons of brainstem that does not cross the blood brain barrier (Mann et al., 1992). Thus, it is de facto a molecule with two distinct functional identities depending on its site of synthesis: a hormone when made in the gut and a neurotransmitter when made in the brain (Walther et al., 2003; Yadav et al., 2008). Although brain-derived serotonin (BDS) has many known roles (Heath and Hen, 1995) its potential function as a regulator of bone mass accrual or other homeostatic processes has not been thoroughly examined yet. This is an important question to address for several reasons. Firstly, the critical role exerted by gut-derived serotonin on bone formation (Yadav et al., 2008) raises questions regarding the role BDS may have in this process. Additionally, in invertebrates where it has been tested genetically, serotonin strongly enhances appetite (Horvitz et al., 1982; Srinivasan et al., 2008).

Here we show that, unlike leptin, BDS favors bone mass accrual and appetite, and decreases energy expenditure following its binding to distinct receptors located on two different

hypothalamic nuclei. Cell-specific gene deletion of the leptin receptor show that leptin regulation of these functions occurs by inhibiting serotonin synthesis in neurons of the brainstem. These results reveal a different map of leptin action in the brain, expand the importance of BDS in physiology, they also identify a molecular basis for the common central control of bone mass and appetite.

## Results

### Low bone mass in mice deprived of serotonin in the brain

Serotonin synthesis is initiated by hydroxylation of tryptophan, a rate-limiting reaction performed by the enzyme tryptophan hydroxylase 2 (Tph2) in the brain (Walther et al., 2003). To determine whether BDS affects bone mass we disrupted *Tph2* by inserting *LacZ* in its locus (Figure S1A) and first used this allele to study *Tph2* pattern of expression.

The location of serotonergic neurons in the present study was defined according to Jensen et al (Jensen et al., 2008) as follows: Dorsal raphe (B4, B6 and B7), median raphe (B5, B8 and B9) and caudal raphe (B1, B2 and B3) nuclei. Together these neurons will be referred thereafter as serotonergic neurons of the brainstem in this manuscript. β-galactosidase staining of the whole brain showed that during embryonic development *Tph2* expression was detected as early as E12.5 in neurons of the dorsal and median raphe nuclei in the brainstem (Figure 1A & data not shown). At E14.5, 15.5 and 18.5, β-galactosidase staining was also detected in neurons of the caudal raphe nuclei of the brainstem (Figure 1A & B) but not in other areas of the brain or in peripheral tissues (Figure S1B–D). To determine whether β-galactosidase staining is a faithful representation of *Tph2* endogenous expression we performed *in situ* hybridization and co-immunolocalization of *Tph2* and β-galactosidase. These experiments revealed a tight concordance between *Tph2* expression and β-galactosidase staining (Figure 1C). After birth, *Tph2* expression measured by real-time PCR was 4 orders of magnitude higher in the brainstem than in other parts of the brain or in peripheral tissues (Figure 1D). Based on these criteria, *Tph2* expression is specific to serotonergic neurons of the brainstem.

*Tph2*−/− mice were born at the expected Mendelian ratio, had a normal size and appearance and were normally fertile (Figure 1H & data not shown). The near complete absence of detectable serotonin in the brain of *Tph2*−/− mice verified that we had successfully inactivated this gene and was consistent with the fact that *Tph1* expression in the brain was not enhanced, at least post-natally, by the *Tph2* deletion (Figure 1E & F). Conversely, blood serotonin levels were normal in *Tph2*−/− mice (Figure 1G). Thus the *Tph2*−/− mouse is an animal model lacking serotonin selectively in the brain. Serum levels of leptin, insulin, corticosterone and T4 as well as body length were also normal in 3 month-old *Tph2*−/− animals (Figure 1H & Figure S1E).

To assess the influence of BDS on bone remodeling histological, histomorphometric and microcomputed tomography (μCT) analyses of bones were performed in 4, 6 and 12 week-old wild type (WT) and *Tph2*−/− mice. The absence of serotonin in the brain resulted, at all time points, in a severe low bone mass phenotype affecting axial (vertebrae) and appendicular (long bones) skeleton while bone length and width were unaffected (Figures 2A–D & data not shown). Three month-old *Tph2*+/− mice also displayed a decrease in bone mass, albeit milder (Figure 2A). This phenotype was secondary to a decrease in bone formation parameters (osteoblast numbers and bone formation rate) and to an increase in bone resorption parameters [osteoclast surface and circulating levels of deoxypridinoline (Dpd), a degradation product of type I Collagen and a biomarker of bone resorption (Eyre et al., 1988)] (Figure 2A & E). Bone mineralization was normal in *Tph2*−/− mice (Figure S2). These results demonstrate that BDS is a positive and powerful regulator of bone mass accrual acting on both arms of bone

remodeling. Since serotonin does not cross the blood brain barrier these observations provide a rare example of a regulation of bone mass by a neuromediator.

## The influence of brain-derived serotonin on bone mass prevails over the one of gut-derived serotonin

That serotonin exerts opposite influences on bone remodeling depending on its site of synthesis was unexpected. Since BDS accounts for only 5% of total serotonin we asked what was its actual contribution to the overall regulation of bone mass accrual by serotonin. To that end we generated mice unable to synthesize serotonin anywhere in their body by inactivating both *Tph1* and *Tph2* (Figure 3A & B). *Tph1−/−*;*Tph2−/−* mice were born at the expected Mendelian ratio and had normal size and life span (data not shown). To our surprise, like the *Tph2−/−* mice, *Tph1−/−*;*Tph2−/−* mice displayed a low bone mass secondary to a decrease in bone formation and to an increase in bone resorption parameters and affecting the axial and appendicular skeleton (Figure 3C & data not shown). By showing that the influence of BDS on bone remodeling prevails over the one exerted by gut-derived serotonin even though it accounts for only 5% of the total pool of serotonin this experiment underscored the importance of BDS in the regulation of bone mass and was an incentive to elucidate its mode of action.

## Sympathetic mediation of brain-derived serotonin regulation of bone mass

The decrease in bone formation and the increase in bone resorption seen in *Tph2−/−* mice is the mirror image of what is observed in mice lacking the *β2 adrenergic receptor* (*Adrβ2−/−* mice) (Elefteriou et al., 2005). This feature suggested that the bone phenotype of the mice lacking serotonin in the brain could be secondary to an increase in sympathetic signaling in osteoblasts. That norepinephrine content in the brain, epinephrine elimination in the urine and *Ucp1* expression in brown fat, 3 markers of the sympathetic tone, were all markedly increased in *Tph2+/−*, *Tph2−/−* and *Tph1−/−*;*Tph2−/−* mice at 6 and 12 weeks of age supported this hypothesis (Figure 3D–F & Figure S3). We also generated *Tph2−/−* mice in which one allele of *Adrβ2* had been inactivated (Figures 3F & 3G). We removed one copy of this gene because *Adrβ2* is the only adrenergic receptor expressed in osteoblasts (Takeda et al., 2002). *Tph2−/−*;*Adrβ2+/−* mice had normal bone formation and bone resorption parameters and a normal bone mass, the same was true for *Tph2−/−*;*Adrβ2−/−* mice (Figure 3G & data not shown). These results indicate that the regulation of bone mass accrual by BDS occurs by decreasing the sympathetic tone.

## Brain-derived serotonin regulates bone mass through the hypothalamus

Since the sympathetic regulation of bone mass requires the integrity of the VMH neurons of the hypothalamus (Takeda et al., 2002) we next asked whether the BDS regulation of bone mass also occurs through a VMH relay.

To search for anatomical connections between *Tph2*-expressing and hypothalamic neurons we used the *Rosa26R-Ecfp* mice (Srinivas et al., 2001). In this latter mouse model the *Ecfp* (Enhanced Cyan fluorescent protein) reporter gene containing a floxed transcriptional blocker cassette inserted between the transcription start site and the ATG is placed downstream of the *Rosa26* promoter. Thus, *Ecfp* can only be expressed after *Cre*-mediated deletion of the transcriptional blocker. We crossed *Rosa26R-Ecfp* mice with *Sert-Cre* transgenic mice that express *Cre* only in *Tph2*-expressing neurons (Zhuang et al., 2005). Ecfp immunostaining in *Sert-Cre*; *Rosa26R-Ecfp* mice showed that axons emanating from *Tph2*-expressing neurons of the brainstem projected to the hypothalamus (Figure 4A) and in situ hybridization performed on adjacent sections demonstrated that those axonal projections reached *Sf1*-expressing VMH neurons (Figure 4A). These findings were confirmed by fluorescent dextran tracing. Anterograde and retrograde labelling in *Tph2+/−* mice showed that VMH neurons were targeted by neuronal projections emanating from *Tph2*-expressing neurons in the brainstem

(Figure 4B–C & Figure S4A–B). This morphological data suggesting that serotonin signals in neurons of the VMH nuclei was an incentive to search for serotonin receptor(s) on these neurons.

Real-time PCR analysis revealed that among the 14 serotonin receptors *Htr2c* was by far the most highly expressed in the hypothalamus albeit it was not the only one (Figure 4D) and double fluorescent *in situ* hybridization experiments showed that *Htr2c* was expressed in *Sf1*-expressing VMH and in *Pomc*-expressing arcuate neurons (Pasqualetti et al., 1998) (Figure 4E and Figure S4C). Moreover, *Ecfp*-positive neuronal arborizations originating from serotonergic neurons of the brainstem project preferentially to the anterior part of the VMH nucleus where *Htr2c* is expressed at its highest level (from Bregma −1.06 mm to −1.34 mm; Figure 4A, right panel). To determine the importance of serotonin signaling through Htr2c in the regulation of bone mass we first analyzed mice lacking *Htr2c* in all cells (*Htr2c*−/− mice). Since *Htr2c*−/− mice develop an increase in food intake and adiposity beyond 14 week of age (Tecott et al., 1995), we analyzed 6 and 12 week-old animals after verifying that at those ages appetite, energy expenditure, body weight, fat pad weights and hormonal profiles were identical in *Htr2c*−/− and WT mice (Figure S4D–H).

Histological analyses uncovered in both 6 and 12 week-old *Htr2c*−/− mice a severe low bone mass phenotype secondary to a decrease in the number of osteoblasts and bone formation rate, and to an increase in the number of osteoclasts and bone resorption parameters (Figure 4F & data not shown). Moreover, *Ucp1* expression in brown fat and urinary elimination of epinephrine were both significantly higher in *Htr2c*−/− mice revealing the existence of a high sympathetic activity (Figures 4G–H). Thus, both in terms of bone remodeling parameters and sympathetic tone, *Htr2c*−/− mice are a phenocopy of *Tph2*−/− mice at time points when no metabolic abnormalities could be found. To establish that it is by signaling through Htr2c that BDS regulates bone mass we generated compound mutant mice lacking one allele of *Tph2* and one allele of *Htr2c* (*Tph2*+/−;*Htr2c*+/− mice). These latter mutant mice presented at 6 and 12 weeks of age the same low bone mass/ high sympathetic activity phenotype than the *Htr2c*−/− and *Tph2*−/− mice (Figure 4F & data not shown). These results support the notion that BDS utilizes the *Htr2c* receptor to regulate sympathetic tone and bone mass independently of the influence it exerts through this receptor on energy metabolism.

To determine whether it is through its expression in VMH neurons that *Htr2c* regulates bone mass we used mutant mice harboring a loxP-flanked transcriptional blocking (loxTB) cassette inserted in the *Htr2c* gene (loxTB *Htr2c* mice) (Xu et al., 2008). In these mice disruption of *Htr2c* transcription can be alleviated, in a cell population of choice, by the Cre recombinase. *Htr2c* re-expression was targeted to VMH neurons by crossing loxTB *Htr2c* mice with *Sf1-Cre* mice (Figure S4J). Histological analyses showed that re-expression of *Htr2c* receptor in VMH neurons ($Htr2c_{SF1}$+/+ mice) rescued entirely the bone mass phenotype observed in the absence of *Htr2c* (Figures 4G–I). Moreover, *Ucp1* expression in brown fat and urinary elimination of epinephrine were also similar between WT and $Htr2c_{SF1}$+/+ mice and levels of glutamate, an inhibitor of sympathetic tone that were suppressed in *Htr2c*−/− hypothalami were partially restored in $Htr2c_{SF1}$+/+ hypothalami (Figures 4G, 4H & 4J). These findings echo previous observations indicating that serotonin attenuates activation of noradrenergic neurons in the locus coeruleus (Aston-Jones et al., 1991). Taken together, the results presented so far indicate that BDS acts on VMH neurons, through Htr2c, to decrease sympathetic activity and thereby favors bone mass accrual.

## Leptin inhibits bone mass accrual by decreasing brain-derived serotonin synthesis

Although leptin and serotonin exert opposite influences on bone mass accrual, several features suggested that they might operate in the same pathway. For instance serotonin, like leptin, uses the sympathetic tone to regulate bone mass and, also like leptin, it requires VMH neurons

integrity to achieve this function. As shown below, multiple lines of evidence indicate that it is by inhibiting BDS synthesis that leptin prevents bone mass accrual.

First, ObRb, the signaling form of the leptin receptor, is expressed in β-galactosidase-positive *Tph2*-expressing neurons (Figure 5A). Second, *Tph2* expression increased steadily over time in *ob/ob* mice to eventually reach a level 10 fold higher than what is seen in WT mice at 6 months of age (Figure 5B) and conversely, serotonin content is significantly higher in the brainstem of *ob/ob* mice (Figure 5C). Third, leptin ICV infusion decreased *Tph2* expression in a time- and dose-dependent manner in WT mice (Figures 5D–E). Fourth, co-immunolocalization studies revealed that the phosphorylation of Stat3, a transcription factor mediating leptin signaling, that was increased in β-galactosidase-positive serotonergic neurons of the brainstem following acute leptin ICV infusion in WT mice was dramatically reduced in $ObRb_{SERT}$−/− mice (Figure 5F). In support of these correlative arguments *ob/ob* mice lacking one allele of *Tph2* (*ob/ob*;*Tph2*+/− mice) displayed normal *Tph2* expression, normal serotonin content in the brainstem, normal sympathetic tone and normal bone remodeling parameters and bone mass (Figure 5G–I & Figure S5). These data suggest a model whereby leptin regulates bone mass accrual through a double inhibitory loop. Leptin inhibits synthesis of BDS, which in turn reduces, by signaling in VMH neurons, the sympathetic tone; as a result leptin prevents bone mass accrual.

## Leptin inhibits the neuronal activity of serotonergic neurons

The mediation of peripheral hormone action on the output of the brain relies on altered circuit activity. Interaction between neuronal circuits hinges on electric properties of neurons, particularly on the generation of action potentials. Thus to test whether leptin directly alters serotonin output from brainstem neurons, we analyzed the responses of serotonin-producing cells to leptin with whole cell patch clamp recording in brain slices containing dorsal raphe (DR) (Supplemental methods). Slices were taken from WT animals and from mice lacking *ObRb* selectively in *Tph2*-expressing neurons ($ObRb_{SERT}$−/− mice). Serotonergic neurons were identified according to their unique properties (long-duration action potential, activation by norepinephrine and inhibition by serotonin itself) (Liu et al., 2002). Since serotonergic neurons are usually quiescent in slices because of the loss of noradrenergic inputs, action potentials in these neurons were restored by application of alpha-1 adrenergic agonist phenylephrine (3 μM) in the bath (Liu et al., 2002). Whole cell patch recording showed that leptin significantly decreased action potential frequency in serotonergic neurons of WT mice, but not in serotonergic neurons of mice lacking *ObRb* in *Tph2*-expressing neurons ($ObRb_{SERT}$−/− mice) (Figure 5J–L). These data show that leptin can alter directly activity of serotonergic neurons in the brainstem and that this effect of leptin is mediated by *ObRb* expressed on these neurons.

## Brain-derived serotonin regulates appetite and energy expenditure

In addition to low bone mass, we consistently observed a significant decrease in fat pad weight in *Tph2*−/− mice (Figure 6A). This surprising observation led us to analyze in greater details energy metabolism in these mutant mice. At both 6 and 12 weeks of age there was a significant decrease in food intake in *Tph2*−/− (~31%) and *Tph2*+/− (~14%) mice compared to WT littermates along with an increase in energy expenditure (as measured by $V_{O2}$, XTOT and Heat production) (Figures 6B–E). In contrast glucose metabolism, serum levels of leptin and other hormones were not affected in *Tph2*-deficient mice (Figure 1H, Figure S1E & Figure S6A–B).

This observation along with the fact that the control of appetite and energy expenditure requires the integrity of the arcuate nuclei of the hypothalamus raised the prospect that axonal projections emanating from *Tph2*-expressing neurons reach arcuate nuclei to regulate these

functions. To address this question we crossed *Rosa26-Ecfp* mice with *Sert-cre* mice and analyzed on adjacent sections expression of *Pomc-1* and *Npy*, two arcuate neuron-specific genes (Figure 6F & Figure S4B). This analysis verified that neurons of the arcuate nuclei were targeted by serotonergic innervations emanating from the brainstem, an observation confirmed in the *Tph2*+/− mice by retrograde labelling of the projections reaching the serotonergic neurons of the brainstem (Figure 6F). Among all serotonin receptors the most highly expressed in arcuate neurons were *Htr1a*, and, to a lower extent, *Htr2b* and *Htr2c* (Figures 6G & Figure S4C). While food intake was not affected in *Htr2c*−/− mice, it was significantly reduced in mice lacking *Htr1a* in all cells (~24% reduction) or lacking *Htr2b* in arcuate neurons only (~10% reduction); fat pad weight was also lower in *Htr1a*−/− and $Htr2b_{POMC}$−/− mice (Figure 6H, 6I & Figure S4D).

We next asked whether expression of genes expressed in hypothalamic neurons and that may mediate leptin regulation of appetite was perturbed in *Tph2*−/− mice. Among those tested the only gene whose expression was significantly increased in *Tph2*−/− mice was *Mc4r* (Figure 6J), a gene whose inactivation in mice and humans cause hyperphagia and obesity (Huszar et al., 1997; Yeo et al., 1998). Two experimental evidences support the notion that the appetite phenotype of the *Tph2*−/− mice was caused, at least in part, by an increase in melanocortin signaling. First, ICV infusion of a Mc4r antagonist (HS014) increased appetite ~50% in *Tph2*−/− mice (Figure 6K); second ICV infusion of a Mc4r agonist (MTII) increased *c-Fos* expression in neurons of the paraventricular and arcuate nuclei of both WT and *Tph2*−/− mice (Figure 6L & Figure S6C). Moreover, *Mc4r* expression was increased ~2 fold in *Htr1a*−/− and ~1.6 fold in $Htr2b_{POMC}$−/− mice, but was unaffected in *Htr2c*−/− mice (Figure 6J & data not shown). Energy expenditure was normal in *Htr1a*−/− and $Htr2b_{POMC}$−/− indicating that serotonin uses other receptors, yet to be identified, to regulate this function (Figure S6D–G). Taken together these results indicate that BDS regulates appetite and energy expenditure and that for the control of appetite this mediation occurs through the Htr1a and Htr2b receptors and involves melanocortin signaling.

## Leptin signaling in serotonergic neurons regulates appetite, energy expenditure and bone mass

Three reasons led us to ask next whether the appetite and energy expenditure phenotypes of the *ob/ob* mice were serotonin-dependent. The first one is that the conjunction of a decrease in appetite and an increase in energy expenditure is the mirror image of what is seen in mice lacking leptin signaling; the second one is that leptin inhibition of serotonin synthesis in the brainstem is the mechanism used by this hormone to inhibit bone mass accrual; the third one is that no molecular mechanisms has been identified so far to explain the common control of bone mass and energy metabolism.

To test this hypothesis we first used *ob/ob*;*Tph2*+/− mice, that have a normal content of serotonin in the brain (Figure 5H). Remarkably, *ob/ob*;*Tph2*+/− mice also had appetite and energy expenditure parameters undistinguishable from WT littermates (Figures 6M–O & data not shown) suggesting that leptin must inhibit BDS synthesis in order to decrease appetite and to increase energy expenditure. Consistent with this hypothesis *ob/ob* mice unable to synthesize serotonin at all in the brain (*ob/ob*;*Tph2*−/−) had even a lower appetite than WT mice; as a result their fat pad weights were significantly smaller than the ones of *ob/ob* littermates (Figures 6M–O).

Next, to establish that serotonegic neurons of the brainstem and BDS are a critically important entry point and target of leptin in the brain, we analyzed bone mass, appetite and energy expenditure in mouse strains lacking *ObRb* in distinct neuronal populations in the brain (Figure S7A–B). This analysis was performed on mice fed a normal diet since leptin signaling-deficient mice develop a massive obesity on this diet. The specificity of *Cre* expression was verified for

each mouse line by crossing them with RosaR26 mice and by in situ hybridization (Soriano, 1999) (Figure S7A–B).

As reported previously mice lacking *ObRb* either in *Sf1*-expressing neurons of the VMH nuclei or in *Pomc*-expressing neurons of the arcuate nuclei had normal sympathetic activity, bone remodeling parameters and bone mass; they also had normal appetite, energy expenditure and body weight (Figures 7A–G & S7A–I) (Balthasar et al., 2004; Dhillon et al., 2006). In contrast $ObRb_{SERT}{}^{-/-}$ mice lacking *ObRb* in serotonergic neurons of the brainstem developed rapidly, a low sympathetic activity, high bone mass phenotype and a similar increase in appetite as *ob/ob* mice; they also had low energy expenditure (Figures 7A–G). As a result $ObRb_{SERT}{}^{-/-}$ mice, when fed a normal diet, developed an obesity phenotype of similar severity and at a similar pace than mice lacking leptin signaling (Figure 7G & Figure S7E). Serotonin in the brain of $ObRb_{SERT}{}^{-/-}$ was elevated to the same extent as in *ob/ob* mice, while it was normal in the brain of $ObRb_{SF1}{}^{-/-}$ mice (Figure 7H). Remarkably for our purpose hypothalamus gene expression analysis by real-time PCR revealed a decrease in *Mc4r* and *Pomc* expression, and an increase in *Npy* and *Agrp* expression in $ObRb_{SERT}{}^{-/-}$ mice that is of similar severity to the one observed in *ob/ob* mice. (Figure 7I).

*ObRb* deletion in *Tph2*-expressing neurons also had an organizational effect on *Pomc*-expressing neurons of the arcuate nuclei. Indeed, the average diameter of *Pomc*-expressing neurons in $ObRb_{SERT}{}^{-/-}$ mice (n=42) was significantly lower than in WT mice (Figure 7J). The lower POMC perikaryal diameter of *ob/ob* mice is associated with a ~50% decrease in the number of perikaryal synapse density of POMC neurons (Pinto et al., 2004). Altered synaptic input organization of POMC neurons was also detected in $ObRb_{SERT}{}^{-/-}$ mice (14.76±1.3 vs 27.31±2.03 synapses per 100 micron perikaryal membrane in $ObRb_{SERT}{}^{-/-}$ and WT mice respectively). Thus, it is likely that the *ob/ob* phenotype of POMC neurons is determined, at least in part, by leptin signaling in serotonergic neurons of the brainstem.

## Discussion

The results presented here demonstrate that in order to regulate bone mass accrual, appetite and energy expenditure leptin needs to inhibit the electrical activity and serotonin synthesis in *Tph2*-expressing neurons of the brainstem (Figure 7K). These results modify the map of leptin signaling in the brain and indicate that the serotonergic neuronal circuitry exerts a more fundamental influence on several homeostatic functions than previously thought. Moreover, they identify BDS as the long sought-after molecular basis for the common control of bone mass and energy metabolism.

### Brain-derived serotonin regulation of bone mass

This effort to complete our understanding of the regulation of bone mass by serotonin led to two unexpected results. The first one is that, depending on its site of synthesis, serotonin regulates bone mass accrual in opposite directions: it inhibits it when synthesized in the duodenum and favors it when acting as a neurotransmitter. To our knowledge this is the first example of a molecule exerting different influences on bone remodeling depending on its site of synthesis. The central function of serotonin is mediated through the Htr2c receptor expressed in VMH neurons. $Htr2c^{-/-}$ mice are markedly osteopenic before any metabolic modification is detectable, indicating that serotonin regulation of bone mass occurs independently of its effects, through Htr2c, on energy metabolism. Given what is known about the molecular signaling of serotonin in osteoblasts (Yadav et al., 2008), future studies are needed to determine whether BDS recruits the same transcription factor(s) in VMH neurons.

The second surprising observation is that although it accounts only for a rather small portion of the total pool of serotonin in the body (~5%) BDS influence on bone remodeling is dominant

over that exerted by gut-derived serotonin. Since BDS synthesis is regulated by leptin these results infer that leptin regulation of bone mass is more important than the one exerted by gut-derived serotonin (Yadav et al., 2008). This observation along with the fact that leptin appears during evolution with a bony skeleton underscores the importance of the regulation of bone remodeling in the panoply of leptin's function; it also predicts that using leptin as a treatment for obesity would favor appearance of osteoporosis.

Patients taking chronically serotonin reuptake inhibitors (SSRIs) have an increased risk of osteoporotic fractures (Richards et al., 2007). Consistent with this observation an animal model reproducing this chronic use of SSRIs, namely mice lacking 5-hydroxytryptamine transporter (5HTT), a molecule responsible for serotonin reuptake by cells, also develop a low bone mass phenotype (Warden et al., 2005). Surprisingly however, this low bone mass phenotype cannot be ascribed to an increase in circulating serotonin levels i.e. to gut-derived serotonin, since serotonin is undetectable in the plasma of these mice (data not shown). The low bone mass of *5Htt*−/− mice further supports the notion that BDS exerts a dominant role in the regulation of bone mass.

## Serotonin regulation of appetite and energy expenditure

The study of BDS functions led to other unexpected findings beyond the control of bone mass. Indeed the deletion of *Tph2* shows that BDS is a powerful orexigenic molecule in vertebrates. This function of BDS is similar to the function of serotonin in invertebrates (Nonogaki et al., 1998; Srinivasan et al., 2008). Genetic analysis showed that the orexigenic effect of serotonin occurs through Htr1a and Htr2b receptors, in a *Mc4r*-dependent manner (Heisler et al., 2002; Lam et al., 2008). This role of Htr1a in the regulation of appetite is consistent with the orexigenic action of Htr1a agonists (Gilbert et al., 1988; Neill and Cooper, 1988). The demonstration that it is through its expression in arcuate neurons that *Htr1a* regulates appetite will need generation of a cell-specific deletion of this gene.

The fact that removal of the ligand and addition of a pharmacological agonist of a receptor result in the same phenotype, i.e. anorexia, could have several explanations (Vickers et al., 1999). For instance, it is possible that signaling through some serotonin receptors may antagonize signaling through others. Likewise, it should be noted that in most settings the effect of pharmacologic agents are acute while by definition the effect of gene deletion is chronic. Lastly, it is also possible that different serotonin receptors regulate differently food intake possibly even on the same neuron (Xu et al., 2008).

## Brain-derived serotonin as a target of leptin

Several lines of evidence suggested initially that leptin regulates bone mass, appetite and energy expenditure following binding to its receptors located on hypothalamic neurons. Chief among those are the facts that the hypothalamus is a known regulator of most homeostatic functions and that *ObRb* is highly expressed in various hypothalamic neuronal populations (Elmquist et al., 2005). Surprisingly however, deletion of *ObRb* in those hypothalamic neuronal populations did not affect any of these three functions (Balthasar et al., 2004; Dhillon et al., 2006). These latter findings suggested the following hypothesis: leptin would act in other parts of the brain where its receptor is expressed to affect synthesis of neurotransmitter(s), which would then act in hypothalamic neurons. That serotonin is the initial target in the brain of leptin regulation of bone mass accrual was an incentive to test whether it could also be implicated in the leptin regulation of appetite and energy expenditure.

We show here, through cell-specific gene deletion of *Tph2* or the leptin receptor, that BDS enhances appetite and decreases energy expenditure and that leptin regulates these functions by inhibiting BDS synthesis. Thus leptin does use the same mechanism to regulate bone mass

accrual, appetite and energy expenditure. We remain aware however, that these data do not exclude formally the possibility that leptin acts also on hypothalamic neurons at a level not detectable using cell-specific gene deletion experiments. For instance, our study does not rule out an involvement of *Agrp*-expressing neurons in the control of various aspects of energy metabolism (van de Wall et al., 2008). This is especially true since *Agrp* expression is similarly increased in $ObRb_{SERT}{}^{-/-}$ and *ob/ob* mice. By identifying brainstem serotonergic neurons as an initial target of leptin this study provides a cellular and molecular explanation for the apparent contradiction between the fact that chemical lesioning of hypothalamic neurons hampers leptin signaling while inactivating *ObRb* in these neurons does not. A question raised by our work is to know what are the functions of the leptin receptor expressed in hypothalamic neurons? At the present time it seems that *ObRb* expression on these neurons is mainly needed for the regulation of insulin secretion and glucose metabolism (Coppari et al., 2005; Hinoi et al., 2008; van de Wall et al., 2008) a function not affected by serotonin or leptin signaling in serotonergic neurons (Figure S1). Indeed it is important to underscore that serotonin is not implicated in all functions of leptin, for instance *ob/ob*;*Tph2*+/−, as *ob/ob* mice, are sterile.

### Co-regulation of bone remodeling and energy metabolism

Why would bone remodeling and energy metabolism need to be co-regulated in the first place? To answer this question, one needs to look at what was the original purpose of bone remodeling earlier during evolution. Through its ability to constantly renew bone the original function of bone remodeling was to repair micro and macro damages, i.e. fractures of bones. This function was, early on, absolutely necessary to maintain mobility and therefore to assure survival. In addition, bone remodeling is characterized by two opposing processes: destruction followed by de novo formation; these two cellular events are costly in terms of energy; even more so if one takes into account that they occur simultaneously in multiple locations. Thus, for bone remodeling to occur there must be a constant supply of energy channeled to osteoclasts and osteoblasts. This view of bone remodeling predicts that there should be one or several hormones, appearing during evolution with this function, and regulating it and energy metabolism. To date leptin is the only known hormone fulfilling all these criteria. This view of bone remodeling also implies that the metabolic importance of the skeleton that begins to be unraveled (Lee et al., 2007) has not been fully characterized yet.

## Experimental Procedures

### Mice Generation

*Tph2-LacZ* mice were generated by embryonic stem cell manipulations following standard protocols to obtain *Tph2*+/− mice. *Tph2*+/− mice were intercrossed to obtain the WT, *Tph2* +/− and *Tph2*−/− mice for analysis. Generation of *Tph1*−/−, *Htr2c*−/−, loxTB *Htr2c*, *Htr1a* −/−, $ObRb^{fl/fl}$, $Htr2b^{fl/fl}$, *Sf1-Cre* and *Sert-Cre* mice was previously reported (Balthasar et al., 2004; Dhillon et al., 2006; Klemenhagen et al., 2006; Tecott et al., 1995; van de Wall et al., 2008; Xu et al., 2008; Yadav et al., 2008; Zhuang et al., 2005). WT, *Pomc1-Cre* and *ob/ob* mice were obtained from The Jackson Laboratory.

### Histological procedures, immunohistochemistry, in situ hybridization, axonal tracing and microcomputed tomography (μCT) analysis

Sections containing dorsal raphe were from bregma −4.04 to −5.40; median raphe from −4.04 to − 4.48; caudal raphe from −4.84 to −7.48; arcuate from −1.22 to −2.80; VMH from −1.06 to −2.06 and PVN from −0.58 to −1.22 according to Franklin and Paxinos mouse brain atlas. Immunohistochemistry was performed on paraffin-embedded specimens sectioned at 6 μm according to standard protocols. LacZ staining was performed on whole brain and coronal sections obtained from the *Tph2*+/− mice following standard procedures. In situ hybridization on brain sections was performed as described (Oury et al., 2006). Ex vivo axonal tracing was

performed using Rhodamine-conjugated dextrans (Molecular Probes, Eugene, Oregonaxonal; See supplemental methods for details). Bone histomorphometric analyses were performed on undecalcified sections using the Osteomeasure analysis system (Osteometrics, Atlanta). Trabecular bone architecture of proximal tibia was assessed using a μCT system (VivaCT 40, SCANCO Medical AG, Switzerland) as described (Shi et al., 2008). Six to 12 animals were analyzed for each group.

### Bioassays

Serotonin levels in the brain and serum were quantified as described (Yadav et al., 2008). Serum level of total deoxypyridinoline (DPD) cross-links was measured using the Metra tDPD kit (Quidel corp. San Diego, CA). Urinary elimination of catecholamines was measured in acidified spot urine samples by EIA (Bi-CAT, Alpco Diagnostics, Salem, NH) and creatinine (Metra creatinine kit, Quidel corp. San Diego, CA) used to standardize between urine samples.

### Molecular studies

RNA isolation, cDNA preparation and qPCR analysis was carried out following standard protocols (See supplemental methods). Genotypes of all the mice were determined by PCR. All primer sequences for genotyping and DNA probes for southern hybridization are available upon request.

### Electrophysiology, food intake and energy expenditure measurements

Brain slice preparation and electrophysiological recording were performed as described [(Liu et al., 2002; Rao et al., 2007) & supplemental methods]. Food intake was measured using metabolic cages (Nalgene, Rochester, NY) and energy expenditure by indirect calorimetry method as described [(Shi et al., 2008) & supplemental methods].

### Statistical analyses

Statistical significance was assessed by Student's t test or a one way ANOVA followed by Newman-Keuls test for comparison between more than 2 groups. $P<0.05$ was considered significant. Different letters indicate significant differences among groups.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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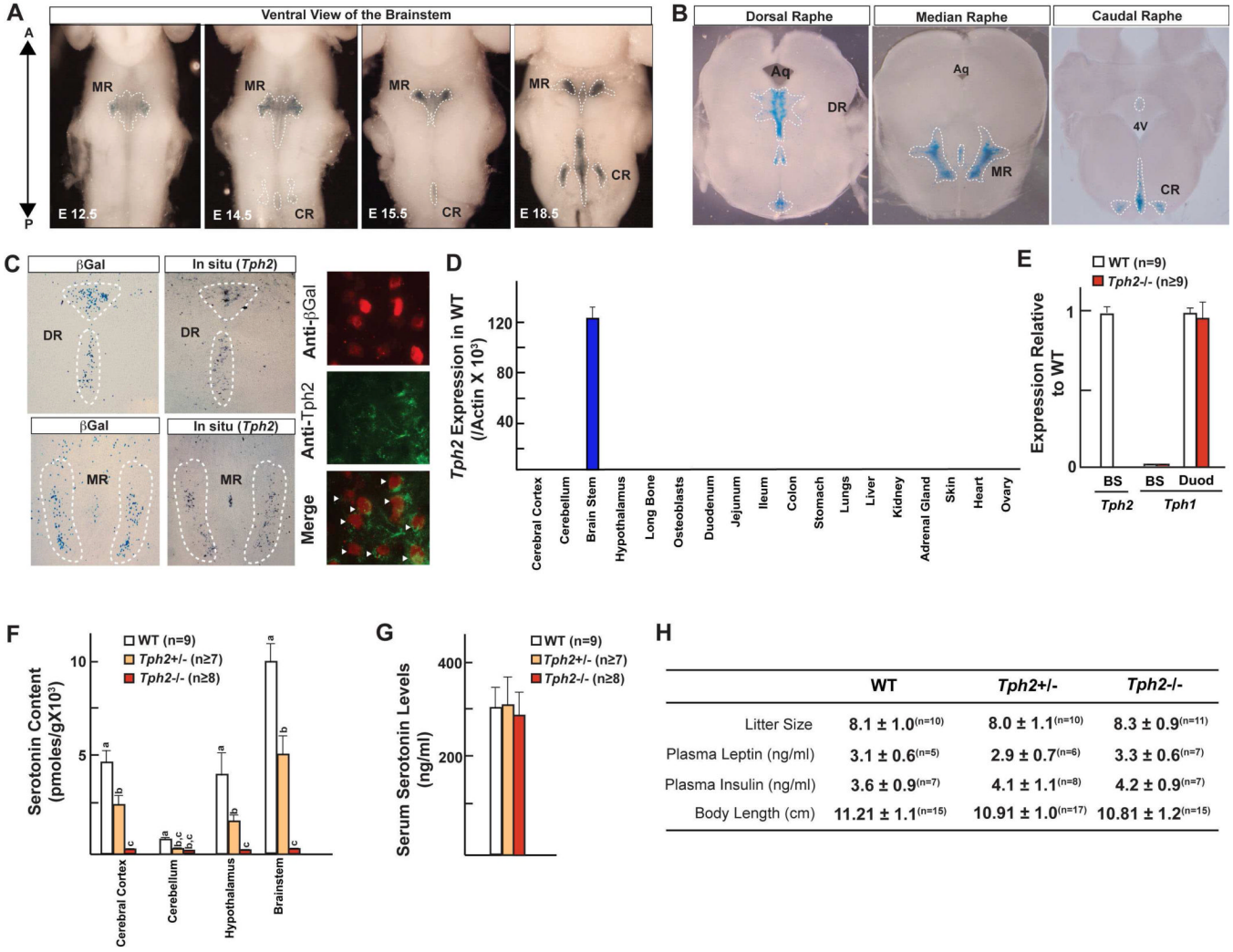



H

| | WT | Tph2+/- | Tph2-/- |
|---|---|---|---|
| Litter Size | 8.1 ± 1.0$^{(n=10)}$ | 8.0 ± 1.1$^{(n=10)}$ | 8.3 ± 0.9$^{(n=11)}$ |
| Plasma Leptin (ng/ml) | 3.1 ± 0.6$^{(n=5)}$ | 2.9 ± 0.7$^{(n=6)}$ | 3.3 ± 0.6$^{(n=7)}$ |
| Plasma Insulin (ng/ml) | 3.6 ± 0.9$^{(n=7)}$ | 4.1 ± 1.1$^{(n=8)}$ | 4.2 ± 0.9$^{(n=7)}$ |
| Body Length (cm) | 11.21 ± 1.1$^{(n=15)}$ | 10.91 ± 1.0$^{(n=17)}$ | 10.81 ± 1.2$^{(n=15)}$ |

**Figure 1. Generation of *Tph2*−/− mice**

(A) β-Galactosidase staining in the mouse brain during embryonic (E12.5–18.5) development. A: Anterior; P: Posterior.

(B) Localization of *Tph2*-expressing neurons in the Dorsal (DR; from Bregma −4.04 to −5.49), Median (MR; from Bregma −4.04 to −4.48) and Caudal raphe (CR; from Bregma −4.84 to −7.48) in coronal sections of a mouse brain.

(C) *Tph2* expression by in situ hybridization, β-galactosidase staining and co-immunolocalization in *Tph2LacZ*/+ mice. Arrowheads indicate Tph2/ β-Gal double positive cells.

(D) Real-time PCR (qPCR) analysis of *Tph2* expression in tissues of WT mice.

(E) qPCR analysis of *Tph2* expression in brainstem (BS) and duodenum (Duod) of WT and *Tph2*−/− mice.

(F) HPLC analysis of serotonin levels in different regions of brain in WT, *Tph2*+/− and *Tph2* −/− mice.

(G) Serum serotonin levels in WT, *Tph2*+/− and *Tph2*−/− mice.

(H) Mean litter size, serum biochemistry and body length in WT, *Tph2*+/− and *Tph2*−/− mice (n is indicated in superscript above each value).

All panels (except F) * $P < 0.05$; ** $P < 0.01$ (Student's t test). Error bars, SEM. Panel F (One way ANOVA, Newman-Keuls test); Different letters on 2 or more bars indicate significant differences between the respective groups ($P < 0.05$).

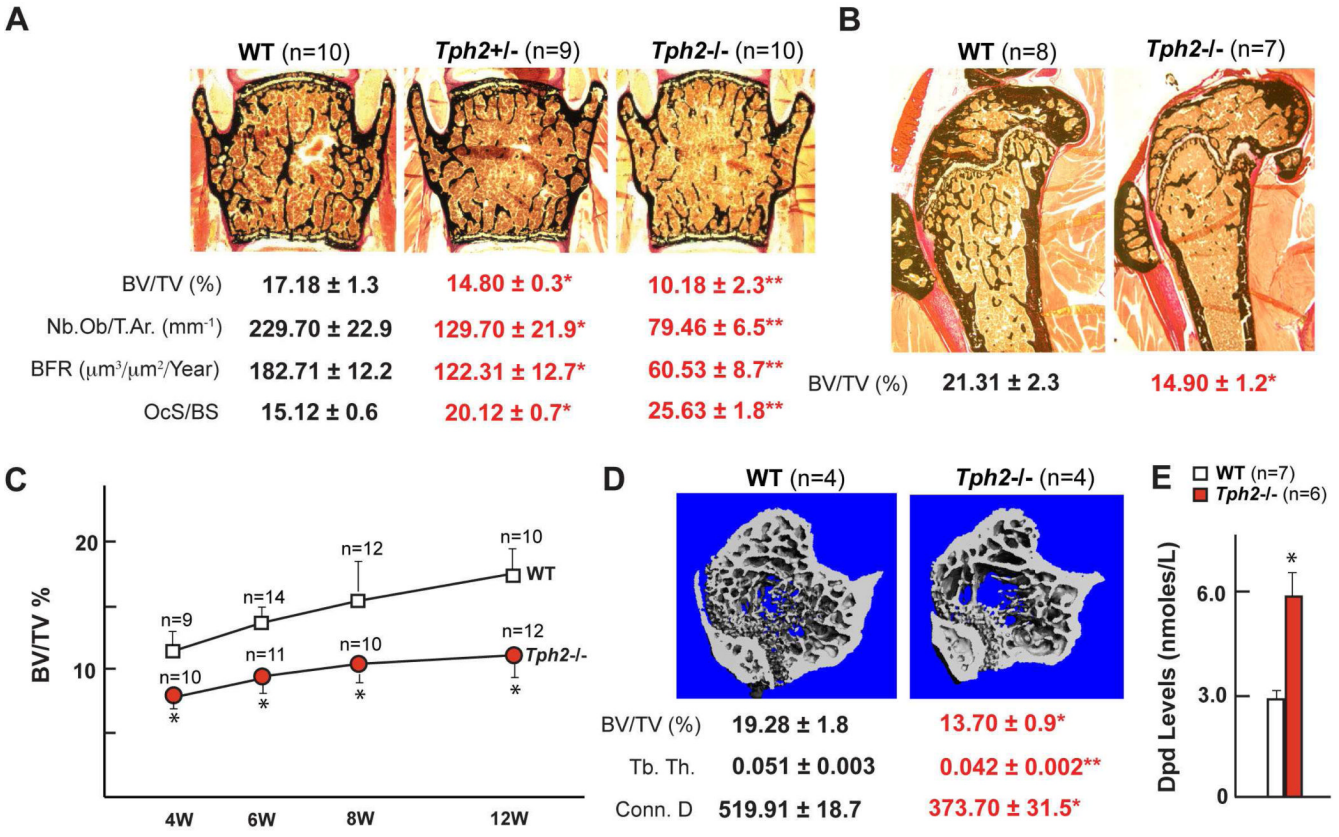



**Figure 2. Low bone mass in *Tph2*−/− mice**

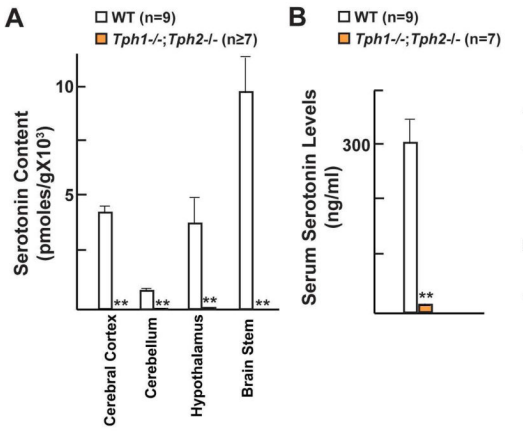
(A–B) Histological analysis of vertebrae (A) and long bones (B) of WT, *Tph2*+/− and *Tph2* −/− mice. Mineralized bone matrix is stained in black by von Kossa reagent. Histomorphometric parameters. BV/TV%, bone volume over trabecular volume; Nb.Ob/T.Ar., number of osteoblasts per trabecular area; BFR, bone formation rate; OcS/BS, osteoclast surface per bone surface.

(C) BV/TV% analysis in WT and *Tph2*−/− mice at 4, 6, 8 and 12 weeks after birth.

(D) Lower bone density in long bones of 12-week-old *Tph2*−/− mice by μCT analysis along with lower Tb.Th (trabecular thickness) and decreased connectivity density (Conn.D).

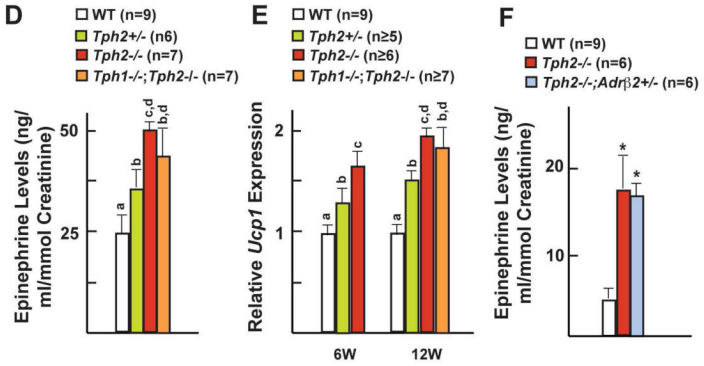
(E) Serum Dpd levels in WT and *Tph2*−/− mice.

All panels * P < 0.05; ** P < 0.01 Error bars, SEM)




C

| | WT (n=7) | Tph1-/- (n=8) | Tph2-/- (n=7) | Tph1-/-;Tph2-/- (n=8) |
|---|---|---|---|---|
| BV/TV (%) | 17.78 ± 1.2 | 22.90 ± 1.4* | 10.98 ± 2.9** | 11.97 ± 1.9* |
| Nb.Ob/T.Ar. ($mm^{-1}$) | 179.90 ± 29.2 | 259.70 ± 21.5* | 99.49 ± 17.7** | 121.60 ± 28.2* |
| BFR ($\mu m^3/\mu m^2$/Year) | 162.91 ± 19.9 | 272.31 ± 12.2* | 70.93 ± 18.9** | 102.91 ± 10.2* |
| OcS/BS | 11.81 ± 3.9 | 14.61 ± 3.2 | 18.13 ± 1.9* | 17.91 ± 1.2* |




G

| | WT (n=8) | Tph2-/- (n=8) | Tph2-/-;Adrβ2+/- (n=9) |
|---|---|---|---|
| BV/TV (%) | 17.29 ± 0.9 | 11.77 ± 1.1* | 18.38 ± 1.2 |
| Nb.Ob/T.Ar. ($mm^{-1}$) | 185.32 ± 12.0 | 114.09 ± 14.9* | 155.23 ± 18.4 |
| BFR ($\mu m^3/\mu m^2$/Year) | 199.27 ± 12.9 | 106.92 ± 19.6* | 166.92 ± 19.8 |
| OcS/BS | 10.01 ± 0.7 | 16.28 ± 2.0** | 11.76 ± 0.9 |

**Figure 3. Brain-derived serotonin inhibits sympathetic activity**

(A–B) HPLC analysis of serotonin levels in different regions of brain and serum serotonin levels in WT and *Tph1−/−;Tph2−/−* mice.

(C) Histomorphometric analysis of vertebrae of WT, *Tph1−/−*, *Tph2−/−* and *Tph1−/−;Tph2−/−* mice.

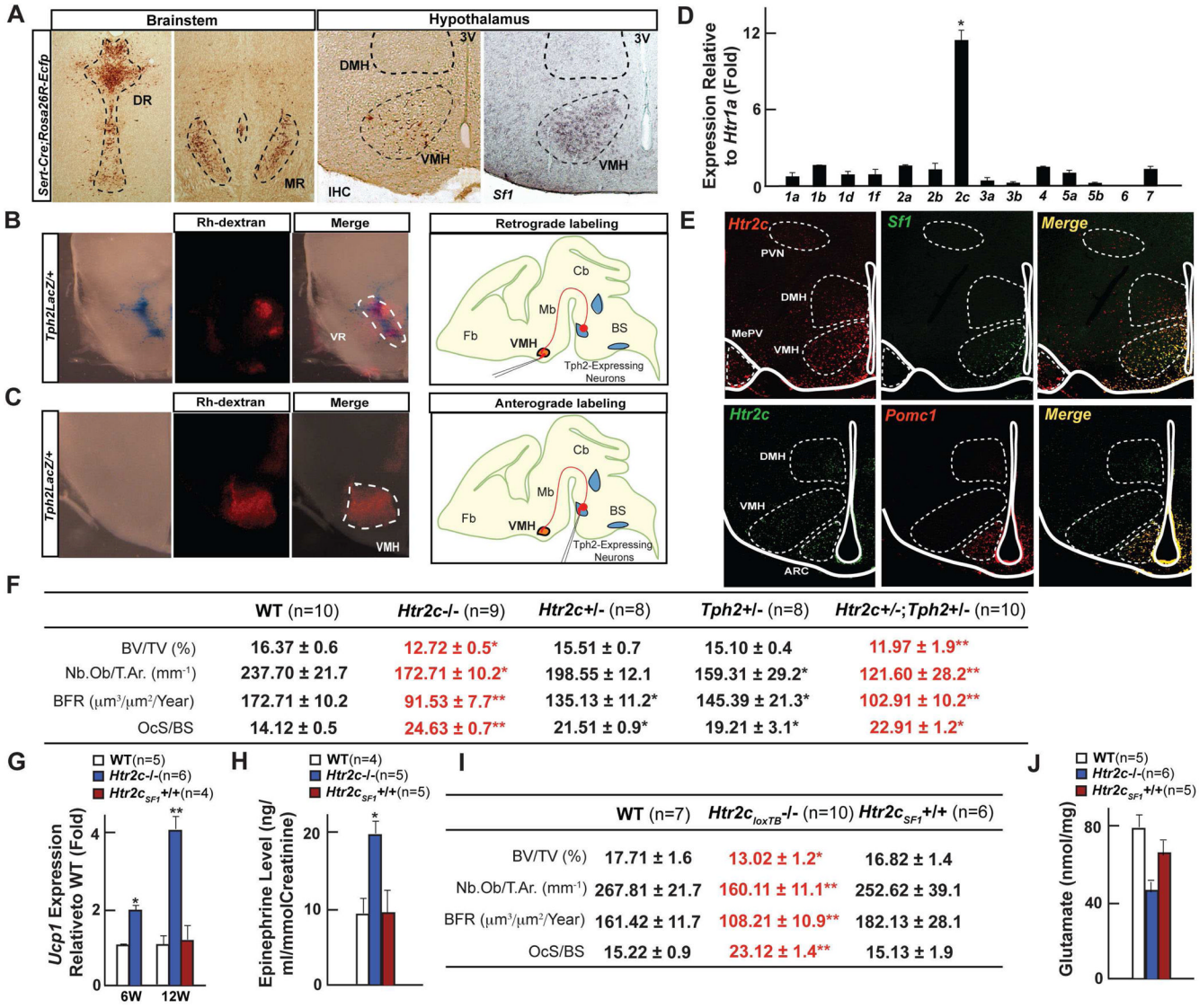
(D) Epinephrine levels in WT, *Tph2+/−*, *Tph2−/−* and *Tph1−/−;Tph2−/−* mice.

(E) qPCR analysis of *Ucp1* expression in brown adipose tissue of WT, *Tph2+/−*, *Tph2−/−* and *Tph1−/−;Tph2−/−* mice.

(F) Epinephrine levels in the urine of WT, *Tph2−/−* and *Tph2−/−;Adrβ2+/−* mice.

(G) Histomorphometric analysis of vertebrae of WT, *Tph2−/−* and *Tph2−/−;Adrβ2+/−* mice.

All panels (except D and E) * $P < 0.05$; ** $P < 0.01$ (Student's t test). Error bars, SEM. Panel D and E (One way ANOVA, Newman-Keuls test); Different letters on 2 or more bars indicate significant differences between the respective groups ($P < 0.05$).




| | WT (n=10) | Htr2c-/- (n=9) | Htr2c+/- (n=8) | Tph2+/- (n=8) | Htr2c+/-;Tph2+/- (n=10) |
|---|---|---|---|---|---|
| BV/TV (%) | 16.37 ± 0.6 | 12.72 ± 0.5* | 15.51 ± 0.7 | 15.10 ± 0.4 | 11.97 ± 1.9** |
| Nb.Ob/T.Ar. (mm$^{-1}$) | 237.70 ± 21.7 | 172.71 ± 10.2* | 198.55 ± 12.1 | 159.31 ± 29.2* | 121.60 ± 28.2** |
| BFR (μm$^3$/μm$^2$/Year) | 172.71 ± 10.2 | 91.53 ± 7.7** | 135.13 ± 11.2* | 145.39 ± 21.3* | 102.91 ± 10.2** |
| OcS/BS | 14.12 ± 0.5 | 24.63 ± 0.7** | 21.51 ± 0.9* | 19.21 ± 3.1* | 22.91 ± 1.2* |

| | WT (n=7) | Htr2c$_{loxTB}$-/- (n=10) | Htr2c$_{SF1}$+/+ (n=6) |
|---|---|---|---|
| BV/TV (%) | 17.71 ± 1.6 | 13.02 ± 1.2* | 16.82 ± 1.4 |
| Nb.Ob/T.Ar. (mm$^{-1}$) | 267.81 ± 21.7 | 160.11 ± 11.1** | 252.62 ± 39.1 |
| BFR (μm$^3$/μm$^2$/Year) | 161.42 ± 11.7 | 108.21 ± 10.9** | 182.13 ± 28.1 |
| OcS/BS | 15.22 ± 0.9 | 23.12 ± 1.4** | 15.13 ± 1.9 |

**Figure 4. Serotonin promotes bone mass through Htr2c receptors in VMH**

(A–C) Analysis of axonal projections emanating from the serotonergic neurons of the brainstem. Coronal sections through the Dorsal (DR), Median (MR) raphe and ventromedial hypothalamus (VMH) nuclei from *Sert-Cre;Rosa26REcfp* mice identifying serotonergic neurons and their axonal projections to VMH neurons through Ecfp immunohistochemistry (A). Retrograde (B) and anterograde (C) Rhodamine dextran labeling (Rh-dextran) in *Tph2LacZ*/+ mice. Coronal sections through the brainstem and hypothalamus showing colocalization of β-galactosidase staining and Rh-dextran fluorescence.

(D) qPCR analysis of serotonin receptor expression in hypothalamus.

(E) Double fluorescence situ hybridization analysis of *Htr2c* expression with *Pomc* or *Sf1* expression in anterior (Top panel) and posterior (Bottom panel) VMH and arcuate nuclei. The third ventricle is outlined by a white line.

(F) Histomorphometric analysis of vertebrae of WT, *Htr2c*−/−, *Htr2c*+/−, *Tph2*+/− and *Htr2c* +/− ;*Tph2*+/− mice.

(G–H) qPCR analysis of *Ucp1* expression in brown adipose tissue (G) and epinephrine levels in urine (H) in WT, *Htr2c*−/− and *Htr2c$_{SF1}$*+/+ mice.

(I) Histomorphometric analysis of vertebrae of WT, *Htr2c$_{loxTB}$*−/− and *Htr2c$_{SF1}$*+/+ mice.

(J) HPLC analysis of glutamate levels in hypothalamus of WT and *Htr2c*−/− mice
All panels (except J) * $P < 0.05$; ** $P < 0.01$ (Student's t test). Error bars, SEM. Panel J (One way ANOVA, Newman-Keuls test); Different letters on 2 or more bars indicate significant differences between the respective groups ($P < 0.05$).

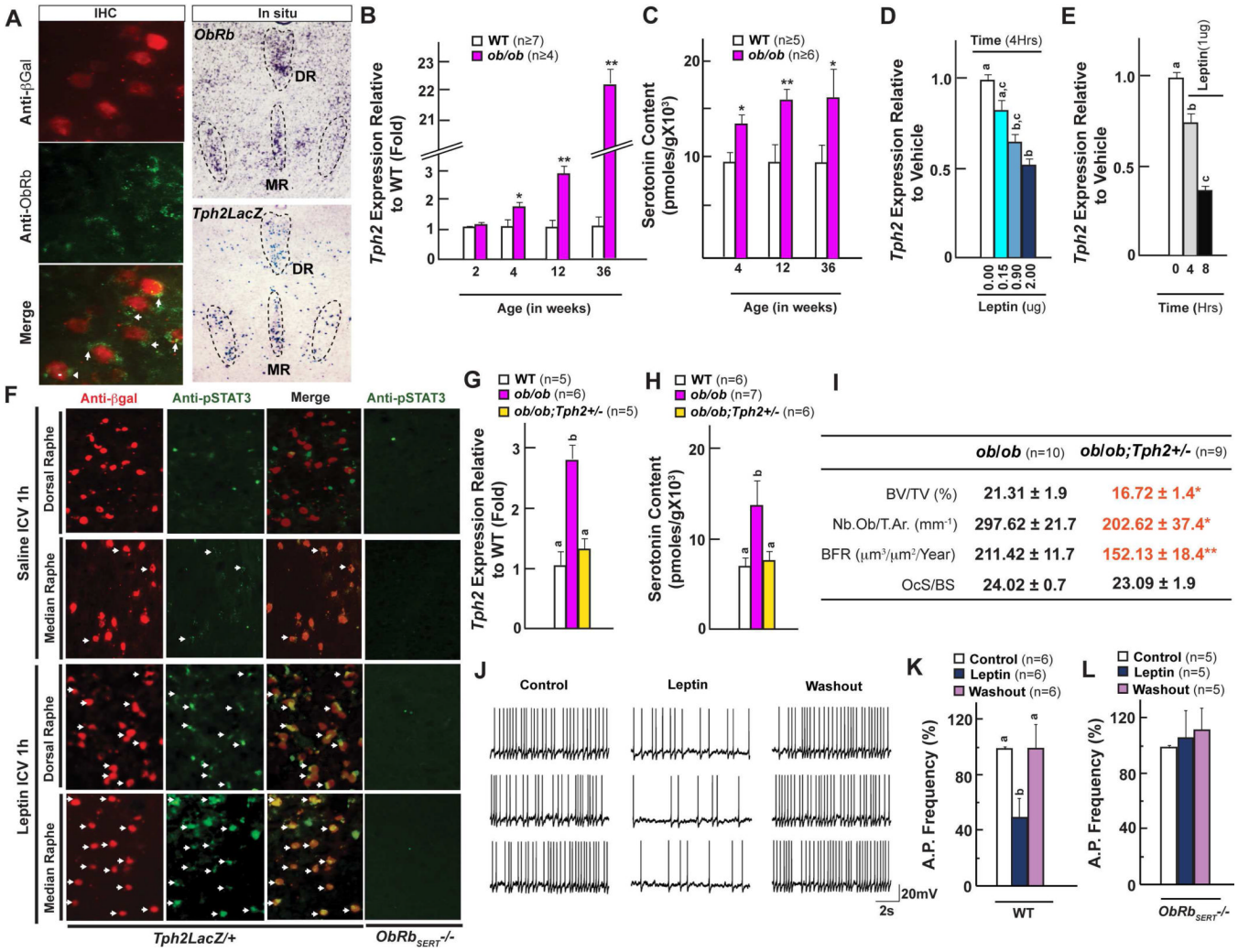



**Figure 5. Leptin inhibits bone mass accrual by inhibiting brain-derived serotonin synthesis**

(A) In situ hybridization analysis and co-immunolocalization of *ObRb* expression in serotonergic neurons.

(B–C) qPCR analysis of *Tph2* expression (B) and brainstem serotonin content (C) at different ages in WT and *ob/ob* female mice.

(D–E) qPCR analysis of *Tph2* expression following intra-cerebroventricular (ICV) infusion of leptin at different doses (D) and at different time points (E) in WT mice.

(F) Immunohistochemical analysis of STAT3 phosphorylation in the dorsal and median raphe following leptin ICV. Arrows indicate pSTAT3/ β-Gal positive cells.

(G–H) qPCR analysis of *Tph2* expression (G) and brainstem serotonin content (H) in WT, *ob/ob* and *ob/ob*;*Tph2*+/− mice.

(I) Histomorphometric analysis of vertebrae of ob/ob and *ob/ob*;*Tph2*+/− mice.

(J) Representative traces of action potentials recorded from WT mice before, during and after the application of leptin (100nM). R.M.P. −43.0 mV.

(K–L) Analysis of serotonergic neuron action potential (AP) frequency in brainstem slices from WT (K) and $ObRb_{SERT}^{-/-}$ (L) mice.

All panels (except D, E, G, H and K) * $P < 0.05$; ** $P < 0.01$ (Student's t test). Error bars, SEM. Panels D, E, G, H and K (One way ANOVA, Newman-Keuls test); Different letters on 2 or more bars indicate significant differences between the respective groups ($P < 0.05$).

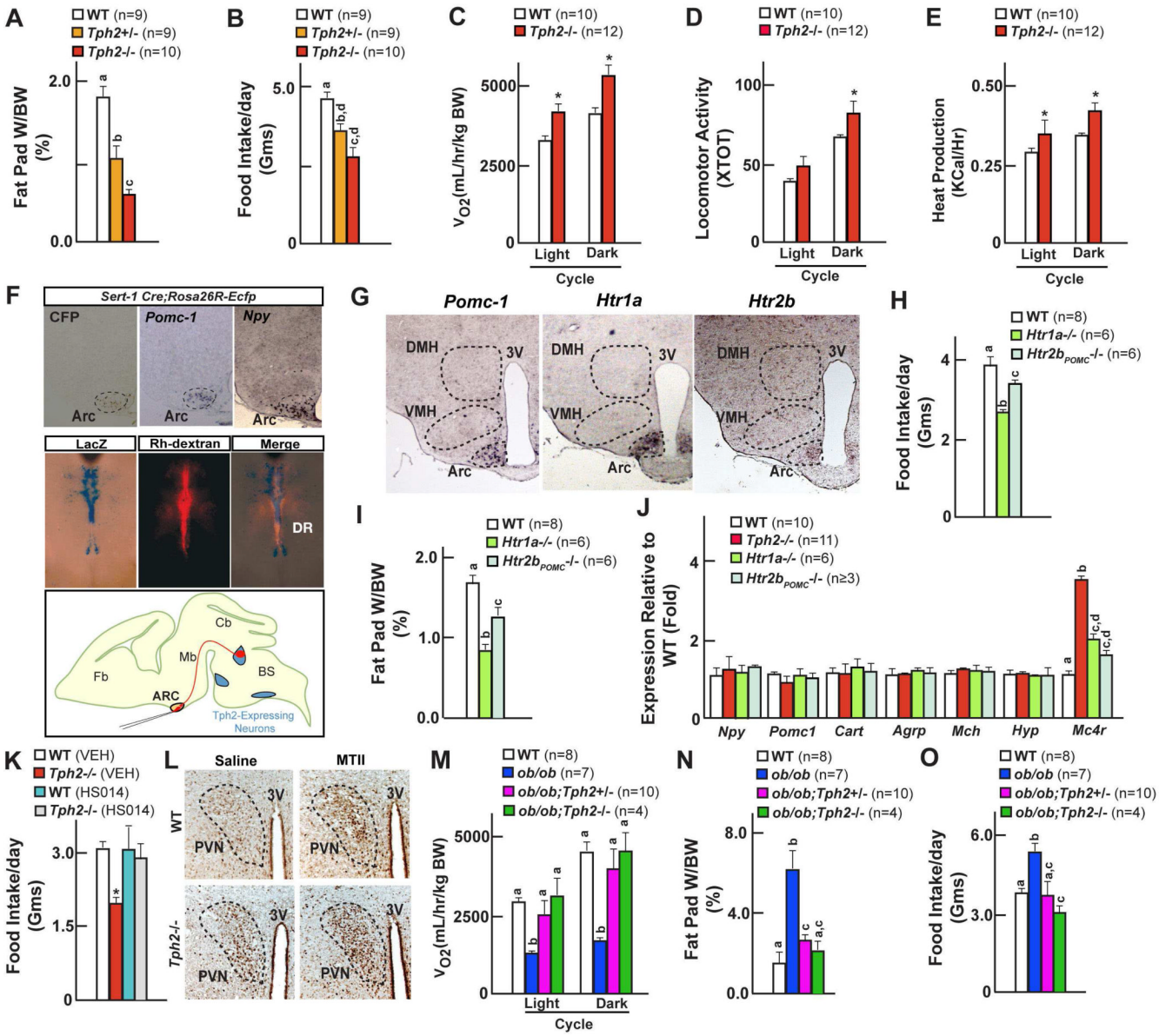



**Figure 6. Serotonin promotes food intake through Htr1a and Htr2b receptors on arcuate neurons**

(A–B) Fat pad weights (A) and food intake (B) in WT, *Tph2*+/− and *Tph2*−/− mice.

(C–E) Energy expenditure in WT and *Tph2*−/− mice; measured by volume of oxygen consumption ($V_{O2}$) (C), locomotor activity (D) and Heat production (E).

(F) Analysis of axonal projections emanating from the serotonergic neurons. Cross of *Sert-Cre* and *Rosa26REcfp* mice identified projections reaching arcuate (Arc) nuclei in the hypothalamus through Ecfp immunohistochemistry colocalized to molecular markers of arcuate neurons (*Pomc-1* and *Npy*) by in situ hybridization. Retrograde Rhodamine dextran labeling of the arcuate neurons identified serotonergic neurons in the brainstem in *Tph2LacZ/+* mice through colocalization of β-galactosidase staining and Rh-dextran fluorescence in serotonergic neurons of the brainstem.

(G) In situ hybridization analysis of *Htr1a*, *Htr2b* in *Pomc1*-expressing arcuate neurons of the hypothalamus. 3V: third ventricle.

(H–I) Food intake (H) and fat pad weights (I) in WT, *Htr1a*−/− and $Htr2b_{POMC}$−/− mice.

(J) qPCR analysis of hypothalamic gene expression in WT, *Htr1a*−/− and $Htr2b_{POMC}$−/− mice.
(K) Food intake in WT, *Tph2*−/− mice before and after Mc4r antagonist (HS014) administration.
(L) cFos induction in paraventricular nucleus of hypothalamus in WT, *Tph2*−/− mice before and after acute administration Mc4r agonist (MTII). 3V: third ventricle.
(M–O) Volume of oxygen consumption (M), fat pad weight (N) and food intake (O) in WT, *ob/ob*, *ob/ob*;*Tph2*+/− and *ob/ob*;*Tph2*−/− mice.
All panels (except A–B, H–J and M–O) * $P < 0.05$; ** $P < 0.01$ (Student's t test). Error bars, SEM. Panels A–B, H–J and M–O (One way ANOVA, Newman-Keuls test); Different letters on 2 or more bars indicate significant differences between the respective groups ($P < 0.05$).

A

| | +/+;*Sf1-Cre* | $ObRb_{SF1}$-/- | +/+;*Pomc-Cre* | $ObRb_{POMC}$-/- | +/+;*Sert-Cre* | $ObRb_{SERT}$-/- |
|---|---|---|---|---|---|---|
| BV/TV (%) | 18.27 ± 1.6 | 17.72 ± 1.9 | 16.52 ± 1.2 | 17.27 ± 0.8 | 15.51 ± 1.7 | 24.17 ± 2.9** |
| Nb.Ob/T.Ar. ($mm^{-1}$) | 207.79 ± 29.8 | 192.68 ± 30.1 | 188.59 ± 32.1 | 181.90 ± 25.2 | 178.39 ± 33.3 | 291.30 ± 31.2* |
| BFR ($\mu m^3/\mu m^2$/Year) | 178.74 ± 19.9 | 199.95 ± 38.2 | 168.99 ± 41.5 | 178.81 ± 22.1 | 198.71 ± 31.2 | 311.20 ± 39.1* |
| OcS/BS | 11.11 ± 2.1 | 12.12 ± 2.2 | 12.19 ± 3.1 | 13.16 ± 4.1 | 13.59 ± 3.7 | 23.27 ± 1.9** |

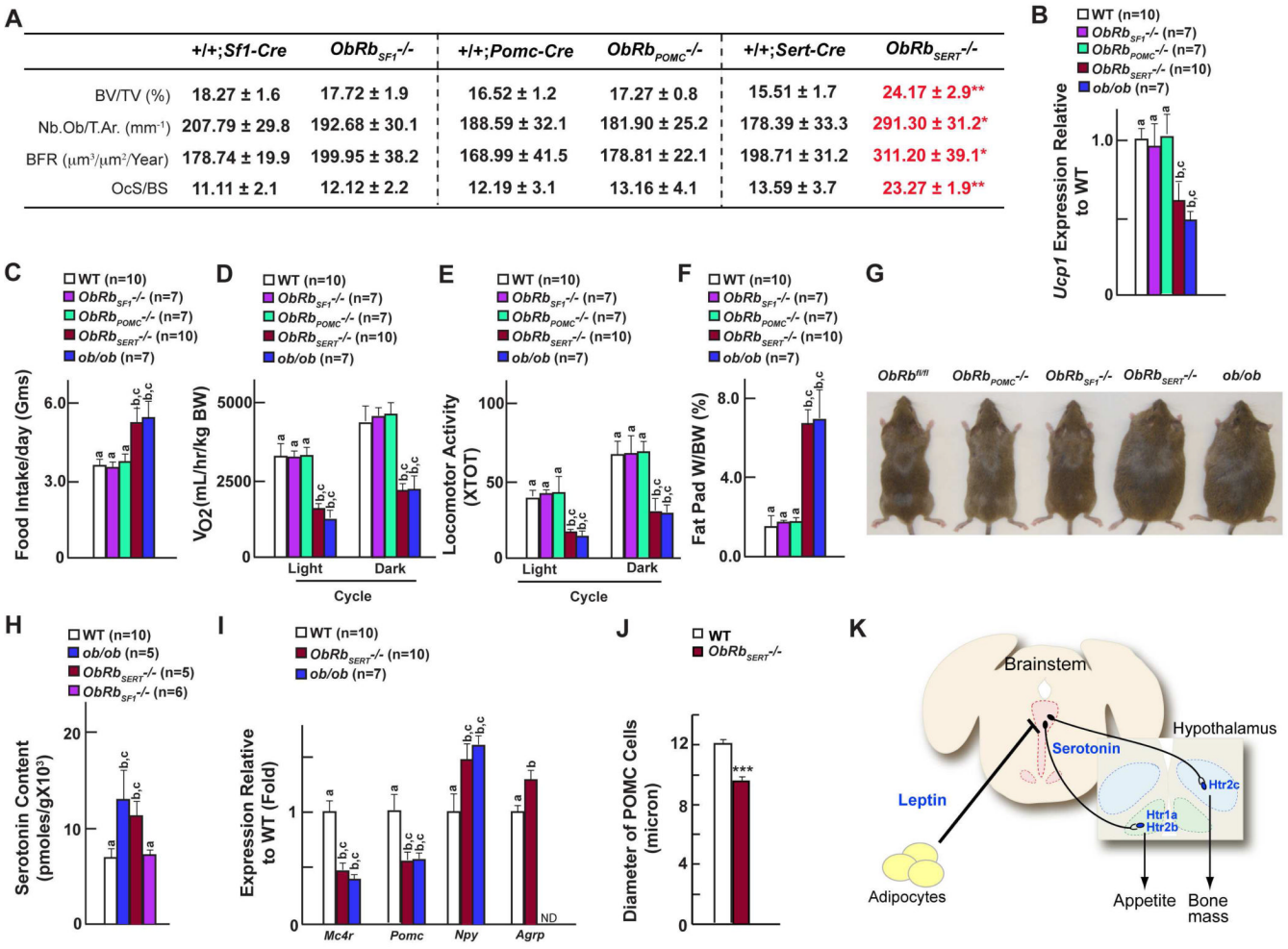



**Figure 7. ObRb expression in serotonergic neurons is necessary and sufficient for leptin regulation of bone mass accrual, appetite and energy expenditure**

(A) Histomorphometric analysis (vertebrae) of +/+;*Sf1-Cre*, $ObRb_{SF1}$−/−, +/+;*Pomc1-Cre*, $ObRb_{POMC}$−/−, +/+;*Sert-Cre* and $ObRb_{SERT}$−/− mice.

(B) qPCR analysis of *Ucp1* expression in brown adipose tissue in WT, $ObRb_{SF1}$−/−, $ObRb_{POMC}$−/− and $ObRb_{SERT}$−/− mice. WT refers to +/+;*Sf1-Cre*, +/+;*Pomc1-Cre* or +/+;*Sert-Cre*.

(C–F) Food intake (C) volume of oxygen consumption (D), locomoter activity (E) and fat pad weights (F) in WT, $ObRb_{SF1}$−/−, $ObRb_{POMC}$−/− and $ObRb_{SERT}$−/− mice.

(G) Representative photomicrographs of WT, $ObRb_{SF1}$−/−, $ObRb_{POMC}$−/− and $ObRb_{SERT}$−/− mice.

(H) Brainstem serotonin content in WT, *ob/ob*, $ObRb_{SERT}$−/− and $ObRb_{SF1}$−/− mice.

(I) qPCR analysis in the hypothalamus in WT, $ObRb_{SERT}$−/− and *ob/ob* mice.

(J) Diameter of *Pomc*-expressing cells in WT and $ObRb_{SERT}$−/− mice.

(K) Model of the leptin-dependent regulation of bone mass and appetite. Leptin inhibits release of brainstem-derived serotonin, which favors bone mass accrual and appetite. Adipocytes are in yellow; serotonergic neurons are in pink; VMH is in blue and arcuate is in green.

All panels (except B–F and H–I) * P < 0.05; ** P < 0.01, *** P < 0.001 (Student's t test). Error bars, SEM. Panels B–F and H–I (One way ANOVA, Newman-Keuls test); Different letters on 2 or more bars indicate significant differences between the respective groups (P < 0.05).