Neuropeptide Y Attenuates Stress-Induced Bone Loss Through Suppression of Noradrenaline Circuits

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ABSTRACT

Chronic stress and depression have adverse consequences on many organ systems, including the skeleton, but the mechanisms underlying stress-induced bone loss remain unclear. Here we demonstrate that neuropeptide Y (NPY), centrally and peripherally, plays a critical role in protecting against stress-induced bone loss. Mice lacking the anxiolytic factor NPY exhibit more anxious behavior and elevated corticosterone levels. Additionally, following a 6-week restraint, or cold-stress protocol, *Npy*-null mice exhibit three-fold greater bone loss compared to wild-type mice, owing to suppression of osteoblast activity. This stress-protective NPY pathway acts specifically through Y2 receptors. Centrally, Y2 receptors suppress corticotropin-releasing factor expression and inhibit activation of noradrenergic neurons in the paraventricular nucleus. In the periphery, they act to control noradrenaline release from sympathetic neurons. Specific deletion of arcuate Y2 receptors recapitulates the *Npy*-null stress response, coincident with elevated serum noradrenaline. Importantly, specific reintroduction of NPY solely in noradrenergic neurons of otherwise *Npy*-null mice blocks the increase in circulating noradrenaline and the stress-induced bone loss. Thus, NPY protects against excessive stress-induced bone loss, through Y2 receptor-mediated modulation of central and peripheral noradrenergic neurons.

INTRODUCTION

Stress is increasingly recognized as a major and growing health issue, associated with negative health outcomes in numerous systems,[**1**] such as obesity and diabetes,[**2**] inflammatory conditions,[**3**] atherosclerosis,[**4**] cancer,[**5**] and Alzheimer's disease.[**6**] As a result, chronic stress has now been implicated not only in psychiatric conditions, but is increasingly associated with somatic disease. One somatic tissue was shown recently to be adversely affected by stress is the skeleton, with several meta-analyses identifying depression as an important risk factor for osteoporosis.[**7-9**] Indeed, the chronic psychological stress associated with depression results in substantial reductions in bone mineral density (7% to 15%) at clinically relevant sites such as the spine and hip, as well as reduced bone formation and osteoporosis in over 40% of these patients.[**10, 11**] Unlike postmenopausal osteoporosis, the effect of depressive stress on bone mass in women is greater in premenopausal than postmenopausal subjects,[**9**] and has been shown to decrease bone accrual in adolescents,[**12**] indicating the potential for lifelong health issues, a challenge amplified by the common and recurrent nature of depression.[**13**] Despite the growing appreciation of stress-induced osteoporosis, the mechanism behind the loss of bone mass during stress and effective treatment regimes remain to be elucidated. Considering that antidepressant medication may also reduce bone mass[**14**] and fracture itself can increase depressive episodes, particularly in the elderly,[**15**] the need to identify mechanisms to ameliorate the problem are clear.

In humans, as in other mammals, specific anxiolytic brain circuitries are activated to combat the negative effects of stress. One of the major anxiolytic neurotransmitters upregulated by stress is neuropeptide Y (NPY). Recent genetic studies have shown a clear relationship between a relative reduction in central NPY levels and depression,[**16**] posttraumatic stress disorder,[**17**] and suicide,[**18**] whereas NPY allele variants inducing elevated NPY responses have been positively associated with stress coping ability in humans.[**19**] Such is the anxiolytic action of NPY, that its actions on the endogenous catecholaminergic and serotoninergic systems have been compared to that of the antidepressants imipramine and fluoxetine.[**20**] These "anti-stress" actions of NPY are also apparent in peripheral tissues. As a cotransmitter in the sympathoadrenomedullary system, NPY is released into the circulation from nerve terminals under conditions of prolonged or intense stress. Under these circumstances, actions of NPY can be amplified to levels sufficient to induce somatic changes. Indeed, NPY has been shown to protect aspects of peripheral energy homeostasis during prolonged stress, with peripheral NPY, Y2 receptors expressed on adipocytes increasing fat deposition,[**21**] thereby ensuring adequate energy supplies during extended periods of stress. Importantly, under unstressed conditions, NPY powerfully regulates the production of skeletal tissue through central and peripheral actions. Indeed, hypothalamus-specific overexpression of NPY dramatically reduces bone mass and osteoblast activity,[**22, 23**] whereas blocking central[**24**] or peripheral[**25**] NPY signaling increases bone mass and osteoblast activity. Thus, the elevation in NPY may contribute to bone loss during stress.

In light of the inhibitory effects of NPY signaling on bone mass and bone formation, the overall elevation in NPY activity associated with chronic stress would predict a negative effect on bone homeostasis. However, NPY has been well described as an anxiolytic agent, acting to reduce the behavioral and neuroendocrine effects of chronic stress.[**16, 19**] Thus, whether NPY is protective or contributory to the bone loss associated with chronic stress has never been studied. To test this, we investigated the role of NPY signaling on bone homeostasis and behavior after chronic stress using several germline and conditional NPY and Y-receptor mutant mouse models. To dissect the relative roles of central and peripheral NPY in the regulation of bone under stress, we used arcuate nucleus–specific *NPY 2r*–null mice and a model in which NPY is expressed exclusively in noradrenergic nerves of otherwise *NPY*-deficient mice.

MATERIALS AND METHODS

This section is available online in the Supporting Information via the following link: http://onlinelibrary.wiley.com/doi/10.1002/ jbmr.2205/suppinfo.

RESULTS

NPY–/– mice are more anxious and show an enhanced behavioral response to stress

In order to clearly demonstrate the role of NPY in chronic stress, we characterized changes in central NPY mRNA and circulating peptide levels in response to chronic stress in mice. Wild-type (WT) mice were exposed to restraint stress for 20 minutes, three times a week for 6 weeks from 10 weeks of age onward. At the end of the stress period, serum NPY levels in the chronic stressed mice were 65% higher compared to that in control, non-stressed mice (Fig. 1*A*). Importantly, NPY mRNA levels in the arcuate nucleus (Arc) of the hypothalamus were also significantly upregulated in the stressed mice compared to the nonstress controls (Fig. 1*B*). The stress-induced changes in NPY production are known to have important effects on the hypothalamic-pituitary-adrenal (HPA) axis.[**26**] In particular, NPY is known to suppress corticotrophin releasing factor (CRF) and corticosterone production. In order to confirm this link in our stress model, we examined CRF production in the paraventricular nucleus (PVN) of WT and NPY-null (*NPY–/–*) mice. In the absence of NPY, CRF production induced by our stress protocol was significantly greater (Fig. 1*C*, *D*) and this was associated with significant increases in corticosterone levels in the *NPY–/–* mice in both the stressed and the non-stressed groups (Fig. 1*E*).

Having established a model of chronic stress that increases endogenous NPY levels, and demonstrated the effect of NPY on HPA responses to stress, we sought to confirm the importance of the NPY/stress relationship in terms of physiological responses. We initially tested the behavioral response to stress in *NPY–/–* mice using established behavioral tests of anxiety and depression. *NPY–/–* mice exhibited normal neurological reflexes and sensory abilities, and had

similar basal motor function and coordination compared to WT controls. The tail suspension test and forced swim test are commonly used to detect depressive-like behaviors and are sensitive to the effect of many antidepressants[**27**]; *NPY*–/– mice exhibited significant increases in immobility times in the tail suspension test and a suggestive effect ($p > 0.06$) in the forced swim test (Fig. 1*F*), indicating a more depressive-like phenotype. Furthermore, an exaggeration of the normal stress-related response was also detected in *NPY*–/– mice in the elevated plus maze task, where *NPY*–/– mice entered at a lesser frequency into the open arms and also spent less time within the open arms (Fig. 1*G*). Taken together, these data show that *NPY*–/– mice have exaggerated stress-like responses and suggest an important role of NPY in regulating stress-induced behavioral responses.

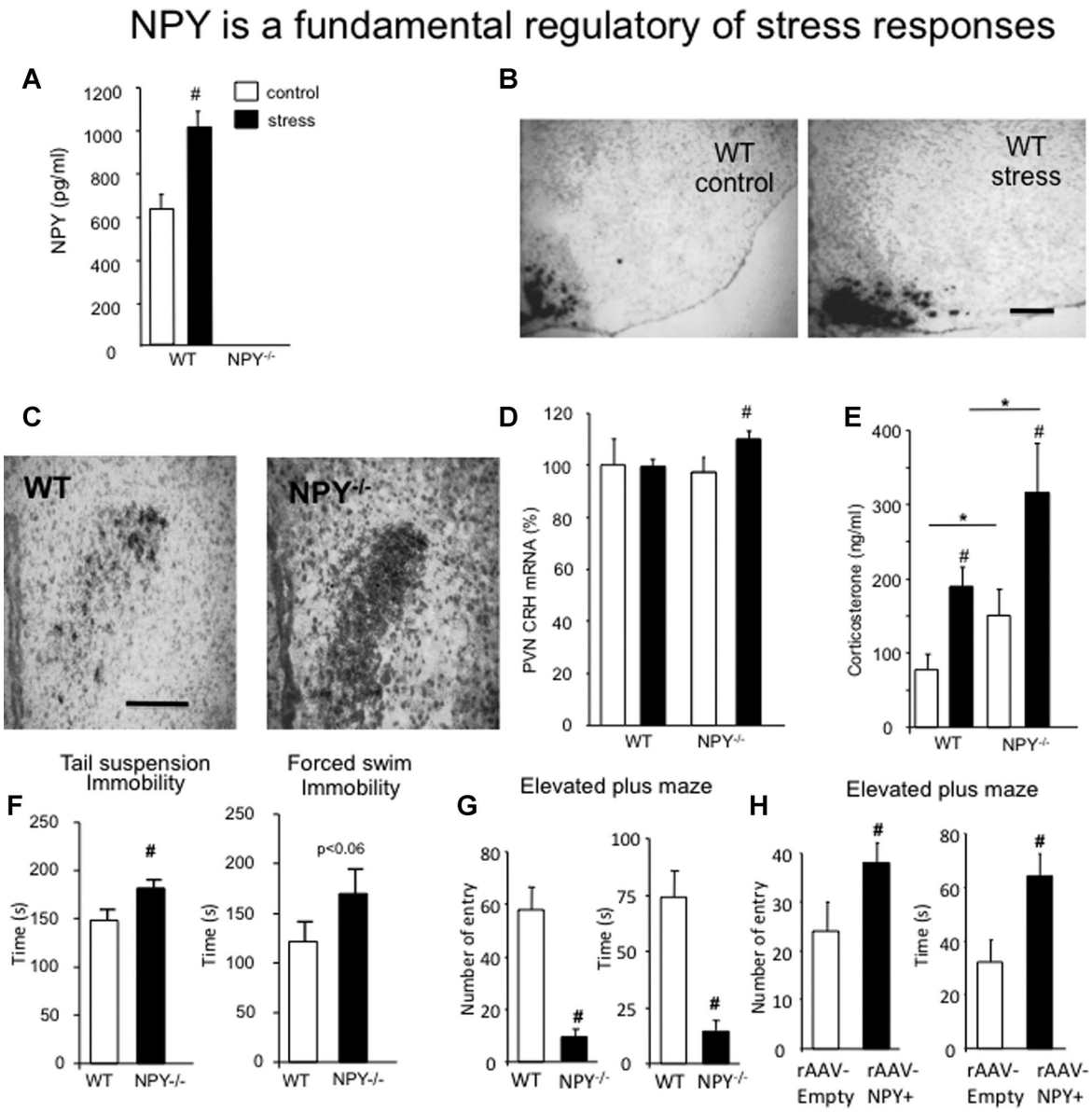

Fig. 1. NPY is upregulated in response to stress. (*A*) Serum NPY levels are increased following exposure to chronic stress in WT mice. Photomicrographs of (*B*) NPY mRNA expression in the Arc of non-stressed and stressed WT mice, and (*C*) CRH expression in the PVN of stressed WT and $NPY^{-/-}$ mice. Scale bar = 40 μm. Quantification of the stress-related increase in (*D*) CRH-positive neurons in the PVN and (*E*) serum corticosterone of WT and $NPY^{-/-}$ mice. #$p < 0.05$ versus non-stress, within genotype; *$p < 0.05$ versus wild type, within stress/non-stress group. $n = 7$–11, data expressed as mean ± SE. Anxiety-like behaviors in $Npy^{-/-}$ mice. (*F*) Increased times of immobility in the depression-related tail suspension test and the forced swim test in $NPY^{-/-}$ mice compared to WT controls. (*G*) In the elevated plus maze, the $NPY^{-/-}$ mice displayed a strongly reduces frequency of entering and time spent in the open arms. (*H*) Viral-mediated overexpression of NPY in the hypothalamus of adult WT mice (rAAV-NPY) resulted in the reversion of the anxiety phenotype seen in the $Npy^{-/-}$ mice. #$p < 0.05$ versus control, $n = 6$–10, data expressed as mean ± SE.

In order to confirm the role of NPY in regulating these behavioral changes, and to explore to importance of Arc NPY-ergic neurons in this response, we employed a hypothalamic NPY overexpression model, using an adenoassociated viral vector expressing NPY (rAAV-NPY) that was injected into the Arc of adult WT mice. Opposite to the increased anxiety in *NPY*–/– mice, NPY overexpression in the Arc significantly reduced open field anxiety, with greater number of entries and longer time spent in the open field compared to control AAV-empty injected mice (Fig. 1*H*). Thus, lack of NPY expression results in increased behavioral indicators of stress and depression, and increased Arc NPY expression is associated with a reduced level of anxiety and improved stress coping ability. Together, this clearly demonstrates the critical role of the hypothalamic NPY in protecting against anxiety-like and depressive-like behavior induced by stress.

NPY signaling is essential for protection against chronic stress–induced bone loss

Having established that arcuate NPY expression regulates the physiological and behavioral responses to chronic stress, we sought to examine the effect of chronic stress on another NPY-responsive and stress-responsive tissue, bone. We have previously identified the NPY system as a potent regulator of bone mass, importantly, through actions of NPY within the arcuate. However, in contrast to any putative stress-protective effect, under unstressed conditions, arcuate NPY, or Y2 receptor signaling suppresses bone formation and reduces bone mass,[**22, 23, 28**] in keeping with whole-body energy conservation. Consistent with this suppressive action, non-stressed control *NPY*–/– or Y2 receptor null mice display a bone anabolic phenotype with greater cancellous bone volume and trabecular number and thickness and greater mineral apposition rate than WT mice (Fig. 2*A*, *C*; Fig. 3*A*, C; Fig. 4*A*). Thus, it was unknown whether NPY would be protective to bone during stress, as suggested by Fig. 1, or suppressive, as evident in non-stressed mice.

In order to explore these influences, the skeletal response to the 6-week chronic stress protocol was examined in WT and *NPY*–/– mice. Suggestive of a specific protective action of NPY on bone during stress, *NPY*–/– mice displayed exaggerated bone loss compared to WT, as evident from the photomicrographs, and chronic stress had a noticeable effect on cancellous bone, particularly in *NPY*–/– mice (Fig. 2*B*). Analysis of the femoral metaphyseal bone revealed a significant loss of cancellous bone volume and removal of trabeculae in stressed WT mice (Fig. 2*C*). Critically, *NPY*–/– mice displayed a threefold greater loss of bone, again owing to loss of trabeculae (Fig. 2*C*), consistent with their greater corticosterone levels (Fig. 1*E*). This result suggests that the anxiolytic effects of NPY extend to peripheral, NPY-responsive tissues, such as bone. Moreover, they indicate the potential of NPY to modulate the stress-mediated osteopenia.

In contrast to the pro-anabolic activity seen in non-stressed *NPY*–/– mice, chronic stress had a strong anti-anabolic effect on bone formation in *NPY*–/– mice (Fig. 2*D*), highlighting the stress-dependence of NPY's regulation of osteoblast activity. Chronic stress caused a reduction in mineral apposition rate (osteoblast activity) and bone formation rate in *NPY*–/– mice with no effect evident in WT mice. Elevation of corticosterone levels is also known to increase bone resorption[**29**]; consistent with this change, resorption indices tended to be higher in the stressed compared to the non-stressed groups (significantly in WT osteoclast number) (Fig. 2*E*). However, the magnitude of resorption changes compared to formation suggests that the major effect of NPY pathways on control of bone mass during stress is via control of bone formation. This is consistent with known actions of this pathway in non-stressed mice.[**22, 23, 28**] The importance of chronic stress to these responses was confirmed in a separate stress model, using 30 minutes of cold exposure (5-mm-deep ice water) three times/week for 6 weeks.[**21**] Again *NPY*–/– mice lost a greater amount of bone and displayed a significant reduction in mineral apposition rate (Fig. 2*F*). These data clearly demonstrate that bone mass and bone formation are protected from the effects of stress by the presence of NPY. We next sought to determine the site(s) of this protective action of NPY on the skeleton.

Arcuate nucleus Y2 receptors are responsible for the osteoprotective effects of central NPY

To establish the signaling axis by which NPY protects bone formation during chronic stress, we focused upon the arcuate nucleus as a putative site of action because this was the site of elevated NPY mRNA expression in the stress models (Fig. 1). The arcuate is a region with a semipermeable blood brain barrier and is thus an important site for feedback control of the HPA axis.[**30**] Because this hypothalamic nucleus is also an area of prominent Y2 receptor expression,[**31**] we first examined the skeletal responses to chronic stress in mice with global deficiency of Y2

receptor (*NPY 2r* –/–). Similar to *NPY*–/– mice, *NPY 2r* –/– mice showed a threefold greater reduction in cancellous bone volume compared to WT mice (Fig. 3*A*). The stress-induced loss of bone in *NPY 2r* –/– mice was again associated with a reduction in mineral apposition rate, without changes in osteoclast surface.

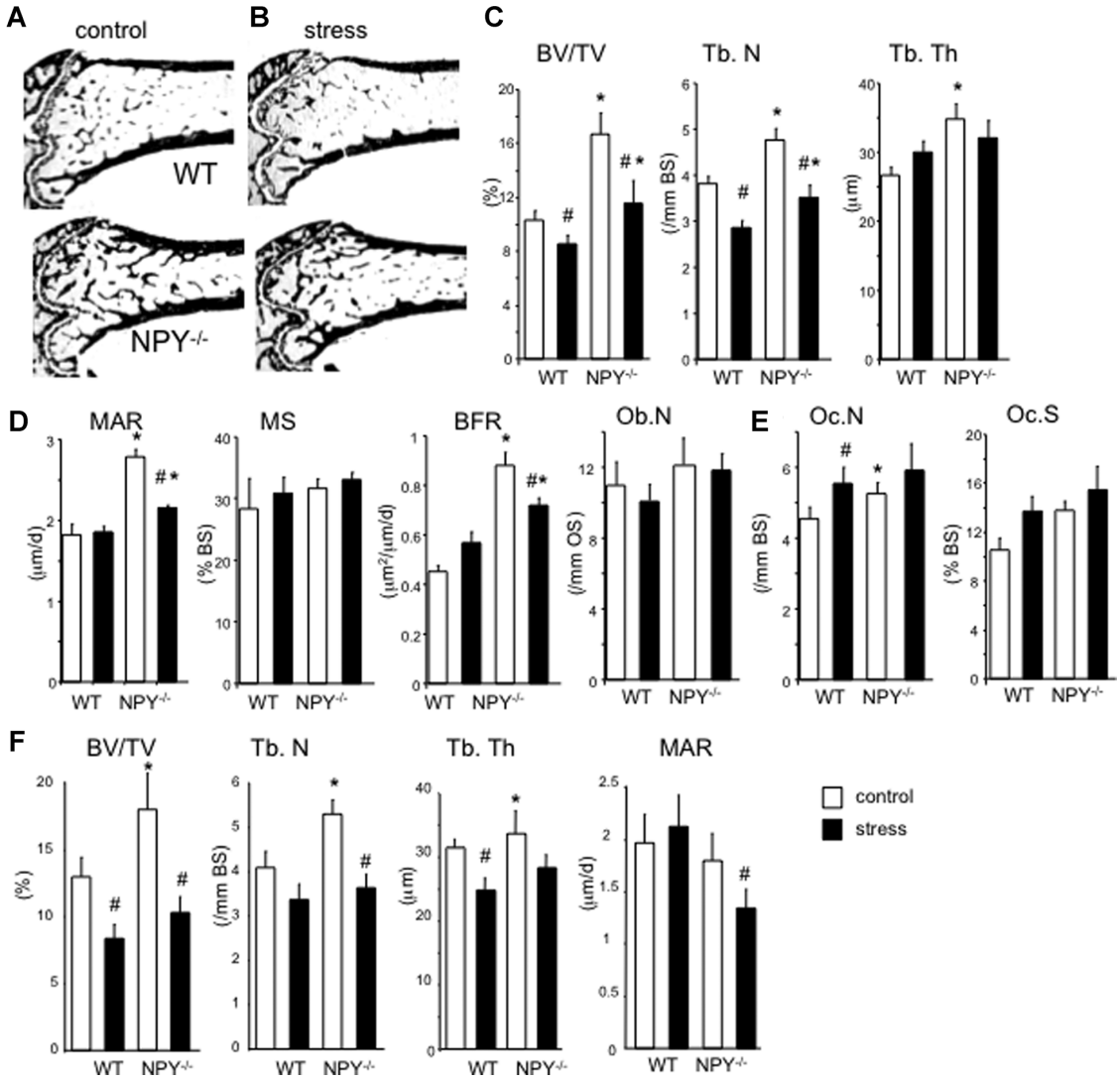

Fig. 2. NPY protects against stress-induced bone loss. Chronic stress induces marked bone loss in the distal metaphysis of *Npy*$^{-/-}$ mice. Following the restraint stress model: (*A*, *B*) Representative photomicrographs of the distal femoral metaphysis of stressed and control WT and *NPY*$^{-/-}$ mice. Histomorphometric analysis of distal femoral metaphysis of stressed and control mice showing: (*C*) cancellous bone volume (BV/TV, %), trabecular number (Tb.N, #/mm), trabecular thickness (Tb.Th, μm); (*D*) bone formation indices mineral apposition rate (MAR, μm/d), mineralizing surface (MS, % BS), bone formation rate (BFR. $\mu m^2/\mu m^3$/d), and osteoblast number (Ob.N, #/mm OS); and (*E*) bone resorption indices osteoclast surface (Oc.S, %BS) and osteoclast number (Oc.N, #/mm) from control and stressed WT and *Npy*$^{-/-}$ mice. The skeletal changes were consistent (*F*) following the cold stress model. #$p < 0.05$ versus non-stress, within genotype; *$p < 0.05$ versus wild-type, within stress/non-stress group. $n = 7$–11, data expressed as mean ± SE.

Interestingly, CRF mRNA levels in the paraventricular nucleus (PVN) of stressed *NPY 2r* –/– mice were not different from stressed WT controls (105.1% ± 1.1% versus 100% ± 3.4% in WT controls), reflected in similar serum corticosterone levels (Fig. 3*B*). This suggests that the exaggerated loss of bone during stress in *NPY 2r* –/– mice is not dependent on increases in CRF levels in the PVN/HPA activity and may involve other pathways. Moreover, the *NPY 2r* –/– mice lacked the stress-induced increase in circulating NPY levels, suggesting that there is a connection of Y2 receptor signaling and NPY release, and that circulating NPY may be involved in the stress-protective effect evident between WT and NPY-deficient mice.

To isolate the specific role of arcuate Y2 receptors, we deleted Y2 receptors selectively from the Arc in adult mice (*Arc Y2r* –/–) via delivery of an adenoassociated viral vector expressing Cre recombinase (rAAV-Cre) targeted to the Arc of *NPY 2r* lox/lox mice, as

described,[**24, 28**] and the skeletal response to the 6-week restraint stress protocol was evaluated. In the non-stressed group, conditional deletion of Y2 receptors selectively from the Arc resulted in greater cancellous bone volume, trabecular thickness, and trabecular number in association with an elevation in bone formation rate and mineral apposition rate with no change in mineralizing surface (Fig. 3*A*, *B*). Importantly, similar to *NPY*–/– and *NPY 2r* –/– mice, *Arc Y2r* –/– mice displayed an enhanced loss of bone following stress, associated with a significant reduction in mineral apposition rate (Fig. 3*C*). This common response to stress indicates that the Y2 receptors in the Arc are a critical site of NPY's action to protect against stress-induced bone loss. However, again, corticosterone levels were not altered between WT and *Arc Y2r* –/– mice (Fig. 3*D*), despite markedly differing bone loss, reinforcing the notion that HPA activity is unlikely to be responsible for the bone loss. Interestingly however, noradrenaline, which is co-stored and co-released with NPY from sympathetic neurons, was significantly increased in the serum of stressed *Arc Y2*–/– mice, compared to WT mice (Fig. 3*D*), suggesting a potential involvement of elevated noradrenergic tone the exaggerated bone loss seen in the germline and arcuate–specific Y2 receptor–deficient mice. Moreover, the lack of change in corticosterone in these Y2 receptor–deficient mice suggests that modulation of catecholamine rather than glucocorticoid action may be the critical axis for NPY's skeletal-protective effects during stress.

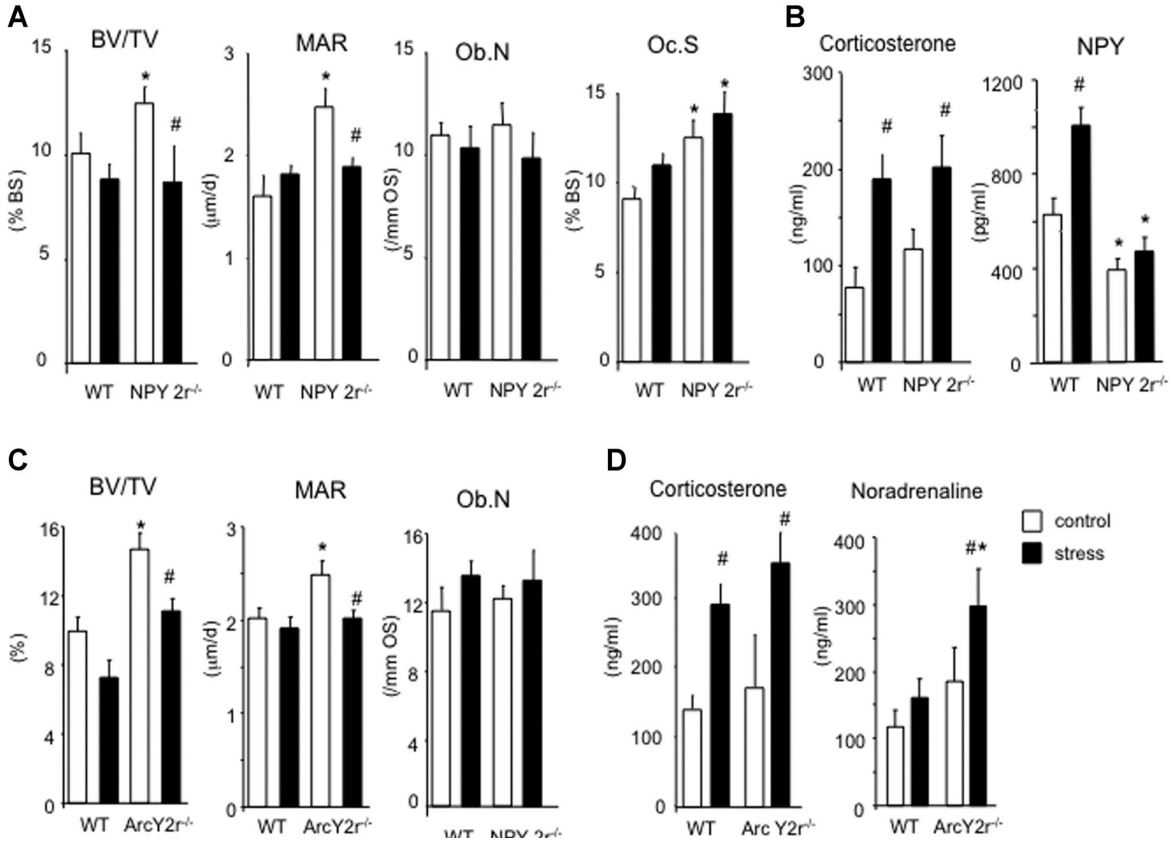

Fig. 3. Stress-induced bone loss is mediated via Y2 receptor signaling. Chronic stress induces marked bone loss in Y2 receptor null mice (*NPY 2r* $^{-/-}$) mice compared to WT. Histomorphometric analysis of (*A*) cancellous bone volume (BV/TV, %), mineral apposition rate (MAR, μm/d), osteoblast number (Ob.N, #/mm OS), and osteoclast surface (Oc.S, %BS) from control and stressed *NPY 2r* $^{-/-}$ mice. (*B*) Serum corticosterone was unaltered between genotypes; however, serum NPY did not increase in stressed *NPY 2r* $^{-/-}$ mice. (*C*) Arcuate-specific Y2 receptor null mice (*Arc Y2r* $^{-/-}$) also displayed greater stress-induced bone loss and MAR reduction compared to WT and no change in Ob.N. (*D*) Serum corticosterone was unaltered between genotypes; however, serum noradrenaline was increased in stressed *Arc Y2r* $^{-/-}$ mice. #$p < 0.05$ versus non-stress, within genotype; *$p < 0.05$ versus wild-type, within stress/non-stress group. $n = 6$–9, data expressed as mean ± SE.

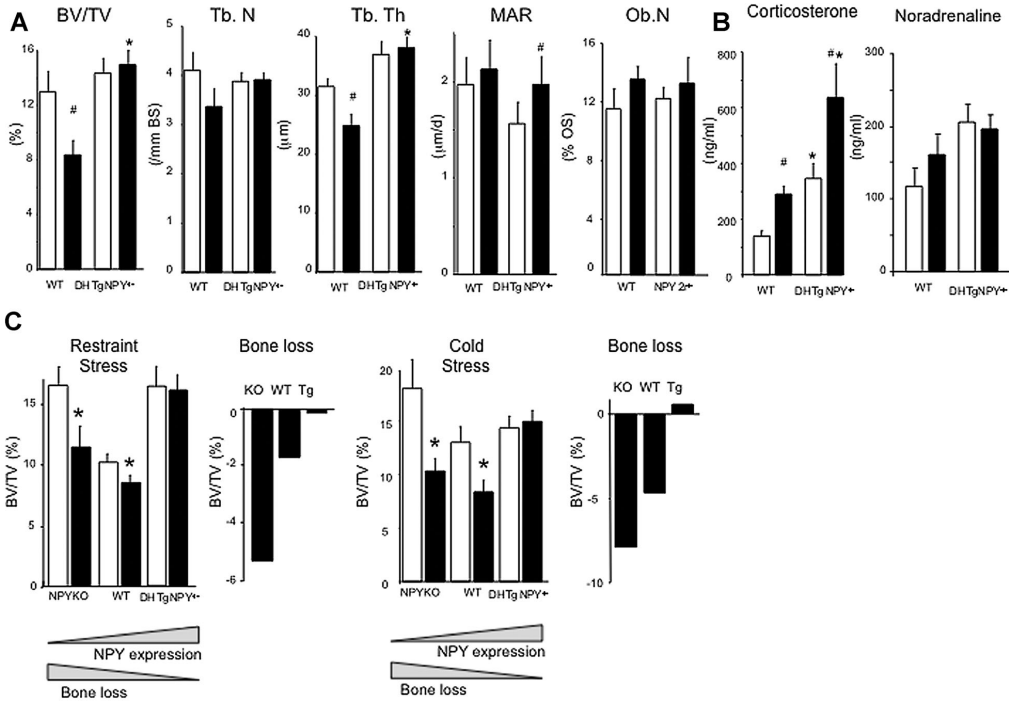

Fig. 4. Noradrenergic neuron–derived NPY protects against stress induced bone loss. Reinstatement of NPY production only in noradrenergic neurons by dopamine beta-hydroxylase–driven NPY production in NPY-null mice (*DH Tg NPY*$^{-/-}$) protects from stress-induced bone loss. (*A*) Histomorphometric analysis of distal femoral metaphysis of stressed and control WT and *DH Tg NPY*$^{-/-}$ mice showing cancellous bone volume (BV/TV, %), trabecular number (Tb.N, #/mm), trabecular thickness (Tb.Th, μm), mineral apposition rate (MAR, μm/d), and osteoblast number (Ob.N, #/mm OS). (*B*) Serum corticosterone was elevated by stress and in *DH Tg NPY*$^{-/-}$; however, serum noradrenaline was not significantly elevated. #$p < 0.05$ versus non-stress, within genotype; *$p < 0.05$ versus wild-type, within stress/non-stress group. $n = 6$–8, data expressed as mean ± SE. (*C*) Relationship between peripheral NPY expression and stress-induced bone loss: increasing peripheral NPY expression is associated with a reduction in bone loss associated with chronic stress, either due to the restraint or the cold-stress protocols.

NPY-mediated activation of the central catecholaminergic system contributes to the protection from stress-induced bone loss

Y2 receptors of the arcuate nucleus are crucial for the bone-protective effect, indicating the action of a central regulatory loop. In order to determine the pathway stemming from this NPY/Y2 receptor signaling in the arcuate, we investigated the nature and role of PVN neurons activated by Arc Y2 receptors. The PVN is a key site for regulating neuroendocrine and autonomic activities involved in stress responses, such as the production of CRH, as is evident in Fig. 1*C*.

In order to confirm Y2-dependent transmission from the Arc to the PVN, we injected the Y2 receptor–preferring ligand PYY3-36 unilaterally into the Arc of WT mice and evaluated neuronal activation through measurement of c-fos expression. PYY3-36 injection induced strong activation of c-fos in the Arc (Fig. 5*A*). Importantly, administration of PYY3-36 to the Arc also lead to a significant increase in c-fos activation in the PVN, specifically on the injected side compared to the contralateral control side, demonstrating that Y2 signaling in the Arc is critical for the activation of PVN neurons.

This was also confirmed by using a different marker, extracellular signal–regulated kinase (ERK) phosphorylation, which acts upstream of c-fos. Injection of PYY3-36 directly into the Arc of WT mice resulted in strong upregulation of phospho-ERK staining in neurons of the PVN, confirming the previous c-fos results (Fig. 5*B*). This signal is absent from brains injected with saline and from PYY3-36 injected *NPY 2r* –/– mice, consistent with this being an Arc-specific, Y2-mediated pathway. In order to examine activation of noradrenergic neurons by Y2 receptor signaling, we performed double immunohistochemistry on PYY3-36 injected brains from WT mice for phospho-ERK (Fig. 5*C*, blue arrows) and tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of catecholamines, such as noradrenaline[**32**] (Fig. 5*C*, black arrows). Histological examination of five brains per group, five sections per mouse, counting ~85 TH-positive neurons per section showed that after injection with PYY3-36 into the Arc found that (55% ± 5.6%) of TH positive neurons were also positive for phospho-ERK (Fig. 5*C*, red arrows). To determine which Y-receptor arcuate-derived NPY controls PVN TH neurons we performed

immunohistochemistry for TH combined with in situ hybridization for Y1 receptor mRNA on WT brain sections. As can be clearly seen in Fig. 5*D*, more than 80% of TH-positive neurons (Fig. 5*D*, black arrows) are also positive for Y1 receptor mRNA expression (Fig. 5*D*, red arrows). This establishes the existence of a functional link between NPY signaling in the arcuate and the catecholamine system in the stress-regulatory region, the PVN. In addition, it indicates the potential of NPY to inhibit TH neurons, thereby attenuating the noradrenaline response to stress.

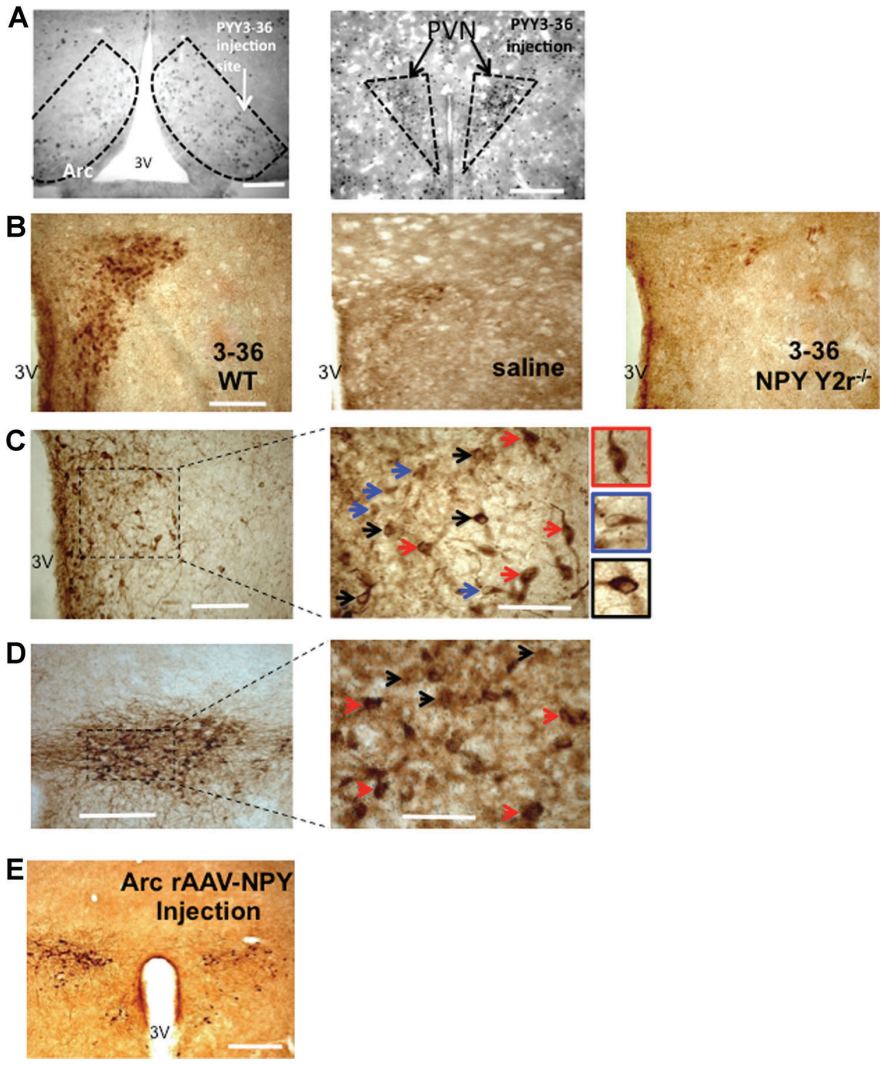

Fig. 5. Hypothalamic-specific Y2 receptors are critical for mediating NPY protective effect on stress induced bone loss. (*A*) Immunohistochemistry of c-fos in the Arc and PVN of the same WT mouse in response to direct unilateral PYY3-36 injection in the Arc. (*B*) phospho-ERK staining in neurons of the PVN after PYY3-36 or saline injection in the Arc of WT or *NPY 2r* $^{-/-}$ mice. (*C*) Double immunohistochemistry in the Arc of WT mice following unilateral PYY3-36 injection into the Arc, showing in high-magnification colocalization (red arrows) of phospho-ERK (blue arrows) and TH (black arrows). Representative neurons are isolated in color-coded panels. (*D*) Double immunohistochemistry in the PVN of WT mice, showing in high-magnification colocalization (red arrows) of Y1 receptor and TH neurons (black arrows). (*E*) Immunohistochemistry of TH-positive neurons in the PVN of conditional Y1 KO mice unilateral injected (right side) with AAV-Cre, followed 3 weeks later by unilateral injection of PYY3-36 into the Arc, showing reduced numbers of TH-positive neurons only on the injected side but not in the contralateral control side. Scale bars = 40 μm (*C*), 200 μm (*D*), 10 μm (*E*), and 60 μm (*F–J*).

We next examined the functional consequences of activation of this axis. The activity of catecholaminergic neurons in the PVN was determined following experimental NPY overproduction specifically in the Arc via unilateral injection of rAAV-NPY. Three weeks after rAAV-NPY injection, the expression of TH protein, was significantly downregulated (Fig. 5*E*). Indeed, the number of TH-positive neurons in the PVN was reduced more than 60% on the injected side compared to the contralateral, control side. The reduced TH-tone following upregulation of signaling through arcuate Y2 receptors demonstrates an inhibitory axis from NPY/Y2 receptors in the arcuate to TH neurons in the PVN. The action of such a pathway is reflected in the serum responses arising from the stress protocol in the opposing model, the

absence of arcuate Y2 receptors, *Arc Y2r* –/–. The *Arc Y2r* –/– mice show a markedly exaggerated noradrenaline response to stress (Fig. 3*D*), owing to the absence of NPY repression of TH neurons, acting specifically through the arcuate Y2 receptors. In this manner we have defined an NPY-mediated loop from arcuate Y2 receptors via Y1 receptors in the PVN to regulate TH neurons and the subsequent levels of noradrenaline in the serum.

Noradrenergic neuron–derived NPY protects against stress-induced bone loss

We have demonstrated a suppression of the catecholamine response by NPY signaling within the hypothalamus, suggesting an important role of noradrenaline in the skeletal response to stress. However, the suppression of bone formation evident after stress may also involve changes in circulating noradrenaline levels. Importantly, not only central but also peripheral sympathetic noradrenergic neurons coexpress NPY. Moreover, as noradrenaline is a secretagogue of NPY in these nerves, their secretion is interrelated and appears to be reciprocal. NPY is known to be a potent inhibitor of noradrenaline release,[**33**] whereas adrenergic antagonism increases NPY release.[**34**] Thus, elevation of adrenergic tone by the stress protocol may inhibit NPY secretion from sympathetic neurons and thereby limit NPY's ability to protect bone under these conditions. Conversely, an elevation of NPY signaling in noradrenergic neurons would likely inhibit noradrenaline release and enhance bone protection during stress.

In order to investigate the actions of NPY in noradrenergic neurons, we developed a transgenic mouse model in which NPY is produced solely in these noradrenergic neurons. For this we crossed our NPY-null mice with a transgenic line expressing NPY under the control of the dopamine beta-hydroxylase (DβH) promoter (*DH Tg NPY*–/–).[**35**] Consistent with a critical contribution of NPY derived from the sympathetic nervous system to the protection of skeletal tissue during chronic stress, cancellous bone volume and osteoblast activity was unaltered in *DH Tg NPY*–/– mice after the 6-week stress protocol (Fig. 4*A*). Lacking NPY in the Arc, and therefore the NPY-induced HPA suppression, *DH Tg NPY*–/– mice display greater HPA responses, as in *NPY*–/– mice (Fig. 1*E*). This is clearly evident in the significant increase in circulating corticosterone levels in *DH Tg NPY*–/– mice compared to WT mice (Fig. 4*B*). As in the Y2 receptor study (Fig. 3*B*, *D*), bone loss was not associated with stress-induced elevation of HPA activity. Critically, the protection from bone loss in *DH Tg NPY*–/– mice was associated with a lack of elevation in noradrenaline. These data indicate that the balance between NPY and noradrenaline in peripheral sympathetic nerves may be crucial for the skeletal response to stress, with greater NPY-ergic activity attenuating noradrenergic tone and bone loss. Indeed, with increasing peripheral NPY expression, from *NPY*–/– to WT to *DH Tg NPY*–/– mice, there is clear preservation of bone mass following chronic stress, both, in the restraint and the cold-stress models (Fig. 4*C*).

In this manner, sympathetically-derived NPY may support the stress-responsive skeletal-protective loop, from the hypothalamus to the osteoblast. The stress-induced NPY mediated Y2 receptor signaling in the Arc inducing activation of CRH and TH neurons in the PVN, subsequently triggering altered noradrenergic neuron activity with suppression of noradrenaline in a presynaptic fashion.

DISCUSSION

In this study we identify NPY as a dominant protector against the deleterious effects of stress on bone mass, and identify a central axis regulating the peripheral actions of stress on bone mass. In addition to greater anxiety-like and depression-like behavior, mice lacking *NPY* exhibit increased activity of the HPA and catecholamine pathways and markedly greater loss of bone under conditions of chronic stress. The stress-protective effect of NPY involves both central and peripheral NPY action. Centrally, stress-induced elevation of NPY signaling in the hypothalamus, resulting from the initial elevation in circulating corticosterone, leads to a suppression of TH neuron activation and reduced CRH production in the PVN, with consequent reduction in catecholamine and glucocorticoid secretion/action. Moreover, specific Y2 receptor signaling in the Arc influences NPY and noradrenaline responses to stress in the circulation, and thereby the balance between bone sparing and bone wasting, respectively. Specific deletion of Y2 receptors from the Arc leads to greater bone loss following stress. This loss of bone in *Arc Y2r* –/– mice is coincident with two major changes: a loss of the stress-induced increase in serum NPY evident in WT mice, and the presence of an elevation in noradrenaline, absent in WT. Regulation of noradrenaline is critical to the skeletal response to chronic stress.[**36**] Our data indicate that NPY

expressed in noradrenergic neurons is the critical mediator of the response to protect bone from the adverse effects of stress. Importantly, this is evidenced by the complete rescue of stress-induced bone loss seen in *Npy*–/– mice by selective reintroduction of NPY in this type of neuron. The NPY derived from these noradrenergic neurons likely acts in a presynaptic fashion to control noradrenaline release by acting on Y2 autoreceptors, which is also evident in neurovascular control.[**37, 38**] This is consistent with the loss of NPY protection to bone loss in the global Y2 knockout mice. Thus it appears that NPY protects bone during times of chronic stress by two interrelated pathways centering on Y2 receptor signaling in the Arc of the hypothalamus and in noradrenergic neurons (Fig. 6). The peripheral pathway modifies the production of NPY by noradrenergic neurons, thereby controlling noradrenaline production and release, which is the critical step in skeletal protection. The central pathway modifies the responses of the TH neurons in the PVN. In addition, this Arc-PVN pathway also modulates the HPA axis, limiting CRH production in the PVN and reducing circulating glucocorticoid levels. This may also contribute to the preservation of bone mass by attenuating the effects of noradrenaline. Glucocorticoids amplify the negative skeletal responses to sympathetic signaling, by upregulating adrenergic receptor expression and response to sympathetic signaling in osteoblasts.[**39**] Thus, in the absence of NPY, noradrenergic production is directly increased, and sensitivity may be indirectly increased, via elevated glucocorticoid production.

In contemporary society, chronic stress and depression are an increasing issue, and there is a growing need to understand their widespread impact on human health. Depression alone affects up to 25% of women and 12% of men.[**13**] As demonstrated herein, chronic stress results in elevations of circulating catecholamine and noradrenaline levels, responses consistent findings in human depression and anxiety.[**40**] It has long been known that many depressed patients exhibit elevated urinary and plasma concentrations of cortisol, with 60% to 70% exhibiting elevated circulating cortisol[**41**] and, as reported more recently, noradrenaline.[**42**] Importantly, these increases in noradrenaline and corticosterone have a described negative impact upon bone mass.[**29, 43, 44**] This is consistent with a growing appreciation of the clinical-relevance of the skeletal impact of stress, such as is evident in major depression.[**7-9**] The identification of reduced NPY levels in subjects with depression,[**16, 45**] and the improved stress-coping in individuals with greater NPY production19 indicate the potential for a causative role for NPY not only in the behavioral aspects of depression, but also the somatic symptoms, such as bone loss. In addition, the coregulation of CRF and TH neurons in the hypothalamus has led to speculation of a feed-forward loop, with noradrenergic neurons in the brain able to activate CRF neurons in the PVN to secrete CRF, and CRF in turn activate noradrenergic neurons.[**46**] This has been speculated to underlie panic and anxiety disorders in depressive illness.[**46, 47**] Although it is the subject of close examination, the existence of such a feed-forward loop was questioned on the grounds that it would need a careful control system in order to limit the escalation of minor stressful stimuli onto anxiety and depression.[**48**] To date, such a control mechanism has not been identified. However, the dual suppressive role of NPY demonstrated herein indicates that NPY may provide a level of control for the CRF/TH feed-forward loop.

Furthermore, our demonstration of greater stress-induced bone loss in mice lacking *Npy* is consistent with known differences between acute and chronic stress responses (Fig. 6*A*). Acutely, NPY stimulates CRH production, raising glucocorticoid levels.[**49**] However, as glucocorticoid exposure continues, this pattern is reversed.[**50**] In *Npy*–/– mice, both CRH and corticosterone levels are increased, reflecting an overall anxiolytic action of this neuropeptide. Thus, after chronic glucocorticoid exposure, NPY acts in a well-described anxiolytic manner to inhibit CRH production,[**50**] placing hypercortisolism as a fundamental trigger for the stress-induced pathways involving central NPY (Fig. 6*B*). However, *NpyY2r* –/– mice were comparable to WT mice with respect to corticosterone responses to stress, indicating that the greater bone loss observed in this model occurs via an alternate pathway. Indeed, whereas corticosterone may act as a trigger for the anxiolytic response of NPY, the bone sparing activities result from inhibition of catecholamine pathways; indicated by inhibition of p-ERK production in TH-positive neurons in the PVN in response to Arc-specific Y2 activation and reduced circulating noradrenaline in WT compared to Y2 receptor–deficient mice. Increased production of noradrenaline is a well-described response to stress.[**51**] Stimulation of adrenoceptors on the osteoblast are known to inhibit bone formation and stimulate bone resorption,[**52-55**] and the elevated noradrenaline levels in Y2 receptor deficient models is consistent with their greater bone loss. These findings, illustrate the complexity and the context-dependent nature of the

relationship between NPY and the HPA axis, reflecting the requirement for the acute anxiogenic nature of the HPA for fight/flight responses and the opposing requirement for anxiolytic actions to protect against chronic HPA activation. In contrast to the effects of acute stress, after chronic stress, clear inverse relationships between NPY signaling, CRH expression, and corticosterone production emerge, defining a complex anxiolytic system designed to attenuate the negative effects of long-term glucocorticoid exposure and inhibit catecholamine production and release.

Taken together, these data highlight the critical role of NPY pathways in the regulation of skeletal tissue, acting in the hypothalamus to attenuate bone loss in times of chronic stress or longer periods of depression. NPY, acting via Y2 receptors in both central and peripheral neural tissues, inhibits noradrenaline and corticosterone production/release, thereby protecting bone from loss during chronic stress. Critically, elevating NPY signaling in noradrenergic neurons was able to completely block the bone loss induced by chronic stress. These insights suggest a potential avenue for therapeutic design that accounts for both primary mood disorders but also the somatic side-effect profile.

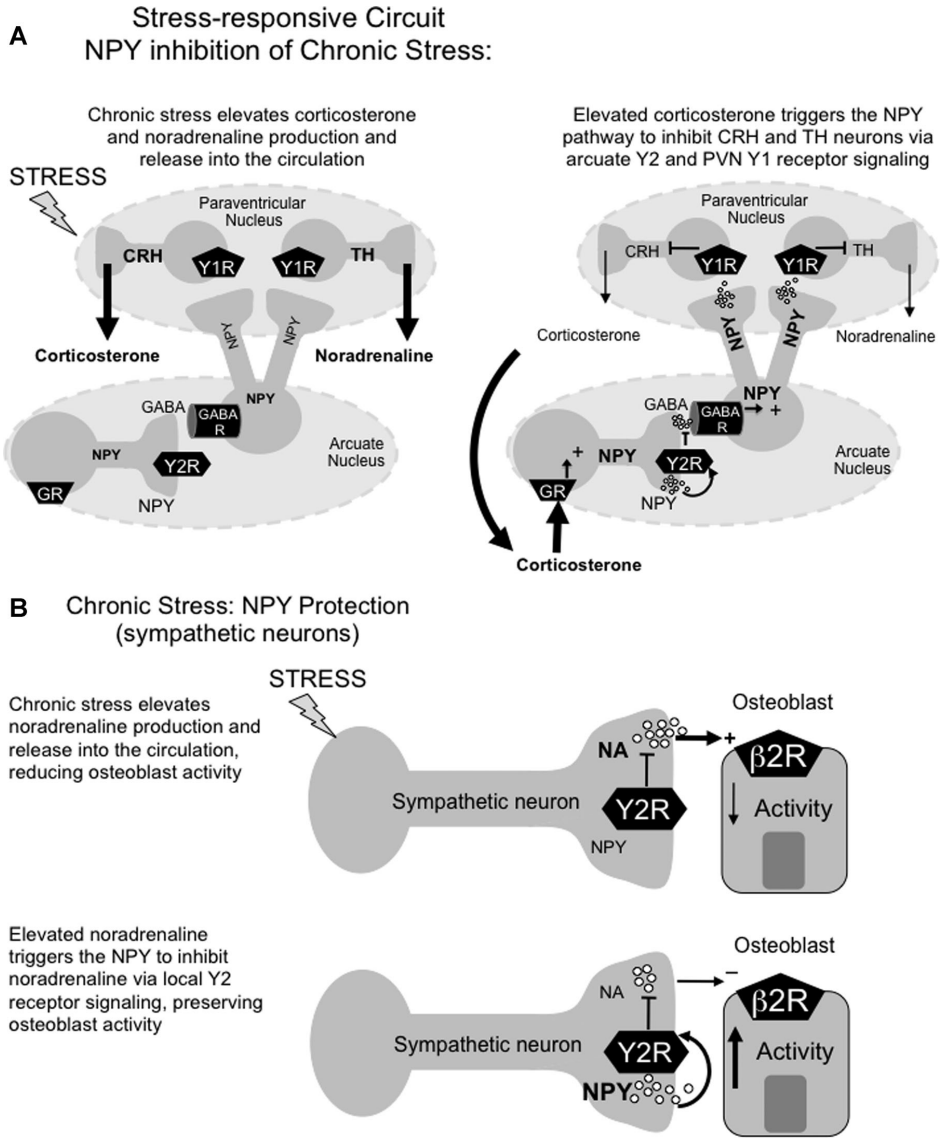

Fig. 6. Schematic model of NPY pathways under normal and stress condition. (*A*) Under normal conditions, energy homeostasis determines central NPY expression, with positive energy balance decreasing hypothalamic NPY and thereby removing the tonic inhibition of osteoblast activity. However, under conditions of chronic stress, NPY is increasingly involved in a stress-protective loop (*A*). Chronic stress drives elevated secretion of corticosterone into the serum, which signals within the arcuate nucleus of the hypothalamus to increase NPY production. Y2 receptors within the NPY-ergic neurons in the arcuate inhibit interneuron GABA signaling, and increase NPY supply to Y1 receptors in the paraventricular nucleus. This increased NPY-ergic tone signals within the PVN to induce a decrease in sympathetic outflow from the brainstem, via TH neurons and a repression of CRH production. (*B*) In addition, the activation of this anxiolytic process raises serum NPY production from sympathetic neurons. This signals via Y2 receptors to inhibit noradrenaline release and its negative effects on osteoblast activity through β2 adrenergic receptors on those cells.

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Authors' roles: Study design: PB, SL, LZ, TK, AS, and HH. Study conduct: PB, SL, LZ, TK, YS, FD, AZ, BH, NL, IPLW, EJDL, RFE, BS, MD, EY, SZ, SR, and ES. Data collection: PB, SL, LZ, TK, YS, FD, AZ, BH, NL, IPLW, EJDL, RFE, BS, MD, and EY. Data analysis: PB, SL, LZ, TK, AS, and HH. Data interpretation: PB, SL, LZ, TK, ES, AS, and HH. Drafting manuscript: PB, SL, LZ, TK, YS, FD, SZ, ES, AS, and HH. Revising manuscript content: PB, SL, LZ, AS, and HH. Approving final version of manuscript: PB and HH.

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