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ARTICLES

Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome

Lydia E Kuo[1], Joanna B Kitlinska[1], Jason U Tilan[1], Lijun Li[1], Stephen B Baker[2], Michael D Johnson[3], Edward W Lee[1,8], Mary Susan Burnett[4], Stanley T Fricke[5], Richard Kvetnansky[6], Herbert Herzog[7] & Zofia Zukowska[1]

The relationship between stress and obesity remains elusive. In response to stress, some people lose weight, whereas others gain. Here we report that stress exaggerates diet-induced obesity through a peripheral mechanism in the abdominal white adipose tissue that is mediated by neuropeptide Y (NPY). Stressors such as exposure to cold or aggression lead to the release of NPY from sympathetic nerves, which in turn upregulates NPY and its Y2 receptors (NPY2R) in a glucocorticoid-dependent manner in the abdominal fat. This positive feedback response by NPY leads to the growth of abdominal fat. Release of NPY and activation of NPY2R stimulates fat angiogenesis, macrophage infiltration, and the proliferation and differentiation of new adipocytes, resulting in abdominal obesity and a metabolic syndrome-like condition. NPY, like stress, stimulates mouse and human fat growth, whereas pharmacological inhibition or fat-targeted knockdown of NPY2R is anti-angiogenic and anti-adipogenic, while reducing abdominal obesity and metabolic abnormalities. Thus, manipulations of NPY2R activity within fat tissue offer new ways to remodel fat and treat obesity and metabolic syndrome.

Stress has been linked to the pathogenesis of many diseases, but its mechanisms of action and role as a risk factor remain unclear. Its rise has paralleled the incidence of obesity[1], leading to serious health consequences. One type of obesity in particular, abdominal/visceral obesity, has been linked to the development of metabolic syndrome, and is a serious risk factor for cardiovascular diseases and diabetes.

In spite of this perceived association between stress and obesity, the nature of the relationship remains uncertain—some people lose weight when stressed, whereas others gain weight. Although there is much evidence that stress and obesity are related to the hypothalamic control of food intake and metabolism, little is known about the peripheral processes by which stress affects adiposity.

Stress stimulates sympatho-adrenomedullary activity, which is responsible for fight-or-flight responses. It is also the body's main mechanism of weight loss, acting through β-adrenoceptor–mediated lipolysis and inhibition of adipocyte proliferation[2] in white adipose tissue (WAT), and stimulation of thermogenesis in brown adipose tissue (BAT)[3]. Paradoxically, sympathetic activity seems to be increased in obese humans[4], indicating that β-adrenergic activity might compensate for other factor(s) that promote weight gain.

One such factor is neuropeptide Y (NPY), a peptide derived from the brain and sympathetic nerves that has potent orexigenic activity, favoring the intake of carbohydrate-rich foods[5]. Increased activity of NPY and its receptors, Y1R, Y2R and Y5R, has been found in the brain in many forms of experimental obesity[5]. In the genetic background of B6.V-*Lep*^{ob}/J mice, which lack leptin (a fat-derived hormone that inhibits appetite and hypothalamic Npy release[6]), knocking out the *Npy2r* gene decreases hyperphagia and body weight, and improves metabolism[7]. Although this supports the idea that NPY-NPY2R signaling has central effects, a few studies have indicated that NPY might also have anti-lipolytic actions through unknown receptors[8]. We therefore focused on the role of NPY and NPY2R in the adipose tissue itself.

We reported previously that NPY is potently angiogenic when signaling through endothelial NPY2R[9] and upon activation of dipeptidyl peptidase IV (DPP4), which generates NPY3–36, an NPY2R-preferring form of NPY[10]. This NPY2R-DPP4 system has an important

[1]Department of Physiology & Biophysics, Georgetown University Medical Center, 3900 Reservoir Rd. NW, BSB 234, Washington, DC 20057, USA. [2]Department of Plastic Surgery, Georgetown University Medical Center, 3900 Reservoir Rd. NW, 1 PHC, Washington, DC, 20007, USA. [3]Department of Oncology, Georgetown University Medical Center, 3970 Reservoir Rd., NW W326A NRB, Washington, DC 20007, USA. [4]Cardiovascular Research Institute, MedStar Research Institute, 108 Irving Street NW, Room 214, Washington, DC 20010, USA. [5]Department of Neuroscience, Georgetown University Medical Center, 3970 Reservoir Rd., NW WB-01 NRB, Washington, DC 20007, USA. [6]Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlarska 3, 833 06, Bratislava, Slovak Republic. [7]Garvan Institute of Medical Research, 384 Victoria St., NSW 2010 Darlinghurst, Sydney, Australia. [8]Present address: Department of Radiology, David Geffen School of Medicine at the University of California, Los Angeles, 10833 Le Conte Ave., Box 951721, Los Angeles, California 90095, USA. Correspondence should be addressed to Z.Z. (zzukow01@georgetown.edu).

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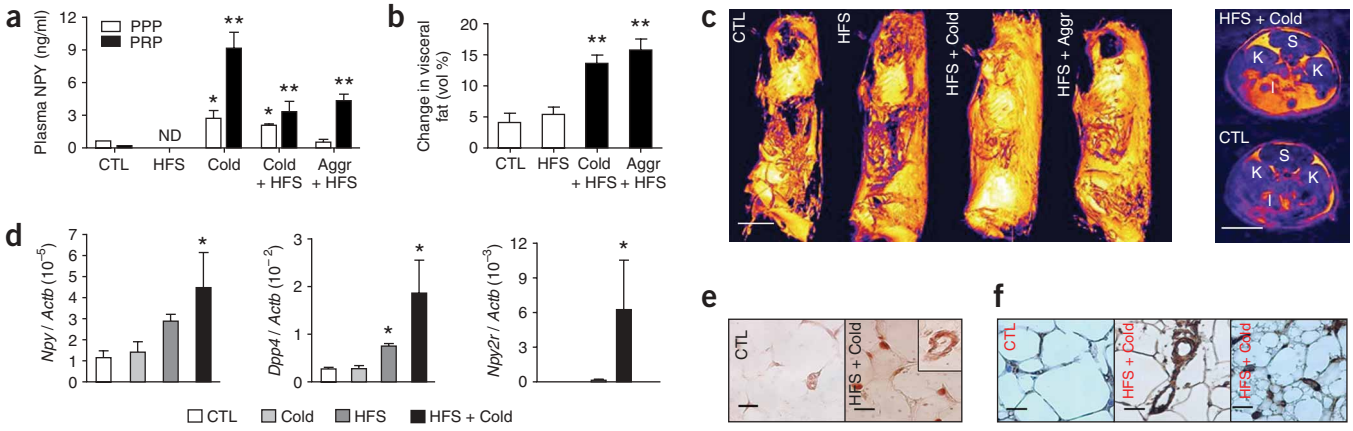

Figure 1 Stress, in the presence of an HFS diet, causes the upregulation of plasma NPY and fat *Npy*, *Npy2r* and *Dpp4* expression, increasing visceral fat growth. (**a**) Levels of NPY-IR in platelet-poor plasma (PPP) and platelet-rich plasma (PRP) from 129X1/SvJ mice under conditions of a HFS diet, cold, cold + an HFS diet, and aggressor (Aggr) + an HFS diet compared to non-stressed standard chow-fed control (CTL). (**b**) Changes in visceral fat measured by 3D-MRI volume reconstruction imaging of fat in mice on HFS diet, cold + HFS, and Aggr + HFS, compared to non-stressed mice on standard chow diet (CTR). (**c**) Representative images of fat, assessed by 3D-MRI, with whole mice at left (rostral end up) and cross-sections at right. Scale bar, 1 cm. S, spine; K, kidney; I, intestines. (**d**) *Npy*, *Dpp4* and *Npy2r* mRNA levels in subcutaneous abdominal fat of HFS and cold stress treated mice as compared to untreated control mice (quantitative real-time PCR). (**e**,**f**) Immunohistochemistry of Npy (**e**) and Npy2r (**f**) localized to vessels and adipocytes in subcutaneous abdominal fat in mice. Scale bars, 20 μm. The results represent average values ± s.e.m. of $n = 6$ mice. $^*P < 0.05$, $^{**}P < 0.01$ by one-way ANOVA with Bonferroni's multiple *t*-test. ND, not detected.

role in neo-vascularization of ischemic tissues[11], retinopathy[12], wound healing[11] and tumors[13]. By acting on NPY1R and NPY5R, NPY also causes vasoconstriction[14], immunomodulation[15] and stimulation of the growth of many cells[15].

NPY is released from sympathetic nerves by various stressors, particularly chronic ones[16]. Its release and actions are modulated by other stress hormones, such as catecholamines[14] and steroids[16]. On the basis of these observations, we hypothesized that NPY stimulates fat growth by exerting angiogenic and immunomodulatory activities in the WAT, and mediates the weight gain associated with stress and a hypercaloric diet.

RESULTS

Stress-induced upregulation of NPY in WAT

We have previously shown in rodents[17] and humans[18] that exposure to cold potently stimulates NPY release acutely and chronically[17]. By contrast, restraint stress does not raise levels of NPY immunoreactivity in the plasma[19]. In this study, we investigated several forms of chronic (2-week) stress in 129SvJ mice, either alone or in combination with a diet that is high in fat and high in sugar (HFS). Water avoidance stress (a mouse standing on a post in room-temperature water) did not elevate plasma Npy (not shown). Exposure to cold water increased Npy-immunoreactivity (IR) levels in platelet-poor and platelet-rich plasma (**Fig. 1**); the latter was attenuated by an HFS diet (**Fig. 1a**). This attenuation could be due to the reported anxiolytic effects of comfort foods that contain high levels of fat and sugar[20], which could decrease stress-induced sympathetic activity[21]. Another stressor, exposure to an aggressive mouse (10 min/d over 14 d) mimicked the effect of cold in HFS-fed mice, increasing Npy levels in platelet-rich plasma (**Fig. 1a**). This indicates that platelets accumulate Npy, as seen with other peptides[16].

We then investigated whether stress-induced activation of the NPY system parallels the effect of a specific stressor on diet-induced obesity (DIO; **Fig. 1b**,**c**). Water avoidance, which did not elevate plasma or platelet Npy, had no effect on fat accumulation in mice fed the HFS diet (not shown). However, aggressor stress and cold stress significantly increased abdominal fat deposits (**Fig. 1b**,**c**).

Two weeks of daily cold exposure alone did not elevate *Npy*, *Npy2r* or *Dpp4* mRNAs in the WAT (**Fig. 1d**). However, when combined with the HFS diet, stress induced marked upregulation of the expression of these genes specifically in the subcutaneous abdominal fat pads, as compared to control mice (**Fig. 1d–f**). The expression of *Npy* and *Npy2r* was co-localized to vessels, interstitial spaces (nerves) and adipocytes in the abdominal adipose tissue (**Fig. 1e**,**f**).

NPY stimulates adipocyte proliferation and differentiation

Human and mouse adipocytes and endothelial cells expressed low levels of NPY2R (**Fig. 2a**, **Supplementary Fig. 1** online). To determine whether neuron-derived NPY promotes the growth of these cells, we co-cultured mouse 3T3-L1 preadipocytes or endothelial cells with sympathetic neuron-derived tumor cells (tyrosine hydroxylase–positive neuroblastomas), which express and release mature NPY (**Supplementary Fig. 1**). Both NPY and neuroblastoma-conditioned medium stimulated the proliferation of preadipocytes and endothelial cells, and this effect was blocked by an NPY2R antagonist (BIIE0246 (1 μM); **Fig. 2a**). Co-culture of endothelial cells and preadipocytes with neuroblastoma cells also markedly upregulated *Npy2r* expression in the endothelial cells and preadipocytes (**Fig. 2a**). This indicates that, in the WAT, sympathetic neuron-derived NPY upregulates the expression of its own growth-promoting receptor NPY2R (as well as the enzyme DPP4), to stimulate proliferation of both endothelial cells and preadipocytes.

NPY also stimulated adipogenesis in preadipocytes that were primed for differentiation (**Fig. 2b**). NPY mimicked the effects of insulin by increasing the secretion of leptin and resistin, and lipid filling of the new adipocytes, and these effects were blocked by an NPY2R antagonist (**Fig. 2b**). Thus, preadipocyte and endothelial NPY2R seem to be an important pathway by which NPY stimulates angiogenesis and adipogenesis *in vitro*.

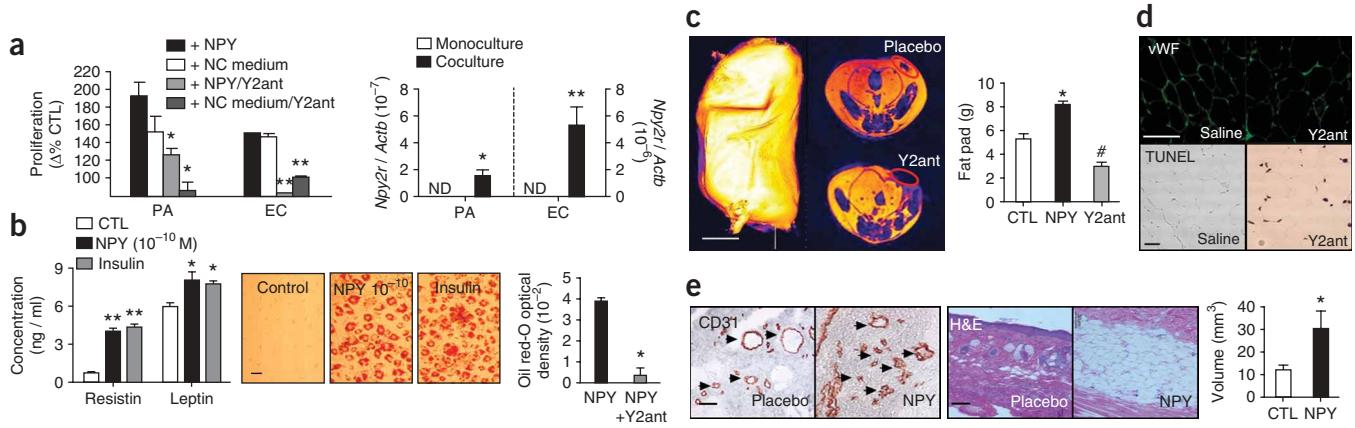

Figure 2 NPY increases fat growth directly by stimulating proliferation and differentiation of preadipocytes and indirectly by angiogenesis in the adipose tissue *in vitro* and *in vivo*. (**a**) Left, proliferation of preadipocytes (PA) and endothelial cells (EC) when treated with neuroblastoma-conditioned medium (NC) containing NPY or synthetic NPY1-36, with or without NPY2R antagonist (Y2ant). $*P < 0.05$, $**P < 0.001$ compared to the control not treated with the antagonist. Right, expression of *Npy2r* in ECs and PAs (quantitative real-time PCR) when cells are co-cultured with sympatho-neural NPY-expressing cells. $*P < 0.05$, $**P < 0.001$ compared to monoculture. ND, not detected. (**b**) Left, adipocyte differentiation markers resistin and leptin (ELISA) measured in media from PAs. Right, lipid oil red-O staining of PAs when treated with insulin or NPY, and quantification of optical density of the staining. Scale bars, 20 μm. $*P < 0.05$, $**P < 0.001$, as compared to non-treated control. For both panels **a** and **b**, ANOVA with Bonferroni's multiple *t*-test was used; $n = 6$ wells per experiment, repeated three times. (**c**) 3D-MRI volume reconstruction imaging of the fat in B6.V-*Lep*^{ob}/J mice with cross-sections of subcutaneous abdominal intra-fat where NPY pellets (1 μg/14 d) or injections of NPY2R antagonist (1 μM/d/14 d) were administered, compared to saline-injected controls ($*P < 0.05$) tested by one-way ANOVA with Bonferroni's multiple *t*-test. Scale bar, 1 cm. (**d**) Von Willebrand's factor (vWF)$^+$ and TUNEL staining in the saline and NPY2R antagonist-treated fat pads in B6.V-*Lep*^{ob}/J mice. Scale bars, 20 μm. (**e**) CD31$^+$ (left) and H&E (right) staining of human fat xenografts in athymic nude mice treated with placebo or NPY (1 μg/14 d slow-release pellets; $*P < 0.01$ by Student *t*-test). Scale bars, 50 μm. Black arrows indicate vessels. (**c**–**e**) $n = 6$ animals per group, $*P < 0.05$ compared to control by ANOVA with Bonferroni's multiple *t*-test.

NPY increases WAT growth and vascularization *in vivo*

To determine whether NPY-NPY2R signaling increases WAT mass *in vivo*, we used genetically obese B6.V-*Lep*^{ob}/J mice, which show centrally mediated hyperphagia, impaired metabolism and reduced sympatho-adrenergic activity[5]. Unexpectedly, these mice had 200% higher plasma Npy levels (**Supplementary Fig. 1**) and markedly upregulated *Npy* and *Npy2r* expression in subcutaneous abdominal fat, compared with control C57BL/6J mice (**Supplementary Fig. 1**), supporting the idea that the circulating Npy is derived from fat. The weight and volume of adipose tissue in both obese and lean mice were increased by 50% as a result of treatment with subcutaneous abdominal fat-delivered NPY (1 μg per 14 day–release pellet, as previously found to produce localized angiogenesis[11]). By contrast, injections with an NPY2R antagonist (BIIE0246, 1 μmol/d for 14 d) decreased adipose tissue weight and volume in both obese and lean mice by 50% (**Fig. 2c**).

The anti-adipogenic effect of the NPY2R antagonist was associated with decreased vascularity and increased apoptosis (**Fig. 2d**) in the abdominal fat pads. We therefore concluded that NPY stimulated *in situ* adipogenesis and angiogenesis through Npy2r, and that inhibition of Npy2r activity in fat tissue induced atrophy of capillaries and adipocytes.

Similarly, NPY proved to be adipogenic for human WAT, which also expresses NPY and NPY2R in adipocytes and endothelial cells (**Supplementary Fig. 1**). An NPY pellet (1 μg over 14 d) administered locally alongside a human fat xenograft in athymic mice increased the vascularization (density of CD31$^+$ vessels) and 3-month survival of the graft, whereas placebo-treated grafts underwent resorption (**Fig. 2e**).

Stress-induced exacerbation of DIO is mediated by NPY2R

Given that the above studies show that NPY and NPY2R on endothelial cells and adipocytes mediate remodeling of mouse and human fat by an angiogenesis-dependent mechanism, we next investigated whether these signals are involved in the stress-induced augmentation of DIO. Two weeks of an HFS diet alone did not significantly alter interscapular BAT or sternal and abdominal (subcutaneous and visceral, respectively) WAT deposits (**Fig. 3a**,**b**, **Supplementary Fig. 1**). Cold or aggressor stress alone was also ineffective, but when combined with an HFS diet, such stress markedly increased the abdominal fat deposit (**Fig. 3a**,**b**) without changing total body weight, food intake or fecal output (**Supplementary Fig. 1**), compared with unstressed, HFS-fed mice. Conversely, expression of uncoupling proteins (*Ucp1-3*), which are markers of thermogenesis[22], increased in the BAT, skeletal muscles and WAT of cold-stressed, HFS-fed mice compared with non-stressed, HFS-fed controls (**Supplementary Fig. 1**). In cold-stressed, HFS-fed mice the WAT exhibited a BAT-like morphology; the adipocytes had vacuolar cytoplasm (**Fig. 1f**) and expressed BAT-specific *Ucp1* (**Supplementary Fig. 1**). This indicated transformation of white fat to brown fat and enhanced thermogenesis, as previously reported in cold adaptation[22]. Surprisingly, in spite of activated thermogenesis, HFS-fed, cold- or aggressor-stressed animals did not reduce but rather increased their abdominal fat deposits.

Similar to the effects of NPY in mouse and human fat, cold stress in HFS-fed mice increased vascularity (**Fig. 3c**) and infiltration with CD68$^+$ macrophages (**Fig. 3d**) in the abdominal WAT, indicating increased angiogenesis and inflammation. In parallel, the number of small adipocytes (<10 μm) also increased (**Fig. 3e**) compared to control lean mice. These adipocytes were particularly rich in Npy2r-IR (**Fig. 1f**) and coincided with areas of increased Ki67$^+$ cell proliferation —indicating that stress, acting through the Npy-Npy2r system, stimulated the proliferation of new adipocytes.

To determine whether NPY2R mediates stress-activated angiogenesis and adipogenesis, we administered an NPY2R antagonist in either

slow-release pellets (1 μg over 14 d) or daily injections (1 μM/d for 14 d) into the abdominal subcutaneous fat of cold- or aggressor-stressed mice. In both models, after 2 weeks, the NPY2R antagonist reduced the visceral fat deposits by ~40% without significantly changing other deposits (**Fig. 3a**,**b**, **Supplementary Fig. 1**). Similarly, germline *Npy2r*$^{-/-}$ mice were resistant to cold stress-induced augmentation of DIO, further supporting the notion that NPY2R is essential in this process (**Fig. 3a**,**b**). The mechanisms of the anti-adipogenic effect of Npy2r in stressed mice, as in B6.V-*Lep*^{ob}/J mice, involved apoptosis of endothelial cells and adipocytes, as indicated by positive TUNEL (**Fig. 2d**) and active caspase-3 (**Fig. 3e**) staining in these cells (**Supplementary Fig. 1**) and also inhibition of preadipocyte proliferation (as tested by decreased Ki67 staining; **Supplementary Fig. 1**). Apoptotic cells were often surrounded by CD68^{+} macrophages, which formed 'crown-like structures' (**Fig. 3d**,**e**) that marked the areas of increased apoptosis and macrophage phagocytotic activity.

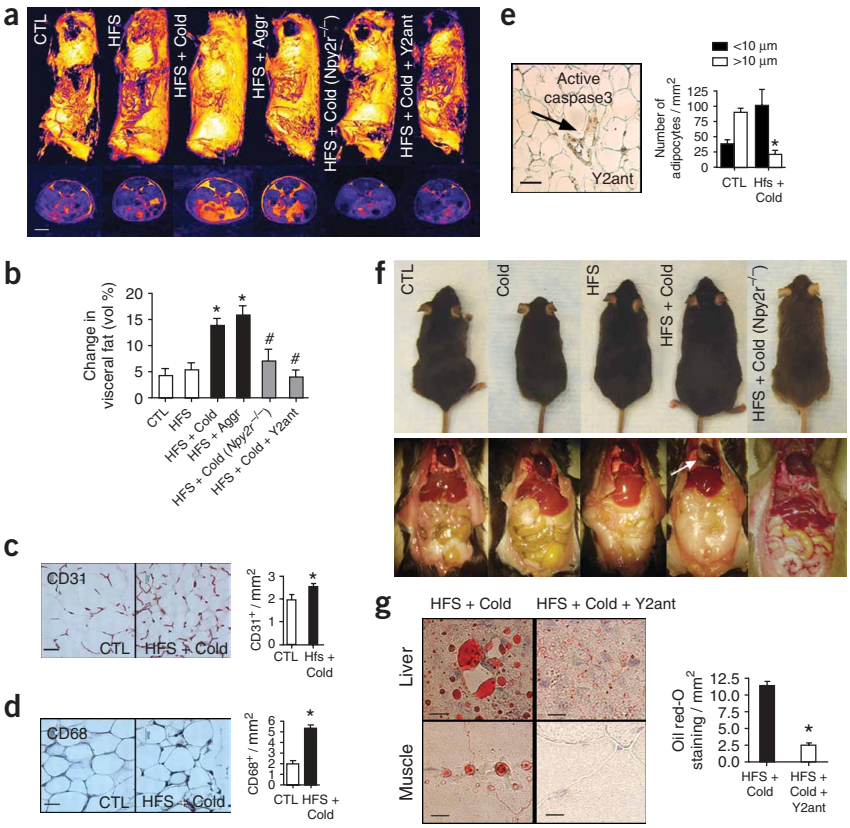

Figure 3 Stress exacerbates DIO by activating NPY and NPY2R in the abdominal fat. (**a**) Representative fat volume-rendered MRI images of 129X1/SvJ mice stressed for 2 weeks. Scale bars, 1 cm. (**a**,**b**) Mice given an HFS diet and exposed to cold or aggressor stress for 2 weeks compared to mice given an HFS diet alone or standard chow-fed controls. This stress effect is compared to germline *Npy2r*$^{-/-}$ and NPY2R-antagonist-treated mice. (**c**,**d**) Abdominal fat from 2-week cold-stressed, HFS-fed mice immunostained for CD31^{+} vascularity (**c**) and CD68^{+} macrophage infiltration (**d**). Scale bars, 20 μm. (**e**) Active caspase-3 staining in mice treated with the NPY2R antagonist and a 'crown structure' (left, arrow). Scale bar, 20 μm. The relative number of small (<10 μm) versus large (>10 μm) adipocytes in stress and HFS-fed mice compared to non-stressed standard chow-fed controls (right). (**f**) C57BL/6J mice after 3 months of HFS-feeding compared with non-stressed HFS-fed mice, mice exposed to cold alone, *Npy2r*$^{-/-}$ mice given HFS and exposed to stress, and standard chow-fed controls. White arrow shows pericardial fat absent in non-stressed HFS-fed mice. (**g**) Liver and skeletal muscle steatosis in C57BL/6J mice which are reduced by treatment with an NPY2R antagonist. Scale bars, 20 μm. All data represents the mean ± s.e.m. of $n = 6$ C57BL/6J and 6 129X1/SvJ mice/group, 3 sections per animal, 6 measurements per section. *$P < 0.05$ compared to control mice in **c**–**g** by Student's *t*-test, and in **b** compared to control (* $P < 0.05$) or to HFS + stress-treated mice (# $P < 0.05$) by one-way ANOVA with Bonferroni's multiple *t*-test.

Stress-induced metabolic syndrome requires NPY2R

Three months of feeding with an HFS diet induced obesity in C57BL/6J and 129X1/SvJ mice (**Fig. 3f**). Daily cold stress augmented this DIO, inducing gross abdominal obesity (**Fig. 3f**), liver and skeletal muscle steatosis (**Fig. 3g**), impaired glucose tolerance, hyperlipidemia (**Supplementary Fig. 2** online), hypertension (data not shown) and increased plasma concentrations of insulin, leptin and resistin (**Supplementary Fig. 2**), indicating the development of metabolic syndrome-like symptoms. Mice treated with an NPY2R antagonist (1 μM/d for 3 months by subcutaneous injection into the abdominal WAT), or germline *Npy2r*$^{-/-}$ mice, were resistant to stress-induced exacerbation of DIO and metabolic-like syndrome (**Fig. 3f**), and had improved glucose tolerance (**Supplementary Fig. 2**) and markedly reduced liver and muscle steatosis (**Fig. 3g**). These changes occurred without the NPY2R antagonist affecting the levels of corticosterone (**Fig. 4a**) and catecholamines (**Fig. 4b**) in the fat, the two systems that stimulate[23] and inhibit[24] fat growth, respectively, raising the question of how the NPY2R system acts on the fat to exacerbate DIO.

Mechanisms of stress-induced augmentation of DIO

Both stress and diet exert multiple hormonal and metabolic effects. Glucocorticoids (cortisol in humans and corticosterone in rodents) mediate stress and also contribute to abdominal obesity, as seen in Cushing's syndrome. In humans, however, chronic stress or DIO is rarely associated with hypercortisolemia[20]—raising questions as to the role of circulating glucocorticoids in these conditions. Similarly, we found that plasma corticosterone levels, although elevated by acute stress, normalized after 2 weeks of daily stress, or when stress was combined with an HFS diet and led to the development of abdominal obesity. Similarly, we found that plasma corticosterone levels were normal in chronically stressed, HFS-fed mice (**Fig. 4a**). However, corticosterone concentration was elevated in the abdominal WAT of these mice (**Fig. 4a**) in parallel with stress-induced upregulation of 11β-hydroxysteroid dehydrogenase-1 (Hsd11b1) expression (**Fig. 4a**). This enzyme, which is highly expressed in visceral fat[25], converts inactive cortisone to cortisol and has been implicated in abdominal obesity[25].

Catecholamines are also stress mediators that impinge on the fat. Stress in HFS-fed mice did not alter norepinephrine in the plasma, but lowered its levels in the fat (**Fig. 4b**). Plasma epinephrine was decreased by an HFS diet, whereas its fat concentration remained unchanged (**Fig. 4b**). These data indicate that chronic stress and an

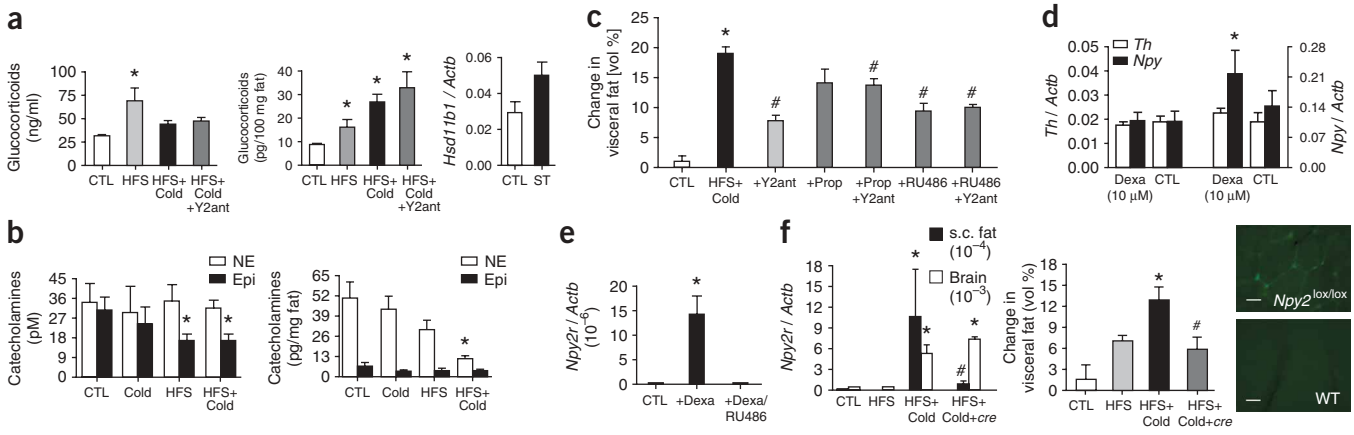

Figure 4 Neurohumoral mechanisms of stress-induced exacerbation of DIO by NPY and NPY2R. (**a**) Glucocorticoids in plasma (left) and in the fat (middle). *Hsd11b1* mRNA in subcutaneous abdominal fat of mice exposed to cold-stress compared to non-stressed control mice (right). (**b**) Norepinephrine (NE) and epinephrine (Epi) levels in plasma (left) and in the fat (right) in cold-stressed, HFS-fed mice, and cold and HFS-fed mice compared to non-stressed standard chow-fed controls. Results represent average values ± s.e.m. from $n = 6$ 129X1/SvJ mice. * $P < 0.05$ by one-way ANOVA compared to controls. (**c**) MRI measurements of visceral fat induced by an HFS diet and cold stress and treated with NPY2R antagonist, RU486, and propranolol (Prop). * $P < 0.05$ by one-way ANOVA compared to control, and # $P < 0.05$ by one-way ANOVA with Bonferroni's multiple *t*-test compared to HFS + cold mice. (**d**) *Npy* versus tyrosine hydroxylase mRNA of dexamethasone-treated (Dexa; 10 μM) sympatho-neural cells (**e**) *Npy2r* mRNA of sympatho-neural cells treated with dexamethasone (Dexa; 10 μM) and Dexa with RU486 (10 μM). $n = 6$ wells per experiment, repeated 3 times, * $P < 0.05$ compared to controls by unpaired *t*-test. (**f**) Changes in *Npy2r* mRNA levels in *Npy2r*$^{lox/lox}$ mice after local injections of GFP-labeled adenoviral vector pAdTrack-*Cre* into the subcutaneous abdominal fat and in the fat volume. GFP expression in the subcutaneous abdominal fat is visualized by fluorescent microscopy. Scale bars, 30 μm. All data represent the mean ± s.e.m. of $n = 6$ mice/group. Comparisons: *$P < 0.001$ compared to control; #$P < 0.001$ for +Y2ant, $P < 0.01$ for +RU486 and +RU486+Y2ant, $P < 0.05$ for +prop+Y2ant and HFS+cold+*Cre* compared to respective HFS + cold-treated mice, by one-way ANOVA with Bonferroni's multiple *t*-test.

HFS diet decrease β-adrenergic activity, which might promote obesity, as seen in mice lacking β-adrenoceptors[26].

To test whether inhibition of β-adrenergic lipolysis augments DIO, we pretreated stressed, HFS-fed mice with propranolol (2 mg/kg/d for 14 d, intra-fat injections), a non-selective β-adrenoceptor antagonist. Surprisingly, propranolol had no effect on its own, nor did it alter the NPY2R antagonist–induced reduction in fat deposits (**Fig. 4c**). This indicates that if decreased β-adrenergic activity contributes to DIO, it is offset by other effects.

To investigate whether corticosterone mediates stress-induced augmentation of DIO, we inhibited glucocorticoid receptors with RU486 (intra-fat injections, 25 mg/kg/d for 5 d). RU486, alone or combined with the NPY2R antagonist, similarly reduced fat accumulation in stressed, HFS-fed mice (**Fig. 4c**). The lack of synergism between NPY2R and either glucocorticoids or β-adrenergic activity indicated that these factors converge on the same pathway (alternatively, a 'floor' effect that limits the overall level of fat reduction could account for the apparent lack of synergism). In order to better understand the interactions between the two sympathetic co-transmitters and glucocorticoids in WAT, we studied these pathways *in vitro*.

When we treated cultured sympathetic neural cells with dexamethasone (10 μM), but not isoproterenol, a β-adrenergic agonist (not shown), the expression of *Npy* and *Npy2r* more than doubled, whereas the expression of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis, was not changed. (**Fig. 4d,e**).

WAT-derived NPY mediates stress-induced exacerbation of DIO

To determine whether regional activation of NPY and NPY2R in the abdominal WAT is the final pathway that is activated by stress, we induced a local conditional knockout of *Npy2r*. To reduce *Npy2r* expression in all cells suspected to be involved in the NPY response (endothelial cells, adipocytes, macrophages and sympathetic neurons), we injected *Npy2r*$^{lox/lox}$ mice with an adenoviral construct expressing *Cre*-recombinase under a CMV promoter (pAdTrack-*Cre*) into the subcutaneous abdominal WAT (**Fig. 4f**, **Supplementary Fig. 2**). After 1 week, *Npy2r* expression decreased by 80% in adipocytes and endothelial cells, specifically in the subcutaneous abdominal fat but not in the skeletal muscles or the brain (**Fig. 4f**). This local knockdown of *Npy2r* reduced stress-induced visceral fat by 50% in 2 weeks (**Fig. 4f**).

DISCUSSION

This study has shown, for the first time, the importance of the sympathetic neural transmitter NPY in adipose tissue remodeling and its role in stress-induced augmentation of DIO and metabolic syndrome. So far, stress has been linked to obesity mainly through hypothalamic effects on food intake or peripherally through β-adrenergic[27], glucocorticoid[28] or parasympathetic activity[29]. However, the roles of these factors in chronic stress and human DIO remain uncertain[30]. By contrast, the adrenergic co-transmitter NPY is released primarily in conditions of prolonged activation of the sympathetic nerves and stress, both in mice (this study), rats [14–17,19,31] and in humans[18].

In our study, cold exposure not only increased circulating NPY but, when combined with an HFS diet, markedly upregulated expression of the NPY-NPY2R-DPP4 system in the abdominal WAT. This led to abdominal obesity within two weeks and to a metabolic syndrome-like condition after three months. Initially, we considered increased adiposity as an adaptation to cold, considering the implication of NPY in hibernation[32]. However, aggressor stress exerts similar amplifying effects on DIO, extending our conclusions to another, more socially relevant stress. This implies that chronic physical or psychosocial stressors to which animals do not easily habituate lead to upregulation of the NPY system and augmentation of DIO.

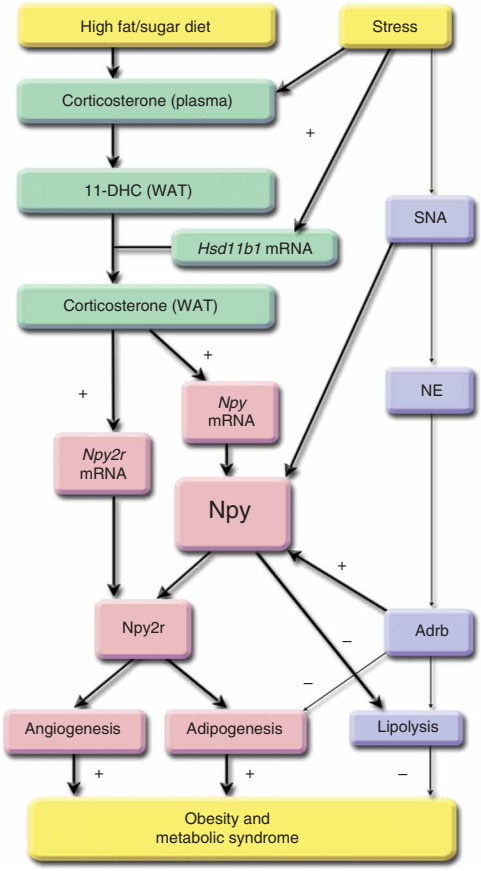

Figure 5 Proposed mechanisms of stress-induced exacerbation of abdominal DIO by activation of the adipose tissue NPY-NPY2R pathway (based on current and other data, as indicated). Stress stimulates release of NPY and norepinephrine from the sympathetic nerves and secretion of corticosterone from the adrenal gland. Additionally, stress activates *Hsd11b1* expression in the WAT. HFS diet, like stress, stimulates corticosterone secretion, increasing plasma corticosterone levels. Both stress and HFS diet elevate glucocorticoids in the visceral fat, which in turn upregulates adipose tissue expression of NPY and NPY2R on endothelial cells and adipocytes. More NPY is available for stress-induced release from the sympathetic nerves in the WAT. This then acts on upregulated NPY2R, leading to angiogenesis and adipogenesis, and also, via unknown receptors, inhibits β-adrenergic lipolysis[4]. Stress and HFS diet also hyperactivate the adrenergic system, which in the long run, depletes norepinephrine in the adipose tissue, decreasing adipocyte β-adrenoceptor stimulation and promoting obesity (reduced lipolysis[8] and anti-adipogenic actions[4]), but also opposes obesity through presynaptic β-adrenoceptors[45] on the sympathetic nerves, which decreases NPY release. Prolonged activation of NPY-NPY2Rs in adipocytes and endothelial cells leads to abdominal obesity and metabolic syndrome by increasing the growth of abdominal adipose tissue directly, by stimulating its adipogenesis, and indirectly, by augmenting angiogenesis. 11-DHC, 11-dehydrocorticosterone.

In humans, stress-induced increases in NPY are particularly high in individuals with an NPY signal peptide gene polymorphism[33]. In Northern Europeans, this common mutation is associated with a greater incidence of atherosclerosis, obesity and diabetic retinopathy[12], indicating that NPY is involved in metabolic diseases. We propose that stress-induced and genetically modulated increases in NPY raise the risk for DIO and metabolic syndrome by upregulating NPY and NPY2R in the abdominal fat (**Fig. 5**).

The NPY-NPY2R system acts indirectly through angiogenesis, regulating endothelial cell proliferation and apoptosis, and directly, by modulating preadipocyte proliferation, adipocyte apoptosis and differentiation. The role of angiogenesis in stimulating fat growth is established[34,35] and has already led to anti-angiogenic drugs being proposed as a therapy for obesity[34,35]. Our study, however, has revealed an endogenous pathway by which angiogenesis and adipogenesis are coordinated during fat remodeling, when stimulated by stress and an HF diet.

In the long term, stress and an HFS diet lead to gross obesity (**Fig. 3f**) and symptoms of metabolic syndrome: glucose intolerance, hyperlipidemia, hyperinsulinemia (**Supplementary Fig. 2**), liver and skeletal muscle steatosis (**Fig. 3g**), and hypertension (not shown). Remarkably, most of these symptoms are greatly reduced by inhibition of NPY2R within the fat. Although exact mechanisms of the beneficial metabolic effects of blocking NPY2R in the abdominal WAT still needs to be determined, they occurred in the presence of increased thermogenesis and without any discernible effects on food intake, which remained as increased as it was in untreated HFS-fed stressed mice (**Supplementary Fig. 1**). This underscores the contribution of the abdominal fat and its NPY-NPY2R pathway to overall regulation of body's metabolism and development of the metabolic syndrome.

Glucocorticoids are also important in stress-mediated exacerbation of DIO, and our data suggests that they do so by acting as an upstream modulator of NPY-NPY2R signaling. An HFS diet increases plasma corticosterone levels, and stress increases the conversion of this inactive steroid to an active form by upregulating Hsd11b1 activity specifically in the abdominal fat. The role of NPY2R as the pathway downstream from glucocorticoids is supported by the effect of the NPY2R antagonist on corticosterone levels in the WAT (**Fig. 4a**). The NPY2R antagonist, which blocks the effect of stress on DIO (**Fig. 4a**), elevates rather than reduces steroid concentration, contrary to what would have been expected if glucocorticoids were the final mediators of fat remodeling. Glucocorticoids also upregulate *Npy* and *Npy2r* expression and increase Npy concentration, particularly in the abdominal fat (**Fig. 5**). Similar glucocorticoid-mediated *NPY* expression has been found in many cells, including sympathetic nerves[36], and during stress[37]. As the synthesis of catecholamines is not upregulated and their concentration in fat decreases during chronic stress and HFS diet, this shifts the balance between the two neurotransmitters away from the lipolytic β-adrenergic system, to the adipogenic NPY-NPY2R pathway (**Fig. 5**).

Although the cells that are responsible for increased NPY production in the fat have not been identified, it is possible that in addition to sympathetic nerves, macrophages might also be involved. We[38] and others[39] have observed the induction of *NPY* expression in immune cells by antigens[39] or stress[15], and suggested that it contributes to inflammation[40] and vessel remodeling[38]. Visceral obesity in both mice and humans has been associated with the infiltration of fat tissue by macrophages and increased production of inflammatory cytokines and adipokines[41,42]. In our studies, stress and exogenous NPY increased the release of the inflammatory adipokine resistin. Resistin has been implicated in glucose intolerance and insulin resistance[43], and is a plausible secondary mediator of the metabolic abnormalities that develop after 3 months of stress and an HFS diet.

The role of β-adrenergic activity in stress-induced augmentation of DIO seems to be indirect. The depletion of norepinephrine in the abdominal WAT indicates downregulation of the system (decreased release and receptor desensitization)[44] owing to prolonged stress and an HFS diet (**Fig. 5**). The loss of this fat-burning mechanism could promote obesity. However, decreased β-adrenergic signaling might also oppose obesity by reducing NPY release, regulated by presynaptic facilitatory β-receptors (**Fig. 5**), as shown in other preparations[45].

Thus, glucocorticoids and the adrenergic system seem to converge on the NPY-NPY2R pathway. This might explain the lack of synergism between these receptor antagonists and the NPY2R blocker, although a floor effect might also mask any further benefits of combining these antagonists.

The importance of adipose-tissue-derived NPY-NPY2R as the final pathway in stress-induced amplification of DIO is demonstrated by pharmacological and genetic NPY2R inactivation. The stress effect was absent not only in mice treated with local administration of an NPY2R antagonist and in germline *Npy2r*$^{-/-}$ mice, but also in mice with regional fat-targeted *Npy2r* knockdown (**Figs. 3**,**4f**). Several lines of evidence support the relevance of our findings to humans. First, human WAT expresses *NPY2R*, which is angiogenic and adipogenic, as shown in the model of human fat xenografts in nude mice (**Fig. 2e**). Second, a common silent *NPY2R* gene variant recently found in the Swedish population seems to protect it against obesity[46], whereas the gain-of-function Leu7Pro7 *NPY* gene polymorphism is associated with human obesity[47]. Finally, our preliminary studies indicate that the NPY-NPY2R adipogenic system also operates in non-human primates (data not shown). The effectiveness of NPY2R blockade in causing fat loss opens new possibilities for the treatment and prevention of obesity and metabolic syndrome using NPY2R-selective antagonists in overweight patients. Fat-targeted blockade of the NPY-NPY2R pathway could also complement anti-obesity therapy with centrally acting appetite suppressants. In addition, NPY-NPY2R agonists could potentially enhance the retention of autologous fat grafts, and NPY2R antagonists might be useful for nonsurgical localized elimination of fat. Although the popularity of injecting compounds for fat-melting (pharmacologic lipolysis) or augmentation has been increasing[48], there are currently no scientifically sound compounds that have been approved for use in humans for fat remodeling. Our study provides support for NPY-NPY2R-based drugs as candidates for such 'liporemodeling'.

Finally, our findings provide evidence that stress is not 'just in the mind' but rather affects body weight and metabolism by activating neurogenic angiogenesis and fat remodeling through an NPY-NPY2R-dependent pathway that is localized in the WAT.

METHODS

Proliferation assay. We cultured 3T3-L1 preadipocyte cells in DMEM (10% FCS), d-biotin (8 μg/ml) and pantothenate (8 μg/ml). SK-N-BE(2) neuroblastoma cells (ATCC) were cultured in EMEM/F12K (10% FBS). HMVEC endothelial cells (Cambrex) were cultured in EBM2 (Cambrex). We treated cells with NPY (10^{-14} to 10^{-8} M), the NPY2R antagonist BIIE0246 (1 μM), dexamethasone (10 μM), neuroblastoma-conditioned medium (24 h incubation with 70% confluent SK-N-BE(2) cells), or combinations of these treatments. We treated cells for 24 h in low serum medium (0.25% FBS); 4 h after treatment we added 0.5 μCi [^{3}H]thymidine to each well. We harvested cells after 24 h in a 96-well harvester (Tomtec) and counted them in a Betaplate Liquid Scintillation Counter (Wallac).

Co-culture. We performed co-culture in a Costar Transwell system. We cultured cells on plates or 0.4 μm polyester membrane inserts. We combined inserts with wells containing other cells at 30% confluence. After treatment, we harvested the cells for RNA isolation. For the mitogenic assay, we first growth-arrested the cells in serum-free medium for 24 h, and then placed inserts into the plates and treated them with the desired factors in 0.25% FBS. After 48 h, we counted the cells with a Beckman Coulter Counter. In both co-culture models, we harvested the medium for leptin (R&D Systems), resistin (R&D Systems) and NPY (Bachem) ELISA.

Quantitative real-time RT-PCR. We isolated RNA using Tri Reagent (Sigma) and synthesized cDNA using the iScript cDNA synthesis kit (Bio-Rad). We performed quantitative real-time RT-PCR using the ICycler iQ Detection System (Bio-Rad). We amplified cDNA for 40 cycles using the TaqMan PCR Reagent Kit and pre-designed primers and fluorescein-labeled probes from Applied Biosystems for human NPY, NPY1R, NPY2R, NPY5R and DPP4, as described[13], according to the manufacturer's procedure. We analyzed results using software provided by Bio-Rad and we calculated expression levels by the comparative C_T method using *Actb* as an endogenous reference gene, according to Applied Biosystems' ABI PRISM 7700 User Bulletin no. 2.

Apoptosis assays. We performed a TUNEL reaction using an *in situ* Cell Detection Kit (Roche Diagnostic) and converted the signal to visible light using an AP-converter. We measured the density of TUNEL-positive cells using NIH ImageJ software. We confirmed TUNEL results with immunohistochemistry for active caspase-3 (Abcam).

Plasma composition. We collected blood samples from the abdominal vena cava of anesthetized mice into EDTA- or heparin-treated tubes. We centrifuged the samples to separate platelet-rich and platelet-poor fractions, as described[38]. We assayed plasma for NPY (Bachem Laboratories: S-1145), leptin, corticosterone, resistin (R&D systems: DY498, DE3600, MRSN00), insulin and adiponectin (Linco Research: EZRMI-13K, EZMADP-60K) using commercially available kits and performed the assays according to the manufacturers' procedures. We quantified plasma levels of leptin, insulin and resistin using a Mouse Adipokine LINCOplex kit (Linco: MADPK-71K), measured plasma lipids by Penn Medical Laboratories, measured glucose using a FreeStyle Thermosense portable glucometer and measured plasma norepinephrine and epinephrine by radioenzymatic assay[19].

Tissue composition. We extracted corticosterone from snap-frozen fat using methanol extraction as suggested by R&D Systems. We added [H^3]testosterone (1,500 c.p.m.) before the extraction to calculate percent yield. We extracted catecholamines in the fat by sonication in ice-chilled 0.1 M perchloric acid (PCA) and assayed for norepinephrine and epinephrine using a single-isotope radioenzymatic, thin-layer chromatographic procedure as described[19,37].

Immunohistochemistry. We either postfixed tissues with 10% formaldehyde and paraffin-embedded them or embedded the tissues in tissue-freezing medium and flash-froze them in liquid nitrogen for frozen sections. We performed immunostaining using the following primary antibodies: rabbit polyclonal antibody to NPY (Bachem Laboratories) and mouse monoclonal antibody to tyrosine hydroxylase (Immunostar), rabbit antibody to NPY2R (gift from AstraZeneca), goat antibody to DPP4/CD26 (R&D systems), rat antibody to mouse CD31/Pecam1 (BD Pharmingen), mouse monoclonal antibody to CD31/Pecam1 (Abcam), rabbit antibody to human vWF (DAKO), mouse antibody to human CD68 (DAKO), rabbit polyclonal antibody to mouse Ki67 (Abcam), and rabbit polyclonal antibody to active caspase-3 (Abcam). We verified the specificity of the antibodies by doing control experiments in parallel but omitting the primary antibody (data not shown). We tested the specificity of the NPY2R antibody in tissues from *Npy2r*$^{-/-}$ mice (data not shown). Images were captured on a Nikon eclipse E 600 microscope.

Use of animals and human samples. All use of animals and human tissue samples in this study was approved by and in accordance with the Georgetown University Animal Care and Use Committee (GUACUC) which is also accredited by the Association for Assessment and Accreditation of Laboratory Care (AAALAC), and the Institutional Review Board at the Georgetown University Medical Center and Hospital, respectively.

Animals. We used male 6–8-week-old C57BL/6J, 129X1/SvJ, B6.V-*Lep*^{ob}/J (from JAX Laboratories), C57/129Sv (bred in house), athymic nude (Taconic), *Npy2r*$^{-/-}$/129Sv (AstraZeneca[49]) and *Npy2r*$^{lox/lox}$/ C57/129Sv mice (Garvan Institute[7]).

Human fat implantation. We harvested human fat from lipoaspiration procedures and loaded the fat into 1-ml syringes. We anesthetized athymic nude mice (Taconic) and implanted slow-release pellets (Innovative Research of America) subcutaneously. We injected 100 μl of fat close to the pellets using a 16-gauge Coleman infiltration cannula (Byron Medical) attached to the syringe. We closed the skin wound with medical cyanoacrylate.

MRI. We used a Bruker 7-Tesla small-animal magnetic resonance imaging system with a 12-cm radiofrequency coil to visualize and non-invasively quantify various fat deposits. We optimized and implemented a 3D imaging protocol for high-contrast fat imaging. More specifically, we used a 3D rapid acquisition with relaxation enhancement (RARE) imaging sequence: TE 5.9, TR 200, RARE factor 8, flip angle 60, matrix 256 × 128 × 128, 7 × 3x × 3 cm to 9 × 3 × 5 cm (cranial-caudal AP LR). Using this protocol, we produced a 3D-reconstructed image using a maximum intensity projection algorithm with an intensity threshold that shows fat as the brightest signal and disregards signals from other tissues. We calculated the total body fat and separated specific fat deposits using thresholding and voxel count plug-ins from NIH ImageJ software and a VolumeJ plug-in to create three-dimensional fat images. We used an animal management system in conjunction with the MRI to record core, skin, ambient and water-blanket temperature measurements for monitoring during imaging. We maintained the core temperature of the animal during imaging and anesthesia induction using a water blanket maintained at 37 °C.

Oil Red-O staining. We stained lipid droplets in mature adipocytes and 4% paraformaldehyde-fixed cells using an Oil Red-O solution (0.36% Oil Red-O solution in 60% isopropanol, Chemicon Adipogenesis Assay kit). We used Oil Red-O in propylene glycol (Newcomer Supply) to stain lipids in liver and muscle frozen sections.

High fat diet. We fed mice either a standard chow (SC) diet—28% protein, 60% carbohydrate (4% sucrose), 12% fat (kcal%; 4 kcal/g)—or a high fat and high sugar (HFS) diet—20% protein, 35% carbohydrate (17% sucrose), 45% fat (kcal%; 4.7 kcal/g) from Research Diets, Inc. (D12451) for between 14 d and 3 months.

Stress. We carried out cold stress as described[16]. Briefly, we placed mice in 0.5 cm ice-cold water for 1 h per day for between 14 d and 3 months. Restraint stress was carried out using an approved animal holder to restrain the mouse for 1 h and then they were returned to their home cages. In water avoidance stress, we placed mice on a 10 cm post stably positioned on the cage floor and surrounded by room temperature water deep enough for the mouse to swim in for 1 h daily for 2 weeks. Aggressor stress (also known as social-defeat stress) involved an aggressor mouse (an alpha-mouse), which was first placed in an empty clean cage and was allowed to establish its territory. A naive mouse (passive mouse) was then placed in the cage with the alpha-mouse for 10 min daily for 2 weeks. All stress tests were done daily between 9:00 and 11:00 a.m. We returned stressed mice to their home cages with free access to food and water, and they showed no signs of difficulty in grooming or eating. Control, non-stressed mice were kept in a separate room, in the same animal facility.

Glucose tolerance test. We injected unanesthetized mice (JAX Laboratories) intraperitoneally after an overnight 17 h fast with 1.5 g of 50% glucose solution per kg of body weight. We obtained blood samples from the tail vein 30 min before the glucose challenge and then again 0, 30, 60 and 90 min after the glucose challenge. We measured blood glucose concentrations with a FreeStyle portable glucose meter (TheraSense).

Conditional knockout mice. We amplified and determined the titer of adenoviral vector pAdTrack-Cre using 293 cells and the standard methods recommended for the AdEasy system. $Npy2r^{lox/lox}$ mice were injected with 100 µl of viral supernatant in sterile PBS (approximately 3×10^7 infectious particles) subcutaneously into the abdominal fat pads of $Npy2r^{lox/lox}$ or wild-type mice. Adenovirus was labeled with green fluorescent protein and the local injection of virus to the fat pad was visualized by fluorescence microscopy.

Statistical analysis. We analyzed the effect of treatment (NPY, HFS diet, stress or antagonists) as compared to respective controls in MRI, ELISA and *in vitro* studies using a one-way ANOVA with Bonferroni's multiple *t*-test to compare between treatment groups, with $P \leq 0.05$ considered statistically significant. We analyzed the effect of antagonist versus saline in vWf staining by one-way repeated measures ANOVA with post-hoc Dunnett's test, with $P < 0.05$ considered statistically significant. We used unpaired Student *t*-tests to analyze the effects of a single treatment compared to control in immunohistochemistry and qRT-PCR, with $P < 0.05$ considered statistically significant. All analyses were performed with Prism 4.03 (GraphPad Software).

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

L.E.K. carried out most of the experiments (as her PhD thesis work); developed stress models; established adipocyte-endothelial-neuronal co-cultures; together with S.T.F., adapted the MRI technique to the assessment of fat volumes; prepared most of the figures; and wrote major parts of the manuscript. J.U.T. contributed to many experiments using stress models, and assisted with harvesting tissues and adenoviral vectors. J.B.K. designed primers for RT-PCR; carried out molecular analyses; assisted with adipocyte-endothelial-neuronal co-cultures; and made major contributions to experimental design, data analyses, interpretation and presentation, and manuscript writing. L.L. performed NPY ELISA measurements, developed and carried out immunocytochemistry protocols, and contributed to data analyses and interpretation. S.B.B. provided human fat tissue derived from plastic surgeries that he had performed, together with M.D.J.; established a xenograft model of human fat growth in nude mice; and made major conceptual contribution to the clinical significance of the study for fat grafting and remodeling. M.D.J. contributed to all aspects dealing with human fat, and was instrumental in the adenoviral work by providing the vectors, measuring viral titers and training L.E.K. in experimental techniques. E.W.L. started the project and carried out experiments on genetically obese B6.V-Lep^{ob}/J mice. M.S.B. carried out resistin analyses and contributed to discussions of stress effects on inflammation and metabolic syndrome. H.H. developed and provided the $Npy^{-/-}$ and $Npy2r^{lox/lox}$ mice for the study, trained J.U.T. in preparing adenoviral vectors, and contributed to discussion of the results. S.T.F. developed the MRI protocol for analyzing fat and contributed to discussion of the results. R.K. supervised catecholamine assays and contributed to analyses, interpretation and discussion of the data dealing with glucocorticoids and adrenergic system. Z.Z. developed the idea for and supervised the study, designed protocols, developed collaborations and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/naturemedicine.

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Corrigenda: Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome

Lydia E Kuo, Joanna B Kitlinska, Jason U Tilan, Lijun Li, Stephen B Baker, Michael D Johnson, Edward W Lee, Mary Susan Burnett, Stanley T Fricke, Richard Kvetnansky, Herbert Herzog & Zofia Zukowska

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The version of this article initially published contained several typographical errors affecting figure citations, units of measure and figure legends, none of which change the scientific conclusions of the manuscript in any way. In addition, the authors incorrectly stated that they had no competing financial interests. A proper description of these competing interests, as is required by journal policy, has now been attached to the HTML version of the article, and the typographical errors have been corrected in the HTML and PDF versions.