

OBESITY

Cleavage of the leptin receptor by matrix metalloproteinase–2 promotes leptin resistance and obesity in mice

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Obesity and related morbidities pose a major health threat. Obesity is associated with increased blood concentrations of the anorexigenic hormone leptin; however, obese individuals are resistant to its anorexigenic effects. We examined the phenomenon of reduced leptin signaling in a high-fat diet–induced obesity model in mice. Obesity promoted matrix metalloproteinase–2 (Mmp-2) activation in the hypothalamus, which cleaved the leptin receptor's extracellular domain and impaired leptin-mediated signaling. Deletion of Mmp-2 restored leptin receptor expression and reduced circulating leptin concentrations in obese mice. Lentiviral delivery of short hairpin RNA to silence *Mmp-2* in the hypothalamus of wild-type mice prevented leptin receptor cleavage and reduced fat accumulation. In contrast, lentiviral delivery of *Mmp-2* in the hypothalamus of *Mmp-2*^{-/-} mice promoted leptin receptor cleavage and higher body weight. In a genetic mouse model of obesity, transduction of cleavage-resistant leptin receptor in the hypothalamus reduced the rate of weight gain compared to uninfected mice or mice infected with the wild-type receptor. Immunofluorescence analysis showed that astrocytes and agouti-related peptide neurons were responsible for Mmp-2 secretion in mice fed a high-fat diet. These results suggest a mechanism for leptin resistance through activation of Mmp-2 and subsequent cleavage of the extracellular domain of the leptin receptor.

INTRODUCTION

Obesity is a worldwide health problem with increasing incidence (1). Excessive fat accumulation is associated with diseases such as hypertension, type 2 diabetes, and atherosclerosis, leading to increased morbidity and mortality from cardiovascular complications (1). Despite enormous efforts targeting treatments for obesity, current dietary treatment, behavioral treatment, and pharmacotherapies have limited efficacy (1).

Leptin, a hormone that governs energy expenditure, is predominantly secreted from white adipose tissue and acts in the central nervous system as a negative feedback signal to regulate appetite and metabolism by activating its receptor (ObR) on target cells (2, 3). The ObR has six isoforms (ObRa to ObRf) with an identical extracellular domain (2), and previous studies showed that ObRb is required for the weight-reducing effects induced by leptin (2). Activation of ObR by leptin induces phosphorylation of the receptor and activates downstream signaling proteins such as extracellular signal–regulated kinase (ERK1/2) and signal transducer and activator of transcription 3 (STAT-3). In addition, ObR phosphorylation activates suppressor of cytokine signaling (SOCS3), which reduces ObRb signaling and acts as a negative feedback (4–8).

High-fat diet (HFD)–induced obesity in rodents and in humans is associated with high circulating leptin concentrations concomitantly with reduced leptin receptor signaling (designated as “leptin resistance”)

(3, 4). Resistance to the anorexigenic effects of leptin is demonstrated by an impaired ability to reduce body weight and food intake after exogenous leptin administration (4, 7). Hyperphagia and increased adipose mass in the presence of hyperleptinemia suggest resistance to endogenous leptin (2). In mice, hypothalamic inflammation, glucose dysregulation, and leptin resistance manifest themselves after a short period of unrestricted access to HFD (4–13). Similarly, obesity in humans is characterized by hyperleptinemia and diminished anorexigenic effects in response to exogenous leptin (6), with most cases not attributed to genetic defects in leptin or its receptors (4, 6, 7). Mechanisms that could be responsible for obesity-associated leptin resistance include impaired transport of leptin across the blood-brain barrier and/or impaired leptin signaling in target neurons and downstream signaling in target cells. A previous report showed that impaired leptin transport is acquired during the development of obesity, as a secondary defect (14). Consistent with this notion, the anorexigenic effect of leptin is reduced in obese mice after intracerebroventricular (ICV) administration of the hormone (15). The mechanisms leading to leptin resistance and defective leptin receptor signaling in obesity have not been fully identified. Chronic low-grade inflammation, both systemically and centrally in the hypothalamus, has been linked to the genesis of obesity and suggested to contribute to leptin resistance and glucose disorder, by activation of the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) pathway, induction of SOCS3 and endoplasmic reticulum (ER) stress (4, 5, 7, 9–13). Among other effects, activation of inflammatory pathways leads to activation of proteases (16, 17). Among these, matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that play a role in vascular remodeling, cellular migration, and the processing of extracellular matrix proteins and adhesion molecules (16). Evidence suggests the involvement of MMPs in obesity and inhibition of gelatinases, namely, Mmp-2 and Mmp-9, has been shown to reduce weight gain upon HFD intake (17, 18).

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We tested the hypothesis that HFD-induced obesity elicits the production and activation of specific MMPs within the hypothalamus that in turn might cleave key receptors governing food intake, thus contributing to the pathogenesis of obesity. Our findings show that HFD promoted activation of Mmp-2 in the hypothalamus. Mmp-2 activation induced the cleavage of the extracellular domain of the leptin receptor, thereby impairing leptin-mediated ObR signaling. Using *Mmp-2* and ObR knockout mice and lentivirus delivery systems, we found that Mmp-2 activity in astrocytes and agouti-related protein (AgRP) neurons contributed to leptin resistance in mice. Collectively, our results provide insights into the development of leptin resistance in animal models of obesity.

RESULTS

Mmp-2 activation and leptin receptor cleavage during HFD administration

To elucidate the mechanisms responsible for leptin resistance, we first induced obesity in rats by administration of a HFD for 12 weeks. HFD induced a significant increase in body weight compared to control diet (CD) (HFD, 660 ± 25 g; CD, 510 ± 28 g; $P < 0.05$, $n = 9$;

fig. S1, A and B). Abdominal fat pad weights (bilateral body fat pads from two regions: abdominal and gonadal) were higher in the HFD group (fig. S1C). HFD-induced obesity was associated with increased serum leptin values compared to CD-fed animals (HFD, 56 ± 11 ng/ml; CD, 8 ± 4 ng/ml; $P < 0.001$; fig. S1D).

Total protease activity (serine, aspartic, and cysteine proteases and metalloproteinases) in brain lysate, as measured by fluorescently quenched substrate, was significantly higher in HFD rats compared to CD rats (fig. S2A). In situ zymography, using quenched gelatin as a substrate, indicated that gelatinases were major contributors to the protease activity (fig. S2B). Gel zymography yielded an Mmp-2 activity that was about 2.5 times higher in a HFD brain compared to a CD brain (Fig. 1, A and B). We confirmed this finding by using an Mmp-2/Mmp-9-specific activity assay that showed an almost two-fold increase in the activity of Mmp-2/Mmp-9 in HFD brain lysates compared to CD (fig. S2C). Because gel zymography and enzyme kinetic assays do not localize the Mmp activity, we performed in situ zymography specifically in the hypothalamus using a substrate specific for Mmp-2/Mmp-9. We found higher activity of Mmp-2 in HFD rats compared to CD rats (fig. S2D). Immunohistochemistry and Western blot (WB) analysis using a specific antibody against

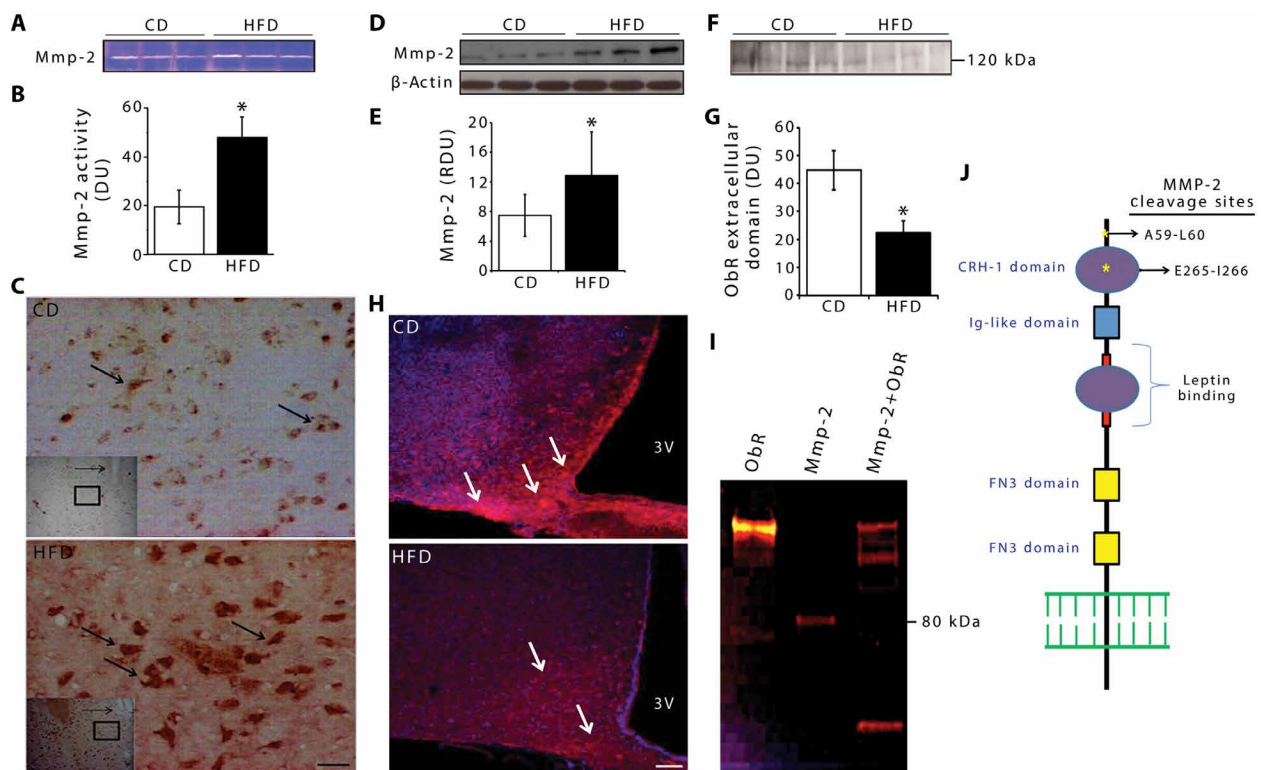


Fig. 1. Mmp-2 activity is increased in brains after HFD intake. (A) Representative gel zymography showing the activity of Mmp-2 in the brain of rats fed a HFD and a CD. (B) Values of the band density in (A) are presented as density units (DU). Data are shown as means \pm SD ($n = 8$ per group). (C) Representative sections of the hypothalamus of rats fed a CD and a HFD after immunolabeling with an antibody against the active form of Mmp-2. The insets show an overview, with black squares delineating the magnified fields. Arrows point to the Mmp-2-positive cells. (D) WB analysis of Mmp-2 in brain homogenates of rats fed a HFD and a CD. Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis, and Mmp-2 (active form) was detected. (E) Mmp-2 and β -actin band densities were determined, and measurements were presented as relative density units (RDU) of the Mmp-2/ β -actin ratio ($*P < 0.05$, HFD versus CD; data are shown as means \pm SD; $n = 9$ per group). (F) Representative WB showing ObR extracellular domain signal in brains of the CD and HFD groups. (G) Optical density measurements of the WB signal and data are presented as DU ($*P < 0.05$, CD versus HFD; data are shown as means \pm SD; $n = 8$). (H) IF imaging of the ObR extracellular domain in HFD and CD. White arrows point to positive staining in CD and HFD. (I) Silver stain of ObR (left), Mmp-2 (middle), and ObR incubated with Mmp-2 (right). (J) Extracellular domain of the leptin receptor. Cytokine receptor homology (CRH-2) is the main binding site for leptin. The immunoglobulin (Ig)-like and fibronectin type 3 (FN3) domains are essential for receptor signaling. Stars in the extracellular domain represent the cleavage sites determined by high-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS).

the active form of Mmp-2 independently confirmed that Mmp-2 was elevated and showed increased activity during HFD administration (Fig. 1, C to E).

Collectively, the results indicate that HFD promotes activation of Mmp-2 in the brain of HFD-fed rats. Although the activity of Mmp-2 induced by HFD is not restricted to the hypothalamus, we focused our studies on this region as it affects food intake through the action of the leptin receptor (4).

To determine the effect of proteolytic activity on the extracellular domain of the leptin receptor (ObR), we analyzed its values using WB analysis and digital immunofluorescence (IF). Antibody specificity against the ObR was confirmed using ObR knockout rats (Koletsky rats) (19) as a negative control (fig. S3, A and B) and ObR-transfected cells as a positive control using multiple methods (fig. S3C). WB analysis of brain homogenate membrane fractions showed that the ObR extracellular domain is reduced by about 50% in HFD compared to CD (Fig. 1, F and G), a result supported by IF (Fig. 1H).

The decreased values of the extracellular domain of the ObR together with increased Mmp-2 activity in the hypothalamus of HFD rats could be either a phenotypic response to HFD per se or a specific cleavage of ObR by Mmp-2 that is induced by HFD. We incubated the recombinant extracellular domain of ObR with active Mmp-2 and looked for the formation of ObR fragments. Protein staining showed the formation of distinct fragments (Fig. 1I). Using LC-MS/MS, we validated these fragments as cleavage products of the ObR extracellular domain. The identified peptides included several semi-trypsinic peptides that appeared only in Mmp-2-treated samples but not in ObR alone (Fig. 1I) and suggested two potential Mmp-2 cleavage sites in the extracellular receptor domain: E265-I266 (EAAE↔IVSA) and A59-L60 (NASA↔LKGA) (Fig. 1J and table S2).

To determine the ability of active Mmp-2 to cleave the extracellular domain of other receptors in the cytokine receptor family, we also tested in vivo the insulin receptor (InsR) and interleukin-6 receptor (IL-6R). Silver staining failed to show any cleavage products of the receptors after treatment with Mmp-2 (fig. S3D).

Mmp-2 attenuates leptin signaling through cleavage of the leptin receptor

To test whether Mmp-2 cleaves the ObR extracellular domain in living hypothalamic cells, we incubated ObR-transfected GT1-7 hypothalamic cells with active Mmp-2 and measured the signal intensity of the extracellular domain of the ObR on the cell membrane. The results showed reduced expression of the ObR extracellular domain after treatment with Mmp-2 by more than 20% ($P < 0.01$; Fig. 2, A and B). WB analysis of the incubation media detected an ObR fragment in the media of cells incubated with Mmp-2 but not in the controls (Fig. 2C).

Because members of the a disintegrin and metalloproteinase (ADAM) family may also cleave the extracellular domain of the ObR, we tested whether ADAM-17 (TACE) and ADAM-10 are activated by Mmp-2 and thus could generate ObR extracellular domain fragments found in the incubation media. CLU-172 mouse hypothalamic cells were incubated with active Mmp-2, and the activity of TACE and ADAM-10 was measured. We found that active Mmp-2 did not induce TACE or ADAM-10 activation (Fig. 2, D and E).

To evaluate the effect of Mmp-2 on leptin signaling, we treated the hypothalamic cells with Mmp-2 (10 ng/ml) for 30 min followed by incubation with leptin (0, 5, 15, and 30 min). ObRb, ERK, and STAT-3 phosphorylation at these time points was significantly at-

tenuated ($P < 0.05$), suggesting reduction of leptin signaling (Fig. 2, F to I). To assure that the proteolytic cleavage is not cell line-specific, we tested leptin signaling in another hypothalamic cell line (GT1-7). Mmp-2 treatment reduced the expression of the ObR extracellular domain and reduced ObRb, ERK, and STAT-3 phosphorylation (fig. S4, A to E).

To determine whether ObR cleavage was specific to Mmp-2, we repeated the above experiment using another gelatinase (Mmp-9). Whereas Mmp-2 reduced the expression of the ObR extracellular domain, incubation with Mmp-9 had no effect (fig. S5).

Mmp-2 is activated upon HFD intake, cleaves the leptin receptor, and contributes to leptin resistance in vivo

To determine the effect of total Mmp-2 depletion on HFD-induced obesity, we used Mmp-2-depleted (*Mmp-2*^{-/-}) mice. The lack of Mmp-2 expression was confirmed by gel zymography (fig. S6). After HFD intake, *Mmp-2*^{-/-} mice were smaller in size, had lower body weight, and reduced weight gain compared to their wild-type (WT) littermates used as control (Fig. 3, A and B). In addition, 24-hour food intake was significantly lower in *Mmp-2*^{-/-} mice compared to controls (1.8 ± 0.17 g and 2.3 ± 0.21 g, *Mmp-2*^{-/-} versus WT, respectively; $P < 0.05$). Similar to rats, WT mice fed a HFD had higher values of active MMP-2 in the brain (Fig. 3, C and D). The activity was confirmed by gel and in situ zymography and found to be located in several brain areas (cortex and amygdala), including the hypothalamus (fig. S7, A to C).

We next analyzed the expression of ObR in *Mmp-2*^{-/-} mice and their controls after HFD intake. WB analysis for the extracellular domain of the ObR showed decreased expression of the receptor in WT-HFD mice compared to controls, whereas this expression was unaffected in *Mmp-2*^{-/-} mice (Fig. 3, E and F). A representative IF labeling of the ObR extracellular domain is presented in Fig. 3G. In addition, while circulating leptin concentration was higher in HFD-fed WT compared to CD-fed WT, its concentration was lower in HFD-fed *Mmp-2*^{-/-} (Fig. 3H). In vivo leptin administration to WT mice fed a HFD resulted in lower values of pSTAT-3 in the hypothalamus compared to controls, suggesting reduced receptor signaling ($P < 0.05$). In contrast, HFD-fed *Mmp-2*^{-/-} mice showed normal STAT-3 phosphorylation (fig. S7D).

Hypothalamic Mmp-2 deletion restores leptin receptor expression and reduces weight gain

Our results so far suggest that Mmp-2 can cleave the leptin receptor and impair leptin-induced signaling. Next, we focused on Mmp-2 activity in relation to leptin signaling in the hypothalamus. We designed lentiviral vectors to deliver *Mmp-2* short hairpin RNA (shRNA) to the hypothalamus and inhibit Mmp-2 expression. All constructs were validated before in vivo experiments by WB, gelatin zymographies, and polymerase chain reaction (PCR; fig. S8, A to C). Experiments with green fluorescence protein (GFP)-tagged viruses confirmed the site of injection to the arcuate nucleus of the hypothalamus, although diffusion around the injection site caused some surrounding cells in the ventromedial hypothalamus to be also infected (fig. S8, D and E). Mice were infected at 8 weeks, and HFD was administered 2 days after infection for 12 weeks. WT mice fed a HFD gained more weight compared to mice with hypothalamic *Mmp-2* deletion (Fig. 4, A and B; $*P < 0.05$, *shMmp-2* arcuate nucleus (ARC) HFD versus WT HFD). Moreover, deletion of hypothalamic *Mmp-2* lowered leptin concentrations that were elevated in WT mice fed a HFD ($*P < 0.05$,

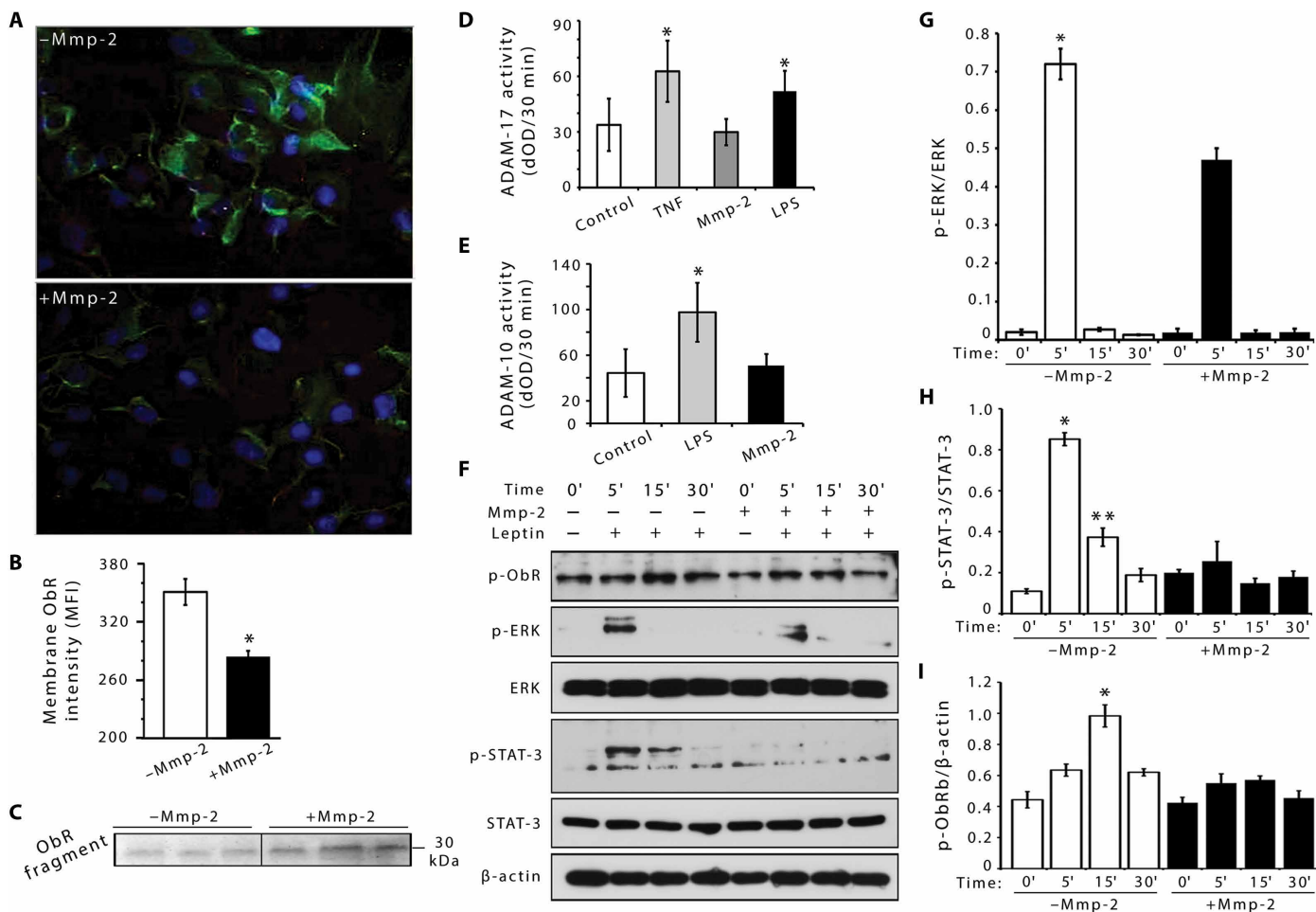


Fig. 2. ObR is cleaved and has reduced signaling capabilities after incubation with Mmp-2. (A) Representative immunolabeling of the ObR extracellular domain (green) in mouse hypothalamic cells exposed to Mmp-2. DAPI (4',6-diamidino-2-phenylindole; blue) shows cell nuclei. (B) Measurement of the ObR extracellular domain density on cells treated with/without Mmp-2 is presented as mean fluorescent intensity (MFI) \pm SD [$*P < 0.05$, cells treated with Mmp-2 (+Mmp-2) versus without Mmp-2 (–Mmp-2); $n = 6$]. (C) The incubation media of cells treated with/without MMP-2 analyzed for ObR fragments by WB. (D and E) Activation of ADAM-17 (D) and ADAM-10 (E) by Mmp-2 in hypothalamic cells. Activity was recorded for 30 min and presented as the difference in optical density (dOD \pm SD; $n = 6$). $*P < 0.05$. TNF, tumor necrosis factor; LPS, lipopolysaccharide. (F) Representative WB of p-ObR, p-STAT-3, p-ERK, and corresponding total protein in cells treated with Mmp-2 (last four lanes) and their respective controls (first four lanes). Leptin was added to the cells after Mmp-2 removal for selected time points (indicated in the figure). (G) p-ERK/ERK ratio in control and Mmp-2–treated cells after leptin stimulation. $*P < 0.05$, untreated versus Mmp-2–treated cells at $t = 5$ min ($n = 3$ in each group). (H) p-STAT-3/STAT-3 ratio in untreated and Mmp-2–treated cells after leptin stimulation. $*P < 0.05$, untreated versus Mmp-2–treated cells at $t = 5$ min; $**P < 0.005$, untreated versus Mmp-2–treated cells at $t = 15$ min ($n = 3$ in each group). (I) p-ObR/ β -actin ratio in untreated and Mmp-2–treated cells after leptin stimulation. $*P < 0.05$, untreated versus Mmp-2–treated cells at $t = 15$ min ($n = 3$ in each group).

shMMP-2-HFD versus WT-HFD; Fig. 4C). In control mice, weight gain and leptin values were comparable to WT mice on a HFD (Fig. 4, A to C). IF analysis of Mmp-2 and ObR in the groups infected with the lentivirus expressing the shRNA for *Mmp-2* confirmed low expression of Mmp-2 in the infected group concomitantly with increased expression of the extracellular domain of the ObR. In addition, in cells where Mmp-2 was expressed (seen as granular and diffuse staining due to protease secretion), the expression of the ObR extracellular domain was markedly reduced (Fig. 4, D and E).

To confirm the results obtained from deletion of hypothalamic Mmp-2 in WT mice, we reintroduced the protease to the hypothalamus of *Mmp-2*^{–/–} mice by ICV injections of lentivirus expressing *Mmp-2* into the hypothalamus (*Mmp-2*^{–/–}/L-*Mmp-2*). In line with our hypothesis, mice lacking Mmp-2 gained less weight than the same mice infected with *Mmp-2* in the hypothalamus ($*P < 0.05$, *Mmp-2*^{–/–}/

L-*Mmp-2*-HFD versus *Mmp-2*^{–/–}-HFD; Fig. 4, F and G). Moreover, leptin values, which were low in *Mmp-2*^{–/–} mice fed a HFD, increased in *Mmp-2*^{–/–}/L-*Mmp-2*-HFD ($*P < 0.05$; Fig. 4H), suggesting enhancement of leptin resistance in these mice.

MMP-2 mediates weight gain and is controlled by NF- κ B

IF labeling of Mmp-2 and ObR in the experimental group demonstrated that infected cells expressing Mmp-2 have lower label densities of the ObR extracellular domain, confirming reduction of its expression in *Mmp-2*–expressing cells (Fig. 5A). To show that it is the cleavage of ObR by MMP-2 that is responsible for the decreased ObR signaling and increased weight gain, we mutated the ObR at the two cleavage sites recognized by Mmp-2, forming an MMP-2 cleavage-resistant receptor. After confirming that our mutated receptor is resistant to Mmp-2 but still induces STAT-3 and ERK phosphorylation

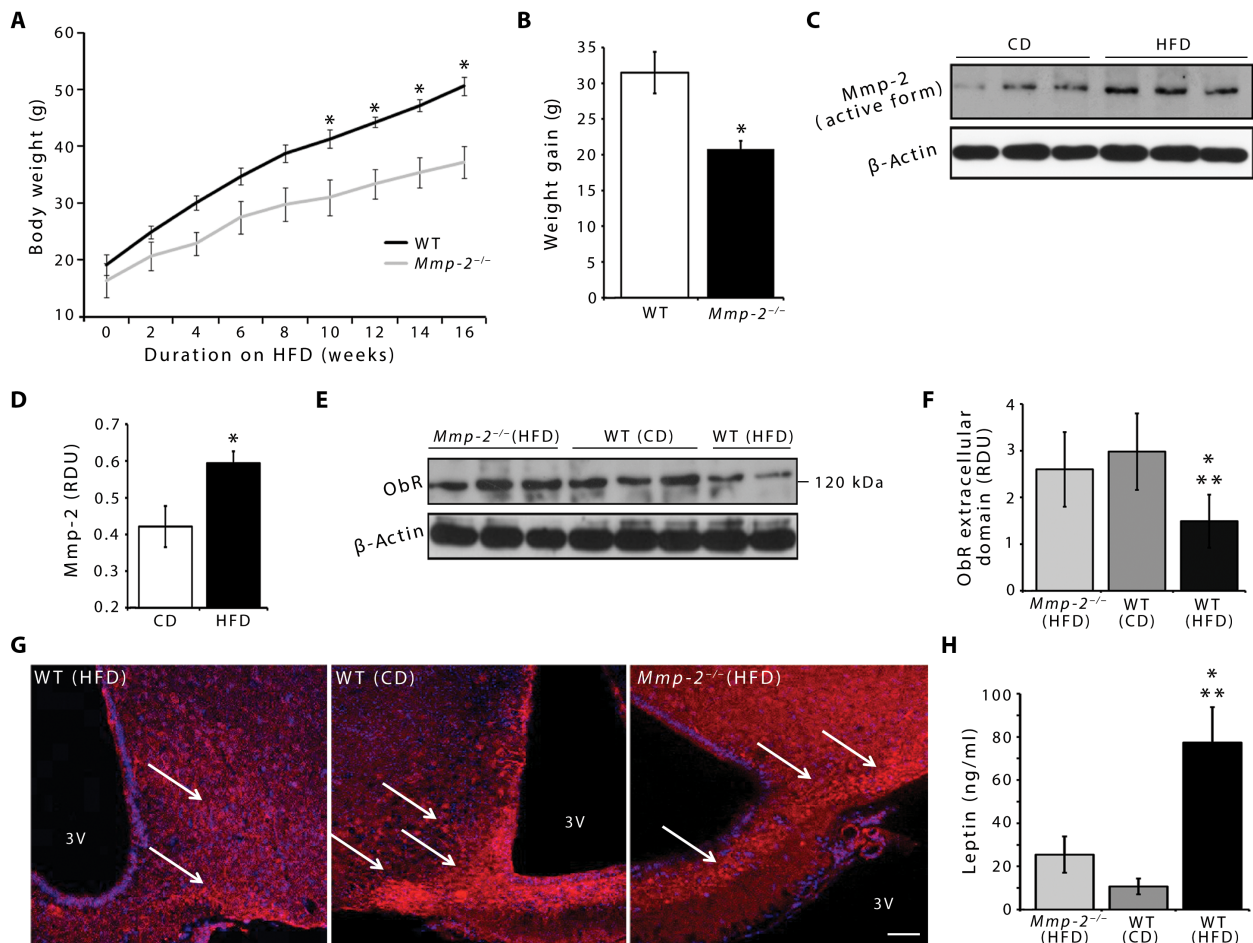


Fig. 3. *Mmp-2*^{-/-} mice are protected from obesity and leptin resistance. (A) Body weight in WT and *Mmp-2*^{-/-} mice fed a HFD ($n = 8$). (B) Weight gain in WT and *Mmp-2*^{-/-} mice after HFD intake (means \pm SD; $*P < 0.05$, $n = 8$). (C) Representative WB for the detection of active *Mmp-2* in brain homogenate of mice fed a CD or a HFD. (D) Concentrations of *Mmp-2* were quantified and are presented as RDU \pm SD normalized against actin. $*P < 0.05$, CD versus HFD. (E) Representative WB of the ObR extracellular domain in *Mmp-2*^{-/-} mice fed a HFD, WT mice fed a CD, and WT mice fed a HFD. (F) The density of the bands was determined relative to β -actin, and results are presented as RDU \pm SD. $*P < 0.05$, WT (HFD) versus *Mmp-2*^{-/-} (HFD); $**P < 0.05$, WT (HFD) versus WT (CD); $n = 8$. (G) Immunolabeling using an antibody against the extracellular domain of the ObR in the hypothalamus of WT mice fed a HFD, WT mice fed a CD, and *Mmp-2*^{-/-} mice fed a HFD. White arrows point to ObR staining. Scale bar, 20 μ m. (H) Serum leptin concentrations measured with enzyme-linked immunosorbent assay after HFD (WT and *Mmp-2*^{-/-}) and CD intake (WT) [$*P < 0.001$, WT (HFD) versus WT (CD); $**P < 0.001$, WT (HFD) versus *Mmp-2*^{-/-} (HFD); $n = 9$ per group].

after leptin administration (fig. S9, A to C), we used a genetic mouse model of obesity (db/db mice) infected with the mutated ObR and followed the weight gain compared to controls. Our results show that infected mice gained less weight than the noninjected db/db mice ($P < 0.05$ versus control db/db; Fig. 5B). This effect, which lasted about 2 weeks after infection, was abolished when the mice reached the weight of controls, suggesting either a compensation mechanism or low infection yield (Fig. 5B).

To understand whether *Mmp-2* is under the control of *NF- κ B*, we treated GT1-7 cells with *TNF α* , followed by quantitative PCR (qPCR), and we determined the expression of *Mmp-2* and that of known *NF- κ B* target genes (table S1). We found that *TNF α* application increased *Mmp-2* expression (Fig. 5C). After inhibition of *NF- κ B* activation using an *NF- κ B* kinase subunit beta (IKK2) inhibitor, the expression of *NF- κ B* representative target genes and that of *Mmp-2* were decreased (Fig. 5C). These results suggest up-regulation of *Mmp-2* in response to *NF- κ B* activation. Because hypothalamic inflammation precedes weight gain and affects glucose regulation in

obesity and *Mmp* activation is downstream of inflammation, we inhibited *Mmps* specifically in the hypothalamus of HFD mice using Dox injections and measured its effect on glucose values. Our results (Fig. 5D) showed elevated glucose values in HFD that were significantly reduced, when hypothalamic *Mmps* were inhibited ($P < 0.05$, HFD-Dox versus HFD-saline; $n = 8$).

To distinguish cell types responsible for *Mmp-2* production and secretion in the hypothalamus upon HFD intake, double IF was carried out. It showed no colocalization with neurons expressing POMC, SF1, or glial cells expressing Iba1. In contrast, we found that *Mmp-2* colocalized with AgRP neurons and with the astrocytic marker GFAP, suggesting that astrocytes and AgRP neurons are the main source of *Mmp-2* in mouse hypothalamus upon HFD intake (Fig. 5E).

DISCUSSION

The mechanism of leptin resistance in obesity is not completely elucidated. Here, we identify a mechanism for the development of

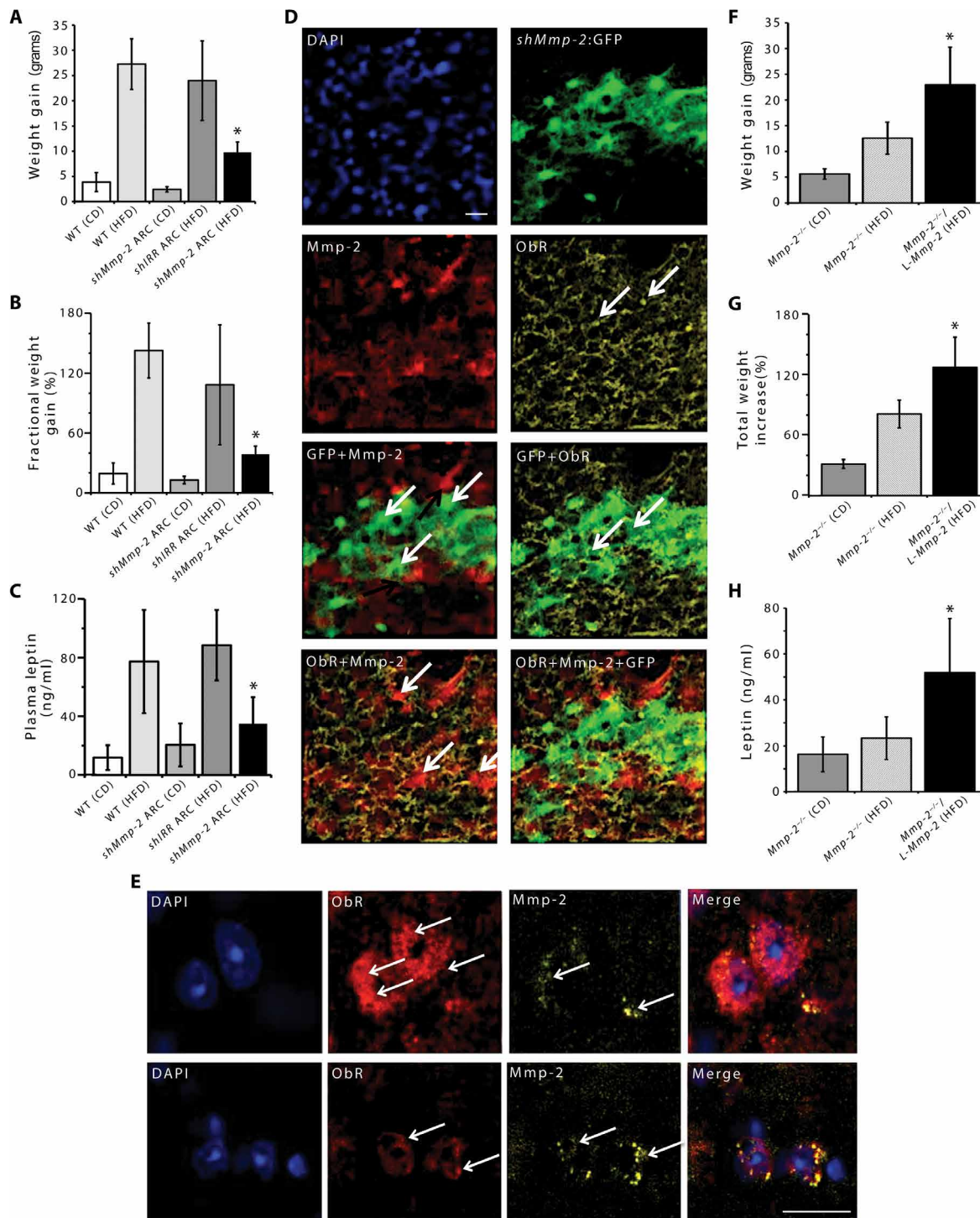


Fig. 4. Hypothalamic Mmp-2 mediates weight gain and plasma leptin. (A) The ARC part of the hypothalamus of WT mice was infected with viruses carrying shRNA to inhibit MMP-2 (shMMP-2-ARC) or an irrelevant sequence (shIRR-ARC), and mice were subjected to either CD or HFD. The shMMP-2-ARC on HFD gained significantly less weight compared to WT on HFD ($*P < 0.05$; $n = 8$). (B) Weight increase as a fraction of body weight (before the start of HFD/CD) ($*P < 0.05$, WT fed HFD versus *shMmp-2* ARC fed HFD; $n = 8$). (C) Plasma leptin values in the experimental groups ($*P < 0.05$ *shMmp-2* versus WT fed HFD; $n = 8$). (D) IF images showing the expression pattern of Mmp-2 (red) and ObR (yellow) in the hypothalamus of mice infected with *shMmp-2* (GFP-green); blue indicates nuclear DAPI labeling. White arrows point to ObR-positive cells (second row, right), leti-infected cells (third row, left and right), and Mmp-2-positive cells (fourth row, left). Note that, in the presence of Mmp-2, ObR is reduced. Scale bar, 20 μ m. (E) IF labeling (high magnification) of the ObR extracellular domain (red) and Mmp-2 (green) in the hypothalamus of obese mouse. (F) *Mmp-2*^{-/-} were reintroduced with MMP-2 (*Mmp-2*^{-/-}/L-MMP-2) and were subjected to either CD or HFD (means \pm SD; $*P < 0.05$ versus *Mmp-2*^{-/-} fed a HFD; $n = 7$). (G) Total weight increase as percentage of body weight (before the start of HFD/CD versus 16 weeks of CD or HFD). (H) Concentrations of plasma leptin after HFD intake in mice reintroduced with Mmp-2 compared to *Mmp-2*^{-/-} on the same diet.

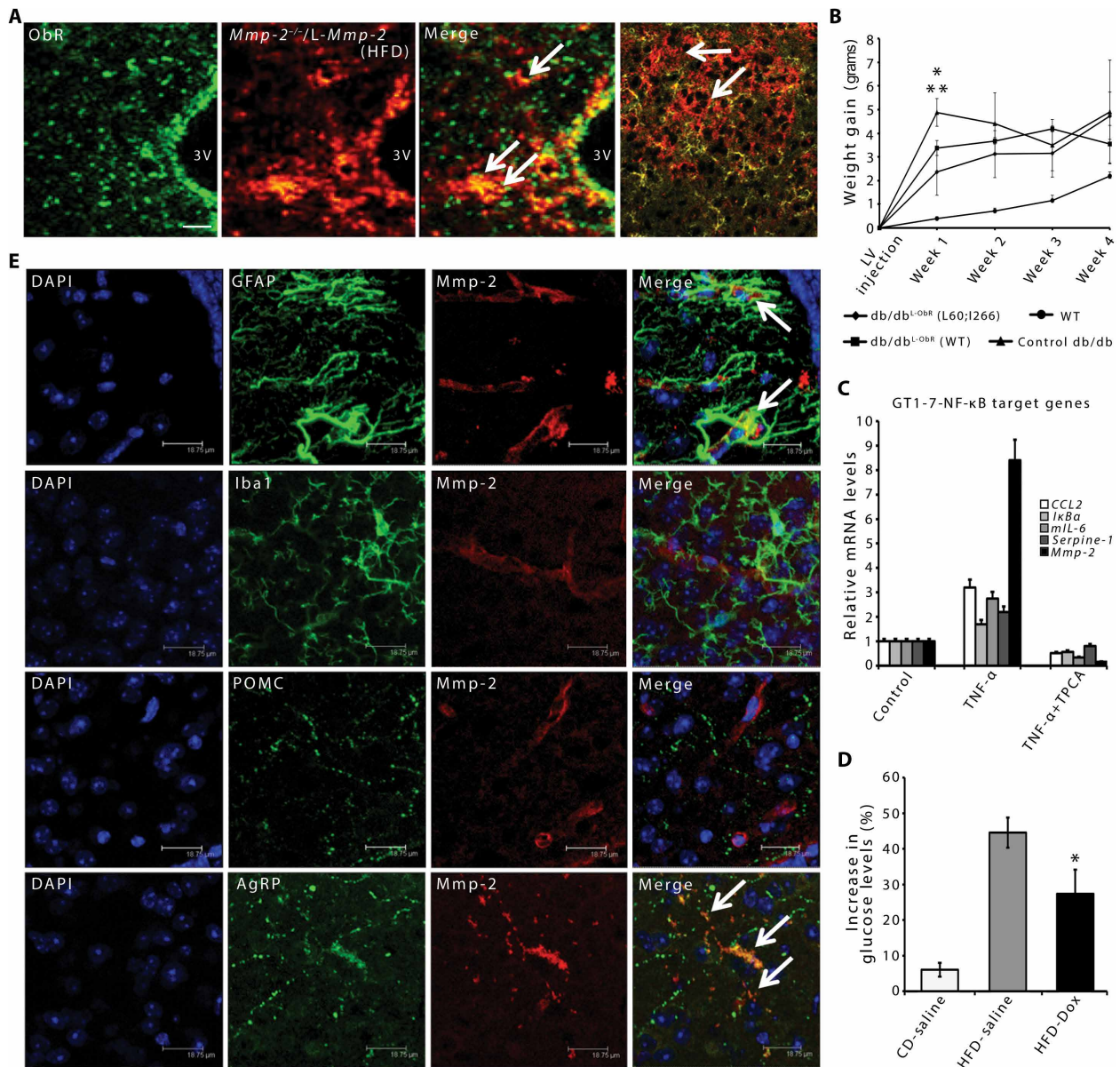


Fig. 5. MMP-2 mediates weight gain and is controlled by NF- κ B. (A) Representative confocal images showing reduced expression of ObR in cells of *Mmp-2*^{-/-} mice after reintroduction of Mmp-2 to the ARC part of the hypothalamus. White arrows point to locations with high expression of Mmp-2 but reduced expression of ObR. Scale bar, 20 μ m. (B) Weight gain in db/db mice infected with MMP-2-resistant ObR [means \pm SD; * P < 0.05, db/db^{L60;1266} (L60;1266) versus db/db^{L60;1266} (WT); ** P < 0.01, db/db^{L60;1266} (L60;1266) versus db/db; n = 6]. (C) Reverse transcription (RT) qPCR for NF- κ B target genes and *Mmp-2* in GT1-7 cells treated with TNF α to activate NF- κ B and with the IKK2 inhibitor TPCA. Results are presented as relative to glyceraldehyde-3-phosphate dehydrogenase. (D) Changes in blood glucose concentrations in mice fed a CD or a HFD treated with doxycycline (Dox) or saline. Data are presented as percent increase at day 5 versus day 0 (means \pm SD, n = 8). (E) IF imaging of hypothalamus from obese mice labeled with glial fibrillary acidic protein (GFAP), Iba1, pro-opiomelanocortin (POMC), and AgRP and colocalized with *Mmp-2*. Colocalization was found in astrocytes (GFAP-positive; white arrows, upper right) and AgRP neurons (white arrows, lower right).

leptin resistance in rodent models that involves the cleavage of the ObR extracellular domain by Mmp-2.

In the hypothalamus, leptin has anorexigenic properties and increases energy expenditure (4, 8). However, one of the hallmarks of obesity is a reduced ability of leptin to suppress appetite and promote energy expenditure (3, 4). Thus, the term leptin resistance describes the apparent paradox of elevated circulating leptin in most obese individuals. Several mechanisms contributing to this phenomenon have been suggested and include increased SOCS3 activity,

ER stress, and hypothalamic inflammation (4, 7, 9–13). Our results in rodent models of obesity show that cleavage of the extracellular domain of the ObR by Mmp-2 might also contribute to leptin resistance in obesity. On a broader view, proteolytic receptor cleavage and loss of cell functions might be a pathophysiological process that leads to multiple comorbidities in the metabolic syndrome (20).

Chronic low-grade inflammation plays a role in the genesis of obesity, and HFD induces hypothalamic inflammation and activation of NF- κ B (9–11). Previous studies showed that NF- κ B inhibition in vivo

protects against obesity and leptin resistance (9). We found that NF- κ B activation in hypothalamic cells increased Mmp-2 expression, suggesting a role for Mmp-2 activity in the progression of hypothalamic inflammation and obesity. In obesity, leptin itself plays a proinflammatory role, promotes the production of proinflammatory cytokines in the brain, and up-regulates and activates MMPs that in turn contribute to tissue remodeling and protein degradation (21, 22).

Rats fed a HFD showed increased plasma leptin concentration and increased hypothalamic Mmp-2 activity. Mmp-2 activity is found in conditioned media of adipocytes, and there is high expression of the protease in adipose tissue of obese animal models (23, 24). Yet, while these studies focused on the contribution of Mmp-2 to adipogenesis, its possible role as a protease capable of cleaving the leptin receptor and promoting leptin resistance was not addressed.

Increased hypothalamic MMP-2 activity concomitantly with reduced expression of the ObR extracellular domain in HFD rats raises the question whether MMP-2 directly cleaves the extracellular domain of the ObR. The structure of the extracellular domain includes a conserved cytokine receptor and FN3 domains separated by an Ig domain, together referred to as cytokine receptor homologous domain (CRH1). There are two additional FN3 domains in the extracellular portion of the receptor. The CRH domain close to the membrane is involved in ligand binding, whereas the rest of the domains stabilize the receptor (25). Incubating active Mmp-2 with the recombinant extracellular domain of the ObR resulted in formation of ObR fragments from cleavage sites known to be recognizable by Mmp-2 (26). Coexpression of a mutated receptor in the leptin's binding site with WT ObRb inhibits normal signaling by the receptor (25). In that respect, a cleaved receptor as shown herein would further augment the reduction of leptin signaling even if intact receptors are still present. Formation of ObR fragments in conditioned media of cells treated with Mmp-2 served to verify Mmp-2-mediated cleavage of the ObR in a more biological setting. Our studies point to cleavage sites in the CRH1 domain. This result seems to be in contrast to others who have shown that mutation (Q223R) in this domain is dispensable for ObR signaling (27). Yet, the obese phenotype of fatty Zucker rats results from the Q269P mutation in the CRH1 domain (28). Moreover, the single-nucleotide polymorphism Q223R is reported to relate to obesity in certain populations (29). In light of the unclear role of the CRH1 domain in ObRb signaling in obesity, we propose that the CRH1 domain plays an important role in stabilizing the ObR molecule *in vivo*. Thus, although point mutations may have little effect on the receptor signaling, others might destabilize its molecular structure. Furthermore, mutations might generate cleavage sites for proteases. Cleavage may also expose cleavage sites within the receptor that are otherwise not accessible in the intact protein. Finally, the *in vivo* data supporting lack of significance for the Q223R mutation in the ObR were drawn from a single mouse strain (129P3/J). It is well known that the phenotypic "penetrance" of mutations varies depending on the strain(s) in which the mutation is segregated; thus, the importance of this mutation in ObRb signaling *in vivo* merits further exploration.

ADAMs, specifically ADAM-10 and ADAM-17, are involved in ObR cleavage (30, 31). Both enzymes share an identical catalytic domain, and ADAM-17 heterozygous mice are protected from obesity and insulin resistance (32, 33). We measured the activation of ADAM-10 and ADAM-17 by Mmp-2 as a possible mechanism to enhance ObR cleavage and found that Mmp-2 could not activate these enzymes. Therefore, our *in vivo* evidence showing the effect

of hypothalamic knockdown of *Mmp-2* on the ObR expression and the development of obesity suggests that Mmp-2 might exert a direct action on the leptin receptor. However, an alternative explanation for our results is that, *in vivo*, other proteases active in the hypothalamus or being activated by Mmp-2 may also cleave the ObR. When testing other related receptors such as IL-6R and InsR, we found that they are not cleaved by Mmp-2, although cleavage of the InsR extracellular domain by an MMP was proposed as a possible mechanism for diabetes in a genetic model of hypertension (34).

We found that Mmp-2-treated cells are impaired in their response to leptin. It is important to note that Mmp-2-treated cells showed STAT-3 phosphorylation without addition of leptin. STAT-3 can be activated by stressful conditions (35); thus, this basal activation could be explained by effects of starvation and activation directly or by other formed peptides in Mmp-2-treated cells. Still, upon stimulation with leptin, phosphorylation of STAT-3 was reduced compared to Mmp-2-untreated cells at all time points.

Previous studies addressing MMP-2 in obesity have shown that *Mmp-2*^{-/-} mice on HFD are leaner with reduced fat mass (18) and that WT mice treated with a gelatinase inhibitor have reduced body weight and adipose tissue mass (18). Another gelatinase (MMP-9) correlates with body weight in humans (36). Yet, we found no effect of Mmp-9 on the ObR, and others showed no role for this enzyme in weight gain and adipose tissue development (36), further supporting our finding that, at least in rodents, ObR cleavage is at least partially Mmp-2-specific.

We found that, in obese WT mice, leptin values were elevated together with decreased expression of the ObR and reduced p-STAT-3. In contrast, *Mmp-2*^{-/-} mice fed a HFD had reduced values of circulating leptin and comparable expression of ObR and p-STAT-3 to control mice, further supporting the role of Mmp-2 in mediating leptin resistance in mice.

On the basis of the results obtained in *Mmp-2*^{-/-} mice fed a HFD, we propose a possible mechanism for leptin-mediated Mmp-2 activation and cleavage of the ObR: Leptin increases the expression and activity of Mmp-2 (37), and the *MMP-2* promoter can be bound by STAT-3 (38), thereby stimulating its transcription. We therefore suggest that high concentrations of leptin together with hypothalamic inflammation might cause elevated expression and activation of MMP-2, which in turn cleaves the leptin receptor on ObR-expressing cells. Moreover, because hypothalamic inflammation and glucose dysregulation upon HFD intake occurs before significant weight gain and MMP activation is downstream of inflammation, it is possible that hypothalamic MMP inhibition might improve glucose metabolism upon HFD intake. We found that injections of the MMP inhibitor Dox to the hypothalamus inhibited the elevation in glucose values upon HFD intake, further supporting the role of hypothalamic inflammation in the progression of obesity and its comorbidities.

Whereas our results support cleavage of the ObR extracellular domain as a mechanism for leptin resistance and obesity, mice that are heterozygous for leptin receptor expression ObRb(db/+) have normal phenotype in regard to body weight (39), despite their overall lower ObRb expression. This raises the question of whether our model, in which the leptin receptor is cleaved, is in fact analogous to mouse models with lower expressed protein. With a normal diet, ObRb(db/+) mice have higher values of circulating leptin, decreased energy expenditure, and greater body fat mass (39, 40). In addition, the leptin signal is attenuated in these animals as a result of a reduced number of intact receptor isoforms, suggesting that heterozygosity

of the leptin receptor may play a role in susceptibility to environmental conditions favoring obesity (39–41). Cleaved receptor fragments may have other physiological effects not seen in the ObR heterozygous mice. Soluble ObR may, to some degree, limit the bioavailability of leptin and decrease receptor signaling. In the presence of proteolytic activity, other isoforms of the ObR may also be cleaved, thereby modulating pathways such as leptin transport from the circulation, a biological effect that is not present in the [ObRb(db/+)] model. It is also possible that ObRb heterozygous mice have adapted to a reduced receptor expression during development.

If Mmp-2-mediated cleavage of the ObR is a key event mediating leptin resistance and obesity, then introducing an Mmp-2-resistant receptor to ObR-deficient mice should rescue the obese phenotype. Weight loss was higher in db/db mice infected with a mutated receptor resistant to Mmp-2 cleavage than db/db mice or db/db infected with a WT receptor. These results confirm the importance of Mmp-2 in cleaving the ObR: When a WT receptor was introduced into db/db mice, the rate of weight gain was higher than when a mutated receptor was introduced, which might be due to the cleavage of the WT receptor by Mmp-2 and thus decreased leptin signaling. Altogether, these results suggest that Mmp-2 activity in the hypothalamus promotes obesity and leptin resistance in rodents.

To identify the cell type(s) producing Mmp-2 upon HFD intake, we colocalized Mmp-2 with different cell and neuronal markers in the hypothalamus. We found AgRP neurons and astrocytes to be the main source of Mmp-2. When activated, AgRP neurons stimulate appetite, an action that is inhibited by leptin (42). As the ObRb is cleaved on these cells, leptin binding and signaling would be impaired, resulting in activation and increased feeding behavior. Recent studies have shown that astrocytes express the ObR and were suggested to act as transporters of leptin from the bloodstream to target neurons (43). After prolonged HFD, astrocytes become activated and release inflammatory mediators into the surrounding milieu, contributing to hypothalamic inflammation associated with obesity (44). Thus, these results suggest that HFD activates astrocytes to induce inflammation and might promote Mmp-2 secretion. Mmp-2 could then cleave the ObR on adjacent neurons, thereby attenuating leptin signaling.

Our study has several limitations: *Mmp-2*^{-/-} mice are known to be smaller at birth and to have slower growth rates than WT (45). Moreover, deficiency of *Mmp-2* may also affect other organs and other proteinases that depend on Mmp-2 for their activation. Our results may explain these differences, because in the absence of hypothalamic Mmp-2, there might be increased ObR availability for leptin binding and signaling. Moreover, to date, there is no antibody available that is specifically designed for use in IF to label the extracellular domain of the ObR. Therefore, IF evidence demonstrating the extracellular ObR reduction was independently investigated by physiological and molecular manipulation of the receptor to confirm that the receptor is cleaved by Mmp-2. Our molecular approach in vivo involved infection of the hypothalamus with lentivirus vectors to silence/express proteins. Although this approach allows for tissue-specific manipulations, one cannot control the yield of infection. Yet, the fact that we did not get complete hypothalamic infection suggests that the overall physiological effects measured in these studies might be even greater. Finally, we used recombinant receptors to study ObR extracellular domain cleavage. In vivo, other enzymes may also be activated and contribute to ObR cleavage directly or by acting upstream via Mmp-2 activation. Such actions of other enzymes can result in cleavage of the receptor at sites that

were not identified in this study and result in receptor fragments different from those detected herein.

In conclusion, our results support a mechanism that contributes to the development of obesity and leptin resistance (fig. S10). With the current obesity epidemic, this study presents a possible strategy for the development of new treatment modalities aimed at reducing chronic systemic and hypothalamic inflammation, consequently reducing the activity of MMPs, specifically MMP-2, and thereby restoring leptin sensitivity and decreasing weight gain.

METHODS

Study design

The objective of this study was to determine protease activity in the hypothalamus of obese mice and rats as a mechanism contributing to leptin resistance. We hypothesized that upon HFD intake, increased MMP activity within the brain promotes cleavage of the extracellular domain of the leptin receptor, leading to decreased leptin sensitivity. Using rodents, lentiviral vectors, and in vivo analysis, we analyzed the effect of Mmp-2 on the ObR cleavage, mapped the cleavage site, and used a mutated receptor in vivo to show that Mmp-2-resistant ObR restores leptin sensitivity. Unless otherwise specified, all in vitro studies were based on at least three repeats and in vivo data on $n = 8$ to achieve significant differences between groups.

Animals

All animal protocols were reviewed and approved by the University of California San Diego Animal Subjects Committee, and the experiments were performed in adherence to the National Institutes of Health Guidelines on the Use of Laboratory Animals.

Rat studies

Young (4 weeks old) male Wistar rats were divided into two groups ($n = 9$ per group), an experimental group provided with HFD for 16 weeks to induce obesity (Harlan TD.96001, 40.9% kcal from fat) and a control group provided with normal CD. At 16 weeks, rats were euthanized [120 mg/kg, intraperitoneally (ip); Beuthanasia], and organs were collected for analysis. For validation of ObR IF staining in vivo, Koletsky rats (complete ObR knockout) were used as a negative control.

Mice studies

C57BL/6 mice served as WT controls. Gelatinase A knockout mice (*Mmp-2*^{-/-}) [100% genetic background of C57BL/6; provided by F. Kheradmand (Baylor College of Medicine)], WT mice infected with lentivirus for hypothalamic silencing of *Mmp-2* (*Sh-Mmp-2*) and their respective controls infected with a virus carrying irrelevant sequences (*Sh-IRR*), and *Mmp-2*^{-/-} mice were reintroduced with Mmp-2 to the hypothalamus (*Mmp-2*^{-/-}/L-*Mmp-2*). To show that the Mmp-2-mediated effect on weight gain is restricted to the hypothalamus, a control WT group was infected with lentivirus to knock down *Mmp-2* expression in the hippocampus ($n = 3$). To show that Mmp-2-mediated cleavage of the leptin receptor is a key event in the progression of leptin resistance, we used the obese db/db mouse model (mice homozygous for the diabetes spontaneous mutation *Lepr* db) infected with a WT form of ObRb and compared them to db/db mice infected with a mutated form of ObRb that is resistant to cleavage by Mmp-2 (see below for specifics). In selected experiments, WT mice fed a CD and a HFD were injected with Dox into

the hypothalamus (bilateral, same coordinates as for the lentivirus injections; 5 µg/µl) to inhibit Mmps, and blood glucose values were measured as previously described (34).

For in vivo pSTAT-3 measurements in the hypothalamus, WT mice fed a CD and a HFD and *Mmp-2*^{-/-} mice fed a HFD were restricted from food for 4 hours followed by administration of leptin (25 µg/kg, ip). An hour after leptin administration, mice were euthanized and pSTAT-3 signal was calculated per positive cell in the hypothalamus. Intensity of the signal was digitally recorded as previously described (46) and presented in DU ± SD. The mice were bred in the animal housing facility of the Department of Bioengineering, University of California San Diego. Determination of *Mmp-2*^{-/-} and db/db in new litters was carried out using gelatin zymography and RT-PCR. Animals were provided HFD for 12 weeks to induce obesity. At 12 weeks, mice were euthanized for tissue collection.

Generation of *Mmp-2*-resistant ObR mutants

WT ObR coding region [a gift from C. Bjørnbæk (Beth Israel Deaconess Medical Center)] was used for mutating MMP-2 cleavage sites using a site-directed mutagenesis kit. Mutated receptors were tested in vivo for their resistance to cleavage by MMP-2 and their ability to bind leptin and produce a downstream signal (fig. S9).

Lentivirus studies

Lentiviruses were produced as described previously (47). The shRNA sequences used were as follows: 5'-GACAAGTTCTGGAGATAC-3' (MMP-2) and 5'-TGATGATGCCAAACGACAA3' (IRR, control). The mouse *Mmp-2* coding region (OriGene) and the ObRwt and ObR mutants were cloned into a lentiviral vector under a cytomegalovirus promoter followed by a 2A-GFP cassette inserted by PCR. Biological viral titer was determined by infecting human embryonic kidney (HEK) 293T cells with serial diluted viruses. All constructs were validated in cell cultures before in vivo experiments.

Lentivirus intracerebral injections

Lentiviruses (1 µl; 1 × 10⁹ IU) were stereotaxically injected into anesthetized mice, targeting the arcuate nucleus on both sides of the hypothalamus. Each virus was delivered (0.25 µl/min) in two injections into the ARC at coordinates relative to the bregma: anteroposterior (AP, 1.3 mm), lateral (ML, ±0.3 mm), and dorsoventral (DV, 6 mm) (48) (fig. S8, D and E). The needle was left in place for another 5 min and then slowly withdrawn. Coordinates used to inject the hippocampus (HP) region were as follows: AP, 2.0; ML, 1.5; and DV, 2.3 (49).

Statistics

All statistical results were presented as means ± SD. An unpaired two-tailed Student's *t* test was used for comparisons between two groups. Analysis of variance (ANOVA) was used to test for differences in outcomes of interest among groups. Results were determined to be significant at *P* < 0.05. Bonferroni's post hoc multiple comparison test was used to determine significance between individual groups. All analyses were performed using the Statistical Package for the Social Sciences (SPSS version 18). The number of animals used was estimated to minimize the use of experimental animals required to obtain a statistically conclusive result, assuming equal variances among groups ($\alpha = 0.05$ and $\beta = 1 - 0.9$). Animals were randomly assigned to study or control groups. No animals were excluded from analysis, and studies were not blinded.

SUPPLEMENTARY MATERIALS

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Supplementary methods

Fig. S1. HFD induces obesity in rats.

Fig. S2. Enzymatic activity is enhanced in brains of rats fed a HFD.

Fig. S3. Validation of ObR-specific labeling.

Fig. S4. MMP-2 cleaves the extracellular domain of the ObR and impairs leptin-mediated signaling in the GT1-7 hypothalamic cell line.

Fig. S5. Cleavage of the ObR extracellular domain is MMP-2-specific.

Fig. S6. Identification of *Mmp-2*^{-/-} mice using gel zymography.

Fig. S7. Confirmation of enhanced of *Mmp-2* activity in the brain of mice fed a HFD by gel zymography with gelatin substrate.

Fig. S8. Confirmation of MMP-2 shRNA and complementary DNA constructs.

Fig. S9. Verification of mutated ObR functionality.

Fig. S10. Summary diagram of HFD-induced extracellular leptin receptor cleavage by MMP-2 in the hypothalamus contributing to leptin resistance and weight gain.

Table S1. List of primers used to detect induction of NF-κB target genes and *Mmp-2*.

Table S2. Raw data (provided as a separate Excel file).

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Cleavage of the leptin receptor by matrix metalloproteinase–2 promotes leptin resistance and obesity in mice

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Restoring leptin's effects in obesity

Obesity is the most common metabolic disease in the developed world. Although obese individuals have increased plasma concentrations of the anorexigenic hormone leptin, they are refractory to its anorexigenic effect. Mazor *et al.* showed that, in rodents, obesity induced matrix metalloproteinase–2 (Mmp-2) activation in the hypothalamus. In turn, Mmp-2 activation reduced leptin-mediated signaling by promoting leptin receptor degradation. Mmp-2 deletion in the hypothalamus increased leptin receptor expression and reduced fat accumulation in mice fed a high-fat diet. The results suggest that targeting Mmp-2 might be an effective strategy for treating obesity by restoring the anorexigenic effects of leptin.

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