The Thermogenic Effect of Leptin Is Dependent on a Distinct Population of Prolactin-Releasing Peptide Neurons in the Dorsomedial Hypothalamus

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SUMMARY

Leptin is a critical regulator of metabolism, which acts on brain receptors (Lepr) to reduce energy intake and increase energy expenditure. Some of the cellular pathways mediating leptin’s anorectic actions are identified, but those mediating the thermogenic effects have proven more difficult to decipher. We define a population of neurons in the dorsomedial hypothalamic nucleus (DMH) containing the RFamide PrRP, which is activated by leptin. Disruption of Lepr selectively in these cells blocks thermogenic responses to leptin and causes obesity. A separate population of leptin-insensitive PrRP neurons in the brainstem is required, instead, for the satiating actions of the gut-derived hormone cholecystokinin (CCK). Global deletion of PrRP (in a loxSTOPlox-PrRP mouse) results in obesity and attenuated responses to leptin and CCK. Cre-recombinase-mediated reactivation of PrRP in brainstem rescues the anorectic actions of CCK, but reactivation in the hypothalamus is required to re-establish the thermogenic effect of leptin.

INTRODUCTION

Leptin is an adipokine, produced in proportion to white adipose tissue mass, which is critical for metabolic homeostasis. Deficiency in either leptin or its receptor, Lepr, leads to obesity due to increased feeding (Alting Prins et al., 1986; McLaughlin and Baile, 1981) and reduced energy expenditure (through a decrease in core body temperature) (Trayhurn, 1979; Trayhurn et al., 1977). Leptin acts centrally to influence energy balance, since rescue of brain Lepr expression in otherwise Lepr-deficient mice reverses their obese and diabetic phenotype (de Luca et al., 2005). Substantial progress has been made in the identification of central cellular pathways involved in mediating the effects of leptin on energy intake. These include neurons of the arcuate hypothalamic nucleus, which contain either proopiomelanocortin (POMC) or neuropeptide Y (NPY)/agouti-related peptide (Galthasar et al., 2004; Mercer et al., 1996; van de Wall et al., 2007), as well as neurons in the ventromedial hypothalamic nucleus that contain melanocortin (Cutter and Luckman, 2002). PrRP was originally described following deorphanization of the receptor GPR10 (Hinuma et al., 1998), but it was misnamed, as it has little or no physiological role in the control of prolactin (Dodd and Luckman, 2006; Lawrence et al., 2000, 2002). PrRP is a ligand for the orphaned receptor GPR10 (Dhillon et al., 2006; Hawke et al., 2009). However, selective deletion of Lepr in first-order sensing neurons in the arcuate and ventromedial nuclei produces relatively mild obese phenotypes, suggesting additional populations of leptin-sensing neurons and, in particular, populations that mediate the important effects of leptin on adaptive thermogenesis and energy expenditure. It is hypothesized that Lepr-containing neurons of the dorsomedial hypothalamic nucleus (DMH) are an integral part of central thermogenic circuitry and important in leptin’s actions on energy expenditure (Enriori et al., 2011; Zhang et al., 2011), but the phenotypic identity of these neurons has not been confirmed.

We have proposed previously an important role for the RFamide prolactin-releasing peptide (PrRP) in energy homeostasis and, specifically, in brainstem pathways mediating the actions of the satiety factor CCK (Bechtold and Luckman, 2006; Lawrence et al., 2000, 2002). PrRP was originally described following deorphanization of the receptor GPR10 (Hinuma et al., 1998), but it was misnamed, as it has little or no physiological role in the control of prolactin (Dodd and Luckman, 2013; Samson et al., 1998). Genetic deficiency of either PrRP or GPR10 results in late-onset obesity and the loss of anorectic responses to CCK (Bechtold and Luckman, 2006; Gu et al., 2004; Takayanagi et al., 2008; Watanabe et al., 2005). Importantly, central injection of PrRP causes a reduction in food intake and increases in energy expenditure and core body temperature, which are additive with the effects of leptin (Ellacott et al., 2002; Lawrence et al., 2000, 2004). We have, thus, hypothesized that PrRP, in addition to mediating the satiating actions of CCK, is a critical target for leptin’s thermogenic signaling to the brain.

RESULTS

The Expression of PrRP Is Regulated by Energy Status in Mice

PrRP is expressed in three distinct neuronal populations: one in the caudal DMH and two in separate regions of the brainstem—the nucleus of the tractus solitarius (NTS) and the ventrolateral medulla (VLM). As in the rat (Chen et al., 1999), the PrRP-expressing neurons in the mouse brainstem colocalize the...
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To explore leptin signaling via PrRP neurons, we generated a mouse in which the PrRP gene drives expression of IRES-Cre recombinase. Homologous recombination in embryonic stem cells was used to generate a line of heterozygous PrRP-Cre mice, which were then crossed with an eGFP (Z/EG) reporter mouse to produce PrRP-Cre::eGFP offspring. Targeted recombination is confirmed since eGFP in these mice is restricted exclusively to PrRP neurons (Figure S1 available online). As Lepr is linked to the STAT3 intracellular signaling pathway, the phosphorylated transcription factor (pSTAT3), which migrates to the cell nucleus upon activation, can be used as a measure of leptin signaling (Münzberg et al., 2003). A single, systemic injection of leptin (5 mg/kg body weight; intraperitoneal [i.p.]) causes a robust induction of pSTAT3 in PrRP-Cre::eGFP neurons in the DMH (Figures 2E and 2F) but none in PrRP-Cre::eGFP neurons in the NTS (Figure S2).

We next utilized a transgenic “floxed” leptin receptor gene mouse (Lepr<sup>lox/lox</sup>) (McMinn et al., 2004) in order to selectively knock out Lepr only in cells expressing PrRP. Heterozygous PrRP-Cre mice were crossed with Lepr<sup>lox/lox</sup> mice, and their offspring were mated to produce the homozygous littermates required for phenotyping experiments. Compared with wild-type (wt), single transgenic PrRP-Cre, and Lepr<sup>lox/lox</sup> control littermates, the PrRP-Cre::Lepr<sup>lox/lox</sup> mice display late-onset obesity (Figure 3A). The difference in body weight at 16 weeks of age (approximately +17%) is comparable with that of mice lacking Lepr in either POMC or SF-1 neurons (Dhillon et al., 2006) but is still significantly less than that reported for complete Lepr-deficient db/db mice (+60%) (Hummel et al., 1966). The obesity in PrRP-Cre::Lepr<sup>lox/lox</sup> mice is not due to greater food intake, as there was no difference between littermates at any age, but is instead due to lower energy expenditure (Table S1). These mice have slightly lower average core body temperature over the 24 hr period, measured remotely in freely behaving mice by radiotelemetry (Table S1; a surrogate measure of adaptive thermogenesis and energy expenditure), as seen in complete leptin-receptor-deficient db/db mice (Trayhurn, 1979).
Furthermore, the action of leptin to increase core body temperature is completely blocked in preobese (6 weeks of age), PrRP-Cre::Lepr\textsuperscript{lox/lox} mice (Figure 3B and 3C). As leptin is thought to activate sympathetic output to brown adipose tissue, we measured gene expression for uncoupling protein, UCP-1. An approximate 2-fold increase in Ucp-1 mRNA, following injection of leptin, is blocked in PrRP-Cre::Lepr\textsuperscript{lox/lox} mice (Figure S3A). Importantly, although the reduction in night-time feeding after leptin is slightly attenuated in PrRP-Cre::Lepr\textsuperscript{lox/lox} mice, these mice still respond normally to the satiating effect of CCK (20 \textmu g/kg, i.p.) (Figures 3D and 3E). The dependence of central Lepr signaling for the metabolic actions of leptin is well established (de Luca et al., 2005), so it is important to demonstrate that Lepr signaling is not generally compromised in the PrRP-Cre::Lepr\textsuperscript{lox/lox} mice. Thus, using quantitative PCR, we demonstrated normal expression of Lepr in different tissues (data not shown). Furthermore, we show that, like their littermates, PrRP-Cre::Lepr\textsuperscript{lox/lox} mice respond to leptin with a robust induction of pSTAT3 generally in the hypothalamus (Figure S3B). Our results strongly implicate an integral role for PrRP neurons, and specifically those in the DMH, in mediating the effects of leptin on thermogenesis.

**Genetic Ablation of PrRP Results in Obesity**

The studies above demonstrate the essential role of PrRP-expressing neurons in the thermogenic effects of leptin. We next set out to demonstrate that PrRP signaling itself is central to the leptin pathway mediating thermogenesis. For this, we generated transgenic mice containing a loxSTOPlox (LSL) codon upstream of the PrRP gene (Figure 4A). First, heterozygous LSL-PrRP mice were crossed to produce homozygous wild-type and LSL-PrRP littermates for phenotype comparisons. The lack of PrRP expression in homozygous LSL-PrRP mice was confirmed by both immunohistochemistry and by relative quantitative PCR (Figure S4). The body weights of both male and female homozygous LSL-PrRP mice, which lack expression of PrRP throughout the body, diverge from congenic littermates at 8 to 9 weeks of age, and become significantly obese at approximately 12 weeks (Figures 4B and 4C). By 18 weeks of age, the mice exhibit significantly increased adiposity (without a difference in somatic growth) and are hyperleptinaemic, hyperinsulinaemic, and hyperglycaemic (Table S1). Interestingly, in this model, the obese phenotype appears to be predominantly due to greater energy intake, as no decrease in energy expenditure was measured (Table S1). This is similar to the phenotype of PrRP knockout mice generated previously, but which do not diverge in weight significantly until 18 weeks on normal chow (Takayanagi et al., 2008). LSL-PrRP mice are also sensitive to diet-induced obesity, when maintained on a high-energy diet (60% energy as fat) from 4 weeks of age (Figures 4D and 4E). Here, as with PrRP knockout mice, their body weights diverge significantly from wild-type littermates within 2 weeks of the change in diet.

Preobese (6 weeks of age), LSL-PrRP mice maintained on normal chow were tested for their responses to both leptin and CCK. LSL-PrRP mice fail to increase core body temperature in response to systemic leptin administration (Figures 5A and 5B) or to induce brown adipose Ucp-1 (data not shown), supporting our view that PrRP mediates leptin’s thermogenic effect, though not pinpointing the source of PrRP in this role. The injection of either leptin or CCK causes a dose-dependent decrease in normal, night-time food intake in wt littermates (Figures 5C and 5D). By contrast, LSL-PrRP mice display no significant effects of either leptin or CCK on food intake.

**Selective Rescue of Brain PrRP Identifies Key Role of Hypothalamic Neurons**

Having used the LSL-PrRP mouse to demonstrate that PrRP is critical for leptin-induced thermogenesis, we then used Cre-recombinase technology to reactivate PrRP expression, since crossing LSL-PrRP mice with mice possessing tissue-specific Cre leads to excision of the upstream STOP codon at loxP sites (Figure 4A). As PrRP is expressed in peripheral tissues (the pituitary and adrenal glands) that could have important metabolic consequences, we first crossed LSL-PrRP mice with nestin-Cre mice in order to rescue expression of PrRP only in the brain. First, we demonstrated that LSL-PrRP mice contain no immunoreactivity for PrRP in any of the three brain regions (DMH, NTS, or VLM) but that PrRP immunoreactivity is rescued in the crossed nestin-Cre::LSL-PrRP mice (Figures S5 and S5B). Furthermore, relative quantitative PCR was used to demonstrate selective reactivation of the PrRP gene in the three brain regions of nestin-Cre::LSL-PrRP mice, but not in either the pituitary or adrenal glands (Figure S5B). Next, we crossed LSL-PrRP mice with TH-Cre to reactivate the PrRP gene in TH-positive cells in the NTS and VLM of the brainstem, but not in the DMH. Selective rescue of PrRP only in the brainstem (not in the hypothalamus, pituitary, or adrenal) was confirmed with immunohistochemistry and PCR (Figure S5).

One caveat with these studies is that the Cre lines might have a nonselective metabolic phenotype themselves and, therefore, we were extremely careful to make sure that Cre-expressing crosses were phenotyped using the relevant littermate controls (i.e., homozygous wild-type, homozygous LSL-PrRP, and/or heterozygous nestin-Cre/TH-Cre). Both the nestin-Cre and TH-Cre mice had the same body weight curves as their respective wild-type littermates. In both crosses, the homozygous LSL-PrRP littermates show divergent body weights at 8 to 9 weeks of age on normal chow when compared with the wild-type and the Cre-expressing mice; however, the obesity is fully reversed in the nestin-Cre::LSL-PrRP and TH-Cre::LSL-PrRP mice (Figures 6A and 6B). As previously noted, the obese phenotype of LSL-PrRP mice appears to be dependent on increased food intake, and this is reversed when PrRP expression is rescued in the brain (24 hr food intake in wild-type, nestin-Cre, LSL-PrRP, and nestin-Cre::LSL-PrRP littermates was as follows: 5.3 ± 0.1, 5.4 ± 0.1, 5.8 ± 0.1, and 5.3 ± 0.1g; *p < 0.05 LSL-PrRP versus all other groups). Preobese LSL-PrRP mice show neither the thermogenic response to leptin nor the anorectic response to either leptin or CCK (Figures 6C–6J), thus confirming results from our earlier experiment. However, all responses to either leptin or CCK are rescued in the nestin-Cre::LSL-PrRP littermates, highlighting the importance of brain-expressed PrRP, rather than that produced in peripheral tissues, in mediating the metabolic actions of the two hormones. As predicted, reactivation of only brainstem expression in the TH-Cre::LSL-PrRP mice rescues the response of these mice to the satiating effects of CCK (Figure 6J) and reduces hyperphagia (24 hr food intake in...
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A. Lepr-Cre::eGFP 20X
B. Merge 20X
C. Merge 20X
D. DMH NTS
E. PrRP-GFP pSTAT3 Merge
F. % pSTAT3 positive PrRP neurons

(legend on next page)
Cell Metabolism
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A

B

C

D

E

Figure 2. PrRP Neurons in the DMH Are Directly Responsive to Leptin

(A–C) (A) Neurons in the DMH immunofluorescent for PrRP (red) in Lepr-cre::eGFP (green) mice, and the merged image with digital zoom in adjacent panels. Representative merged images for (B) the NTS and (C) the VLM. PrRP and Lepr colocalize in cells of the DMH (merged yellow, indicated by white arrowheads) but not in the NTS or VLM. Blue arrowheads indicate cells expressing only PrRP. In the VLM, PrRP and Lepr neurons are not adjacent. 3V, third ventricle; AP, area postrema; Arc, arcuate nucleus; cc, central canal.

(D) Anatomical maps showing the distribution of Lepr-containing PrRP neurons in the DMH and NTS. Representative merged images for (B) the NTS and (C) the VLM. PrRP and Lepr colocalize in cells of the DMH (merged yellow, indicated by white arrowheads) but not in the NTS or VLM. Blue arrowheads indicate cells expressing only PrRP. In the VLM, PrRP and Lepr neurons are not adjacent. 3V, third ventricle; AP, area postrema; Arc, arcuate nucleus; cc, central canal.

(E) Nuclear pSTAT3 induction in the DMH 60 min after vehicle or leptin administration (5 mg/kg, i.p) to Lepr-cre::eGFP mice. PrRP neurons are immunostained for enhanced GFP. Black arrows indicate single stained PrRP neurons, and yellow arrows indicate PrRP neurons containing pSTAT3 immunoreactivity.

Figure 3. Lepr Receptors on PrRP Neurons in the DMH Mediate the Thermogenic Actions of Leptin

PrRP-cre mice were crossed with Leprflox/flox mice to knock out leptin receptors selectively in PrRP neurons.

(A) Growth curves of wt, Leprflox/flox, PrRP-cre, and PrRP-cre::Leprflox/flox male littermates. PrRP-cre::Leprflox/flox mice, which lack leptin receptor expression in PrRP neurons, diverge in weight at 10 to 11 weeks of age (n = 8 per group, bars represent mean ± SEM; two-way ANOVA repeated-measures, *p < 0.05).

(B and C) Leptin administration (5 mg/kg, i.p.) acutely increases body temperature in Leprflox/flox homozygotes and their (wt) littermates but not in mice lacking expression of leptin receptors on PrRP neurons (PrRP-cre::Leprflox/flox) (6 weeks old, n = 6 per group, bars represent mean ± SEM; two-way ANOVA, ***p < 0.001). Dotted line in (C) represents time of injection.

(D) Leptin reduces nocturnal food intake 4 hr after injection in wt mice; however, this effect is not significant in PrRP-cre::Leprflox/flox mice (6 to 7 weeks old, n = 5 to 6, bars represent mean ± SEM; two-way ANOVA, ‘p < 0.05).

(E) CCK (20 μg/kg, i.p.) causes a robust decrease in nocturnal food intake 1 hr after injection in wt and PrRP-cre::Leprflox/flox mice (6 weeks old, n = 5 to 6 per group, bars represent mean ± SEM; two-way ANOVA, **p < 0.01).

DISCUSSION

Leptin acts on a distributed network of neurons to affect the key determinants of body weight balance: energy intake and energy expenditure (Myers et al., 2009). Genetic isolation of phenotypically identified cell populations has described some first-order, leptin-responsive peptidergic neurons, notably those residing in the ventromedial region of the hypothalamus (Enriori et al., 2011; Zhang et al., 2011). A second-order action of leptin in the hypothalamus has been described (Cannon and Nedergaard, 2004; Scarpace and Matheny, 1998; Trayhurn et al., 1977). Though the neurons in the ventromedial region of the hypothalamus have been partly implicated in adaptive thermogenesis (Kim et al., 2011; Kong et al., 2012; Shi et al., 2013), compelling evidence is available for an important direct action of leptin on neurons of the dorsomedial region of the hypothalamus (Enriori et al., 2011; Zhang et al., 2011).

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POMC, NPY/Agrp, and SF-1/PACAP (Balthasar et al., 2004; Dhillon et al., 2006; Hawke et al., 2009; van de Wall et al., 2007). POMC and NPY/Agrp neurons engage downstream targets, such as in the paraventricular hypothalamic nucleus, that possess the melanocortin receptor (MC4R). However, this pathway is dissociated from that which mediates the actions of leptin on energy expenditure (Balthasar et al., 2005; Haynes et al., 1999). This distinct action of leptin is achieved by activating sympathetic output from the brain to increase nonshivering, adaptive thermogenesis by brown adipose tissue (Cannon and Nedergaard, 2004; Scarpace and Matheny, 1998; Trayhurn et al., 1977). Though the neurons in the ventromedial region of the hypothalamus have been partly implicated in adaptive thermogenesis (Kim et al., 2011; Kong et al., 2012; Shi et al., 2013), compelling evidence is available for an important direct action of leptin on neurons of the dorsomedial region of the hypothalamus (Eniori et al., 2011; Zhang et al., 2011).
Neurons in the DMH are part of the central circuitry that regulates brown-adipose-dependent thermogenesis in response to both leptin and cold stimulation (Enriori et al., 2011; Morrison et al., 2008; Zhang et al., 2011), but until now, their identity has remained undetermined. Here, we have defined a single population of first-order neuron that is critical for leptin’s actions on adaptive thermogenesis. PrRP neurons in the DMH are sensitive to energy status, possess Lepr, and respond to stimulation by leptin. As all of leptin’s effects on metabolism are mediated by brain-expressed Lepr (de Luca et al., 2005), and because the population in the DMH are the only PrRP neurons in the mouse to have Lepr, we can conclude that the obese phenotype of PrRP expression). Growth curves of wt and LSL-PrRP (B) males and (C) females when fed a high-energy diet. Dotted line represents transition from standard chow to high-energy diet. Body weights were significantly higher in the LSL-PrRP animals when compared with wt littermates at 12 weeks of age (see Table S1 for biometric data at culling and Figure S4 for validation of lack of PrRP expression). Growth curves of wt and LSL-PrRP (D) males and (E) females when fed a high-energy diet. Two-way ANOVA repeated measures; *p < 0.05; **p < 0.01.

knock-out of either PrRP or its cognate receptor, GPR10, in mice produces an obese phenotype (Gu et al., 2004; Takayanagi et al., 2008). Interestingly, a natural mutation of GPR10 also occurs in the Otsuka Long-Evans Tokushima Fatty (OLETF) rat strain, which is commonly studied as it also has a mutation in the CCK1 receptor (Funakoshi et al., 1995). However, the obesity and diabetes of the OLETF rat is completely reversed in congenic rats with the wild-type GPR10 allele, and so caution should be applied in using this animal as a selective CCK1 receptor mutant (Watanabe et al., 2005). Here, we reiterated the obese phenotype in our LSL-PrRP mouse but then reversed the phenotype by reinstating expression not only in the brains of nestin-Cre::LSL-PrRP mice but also in brainstem selective TH-Cre::LSL-PrRP mice. These results first demonstrate that there is not complete redundancy in leptin-sensitive metabolic pathways in the brain. Second, even though brainstem PrRP neurons are involved in satiety, but are not regulated directly by leptin, there are powerful links between forebrain and brainstem circuits that act in concert to regulate body weight (McMinn et al., 2000; Myers et al., 2009; Yang et al., 2009). It is likely that, due to the integrated nature of central circuits involved in regulating metabolism, modifying a single element may have relatively strong overall effects. It is noteworthy that manipulating leptin signaling in PrRP DMH can slightly attenuate the anorexic response to leptin, even though the major influence of leptin on food intake is probably at the level of the ventromedial hypothalamus (Baldaasaran et al., 2005; van de Wall et al., 2007).

The DMH is an integral part of the circuitry regulating body temperature, receiving input from peripheral and central sensors, and providing output to presympathetic neurons in the midbrain and spinal cord that innervate heat-producing brown

Figure 4. Transgenic Mice Expressing a Transcriptionally Silenced PrRP (LSL-PrRP) Allele Are Obese

(A–E) (A) A PrRP inducible knock-in allele was generated by inserting a loxP-flanked stop codon between the transcription initiation (+1) and the ATG of the PrRP coding sequence. Tissue-specific coexpression of Cre-recombinase will remove the stop codon and rescue PrRP transcription. Growth curves of wt and LSL-PrRP (B) males and (C) females when fed standard chow. Body weights were significantly higher in the LSL-PrRP animals when compared with wt littermates at 12 weeks of age (see Table S1 for biometric data at culling and Figure S4 for validation of lack of PrRP expression). Growth curves of wt and LSL-PrRP (D) males and (E) females when fed a high-energy diet. Dotted line represents transition from standard chow to high-energy diet. Body weights were significantly higher in the LSL-PrRP animals, when compared with wt littermates, 2 to 3 weeks later (n = 6 per group, bars represent mean ± SEM; two-way ANOVA repeated measures; *p < 0.05; **p < 0.01).
adipose tissue (Morrison et al., 2008). As well as initiating adaptive thermogenesis in response to cold stimulation, the same circuitry is engaged at the level of the DMH in response to obesogenic diets—an effect that is driven by leptin produced from white adipose tissue (Cannon and Nedergaard, 2004; Enrion et al., 2011; Zhang et al., 2011). NPY neurons in the DMH have been implicated in adaptive thermogenesis (Chao et al., 2011; Lee et al., 2013), though unlike arcuate NPY neurons, they do not contain leptin receptors and do not respond to leptin with an increase in pSTAT3 (Bi et al., 2003; Draper et al., 2010). The knock-down of NPY in the DMH by adeno-associated virus-delivered RNAi enhances the thermogenic capacity of brown adipose tissues (Chao et al., 2011), which suggests that it could have an opposing function compared with DMH PrRP. It would be interesting to hypothesize a “yin-yang” relationship between NPY and PrRP neurons in the DMH, similar to that seen with NPY and POMC neurons in the arcuate nucleus. Leptin and other metabolic signals may affect DMH NPY neurons indirectly via PrRP neurons, which together generate thermogenic output to presymptomatic neurons. Indeed, the increased expression of DMH NPY mRNA noted in obese OLETF rats (Bi et al., 2001) might be a direct consequence of a lack of PrRP-GPR10 signaling in this model (Watanabe et al., 2005). In the current studies, we have also confirmed the role of brainstem PrRP neurons, which together generate thermogenic output to upstream targets in the hypothalamus (Dodd and Luckman, 2013).

RFamides have evolutionarily conserved functions in feeding behavior and in energy balance, but their role in mammalian systems has received relatively little attention (Bechtold and Luckman, 2007). Populations of PrRP neuron in the hypothalamus and brainstem have distinct, nonredundant functions in both arms of body weight regulation: mediating the thermogenic actions of leptin and the satiating actions of CCK, respectively. Thus, they can be added as unique pieces to the distributed brain network affecting whole-body energy homeostasis.

**EXPERIMENTAL PROCEDURES**

**Animals**

All animal procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 (UK) and approved by the University of Manchester Animal Welfare and Ethics Review Board. Mice had access to normal chow (Beekay International, Hull) and water ad libitum unless stated otherwise. For some phenotyping studies, mice were fed on a high-energy diet (5.16 kcal/g, 18.3% protein, 60.9% fat by energy; Research Diets, New Brunswick). All mouse colonies were group housed in a temperature- (22°C ± 1°C) and humidity-controlled (45% ± 10%) environment on a 12:12 hr light/dark cycle. Experimental animals were singly housed for procedures requiring individual measurements of food intake or energy expenditure.

**Transgenic Mice**

BAC genomic library clones (rtgV, GenOway, Lyon) were used for the generation of targeting construct to generate heterozygous PrRP-Cre and LSL-PrRP mice. Briefly, the rtgV BAC clone collection, containing genomic fragments of 15–25 kb in size, was screened by PCR using two PrRP primer pairs. The first primer pair amplifies a genomic fragment of PrRP (official gene name Prth; accession number NM_001101847), enabling screening for the presence of the genomic fragment corresponding to the small homology arm used for targeting vector construction. The second primer pair amplifies a genomic fragment of the PrRP gene enabling screening for the presence of the genomic fragment corresponding to the homologous arm, which were cloned into a targeting vector. The PrRP-Cre targeting vector contained an IRES-Cre transgene inserted just downstream of the endogenous STOP codon in PrRP exon 2. The LSL-PrRP targeting construct incorporated loxp sites flanking a transcription STOP cassette just upstream the endogenous PrRP gene and a neomycin-resistance cassette (Figure 4A). The linearized targeting construct was transfected into embryonic stem cells, and correctly targeted clones were injected into blastocysts. High-percentage male chimeras (chimerism rate >50%) were mated with wild-type C57BL/6J mice to produce heterozygous offspring. F1 mice identified by PCR were further verified by Southern blot analysis. Heterozygous LSL-PrRP mice were mated with nestin-Cre (B6.Cg-Tg(Nes-cre)1Kln/J, C57BL/6, The Jackson Laboratory, Maine) and TH-Cre (B6.Cg-Tg(Th-cre)1Tmd/J, C57BL/6, The Jackson Laboratory) mice for the conditional rescue of PrRP. LSL-PrRP::eGFP mice are homozygous for Lepr-ires-Cre and the reporter gene, RosaGFP (Leshan et al., 2009).

The generation of the other transgenic mice has been described elsewhere. Heterozygous PrRP-Cre mice were also mated with Lepr<sup>inn/flox</sup> mice. This allowed for the conditional excision of leptin receptors in cells expressing Cre (Balthasar et al., 2004). The Lepr-Cre::eGFP mice are homozygous for Lepr-ires-Cre and the reporter gene, Rosa<sup>GFP</sup> (Leshan et al., 2009).
Laser-Capture Microdissection
Male, outbred CD1 mice (8 weeks old, Charles River, Sandwich) were subjected either to a 24 hr fast or 8 hr feeding on the high-energy diet. At the end of the experiments, mice were sacrificed, and the whole brain was isolated and frozen. Fifteen micrometer coronal sections were cut by cryostat and mounted on sterile RNase-free, membrane-coated glass slides (PALM Membrane Slides; PALM Microlaser Technologies, Bernried). Each slide was immediately placed on dry ice. Within 24 hr of sectioning, the frozen sections were thawed and fixed for 30 min in 95% ethanol. All solutions were prepared with RNase-free water. Laser-capture microdissection was performed using a PALM MicrolaserSystem (PALM Microlaser Technologies; Jovancovic et al., 2010). The DMH was microdissected from sections –1.82 to –2.20 mm from the brain and the NTS/VLM from –7.40 to –8.00 mm from bregma (Paxinos and Franklin, 2001). Following microdissection, the captured samples were stored at –80°C prior to RNA isolation.

Relative Quantitative Real-Time PCR
RNA was extracted using Trizol reagent (Life Technologies, Paisley), and total RNA quantity and quality determined using a NanoDrop 3300 (Thermo Scientific, Wilmingston). mRNA were reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington) and processed for quantitative real-time PCR using the QuantFast SYBR Green PCR Kit (QIAGEN, Manchester); β-actin was used for normalization. Relative quantification was achieved using the ΔΔCt method. The reactions were performed in an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Warrington).

Primers Used in Genotyping and Real-Time Quantitative PCR
Genotyping: LSL-PrRP fixed allele: (F) 5’-CAG GCA CCA CAC ACA CAC GAT GTA CAT C-3’; (R) 5’-GGG ATT TTT GGT CAT CTT GGA-3’; Cre allele: (F) 5’-GCC GTG CAA GGG ATT TTT GGA GCA-3’; (R) 5’-ATG GCT AAT CGC CAT CTT CCA GCA-3’;
Lepr fixed allele: (F) 5’-AAT GAA AAA GTT GGT TTT GGA-3’; (R) 5’-CAG TGT GGA CAT GAA CAC AAC ACT-3’; egFP: (F) 5’-AAG TTC ATC TGC ACC ACC-3’; (R) 5’-TCC TGG AAG ATG CGT CG-3’.

Relative quantitative PCR: PrRP: (F) 5’-TGG TGC TGC TAG GCT TAG TC-3’; (R) 5’-GGT GTA CCA GCA AGG AGT GA-3’; Ucp-1: (F) 5’-ATG GCC ACA CCT CCA ATT-3’; (R) 5’-TTT CTC GTC ACT CGT GAT TG-3’; β-actin: (F) 5’-AGA GGA AAA TCG TGC GTG AC-3’; (R) 5’-CAA TAG TGA TCA CTT GGC GGT C-3’.

Feeding Experiments
Mice were assigned randomly to receive i.p. injection of vehicle (0.9% w/v NaCl), CCK-8 sulphated (20 μg/kg; Tocris Bioscience, Bristol), or recombinant murine leptin (i.p. 5 mg/kg) in a volume of 4 ml/kg. Brain sections were processed for pSTAT3 immunohistochemistry (as described above) and subsequently incubated with an egFP antibody (1:1,000; AB13970, Abcam, Cambridge), which was visualized using a FITC-conjugated secondary antibody (1:1,000; Vector Laboratories, UK). The sections were photographed using a fluorescence microscope (Zeiss Axioskop, Carl Zeiss AG, Oberkochen) and assessed for pSTAT3/egFP colocalization. pSTAT3 immunoreactivity was photographed under a bright field, while the egFP fluorescent immunostain was photographed using an FITC filter. The pictures were merged using Adobe Photoshop 7.0 software, and colocalization was quantified using the number of pSTAT3-expressing cell nuclei was quantified only in brain regions containing egFP expression.

Statistical Analysis
Data are presented as means ± SEM. Statistical analyses were performed using Prism statistical package (GraphPad Software Inc, San Diego). Unpaired two-way t tests were used throughout to compare two distinct groups. When more than two groups were compared, a one-way or two-way ANOVA followed by Bonferroni’s multiple-comparison post hoc tests were used.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2014.07.022.
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