POMC neurons expressing leptin receptors coordinate metabolic responses to fasting via suppression of leptin levels

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Abstract

Leptin is critical for energy balance, glucose homeostasis, and for metabolic and neuroendocrine adaptations to starvation. A prevalent model predicts that leptin's actions are mediated through pro-opiomelanocortin (POMC) neurons that express leptin receptors (LEPRs). However, previous studies have used prenatal genetic manipulations, which may be subject to developmental compensation. Here, we tested the direct contribution of POMC neurons expressing LEPRs in regulating energy balance, glucose homeostasis and leptin secretion during fasting using a spatiotemporally controlled *Lepr* expression mouse model. We report a dissociation between leptin's effects on glucose homeostasis versus energy balance in POMC neurons. We show that these neurons are dispensable for regulating food intake, but are required for coordinating hepatic glucose production and for the fasting-induced fall in leptin levels, independent of changes in fat mass. We also identify a role for sympathetic nervous system regulation of the inhibitory adrenergic receptor (ADRA2A) in regulating leptin production. Collectively, our findings highlight a previously unrecognized role of POMC neurons in regulating leptin levels.

Introduction

Pro-opiomelanocortin (POMC) neurons of the arcuate nucleus of the hypothalamus (ARC) are critical regulators of energy balance and glucose homeostasis (Mercer, Hentges et al. 2013, Gautron, Elmquist et al. 2015). These neurons consist of a heterogeneous population with respect to neurotransmitters used and the receptors expressed (Hentges, Otero-Corchon et al. 2009, Williams, Margatho et al. 2010, Lam, Cimino et al. 2017). Electrophysiology and immunohistochemistry studies have established that ~30% of hypothalamic POMC neurons are responsive to leptin (Cheung, Clifton et al. 1997, Ernst, Wunderlich et al. 2009, Williams, Margatho et al. 2010). Given the role of POMC neurons and leptin in metabolism, a conventional model indicates that a subset of POMC cells that expresses the leptin receptor (LEPR) are mediating the metabolic actions of leptin (Cheung, Clifton et al. 1997, Balthasar, Dalgaard et al. 2005). This idea was supported by early observations that prenatal manipulations of LEPR-expressing POMC neurons mildly affect body weight (Munzberg, Huo et al. 2003, Balthasar, Coppari et al. 2004, Huo, Gamber et al. 2009, Berglund, Vianna et al. 2012, Huang, Kong et al. 2012, Mercer, Hentges et al. 2013). However, POMC neurons share developmental origins with other cell types, including subsets of NPY/AgRP neurons (Padilla, Carmody et al. 2010, Lam, Cimino et al. 2017). As such, it is possible that developmental compensation, or *Lepr* deletion from non-POMC neurons, are behind the phenotypes observed with conventional transgenic models (Bouret, Draper et al. 2004, Lam, Cimino et al. 2017). In addition, although it was repeatedly suggested that leptin's anorexigenic effects act through non-ARC POMC neurons (Myers, Munzberg et al. 2009, Berglund, Vianna et al. 2012, Berglund, Liu et al. 2013), the direct contribution of LEPR-expressing POMC neurons on glucose homeostasis has been difficult to dissect due to inevitable alterations of fat mass resulting from prenatal deletions. As such, dissociating the pathways involved in leptin's and melanocortin's effects on adiposity versus glucose homeostasis is key for the development of anti-obesity and anti-diabetes therapies.

The activity and expression of POMC is highly dependent on energy status (Mizuno, Kleopoulos et al. 1998). During obesity, there is an energy surplus and POMC levels are elevated (Schwartz, Seeley et al. 1997, Cowley, Smart et al. 2001). Inversely, during a state of negative energy balance, such as fasting, POMC expression is

decreased (Mizuno, Kleopoulos et al. 1998). Because POMC deficiency causes severe obesity, tremendous efforts have been made to understand a causative role of the POMC neurons in the pathophysiology of both syndromic and diet-induced obesity (Krude, Biebermann et al. 1998, Enriori, Evans et al. 2007). However, relatively little is known about the function of these neurons in the context of low energy levels, despite early suggestions that the effect of fasting to reduce POMC is physiologically relevant (Mizuno, Kleopoulos et al. 1998). In addition, fasting leads to a rapid fall in circulating leptin levels that is out of proportion to the loss in fat mass (Becker, Ongemba et al. 1995, Moinat, Deng et al. 1995, Saladin, De Vos et al. 1995, Ahima, Prabakaran et al. 1996, Flier 1998, Ahima, Kelly et al. 1999). Despite early suggestions that the fall in leptin represent a central physiologic response to fasting required for metabolic adaptations to low energy states, the mechanisms behind fasting-induced reductions in leptin are unknown (Ahima, Prabakaran et al. 1996, Flier 1998, Ahima, Kelly et al. 1999, Flier and Maratos-Flier 2017). Paradoxically, LEPR-null animals do not experience a decrease in leptin levels with fasting, suggesting that LEPRs themselves are required for the starvation-induced fall in leptin (Hardie, Rayner et al. 1996). Together, these observations indicate that neurons expressing LEPRs might play a role in repressing plasma leptin levels during starvation. However, the actual contribution of LEPR-expressing POMC neurons in regulating leptin secretion is unknown.

One way the CNS may regulate leptin is through altering activity of adrenergic receptors expressed by adipocytes. Acute activation of the sympathetic nervous system reduces leptin gene expression and leptin production through a β3-adrenoceptor (ADRB3)-dependent mechanism (Moinat, Deng et al. 1995, Gettys, Harkness et al. 1996, Giacobino 1996, Mantzoros, Qu et al. 1996, Trayhurn, Duncan et al. 1996, Deng, Moinat et al. 1997, Trayhurn, Duncan et al. 1998, Caron, Lee et al. 2018). In addition, forcing the expression of human α2-adrenoreceptor (ADRA2) in mouse adipose tissue results in elevated leptin (Valet, Grujic et al. 2000), suggesting that the ADRA2/ADRB3 balance in adipocytes is critical for leptin regulation. These observations suggest that leptin could regulate its own expression through a negative feedback loop from the brain to the adipose tissue. However, the central pathways and the mechanisms underlying these actions are yet to be fully characterized.

Here, we report that a subset of POMC neurons that express LEPRs directly control glucose homeostasis and are necessary to regulate leptin synthesis,

independent of changes in fat mass. We used a tamoxifen-inducible *Pomc*^{CreERt2} transgenic mouse model to generate mice in which *Lepr* expression is spatiotemporally-controlled in a neuron-specific fashion. Within one week of deleting LEPRs from POMC neurons in adult mice, hepatic glucose production was impaired, while body weight, food intake, and energy expenditure were unaltered. In addition, mice with adult deletion of LEPRs in POMC neurons showed an impairment in the fasting-induced fall in leptin levels. We also identified an important role for adipose tissue ADRA2A in regulating leptin synthesis. Our results support a model predicting that LEPR-expressing POMC neurons coordinate metabolic responses to fasting via suppression of leptin levels.

RESULTS

LEPR-expressing POMC neurons are required for normal liver insulin sensitivity in adult mice

The use of conventional prenatal *Pomc*^{Cre} models was key in deciphering the contribution of many receptors and pathways in glucose and energy homeostasis (Balthasar, Coppari et al. 2004, Caron, Labbe et al. 2016). However, it is now appreciated that prenatal manipulations may lead to compensatory events during development (Padilla, Carmody et al. 2010, Bouret, 2004 #6646). Importantly, there is a subpopulation of cells that express *Pomc*^{Cre} during development, but do not express POMC in adults (Padilla, Carmody et al. 2010). To circumvent these issues, we used a tamoxifen-inducible *Pomc*^{CreERt2} transgenic mouse model (Berglund, Liu et al. 2013) to generate *Pomc*^{CreERt2}::*Lepr*$^{flox/flox}$ mice in which *Lepr* expression is spatiotemporally controlled in a neuron-specific fashion. We first assessed the impact of adult deletion of LEPR-expressing POMC neurons on glucose homeostasis. Fed and fasting glycemia were not different before, or one week after, injection of tamoxifen, indicating that the drug *per se,* did not impair glucose levels (***Figure 1A***). However, adult ablation of LEPRs from POMC neurons resulted in significantly higher fasting glycemia as early as two weeks post-deletion, while fed glycemia was greater at three weeks (***Figure 1A***). This effect was sustained for the entire experimental period. Fed and fasting insulin and glucagon levels were not different between groups (***Figure 1B-C***). Although no changes in glycemia were detectable in the first week, insulin response was already substantially

impaired, as assessed by an insulin tolerance test (***Figure 1D-E***). We did not observe any difference in glycemia following a glucagon stimulation test (***Figure 1-figure supplement 2***).

We further explored the impact of deleting LEPRs in adult POMC neurons on systemic glucose metabolism by performing hypersulinemic-euglycemic clamp assays one week after the deletion in an independent cohort of animals. The glucose infusion rate needed to maintain euglycemia (119.3 ± 3.9 vs 122.0 ± 8.2 mg/dl) was significantly decreased in knock-out animals (***Figure 1F***), further demonstrating whole-body insulin resistance. Importantly, glucose disposal was unaltered, but insulin-induced suppression of hepatic glucose production was drastically impaired in the clamped state (***Figure 1G-H***). Moreover, the ability of insulin to suppress lipolysis during the clamped state was unaltered, suggesting that insulin resistance occurred specifically in the liver (***Figure 1I***). Deletion of LEPRs in POMC neurons in adult mice did not affect fed or fasting levels of insulin, NEFA and triglycerides (data not shown), again suggesting that impaired liver insulin sensitivity, but presumably not impaired insulin secretion, contributes to systemic insulin resistance. Altogether, these data demonstrate that LEPR-expressing POMC neurons directly regulate liver metabolism in adult mice. This is in agreement with previous findings (Hill, Elias et al. 2010, Berglund, Vianna et al. 2012) in which LEPRs were deleted during development. We found that insulin resistance can be detected one week post-deletion (***Figure 1D-I***), however blood glucose levels did not rise until two weeks post-deletion (***Figure 1A***). These findings suggest that deletion of LEPRs in adult POMC neurons impairs liver insulin sensitivity, and the resulting hepatic insulin resistance leads to the development of hyperglycemia.

LEPR-expressing POMC neurons are dispensable for the regulation of energy balance in adult mice

It has generally been assumed that LEPR-expressing POMC neurons are important for feeding and weight regulation (Cheung, Clifton et al. 1997, Balthasar, Dalgaard et al. 2005), despite evidence that other subsets of POMC neurons are more likely to regulate energy balance (Berglund, Liu et al. 2013). Because prenatal deletion of *Lepr* in POMC neurons impairs body weight and fat mass, the direct contribution of these neurons in regulating glucose homeostasis has always been hard to dissect. Here, we show that deleting LEPRs from POMC neurons in adult mice does not affect body

weight or body composition (***Figure 2A-C***). Four weeks following the deletion, we evaluated food intake and energy expenditure using metabolic cages. We observed that food intake was unchanged in mice lacking LEPRs in adult POMC neurons (***Figure 2D***). Moreover, oxygen consumption, respiratory exchange ratio (VCO_2/VO_2) and physical activity were all unaltered (***Figure 2E-G***). These results suggest that LEPR-expressing POMC neurons regulate liver insulin sensitivity independently of changes in body weight.

Fasting reduces *Pomc* mRNA expression in the ARC (Mizuno, Kleopoulos et al. 1998), and this reduction contributes to the promotion of hunger (Mercer, Hentges et al. 2013). We found that adult deletions of LEPRs in POMC neurons did not affect fed or fasting levels of *Pomc* mRNA (***Figure 3A***). Another population of hypothalamic neurons that regulate energy balance and glucose homeostasis are the orexigenic neuropeptide Y (NPY) / agouti-related peptide (AgRP) neurons (Schwartz, Woods et al. 2000, Morton, Cummings et al. 2006). During fasting, the activity of these neurons increases, which promotes food-seeking and eating behaviors (Takahashi and Cone 2005). Moreover, leptin inhibits NPY/AgRP neurons and fasting relieves this inhibition (Schwartz, Seeley et al. 1996). Interestingly, mice with adult deletions of LEPRs in POMC neurons had blunted mRNA levels of *Npy* and *Agrp* in response to starvation (***Figure 3B-C***). This suggests that despite normal food intake in unrestrained conditions (***Figure 2***), fasting-induced hyperphagia might be impaired in mice lacking LEPR in adult POMC neurons. However, we found that mice consumed the same amount of food when access to laboratory chow was restored after a 48-h fast (***Figure 4A***). Interestingly, feeding-induced hyperglycemia was higher in mice lacking LEPRs in adult POMC neurons (***Figure 4B***). Together, these results reinforce the idea that LEPR-expressing POMC neurons are dispensable for the regulation of energy balance in adult mice. Moreover, these data further demonstrate impaired glucose homeostasis when LEPRs are deleted from adult POMC neurons. At this point, it remains unclear whether manipulating LEPR-expressing POMC neurons results in dysfunction of NPY/AgRP neurons or if the receptors themselves are critical for the fasting response.

LEPR-expressing POMC neurons are required for the fasting-induced fall in leptin levels independent of changes in fat mass in adult mice

Fasting leads to a rapid fall in circulating leptin levels, despite no initial changes in fat mass (Becker, Ongemba et al. 1995, Moinat, Deng et al. 1995, Saladin, De Vos et al.

1995, Ahima, Prabakaran et al. 1996, Flier 1998, Ahima, Kelly et al. 1999). However, this regulation in leptin levels is blunted in LEPR-null animals (Hardie, Rayner et al. 1996), suggesting that LEPRs *per se* are required for the starvation-induced fall in leptin. In order to better understand the potential contribution of LEPR-expressing POMC neurons in regulating leptin production, we compared the impact of 48 h of fasting in prenatal and adult models. In contrast to prenatal deletions (***Figure 5A-B***), deleting LEPRs in POMC neurons in adult mice did not affect fasting-induced decreases in body weight or fat-mass loss (***Figure 5E-F***). Consistent with previous reports (Moinat, Deng et al. 1995, Trayhurn, Thomas et al. 1995, Ahima, Prabakaran et al. 1996, Hardie, Rayner et al. 1996), fasting induced a robust fall in both circulating leptin and visceral adipose *Lep* mRNA levels in wild-type littermate controls (***Figure 5C-D and G-H***). Strikingly, this effect was prevented in mice with either prenatal (***Figure 5C-D***) or adult (***Figure 5G-H***) deletions of LEPRs in POMC neurons. Although modest, expression of *Lep* in visceral adipose tissue was significantly higher, in fed mice lacking LEPRs in adult POMC neurons (***Figure 5D and H***), suggesting that the deletion may affect leptin regulation even in the fed state. Collectively, these results indicate that LEPR-expressing POMC neurons are required for the starvation-induced fall in leptin, independent of changes in fat mass. Preventing fasting-induced falls in leptin might explain the blunted response observed in *Agrp* and *Npy* expression (***Figure 3B-C***).

Gi-coupled alpha-2A adrenergic receptors (ADRA2A) regulate leptin synthesis

Given that adult deletions of LEPRs in POMC neurons are sufficient to prevent the fasting-induced fall in circulating leptin levels, we next sought to determine how these neurons regulate leptin production in adipose tissue. One way the CNS may negatively regulate leptin is through the activation of ADRB3 (Moinat, Deng et al. 1995, Collins and Surwit 1996, Gettys, Harkness et al. 1996, Giacobino 1996, Mantzoros, Qu et al. 1996, Trayhurn, Duncan et al. 1996, Trayhurn, Duncan et al. 1998, Evans, Agar et al. 1999). In addition, overexpression of ADRA2 in mouse adipose tissue increases leptin levels (Valet, Grujic et al. 2000), suggesting that the ADRA2/ADRB3 balance in adipocytes is critical for regulation of leptin. We first investigated the expression of the nine identified adrenergic receptors in visceral adipose tissue (***Figure 6A***). The expression of most of the adrenergic receptors was unchanged in mice with adult deletions of LEPRs in POMC neurons compared to wild-type littermates. However, the fasting-induced decrease in

Adra2a mRNA expression was not only prevented, but reversed following the deletion of LEPRs in adult POMC neurons (***Figure 6A***). Using an independent cohort, we found that this observation was not only reproducible, but also specific to visceral adipose tissue (***Figure 6B-C***). This result is in line with the fact that visceral but not subcutaneous adipose tissue is the primary source of leptin in rodents (Trayhurn, Thomas et al. 1995). These findings suggest that ADRA2A may be a candidate for mediating the starvation-induced fall in leptin.

The function of ADRA2A in adipocyte physiology and pathophysiology is well known (Lafontan and Berlan 1995, Garg, Sankella et al. 2016). However, its role in leptin synthesis has never been investigated. To functionally validate a role for ADRA2 in regulating leptin expression and production, C57BL/6J mice were intraperitoneally injected with the ADRA2 agonist clonidine, and visceral adipose tissue was collected 1 hour later. Strikingly, clonidine increased *Lep* mRNA expression by 6 fold (***Figure 7A***). In another cohort of C57BL/6J mice, we also observed that clonidine rapidly increased plasma leptin levels (***Figure 7B***). We next sought to evaluate whether clonidine treatment altered leptin production in mice with adult deletions of LEPRs in POMC neurons. Because clonidine affects every ADRA2, including those express in the CNS, we performed the experiment using adipose tissue explants from mice that were fed or fasted for 48 h prior to the euthanasia. In fed animals, we found higher leptin release in knock-out animals (***Figure 7C***), consistent with the higher expression of *Lep* mRNA observed in visceral adipose tissue (***Figure 5D, H***). Furthermore, in the fasted condition, clonidine was effective at inducing leptin release only in adipose tissue explants from mice with LEPRs deleted in adult POMC neurons (***Figure 7C***). These explant studies indicate that this effect is adipose tissue-autonomous and not mediated through central effects. These results are in line with the observation that *Adra2a* mRNA expression increases with fasting in visceral adipose tissue of knock-out animals (***Figure 6A***). Clonidine was ineffective in subcutaneous adipose tissue (***Figure 7D***), again suggesting that the regulation of leptin production is specific to visceral fat. Together, these results suggest a role for ADRA2 as critical regulator of both leptin expression and production. In addition, these data suggest that ablation of LEPRs in adult POMC neurons prevents the starvation-induced fall in leptin by increasing ADRA2A activity in visceral white adipose tissue.

Discussion

Leptin signaling in POMC neurons has been predicted to be key in regulating energy balance and glucose homeostasis (Munzberg, Huo et al. 2003, Balthasar, Coppari et al. 2004, Kievit, Howard et al. 2006, Huo, Gamber et al. 2009, Berglund, Vianna et al. 2012, Huang, Kong et al. 2012, Mercer, Hentges et al. 2013). Our current findings dissociate the effects of LEPR-expressing POMC neurons on glucose homeostasis and changes in energy balance. In addition, our results suggest that POMC neurons are key regulators of leptin levels. This is interesting as one of the questions in leptin biology is the mechanism behind starvation-induced falls in leptin (Friedman 2016, Beshel, Dubnau et al. 2017). Although it may appear paradoxical that a subset of LEPR-expressing POMC cells controls leptin synthesis, previous studies have suggested that LEPRs are required for the starvation-induced fall in leptin (MacDougald, Hwang et al. 1995, Hardie, Rayner et al. 1996, Commins, Watson et al. 2000). This supports previous models that falling leptin is required to activate neuroendocrine responses (Ahima, Prabakaran et al. 1996, Ahima, Kelly et al. 1999). We also identify a role for ADRA2A in regulating leptin levels during starvation. This is in agreement with a report in which expression of human ADRA2A in adipocytes resulted in elevated leptin levels (Valet, Grujic et al. 2000). Collectively, our study highlights a previously unrecognized role of POMC neurons in the regulation of leptin levels and provides a new framework for the understanding of leptin action and regulation in the context of changing states of energy balance.

The current study highlights the ongoing importance of developing more refined transgenic tools, including adult-inducible models. Here, we used a tamoxifen-inducible *Pomc*^{CreERt2} transgenic mouse model to generate mice in which *Lepr* expression is spatiotemporally controlled in a neuron-specific fashion. Recent findings have demonstrated a need for the development of such a tool. First, the central melanocortin pathways are developmentally plastic, and as such developmental and non-developmental compensations might affect the resulting phenotype, inherently limiting the conclusions that can be drawn (Bouret, Draper et al. 2004, Wu, Boyle et al. 2009, Padilla, Carmody et al. 2010, Bouret, Bates et al. 2012, Wu, Clark et al. 2012). In addition, POMC neurons share developmental origin with other cell types, including their NPY/AgRP counterparts (Padilla, Carmody et al. 2010). For instance, over 25% of POMC-positive neurons were shown to express high levels of *Agrp* (Lam, Cimino et al.

2017). Likewise, we recently developed an *Agrp*^{CreERt2} transgenic mouse model to better study the role of AgRP neurons in ghrelin response (Wang, Liu et al. 2014). These inducible tools will allow us to revisit fundamental beliefs about the central melanocortin system.

The canonical effect of leptin action in the brain is to regulate energy balance (Millington 2007, Mercer, Hentges et al. 2013). Despite early evidence that ablating LEPRs only in POMC neurons results in moderate changes in body weight (Balthasar, Coppari et al. 2004), leptin action on POMC neurons in the ARC is considered a prototypical site of action in the control of food intake and energy expenditure. We and others have previously proposed that leptin directly acts on POMC neurons to regulate glucose homeostasis (Huo, Gamber et al. 2009, Berglund, Vianna et al. 2012). There is also evidence that subpopulations of POMC neurons that do not express LEPRs may regulate food intake (Xu, Jones et al. 2008, Williams, Margatho et al. 2010, Berglund, Liu et al. 2013, Campbell, Macosko et al. 2017). It is also possible that the mild obesity observed in previous studies is the consequence of *Lepr* deletion from a proportion of AgRP neurons. Our data indicate that the effects of leptin on energy balance are not through direct actions on POMC neurons.

Here we show that action of leptin on POMC neurons regulates glucose homeostasis independent of its effects on energy balance. Specifically, removing LEPRs from POMC neurons in adult mice resulted in insulin resistance and impaired hepatic glucose production within one week following deletion. This was followed by sustained hyperglycemia, independent of changes in insulin and glucagon levels, in glucose disposal, or in the ability of insulin to suppress lipolysis. Although food intake was unaltered both in *ad libitum* or refeeding conditions, postprandial glycemia was impaired in mice lacking LEPRs in adult POMC neurons. Together, this suggests that altering leptin signaling in POMC neurons results in rapid-onset hepatic insulin resistance (Brown and Goldstein 2008). This specific effect is consistent with many reports showing direct consequences in the liver following genetic manipulations in POMC neurons (Hill, Elias et al. 2010, Xu, Berglund et al. 2010, Berglund, Vianna et al. 2012, Berglund, Liu et al. 2013, Shi, Zhou et al. 2013, Williams, Liu et al. 2014, Caron, Labbe et al. 2016). It was also recently shown that POMC neurons are important for hepatic parasympathic nerve activity in response to leptin (Bell, Harlan et al. 2018). A recent study also stresses the importance of insulin signaling in POMC neurons in regulating adipose tissue

lipolysis and the development of liver steatosis (Shin, Filatova et al. 2017). However, whether POMC neurons regulate glucose and lipid hepatic metabolism directly through the autonomic nervous system, or indirectly by altering metabolic hormone requires further investigation. It is nevertheless clear from our study that LEPR-expressing POMC neurons play a pivotal role in liver metabolism, independently of changes in energy balance.

Our data also highlight an unexpected role for LEPR-expressing POMC neurons in regulating the fasting-induced fall in leptin. We show that the ability of fasting to suppress leptin is impaired in transgenic mouse models with either prenatal or adult deletion of LEPRs in POMC neurons. Although there is a general consensus that leptin levels are tightly correlated to adiposity (Frederich, Hamann et al. 1995, Considine, Sinha et al. 1996), our data suggest that this fasting-dependent regulation is independent of changes in body weight or fat mass. Moreover, this effect appears specific to visceral adipose tissue, which is in line with the fact that leptin is predominantly secreted from visceral white adipocytes in rodents (Trayhurn, Thomas et al. 1995).

However, one important question still remains. In a particular, how does LEPR signalling in POMC neurons regulate adipocyte leptin secretion during fasting? One speculation is that the deletion of leptin receptors reduces POMC activity and renders the neurons less effective at activating downstream targets. Another possibility is more provocative. In particular, we propose that LEPR-expressing POMC neurons are part of a regulatory loop that is important for adaptative responses to fasting. Fasting rapidly alters key metabolic signals and decreases the circulating peripheral hormones (such as insulin) which are required to maintain normal leptin levels (Saladin, De Vos et al. 1995, D'Souza A, Neumann et al. 2017). These changes are all sensed by POMC neurons. However, drops in leptin trigger neuroendocrine responses that promote survival, including the inhibition of the sexual and thyroid axes and activation of the stress axis (Ahima, Prabakaran et al. 1996, Ahima, Kelly et al. 1999). These survival responses are extreme and safeguards may have evolved to ensure that they are not initiated too quickly. LEPR-expressing POMC neurons might represent such a “gatekeeper” to control the inhibition of leptin production. Thus, removing LEPRs from POMC neurons would prevent their ability to sense small fluctuations in leptin levels ultimately blunting the ability to fully suppress leptin levels.

In support of this model, we observed that fasting-induced expression of *Npy* and *Agrp* in the mediobasal hypothalamus was impaired in *Pomc*^{CreERt2}::*Lepr*$^{flox/flox}$ mice, suggesting that the falling leptin might be an important signal activating NPY/AgRP neurons during starvation (Bi, Robinson et al. 2003). Although this impaired response might be a direct consequence of elevated fasting-leptin levels, we did not observe any differences in food intake. Importantly, this does not invalidate the role of these neurons in regulating re-feeding behavior after a fast. However, these results indicate that preventing the normal fall in leptin levels during fasting have major repercussions, not only on the neuroendocrine system (Ahima, Prabakaran et al. 1996), but also on behavioral, metabolic and neuronal responses.

Mechanistically, we show that visceral adipose tissue expression of *Adra2a*, which normally decreases with fasting, is actually increasing in mice lacking fasted mice lacking LEPRs in adult POMC neurons. Interestingly, the expression of *Adra2a* is not altered in subcutaneuous adipose tissue, further supporting visceral-dependent effect. It is noteworthy that the sympathetic regulation differs between different fat depots, both in terms of innervation and outflow (Brito, Brito et al. 2007, Brito, Brito et al. 2008, Nguyen, Barr et al. 2017). These findings also add another layer of complexity to the way the brain regulates peripheral tissues through the activation of GPCRs. Our pharmacological experiments also support the notion that ADRA2 are important for leptin regulation. ADRB3 is well-known to negatively regulate leptin though a cAMP-dependent mechanism (Moinat, Deng et al. 1995, Gettys, Harkness et al. 1996, Giacobino 1996, Mantzoros, Qu et al. 1996, Slieker, Sloop et al. 1996, Trayhurn, Duncan et al. 1996, Deng, Moinat et al. 1997, Trayhurn, Duncan et al. 1998, Caron, Lee et al. 2018). Because ADRB3 is Gs-coupled, we hypothesize that Gi-coupled ADRA2 might have the opposite action on leptin synthesis. Treating mice with an ADRA2 agonist is sufficient to increase both circulating leptin and mRNA levels in visceral fat. We also found that this regulation is tissue-autonomous, as clonidine effectively affected leptin release only in visceral adipose tissue explants from mice lacking LEPRs in adult POMC neurons. From a translational point of view, the observation that ADRA2A activation stimulates leptin production is meaningful. Human adipocytes express high levels of ADRA2A but few or no ADRB3, while murine adipocytes show high levels of ADRB3 and very low number of ADRA2 (Lafontan and Berlan 1993, Lafontan and Berlan 1995). By creating mice that have a human-like pattern of adrenoreceptors, researchers previously established that

the ADRA2/ADRB2 balance in adipocytes is critical for regulating fat mass (Valet, Grujic et al. 2000). Increasing the ADRA2/ADRB3 balance in adipose tissue resulted in increased circulating levels of leptin, suggesting that this balance is also important for regulating leptin production. However, because these mice were obese, the direct contribution of the ADRA2/ADRB3 balance was hard to define. Here, we show that despite no changes in body weight, the ADRA2/ADRB3 balance in adipocyte is still important for leptin regulation.

In conclusion, our study indicates that a subset POMC neurons that express LEPRs directly controls glucose homeostasis and is necessary to control leptin synthesis, independently of changes in fat mass. We also identified an important role for adipose tissue ADRA2A in regulating leptin synthesis. From a conceptual standpoint, our results predict that leptin regulates its own expression through a negative feedback loop between POMC neurons and adipose tissue.

Materials and methods

Animals

Animal work described in this manuscript has been approved and conducted under the oversight of the UT Southwestern Institutional Animal Care and Use Committee (IACUC). Male mice were housed at an ambient temperature of 23 ± 1˚C and maintained on a 12-hour light/dark cycle (lights on 0700-1900) and fed with normal mouse chow diet (Harlan, Teklad Global 16% Protein Rodent Diet 2016; 12% kcal from fat, 3 kcal/g).

Pomc^{Cre} (RRID:IMSR_JAX:005965) mice (Balthasar, Coppari et al. 2004) and *Pomc*^{CreERt2} (RRID:MGI:5569339) mice (Berglund, Liu et al. 2013) were crossed with *Lepr*$^{flox/flox}$ (RRID:MGI:3511747) mice (McMinn, Liu et al. 2004) to generate mice with constitutive deletion of LEPRs in POMC neurons (*Pomc*^{Cre}::*Lepr*$^{flox/flox}$) and adult deletion of LEPRs in POMC neurons (*Pomc*^{CreERt2}::*Lepr*$^{flox/flox}$) respectively. Mice were maintained on a C57Bl/6J (RRID:IMSR_JAX:000664) background at UT Southwestern Medical Center. Adult ablation was induced by tamoxifen. Tamoxifen (0.15 mg/kg; Sigma-Aldrich, T5648) was suspended in corn oil (Sigma-Aldrich, C8267) and was administered intraperitoneally (three injections every 48 h for 5 days) to 10-12-week-old

Pomc^{CreERt2}::*Lepr*$^{flox/flox}$ and *Pomc*^{CreERt2}::*Lepr*$^{+/+}$ (littermate control) mice. Fasting experiments were performed from 0800 to 0800 (48 h) or from 1600 to 0800 (16 h). The efficiency of the recombination following tamoxifen was performed by crossing *Pomc*^{CreERt2} mice with Ai14(RCL-tdT)-D mice (RRID:IMSR_JAX:007914) mice. Validation of the is presented in Figure 1-figure supplement 1.

Immunohistochemistry and validation of the inducible mice

Immunohistochemistry was performed to visualize phospho-Stat3 (Tyr705, Cell Signaling Technology Cat# 9131, RRID:AB_331586), β-endorphin (Phoenix Pharmaceuticals Cat# H-022-33, RRID:AB_2314007), as well as the fluorescent reporter tdTomato (Santa Cruz Biotechnology Cat# sc-33354, RRID:AB_639922) in the brain and pituitary (Scott, Lachey et al. 2009, Williams, Margatho et al. 2010). For leptin-induced Stat3 activation experiments, mice were fasted for 16 h (1600 to 0800) and injected i.p. with mouse recombinant leptin (5 mg/kg; National Hormone and Peptide Program, AFP1783). Mice were anesthetized 45 minutes later using an i.p. injection of chloral hydrate (350 mg/kg) and then perfused transcardially with 0.9% saline followed by 10% neutral buffered formalin.

Assessment of insulin sensitivity and glucose levels

Blood samples were collected from the tail vein and glucose was measured using a glucometer (Bayer's Contour Blood Glucose Monitoring System; Leverkusen, Germany). For insulin tolerance test (ITT), mice were fasted for 4 hours and then administered insulin by intraperitoneal injection (0.75 U/kg body weight, human insulin, Eli Lilly).

Hyperinsulinemic-euglycemic clamps

Hyperinsulinemic-euglycemic clamps were performed on conscious, unrestrained mice as previously described (Holland, Miller et al. 2011). Euglycemia was maintained by variable infusion of 20% dextrose. Steady state was achieved 80 minutes after initiating hyperinsulinemia and maintained for 40 minutes. Additional blood samples were taken before initiating hyperinsulinemia and at the end of the clamp for analysis of insulin and free fatty acids.

Glucagon stimulation test

Glucagon stimulation test was performed in mice fasted for one hour (0800 to 0900). Briefly, human recombinant glucagon (120 µg/kg i.p.) was given and blood glucose monitored every 10 min for one hour.

Assessment of leptin, insulin and glucagon levels

Blood was collected in EDTA tubes. Plasma was isolated by centrifugation (4000 g x 10 min at 4℃) and was stored at -80℃ for further biochemical analyses. Plasma leptin (Mouse / Rat Leptin ELISA, ALPCO, 22-LEPMS-E01), insulin (Mouse Ultrasensitive Insulin ELISA, ALPCO, 80-INSMSU-E01), and glucagon (Mercodia Glucagon ELISA, 10-1281-01) were measured following manufacturer recommendations.

Assessment of body composition

Fat mass and lean mass were assessed by nuclear magnetic resonance (NMR) spectroscopy using a nuclear magnetic resonance (NMR) spectroscopy (Bruker Minispec mq10 NMR 0.23T/10MHz).

Metabolic cages studies

A combined indirect calorimetry system (CaloSys Calorimetry System, TSE Systems Inc.) was used for all metabolic studies. Experimental animals were acclimated for 5 days in a metabolic chamber with food and water. Oxygen consumption (VO2), carbon dioxide production (VCO2), respiratory exchange ration (RER) and food intake were measured after acclimation. Locomotion was measured using a multi-dimensional infrared light beam system.

Quantitative real-time PCR

Total mRNA was isolated from visceral (epidymal) and subcutaneous (inguinal) white adipose tissues using the RNeasy Lipid Tissue Mini Kit (Qiagen, 74104). Total mRNA was isolated from liver using RNA STAT-60 reagent (Tel-Test, Inc). The RNA concentrations were estimated from absorbance at 260 nm. cDNA synthesis was performed using a High Capacity cDNA Kit (Applied Biosystems). mRNA extraction and cDNA synthesis were performed following the manufacturer's instructions. cDNA was diluted in DNase-free water before quantification by real-time PCR. mRNA transcript levels were measured in duplicate samples using a ABI 7900 HT Sequence Detection

System (Applied Biosystems). The relative amounts of all mRNAs were calculated using the ΔΔCT assay. Primers for *18s* (Hs99999901_s1), *Adra1a* (Mm00442668_m1), *Adra1b* (Mm00431685_m1), *Adra1d* (Mm01328600_m1), *Adra2a* (Mm00845383_s1), Adra2b (Mm00477390_s1), *Adra2c* (Mm00431686_s1), *Adrb1* (Mm00431701_s1), Adrb2 (Mm02524224_s1), *Adrb3* (Mm02601819_g1), *Agrp* (Mm00475829_g1), *Lep* (Mm00434759_m1), *Npy* (Mm00445771_m1) and *Pomc* (Mm00435874_m1) were purchased from Applied Biosystems.

Pharmacological activation of ADRA2 *in vivo*

The ADRA2 agonist clonidine hydrochloride (Sigma-Aldrich, C7897) was administered intraperitoneally (1 mg/kg) to 10-12-week-old C57BL/6J mice following 4 hours of fasting. Two independent cohorts were used to evaluate leptin RNA expression and circulating levels.

Ex vivo leptin release assay

Pomc^{CreERt2}::*Lepr*$^{flox/flox}$ and *Pomc*^{CreERt2}::*Lepr*$^{+/+}$ (littermate control) mice were fasted for 48 hours and ~10-20 mg of visceral (epididymal) and subcutaneous (inguinal) white adipose tissues were cultured in 384 wells plate containing 0.200 ml of Krebs-Ringer Bicarbonate Buffer containing 5 mM glucose and 4% fatty acid-free BSA, as described (Caviglia, Betters et al. 2011). Tissues were subsequently treated either with or without 1 µM clonidine hydrochloride (Sigma-Aldrich, C7897) for basal and clonidine conditions respectively, and leptin release was measured by ELISA and corrected to tissue weight.

Statistical analysis

Data are expressed as mean ± SEM. Comparison between 2 experimental conditions were analyzed by Student's unpaired t test. Two-way ANOVA followed by Bonferroni post hoc test was used to compare more than two experimental conditions. All statistical tests were performed using GraphPad Prism (version 7.0), and $p<0.05$ was considered statistically significant.

Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers
strain (Tg(Pomc-cre)1Lowl)	*Pomc*^{Cre} mouse	PMID: 17556551	RRID:IMSR_JAX:010714
strain (Tg(Pomc-cre/ERT2)#Jke)	*Pomc*^{CreERt2} mouse	PMID: 24177424	RRID:MGI:5569339
strain (Leprtm1.1Chua)	*Lepr*$^{flox/flox}$ mouse	PMID: 15389315	RRID:MGI:3511747
strain (Gt(ROSA)26Sortm14(CAG-tdTomato)Hze)	Ai14(RCL-tdT)-D mouse	PMID: 20023653	RRID:IMSR_JAX:007914
antibody (AB_331586)	phospho-Stat3 antibody	Tyr705, Cell Signaling Technology Cat# 9131,	RRID:AB_331586
antibody (AB_2314007)	β-endorphin antibody	Phoenix Pharmaceuticals Cat# H-022-33	RRID:AB_2314007
antibody (AB_639922)	tdTomato antibody	Santa Cruz Biotechnology Cat# sc-33354,	RRID:AB_639922

511

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References

Ahima, R. S., J. Kelly, J. K. Elmquist and J. S. Flier (1999). "Distinct physiologic and neuronal responses to decreased leptin and mild hyperleptinemia." Endocrinology **140**(11): 4923-4931.

Ahima, R. S., D. Prabakaran, C. Mantzoros, D. Qu, B. Lowell, E. Maratos-Flier and J. S. Flier (1996). "Role of leptin in the neuroendocrine response to fasting." Nature **382**(6588): 250-252.

Balthasar, N., R. Coppari, J. McMinn, S. M. Liu, C. E. Lee, V. Tang, C. D. Kenny, R. A. McGovern, S. C. Chua, Jr., J. K. Elmquist and B. B. Lowell (2004). "Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis." Neuron **42**(6): 983-991.

Balthasar, N., L. T. Dalgaard, C. E. Lee, J. Yu, H. Funahashi, T. Williams, M. Ferreira, V. Tang, R. A. McGovern, C. D. Kenny, L. M. Christiansen, E. Edelstein, B. Choi, O. Boss, C. Aschkenasi, C. Y. Zhang, K. Mountjoy, T. Kishi, J. K. Elmquist and B. B. Lowell (2005). "Divergence of melanocortin pathways in the control of food intake and energy expenditure." Cell **123**(3): 493-505.

Becker, D. J., L. N. Ongemba, V. Brichard, J. C. Henquin and S. M. Brichard (1995). "Diet- and diabetes-induced changes of ob gene expression in rat adipose tissue." FEBS Lett **371**(3): 324-328.

Bell, B. B., S. M. Harlan, D. A. Morgan, D. F. Guo and K. Rahmouni (2018). "Differential contribution of POMC and AgRP neurons to the regulation of regional autonomic nerve activity by leptin." Mol Metab **8**: 1-12.

Berglund, E. D., C. Liu, J. W. Sohn, T. Liu, M. H. Kim, C. E. Lee, C. R. Vianna, K. W. Williams, Y. Xu and J. K. Elmquist (2013). "Serotonin 2C receptors in pro-opiomelanocortin neurons regulate energy and glucose homeostasis." J Clin Invest **123**(12): 5061-5070.

Berglund, E. D., C. R. Vianna, J. Donato, Jr., M. H. Kim, J. C. Chuang, C. E. Lee, D. A. Lauzon, P. Lin, L. J. Brule, M. M. Scott, R. Coppari and J. K. Elmquist (2012). "Direct

546 leptin action on POMC neurons regulates glucose homeostasis and hepatic insulin
547 sensitivity in mice." J Clin Invest **122**(3): 1000-1009.

548 Beshel, J., J. Dubnau and Y. Zhong (2017). "A Leptin Analog Locally Produced in the
549 Brain Acts via a Conserved Neural Circuit to Modulate Obesity-Linked Behaviors in
550 Drosophila." Cell Metab **25**(1): 208-217.

551 Bi, S., B. M. Robinson and T. H. Moran (2003). "Acute food deprivation and chronic food
552 restriction differentially affect hypothalamic NPY mRNA expression." Am J Physiol Regul
553 Integr Comp Physiol **285**(5): R1030-1036.

554 Bouret, S. G., S. H. Bates, S. Chen, M. G. Myers, Jr. and R. B. Simerly (2012). "Distinct
555 roles for specific leptin receptor signals in the development of hypothalamic feeding
556 circuits." J Neurosci **32**(4): 1244-1252.

557 Bouret, S. G., S. J. Draper and R. B. Simerly (2004). "Formation of projection pathways
558 from the arcuate nucleus of the hypothalamus to hypothalamic regions implicated in the
559 neural control of feeding behavior in mice." J Neurosci **24**(11): 2797-2805.

560 Brito, M. N., N. A. Brito, D. J. Baro, C. K. Song and T. J. Bartness (2007). "Differential
561 activation of the sympathetic innervation of adipose tissues by melanocortin receptor
562 stimulation." Endocrinology **148**(11): 5339-5347.

563 Brito, N. A., M. N. Brito and T. J. Bartness (2008). "Differential sympathetic drive to
564 adipose tissues after food deprivation, cold exposure or glucoprivation." Am J Physiol
565 Regul Integr Comp Physiol **294**(5): R1445-1452.

566 Brown, M. S. and J. L. Goldstein (2008). "Selective versus total insulin resistance: a
567 pathogenic paradox." Cell Metab **7**(2): 95-96.

568 Campbell, J. N., E. Z. Macosko, H. Fenselau, T. H. Pers, A. Lyubetskaya, D. Tenen, M.
569 Goldman, A. M. Verstegen, J. M. Resch, S. A. McCarroll, E. D. Rosen, B. B. Lowell and
570 L. T. Tsai (2017). "A molecular census of arcuate hypothalamus and median eminence
571 cell types." Nat Neurosci.

572 Caron, A., S. M. Labbe, M. Mouchiroud, R. Huard, D. Lanfray, D. Richard and M.
573 Laplante (2016). "DEPTOR in POMC neurons affects liver metabolism but is

574 dispensable for the regulation of energy balance." Am J Physiol Regul Integr Comp
575 Physiol **310**(11): R1322-1331.

576 Caron, A., S. Lee, J. K. Elmquist and L. Gautron (2018). "Leptin and brain–adipose
577 crosstalks." Nat Rev Neurosci.

578 Caviglia, J. M., J. L. Betters, D. H. Dapito, C. C. Lord, S. Sullivan, S. Chua, T. Yin, A.
579 Sekowski, H. Mu, L. Shapiro, J. M. Brown and D. L. Brasaemle (2011). "Adipose-
580 selective overexpression of ABHD5/CGI-58 does not increase lipolysis or protect against
581 diet-induced obesity." J Lipid Res **52**(11): 2032-2042.

582 Cheung, C. C., D. K. Clifton and R. A. Steiner (1997). "Proopiomelanocortin neurons are
583 direct targets for leptin in the hypothalamus." Endocrinology **138**(10): 4489-4492.

584 Collins, S. and R. S. Surwit (1996). "Pharmacologic manipulation of ob expression in a
585 dietary model of obesity." J Biol Chem **271**(16): 9437-9440.

586 Commins, S. P., P. M. Watson, N. Levin, R. J. Beiler and T. W. Gettys (2000). "Central
587 leptin regulates the UCP1 and ob genes in brown and white adipose tissue via different
588 beta-adrenoceptor subtypes." J Biol Chem **275**(42): 33059-33067.

589 Considine, R. V., M. K. Sinha, M. L. Heiman, A. Kriauciunas, T. W. Stephens, M. R.
590 Nyce, J. P. Ohannesian, C. C. Marco, L. J. McKee, T. L. Bauer and et al. (1996). "Serum
591 immunoreactive-leptin concentrations in normal-weight and obese humans." N Engl J
592 Med **334**(5): 292-295.

593 Cowley, M. A., J. L. Smart, M. Rubinstein, M. G. Cerdan, S. Diano, T. L. Horvath, R. D.
594 Cone and M. J. Low (2001). "Leptin activates anorexigenic POMC neurons through a
595 neural network in the arcuate nucleus." Nature **411**(6836): 480-484.

596 D'Souza A, M., U. H. Neumann, M. M. Glavas and T. J. Kieffer (2017). "The
597 glucoregulatory actions of leptin." Mol Metab **6**(9): 1052-1065.

598 Deng, C., M. Moinat, L. Curtis, A. Nadakal, F. Preitner, O. Boss, F. Assimacopoulos-
599 Jeannet, J. Seydoux and J. P. Giacobino (1997). "Effects of beta-adrenoceptor subtype
600 stimulation on obese gene messenger ribonucleic acid and on leptin secretion in mouse
601 brown adipocytes differentiated in culture." Endocrinology **138**(2): 548-552.

Enriori, P. J., A. E. Evans, P. Sinnayah, E. E. Jobst, L. Tonelli-Lemos, S. K. Billes, M. M. Glavas, B. E. Grayson, M. Perello, E. A. Nillni, K. L. Grove and M. A. Cowley (2007). "Diet-induced obesity causes severe but reversible leptin resistance in arcuate melanocortin neurons." Cell Metab **5**(3): 181-194.

Ernst, M. B., C. M. Wunderlich, S. Hess, M. Paehler, A. Mesaros, S. B. Koralov, A. Kleinridders, A. Husch, H. Munzberg, B. Hampel, J. Alber, P. Kloppenburg, J. C. Bruning and F. T. Wunderlich (2009). "Enhanced Stat3 activation in POMC neurons provokes negative feedback inhibition of leptin and insulin signaling in obesity." J Neurosci **29**(37): 11582-11593.

Evans, B. A., L. Agar and R. J. Summers (1999). "The role of the sympathetic nervous system in the regulation of leptin synthesis in C57BL/6 mice." FEBS Lett **444**(2-3): 149-154.

Flier, J. S. (1998). "Clinical review 94: What's in a name? In search of leptin's physiologic role." J Clin Endocrinol Metab **83**(5): 1407-1413.

Flier, J. S. and E. Maratos-Flier (2017). "Leptin's Physiologic Role: Does the Emperor of Energy Balance Have No Clothes?" Cell Metab **26**(1): 24-26.

Frederich, R. C., A. Hamann, S. Anderson, B. Lollmann, B. B. Lowell and J. S. Flier (1995). "Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action." Nat Med **1**(12): 1311-1314.

Friedman, J. (2016). "The long road to leptin." J Clin Invest **126**(12): 4727-4734.

Garg, A., S. Sankella, C. Xing and A. K. Agarwal (2016). "Whole-exome sequencing identifies ADRA2A mutation in atypical familial partial lipodystrophy." JCI Insight **1**(9).

Gautron, L., J. K. Elmquist and K. W. Williams (2015). "Neural Control of Energy Balance: Translating Circuits to Therapies." Cell **161**(1): 133-145.

Gettys, T. W., P. J. Harkness and P. M. Watson (1996). "The beta 3-adrenergic receptor inhibits insulin-stimulated leptin secretion from isolated rat adipocytes." Endocrinology **137**(9): 4054-4057.

629 Giacobino, J. P. (1996). "Role of the beta3-adrenoceptor in the control of leptin
630 expression." Horm Metab Res **28**(12): 633-637.

631 Hardie, L. J., D. V. Rayner, S. Holmes and P. Trayhurn (1996). "Circulating leptin levels
632 are modulated by fasting, cold exposure and insulin administration in lean but not Zucker
633 (fa/fa) rats as measured by ELISA." Biochem Biophys Res Commun **223**(3): 660-665.

634 Hentges, S. T., V. Otero-Corchon, R. L. Pennock, C. M. King and M. J. Low (2009).
635 "Proopiomelanocortin expression in both GABA and glutamate neurons." J Neurosci
636 **29**(43): 13684-13690.

637 Hill, J. W., C. F. Elias, M. Fukuda, K. W. Williams, E. D. Berglund, W. L. Holland,
638 Y. R. Cho, J. C. Chuang, Y. Xu, M. Choi, D. Lauzon, C. E. Lee, R. Coppari, J. A.
639 Richardson, J. M. Zigman, S. Chua, P. E. Scherer, B. B. Lowell, J. C. Bruning and
640 J. K. Elmquist (2010). "Direct insulin and leptin action on pro-opiomelanocortin
641 neurons is required for normal glucose homeostasis and fertility." Cell Metab
642 **11**(4): 286-297.

643 Holland, W. L., R. A. Miller, Z. V. Wang, K. Sun, B. M. Barth, H. H. Bui, K. E. Davis, B. T.
644 Bikman, N. Halberg, J. M. Rutkowski, M. R. Wade, V. M. Tenorio, M. S. Kuo, J. T.
645 Brozinick, B. B. Zhang, M. J. Birnbaum, S. A. Summers and P. E. Scherer (2011).
646 "Receptor-mediated activation of ceramidase activity initiates the pleiotropic actions of
647 adiponectin." Nat Med **17**(1): 55-63.

648 Huang, H., D. Kong, K. H. Byun, C. Ye, S. Koda, D. H. Lee, B. C. Oh, S. W. Lee, B. Lee,
649 J. M. Zabolotny, M. S. Kim, C. Bjorbaek, B. B. Lowell and Y. B. Kim (2012). "Rho-kinase
650 regulates energy balance by targeting hypothalamic leptin receptor signaling." Nat
651 Neurosci **15**(10): 1391-1398.

652 Huo, L., K. Gamber, S. Greeley, J. Silva, N. Huntoon, X. H. Leng and C. Bjorbaek
653 (2009). "Leptin-dependent control of glucose balance and locomotor activity by POMC
654 neurons." Cell Metab **9**(6): 537-547.

655 Kievit, P., J. K. Howard, M. K. Badman, N. Balthasar, R. Coppari, H. Mori, C. E. Lee, J.
656 K. Elmquist, A. Yoshimura and J. S. Flier (2006). "Enhanced leptin sensitivity and

improved glucose homeostasis in mice lacking suppressor of cytokine signaling-3 in POMC-expressing cells." Cell Metab **4**(2): 123-132.

Krude, H., H. Biebermann, W. Luck, R. Horn, G. Brabant and A. Gruters (1998). "Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans." Nat Genet **19**(2): 155-157.

Lafontan, M. and M. Berlan (1993). "Fat cell adrenergic receptors and the control of white and brown fat cell function." J Lipid Res **34**(7): 1057-1091.

Lafontan, M. and M. Berlan (1995). "Fat cell alpha 2-adrenoceptors: the regulation of fat cell function and lipolysis." Endocr Rev **16**(6): 716-738.

Lam, B. Y. H., I. Cimino, J. Polex-Wolf, S. Nicole Kohnke, D. Rimmington, V. Iyemere, N. Heeley, C. Cossetti, R. Schulte, L. R. Saraiva, D. W. Logan, C. Blouet, S. O'Rahilly, A. P. Coll and G. S. H. Yeo (2017). "Heterogeneity of hypothalamic pro-opiomelanocortin-expressing neurons revealed by single-cell RNA sequencing." Mol Metab **6**(5): 383-392.

MacDougald, O. A., C. S. Hwang, H. Fan and M. D. Lane (1995). "Regulated expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes." Proc Natl Acad Sci U S A **92**(20): 9034-9037.

Mantzoros, C. S., D. Qu, R. C. Frederich, V. S. Susulic, B. B. Lowell, E. Maratos-Flier and J. S. Flier (1996). "Activation of beta(3) adrenergic receptors suppresses leptin expression and mediates a leptin-independent inhibition of food intake in mice." Diabetes **45**(7): 909-914.

McMinn, J. E., S. M. Liu, I. Dragatsis, P. Dietrich, T. Ludwig, S. Eiden and S. C. Chua, Jr. (2004). "An allelic series for the leptin receptor gene generated by CRE and FLP recombinase." Mamm Genome **15**(9): 677-685.

Mercer, A. J., S. T. Hentges, C. K. Meshul and M. J. Low (2013). "Unraveling the central proopiomelanocortin neural circuits." Front Neurosci **7**: 19.

Millington, G. W. (2007). "The role of proopiomelanocortin (POMC) neurones in feeding behaviour." Nutr Metab (Lond) **4**: 18.

Mizuno, T. M., S. P. Kleopoulos, H. T. Bergen, J. L. Roberts, C. A. Priest and C. V. Mobbs (1998). "Hypothalamic pro-opiomelanocortin mRNA is reduced by fasting and [corrected] in ob/ob and db/db mice, but is stimulated by leptin." Diabetes **47**(2): 294-297.

Moinat, M., C. Deng, P. Muzzin, F. Assimacopoulos-Jeannet, J. Seydoux, A. G. Dulloo and J. P. Giacobino (1995). "Modulation of obese gene expression in rat brown and white adipose tissues." FEBS Lett **373**(2): 131-134.

Morton, G. J., D. E. Cummings, D. G. Baskin, G. S. Barsh and M. W. Schwartz (2006). "Central nervous system control of food intake and body weight." Nature **443**(7109): 289-295.

Munzberg, H., L. Huo, E. A. Nillni, A. N. Hollenberg and C. Bjorbaek (2003). "Role of signal transducer and activator of transcription 3 in regulation of hypothalamic proopiomelanocortin gene expression by leptin." Endocrinology **144**(5): 2121-2131.

Myers, M. G., Jr., H. Munzberg, G. M. Leinninger and R. L. Leshan (2009). "The geometry of leptin action in the brain: more complicated than a simple ARC." Cell Metab **9**(2): 117-123.

Nguyen, N. L., C. L. Barr, V. Ryu, Q. Cao, B. Xue and T. J. Bartness (2017). "Separate and shared sympathetic outflow to white and brown fat coordinately regulates thermoregulation and beige adipocyte recruitment." Am J Physiol Regul Integr Comp Physiol **312**(1): R132-R145.

Padilla, S. L., J. S. Carmody and L. M. Zeltser (2010). "Pomc-expressing progenitors give rise to antagonistic neuronal populations in hypothalamic feeding circuits." Nat Med **16**(4): 403-405.

Saladin, R., P. De Vos, M. Guerre-Millo, A. Leturque, J. Girard, B. Staels and J. Auwerx (1995). "Transient increase in obese gene expression after food intake or insulin administration." Nature **377**(6549): 527-529.

Schwartz, M. W., R. J. Seeley, L. A. Campfield, P. Burn and D. G. Baskin (1996). "Identification of targets of leptin action in rat hypothalamus." J Clin Invest **98**(5): 1101-1106.

713 Schwartz, M. W., R. J. Seeley, S. C. Woods, D. S. Weigle, L. A. Campfield, P. Burn and
714 D. G. Baskin (1997). "Leptin increases hypothalamic pro-opiomelanocortin mRNA
715 expression in the rostral arcuate nucleus." Diabetes **46**(12): 2119-2123.

716 Schwartz, M. W., S. C. Woods, D. Porte, Jr., R. J. Seeley and D. G. Baskin (2000).
717 "Central nervous system control of food intake." Nature **404**(6778): 661-671.

718 Scott, M. M., J. L. Lachey, S. M. Sternson, C. E. Lee, C. F. Elias, J. M. Friedman and J.
719 K. Elmquist (2009). "Leptin targets in the mouse brain." J Comp Neurol **514**(5): 518-532.

720 Shi, X., F. Zhou, X. Li, B. Chang, D. Li, Y. Wang, Q. Tong, Y. Xu, M. Fukuda, J. J. Zhao,
721 D. Li, D. G. Burrin, L. Chan and X. Guan (2013). "Central GLP-2 enhances hepatic
722 insulin sensitivity via activating PI3K signaling in POMC neurons." Cell Metab **18**(1): 86-
723 98.

724 Shin, A. C., N. Filatova, C. Lindtner, T. Chi, S. Degann, D. Oberlin and C. Buettner
725 (2017). "Insulin Receptor Signaling in POMC, but Not AgRP, Neurons Controls Adipose
726 Tissue Insulin Action." Diabetes **66**(6): 1560-1571.

727 Slieker, L. J., K. W. Sloop, P. L. Surface, A. Kriauciunas, F. LaQuier, J. Manetta, J. Bue-
728 Valleskey and T. W. Stephens (1996). "Regulation of expression of ob mRNA and
729 protein by glucocorticoids and cAMP." J Biol Chem **271**(10): 5301-5304.

730 Takahashi, K. A. and R. D. Cone (2005). "Fasting induces a large, leptin-dependent
731 increase in the intrinsic action potential frequency of orexigenic arcuate nucleus
732 neuropeptide Y/Agouti-related protein neurons." Endocrinology **146**(3): 1043-1047.

733 Trayhurn, P., J. S. Duncan, N. Hoggard and D. V. Rayner (1998). "Regulation of leptin
734 production: a dominant role for the sympathetic nervous system?" Proc Nutr Soc **57**(3):
735 413-419.

736 Trayhurn, P., J. S. Duncan, D. V. Rayner and L. J. Hardie (1996). "Rapid inhibition of ob
737 gene expression and circulating leptin levels in lean mice by the beta 3-adrenoceptor
738 agonists BRL 35135A and ZD2079." Biochem Biophys Res Commun **228**(2): 605-610.

739 Trayhurn, P., M. E. Thomas, J. S. Duncan and D. V. Rayner (1995). "Effects of fasting
740 and refeeding on ob gene expression in white adipose tissue of lean and obese (oblob)
741 mice." FEBS Lett **368**(3): 488-490.

742 Valet, P., D. Grujic, J. Wade, M. Ito, M. C. Zingaretti, V. Soloveva, S. R. Ross, R. A.
743 Graves, S. Cinti, M. Lafontan and B. B. Lowell (2000). "Expression of human alpha 2-
744 adrenergic receptors in adipose tissue of beta 3-adrenergic receptor-deficient mice
745 promotes diet-induced obesity." J Biol Chem **275**(44): 34797-34802.

746 Wang, Q., C. Liu, A. Uchida, J. C. Chuang, A. Walker, T. Liu, S. Osborne-Lawrence, B.
747 L. Mason, C. Mosher, E. D. Berglund, J. K. Elmquist and J. M. Zigman (2014). "Arcuate
748 AgRP neurons mediate orexigenic and glucoregulatory actions of ghrelin." Mol Metab
749 **3**(1): 64-72.

750 Williams, K. W., T. Liu, X. Kong, M. Fukuda, Y. Deng, E. D. Berglund, Z. Deng, Y. Gao,
751 T. Liu, J. W. Sohn, L. Jia, T. Fujikawa, D. Kohno, M. M. Scott, S. Lee, C. E. Lee, K. Sun,
752 Y. Chang, P. E. Scherer and J. K. Elmquist (2014). "Xbp1s in Pomc neurons connects
753 ER stress with energy balance and glucose homeostasis." Cell Metab **20**(3): 471-482.

754 Williams, K. W., L. O. Margatho, C. E. Lee, M. Choi, S. Lee, M. M. Scott, C. F. Elias and
755 J. K. Elmquist (2010). "Segregation of acute leptin and insulin effects in distinct
756 populations of arcuate proopiomelanocortin neurons." J Neurosci **30**(7): 2472-2479.

757 Wu, Q., M. P. Boyle and R. D. Palmiter (2009). "Loss of GABAergic signaling by AgRP
758 neurons to the parabrachial nucleus leads to starvation." Cell **137**(7): 1225-1234.

759 Wu, Q., M. S. Clark and R. D. Palmiter (2012). "Deciphering a neuronal circuit that
760 mediates appetite." Nature **483**(7391): 594-597.

761 Xu, Y., E. D. Berglund, J. W. Sohn, W. L. Holland, J. C. Chuang, M. Fukuda, J. Rossi, K.
762 W. Williams, J. E. Jones, J. M. Zigman, B. B. Lowell, P. E. Scherer and J. K. Elmquist
763 (2010). "5-HT2CRs expressed by pro-opiomelanocortin neurons regulate insulin
764 sensitivity in liver." Nat Neurosci **13**(12): 1457-1459.

765 Xu, Y., J. E. Jones, D. Kohno, K. W. Williams, C. E. Lee, M. J. Choi, J. G. Anderson, L.
766 K. Heisler, J. M. Zigman, B. B. Lowell and J. K. Elmquist (2008). "5-HT2CRs expressed
767 by pro-opiomelanocortin neurons regulate energy homeostasis." Neuron **60**(4): 582-589.

Figure legends

Figure 1. LEPR-expressing POMC neurons are required for normal liver insulin sensitivity in adult mice. A) Fed and fasting (16 h) glucose one week before, and every week for four weeks after, *Pomc*^{CreERt2}::*Lepr*$^{flox/flox}$ and littermate controls were injected with the last dose of tamoxifen (n=12). B) Fed and fasting (48 h) insulin four weeks after tamoxifen was given (n=4-6). C) Fed and fasting (48 h) glucagon four weeks after tamoxifen was given (n=4-6). D) Glucose excursion during an insulin tolerance test (ITT) only one week following the last injection of tamoxifen (n=5-6). E) Area under the curve for the ITT shown in B (n=5-6). F) Glucose infusion rate (GIR) needed to maintain euglycemia (119.3 ± 3.9 vs 122.0 ± 8.2 mg/dl) during an hyperinsulinemic-euglycemic clamp performed only one week following the last injection of tamoxifen (n=6). G) Glucose disposal (Rd) during the same hyperinsulinemic-euglycemic clamp (n=6). H) Basal and clamped hepatic glucose production (HPG) (n=6). I) Basal and clamped lipolysis rate as assessed by free fatty acid (FFA) (n=6). The data are expressed as the mean ± SEM. ***p < 0.001, **p < 0.01 and *p < 0.05 versus littermate controls.

Figure 2. LEPR-expressing POMC neurons are dispensable for the regulation of energy balance in adult mice. A) Body weight before, and up to four weeks after, *Pomc*^{CreERt2}::*Lepr*$^{flox/flox}$ and littermate controls were injected with tamoxifen (n=12). B) Fat mass and C) Lean mass as assessed by nuclear magnetic resonance (NMR) four weeks following tamoxifen administration (n=12). D) Daily food intake, E) Oxygen consumption (VO_2), F) Respiratory exchange ratio (RER), and G) locomotor activity in CaloSys Calorimetry System cages four weeks after the administration of tamoxifen (n=5). Summary graphs showing average data for light (ZT0-ZT12) and dark (ZT12-ZD24) cycles are presented under each diurnal graph. The data are expressed as the mean ± SEM.

Figure 3. Deletion of LEPRs in POMC impairs fasting-induced expression of orexigenic neuropeptides in the mediobasal hypothalamus. A) *Pomc*, B) *Agrp* and C) *Npy* mRNA expression in mediobasal hypothalamus of fed and fasted (48 h) *Pomc*^{CreERt2}::*Lepr*$^{flox/flox}$ and littermate control mice four weeks after tamoxifen was given (n=8-14). The data are expressed as the mean ± SEM. ***p < 0.001 versus littermate controls.

Figure 4. Deletion of LEPRs in POMC neurons impairs postprandial glycemia. A) Food intake and B) Blood glucose up to six hours after food access was restored to 48-h fasted *Pomc*^{CreERt2}::*Lepr*$^{flox/flox}$ and littermate control mice, four weeks after tamoxifen was given (n=8). The data are expressed as the mean ± SEM. **p < 0.01 and *p < 0.05 versus littermate controls.

Figure 5. LEPR-expressing POMC neurons in adult mice are required for the fasting-induced fall in leptin levels, independent of changes in fat. A) Weight loss, and B) fat-mass loss after a 48-h fast in mice with constitutive (prenatal) deletion of LEPRs in POMC neurons and littermate controls (n=7-10). C) Plasma leptin levels, and D) visceral adipose tissue *Lep* mRNA expression in fed or fasted (48 h) mice with constitutive deletion of LEPRs in POMC neurons and littermate controls (n=7-14). E) Weight loss, and F) fat-mass loss after a 48-h fast in *Pomc*^{CreERt2}::*Lepr*$^{flox/flox}$ and littermate control mice four weeks after tamoxifen was given (n=12). G) Plasma leptin levels, and H) visceral adipose tissue *Lep* mRNA expression in fed or fasted (48 h) in *Pomc*^{CreERt2}::*Lepr*$^{flox/flox}$ and littermate control mice four weeks after tamoxifen was given (n=6-13). The data are expressed as the mean ± SEM. ***p < 0.001, **p < 0.01 and *p < 0.05 versus littermate controls.

Figure 6. Deletion of LEPRs in POMC impairs visceral adipose tissue expression of *Adra2a* with fasting. A) Expression of the nine adrenergic receptors in fed of fasted (24 h – 48 h) *Pomc*^{CreERt2}::*Lepr*$^{flox/flox}$ and littermate control mice four weeks after tamoxifen was given (n=8-14). B) Comparison of the expression of *Adra2a* and C) *Adrb3* in epididymal (eWAT) versus inguinal (iWAT) adipose tissue in an independent cohort of fed of fasted (48 h) *Pomc*^{CreERt2}::*Lepr*$^{flox/flox}$ and littermate control mice four weeks after tamoxifen was given (n=5-6). The data are expressed as the mean ± SEM. ***p < 0.001 and *p < 0.05 versus littermate controls.

Figure 7. Pharmacological activation of ADRA2 stimulates leptin production. A) Visceral adipose tissue *Lep* mRNA expression one hour following an intraperitoneal (1 mg/kg) injection of the ADRA2 agonist clonidine (n=10-12). B) Plasma leptin levels up to two hours following the administration of clonidine in an independent cohort (n=4-7). C) Leptin release from epididymal (eWAT) and D) inguinal (iWAT) adipose tissue explants from fed and fasted (48 h) *Pomc*^{CreERt2}::*Lepr*$^{flox/flox}$ and littermate control mice following the addition of clonidine (1 µM) (n=6). This experiment was

831 performed four weeks after tamoxifen was given. The data are expressed as the mean ±
832 SEM. ***p < 0.001, **p< 0.01, and *p < 0.05 versus littermate controls.

833 **Figure 1-figure supplement 1. Validation of the *Pomc*^{CreERt2} mice.** A)
834 *Pomc*^{CreERt2} mice were crossed with Ai14(RCL-tdT)-D mice, injected with tamoxifen using
835 the described paradigm. Using immunohistochemistry, tdTomato expression (red) was
836 colocalized with β-endorphin (marker of POMC neurons) expression (green) 2 weeks
837 later. B) The inducible Cre model does not recombine in extra-hypothalamic areas,
838 except for a few β-endorphin+ cells located in the nucleus tractus solitarius in the HB
839 and the pituitary. C) Leptin-induced Stat3 activation in the arcuate nucleus of the
840 hypothalamus. Mice were injected with tamoxifen using the described paradigm and
841 leptin (5 mg/kg, i.p.) was administrated in 16h-fasted animals. Animals were decapitated
842 45 minutes later and pStat3 was evaluated by immunohistochemistry. A representative
843 image of n=3 animals per group is shown. CB, cerebellum, CTX, cortex; DR, dorsal
844 raphe; HB, hindbrain; HIP, hippocampus; OB, olfactory bulb; PT, pituitary; SC, spinal
845 cord; STR, striatum.

846 **Figure 1-figure supplement 2. Glucagon stimulation test.**
847 *Pomc*^{CreERt2}::*Lepr*$^{flox/flox}$ and littermate control mice (n=8) were treated with tamoxifen as
848 described. 4 weeks after treatment, they were fasted for one hour and glucagon (120
849 µg/kg, i.p.) was administrated. Blood glucose was then monitored every 10 min for one
850 hour. The data are expressed as the mean ± SEM.

Figure 1

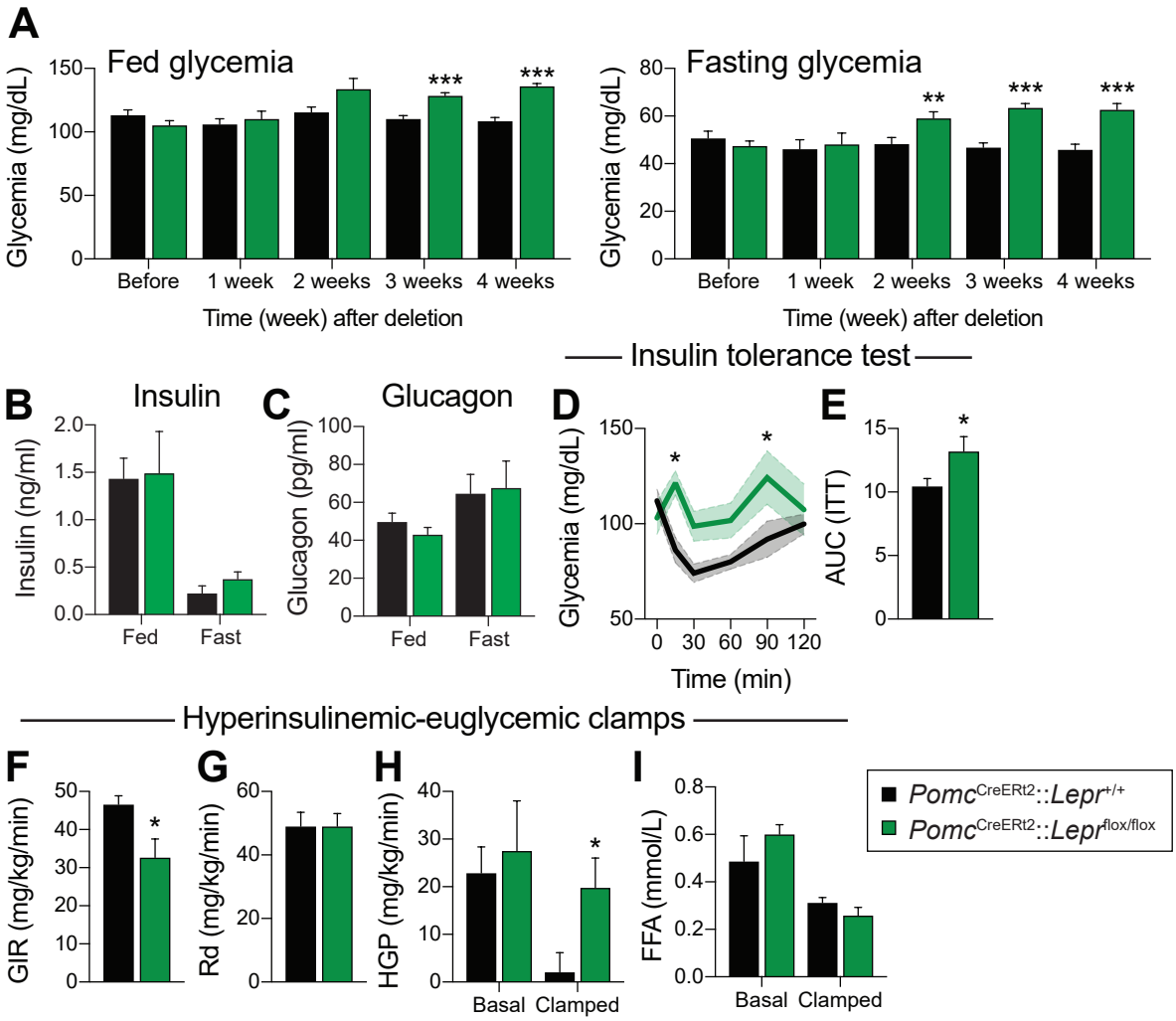

A
Fed glycemia
Glycemia (mg/dL)
150
100
50
0
***
***
Before
1 week
2 weeks
3 weeks
4 weeks
Time (week) after deletion
Fasting glycemia
Glycemia (mg/dL)
80
60
40
20
0
**
***
***
Before
1 week
2 weeks
3 weeks
4 weeks
Time (week) after deletion
Insulin tolerance test
B
Insulin
Insulin (ng/ml)
2.0
1.5
1.0
0.5
0.0
Fed
Fast
C
Glucagon
Glucagon (pg/ml)
100
80
60
40
20
0
Fed
Fast
D
Glycemia (mg/dL)
150
100
50
*
*
0
30
60
90
120
Time (min)
E
AUC (ITT)
15
10
5
0
*
Hyperinsulinemic-euglycemic clamps
F
GIR (mg/kg/min)
50
40
30
20
10
0
*
G
Rd (mg/kg/min)
60
40
20
0
H
HGP (mg/kg/min)
40
30
20
10
0
*
Basal
Clamped
I
FFA (mmol/L)
0.8
0.6
0.4
0.2
0.0
Basal
Clamped
Pomc^CreERt2::Lepr^+/+
Pomc^CreERt2::Lepr^flox/flox

Figure 2

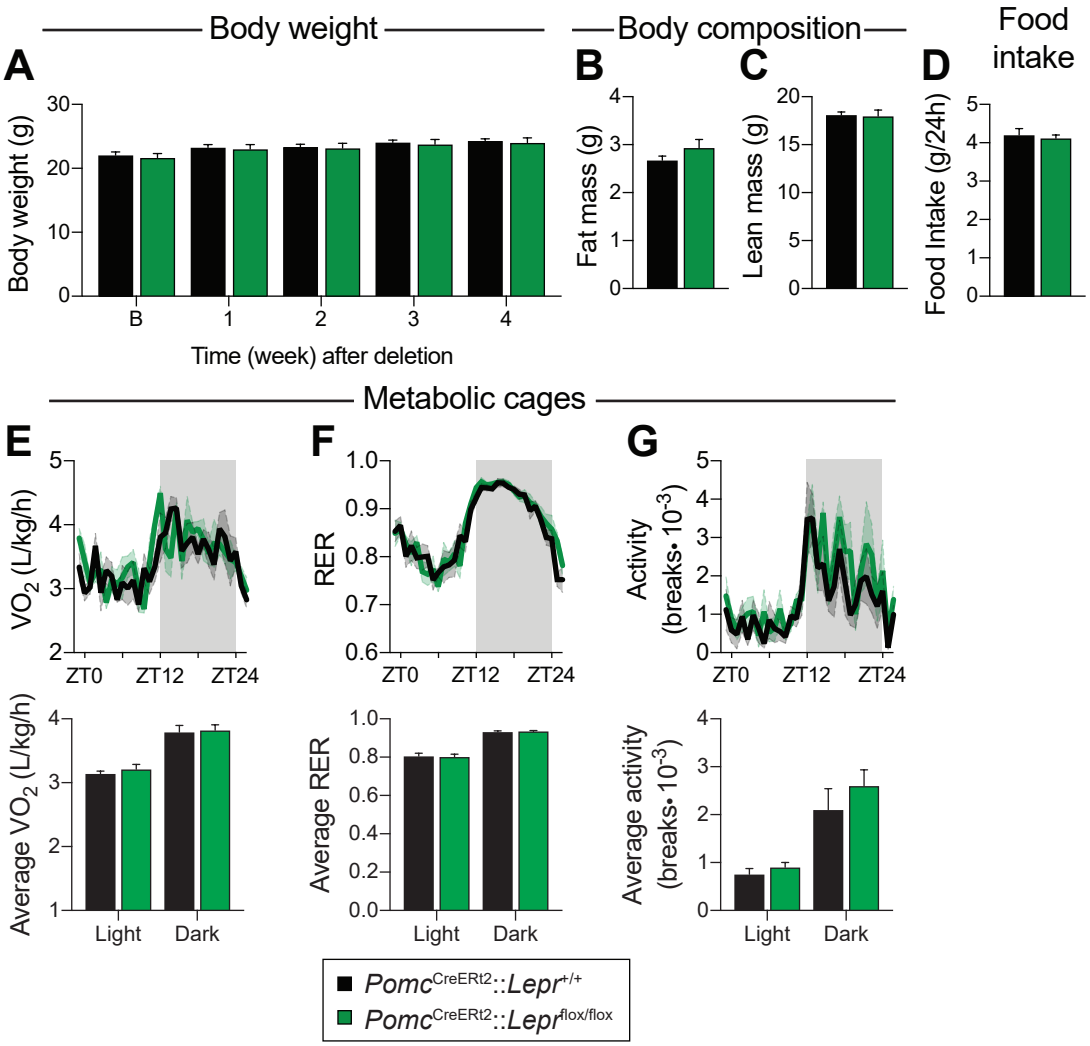

Body weight
Body composition
Food intake
A
B
C
D
Body weight (g)
Time (week) after deletion
Fat mass (g)
Lean mass (g)
Food Intake (g/24h)
Metabolic cages
E
F
G
VO2 (L/kg/h)
RER
Activity (breaks• 10-3)
ZT0
ZT12
ZT24
Average VO2 (L/kg/h)
Average RER
Average activity (breaks• 10-3)
Light
Dark
PomcCreERt2::Lepr+/+
PomcCreERt2::Leprflox/flox

Figure 3

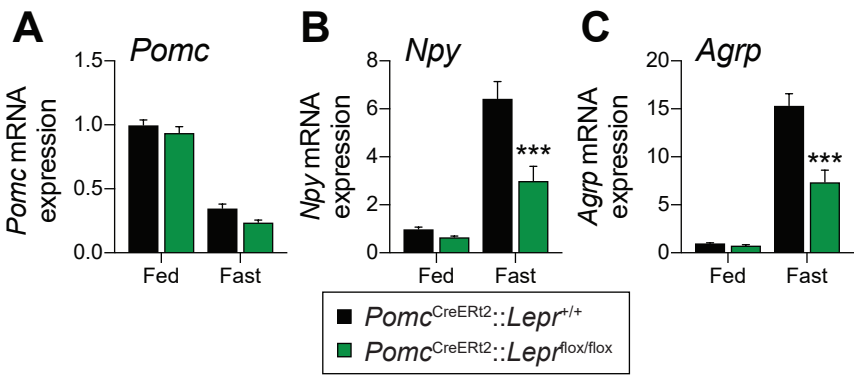

A
Pomc
Pomc mRNA expression
1.5
1.0
0.5
0.0
Fed
Fast
B
Npy
Npy mRNA expression
8
6
4
2
0
***
Fed
Fast
C
Agrp
Agrp mRNA expression
20
15
10
5
0
***
Fed
Fast
Pomc^CreERt2::Lepr^+/+
Pomc^CreERt2::Lepr^flox/flox

Figure 4

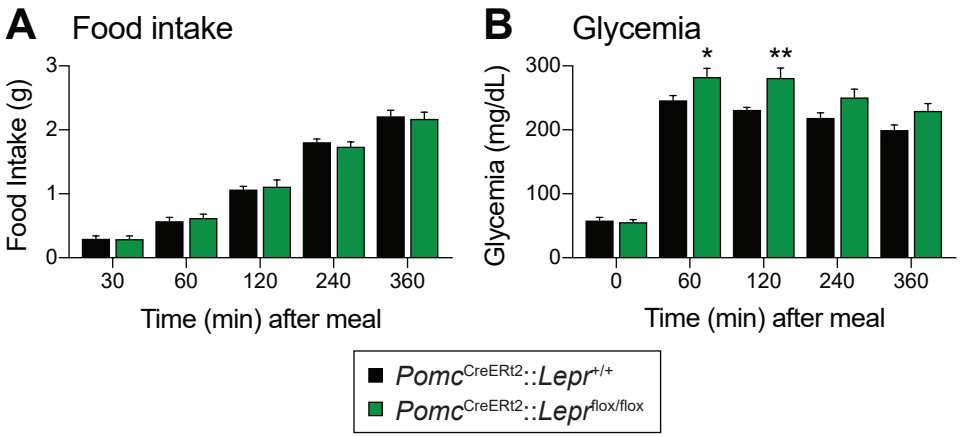

A
Food intake
Food Intake (g)
3
2
1
0
30
60
120
240
360
Time (min) after meal
B
Glycemia
Glycemia (mg/dL)
300
200
100
0
*
**
0
60
120
240
360
Time (min) after meal
Pomc^CreERt2::Lepr^+/+
Pomc^CreERt2::Lepr^flox/flox

Figure 5

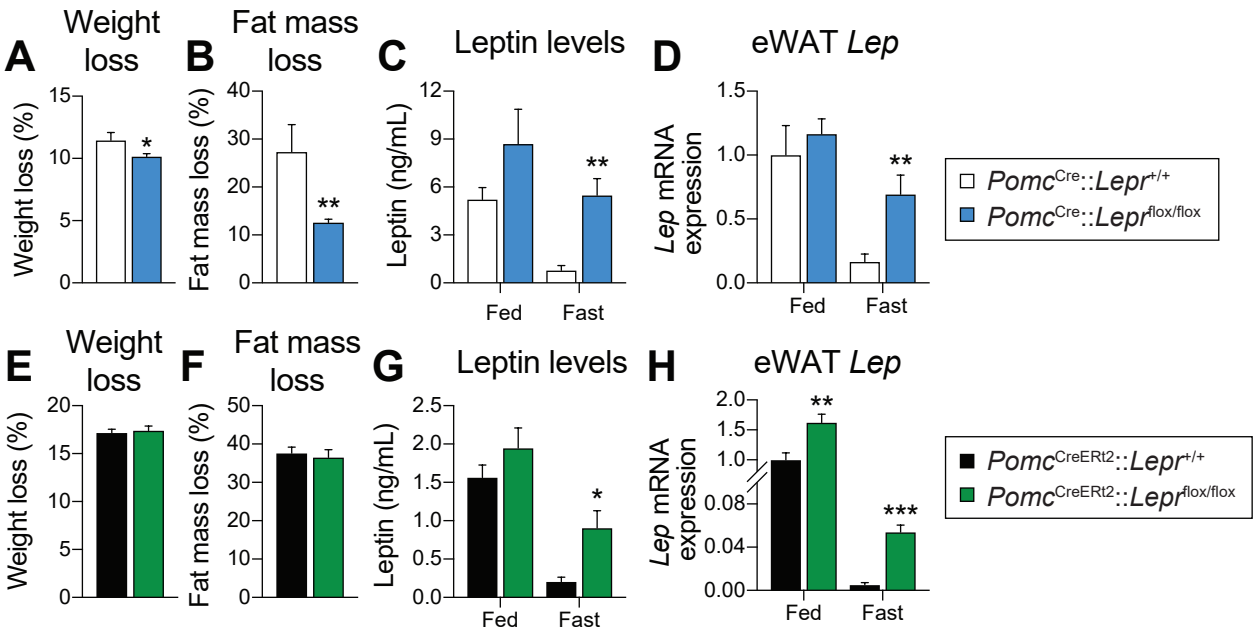

A
Weight loss
Weight loss (%)
B
Fat mass loss
Fat mass loss (%)
C
Leptin levels
Leptin (ng/mL)
Fed
Fast
D
eWAT Lep
Lep mRNA expression
Pomc^Cre::Lepr^+/+
Pomc^Cre::Lepr^flox/flox
E
Weight loss
Weight loss (%)
F
Fat mass loss
Fat mass loss (%)
G
Leptin levels
Leptin (ng/mL)
H
eWAT Lep
Lep mRNA expression
Pomc^CreERt2::Lepr^+/+
Pomc^CreERt2::Lepr^flox/flox

Figure 6

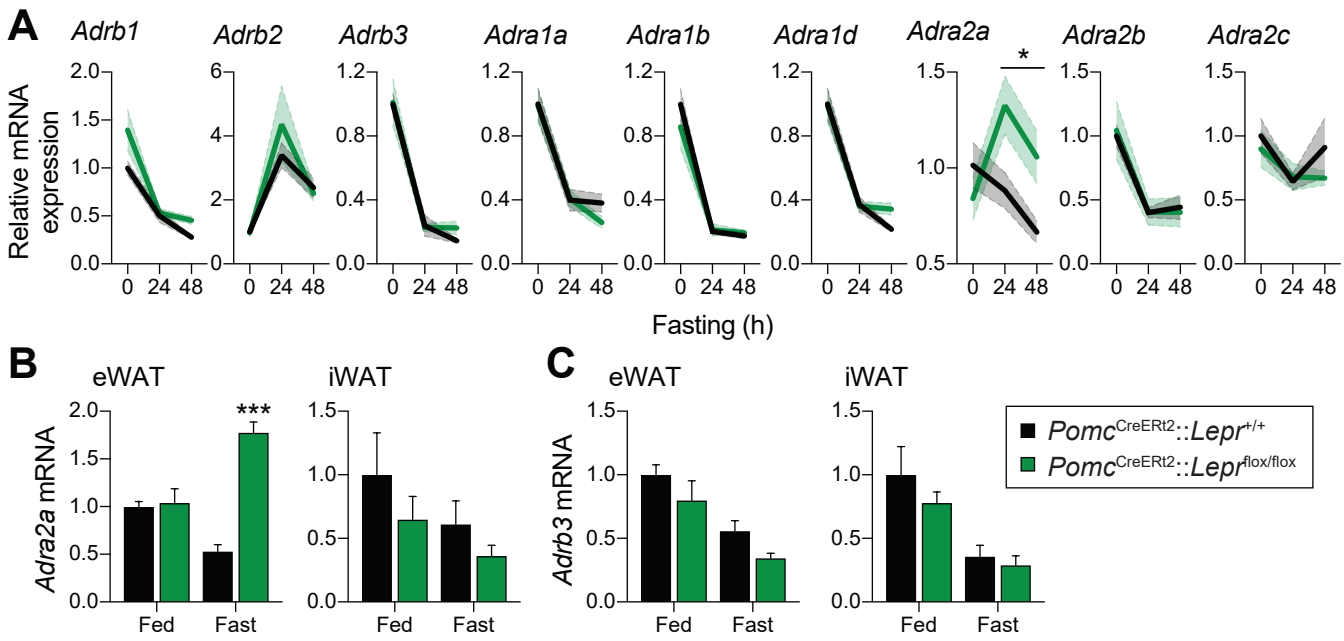

A
Adrb1
Adrb2
Adrb3
Adra1a
Adra1b
Adra1d
Adra2a
Adra2b
Adra2c
Relative mRNA expression
*
Fasting (h)
B
eWAT
iWAT
Adra2a mRNA
***
Fed
Fast
C
eWAT
iWAT
Adrb3 mRNA
Pomc CreERt2 ::Lepr +/+
Pomc CreERt2 ::Lepr flox/flox

Figure 7

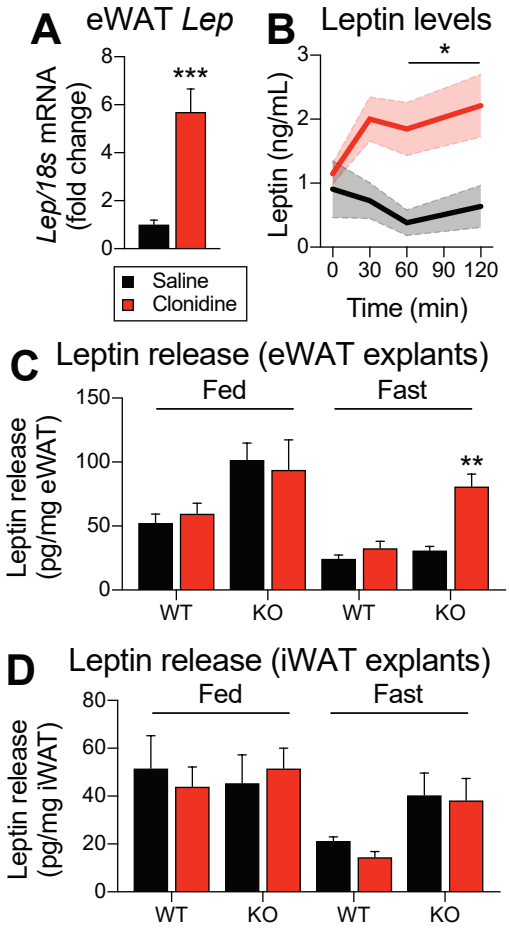
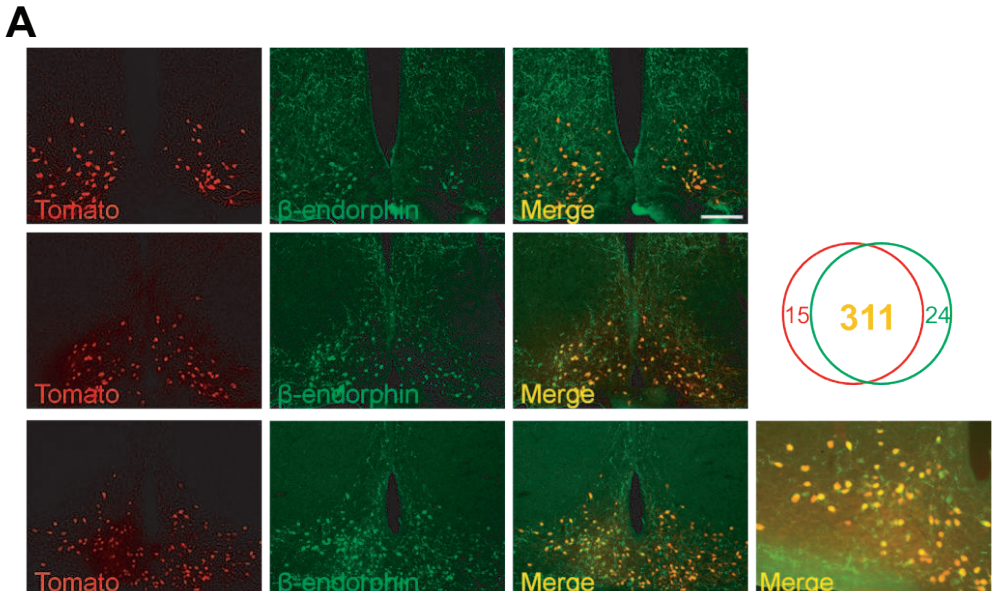
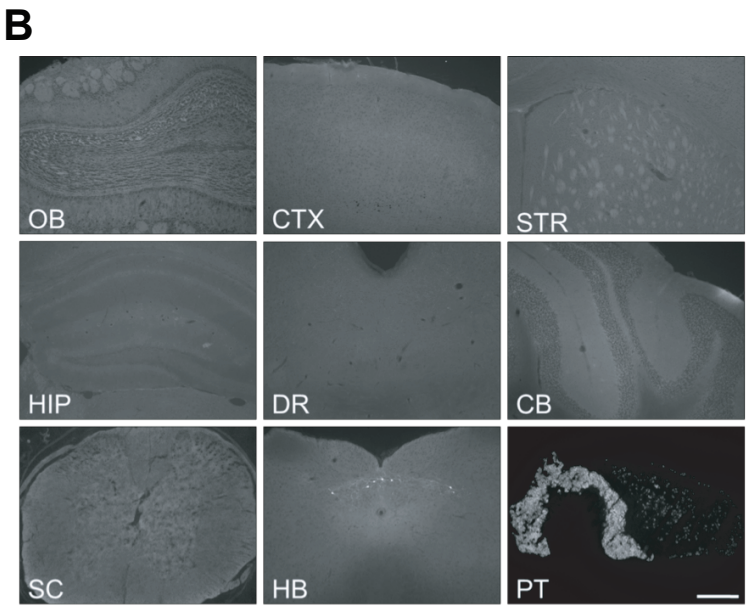

A
eWAT Lep
Lep/18s mRNA (fold change)
***
Saline
Clonidine
B
Leptin levels
Leptin (ng/mL)
*
Time (min)
C
Leptin release (eWAT explants)
Leptin release (pg/mg eWAT)
Fed
Fast
**
WT
KO
D
Leptin release (iWAT explants)
Leptin release (pg/mg iWAT)

Figure 1-supplement 1





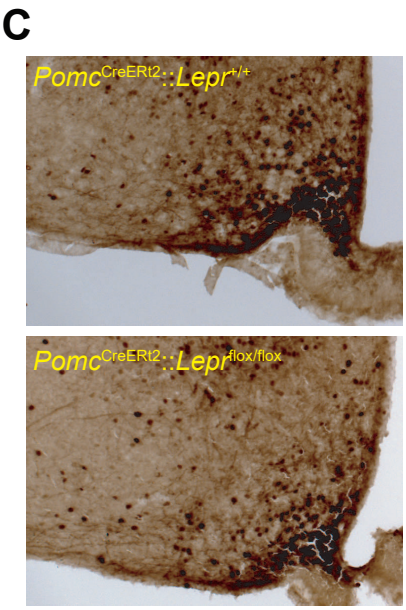


Figure 1-supplement 2