Published in final edited form as:
Neuron. 2011 July 14; 71(1): 142–154. doi:10.1016/j.neuron.2011.05.028.

Leptin Action on GABAergic Neurons Prevents Obesity and Reduces Inhibitory Tone to POMC Neurons

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SUMMARY

Leptin acts in the brain to prevent obesity. The underlying neurocircuitry responsible for this is poorly understood, in part due to incomplete knowledge regarding first order, leptin-responsive neurons. To address this, we and others have been removing leptin receptors from candidate first order neurons. While functionally relevant neurons have been identified, the observed effects have been small suggesting that most first order neurons remain unidentified. Here we take an alternative approach and test whether first order neurons are inhibitory (GABAergic, $VGAT^{+}$) or excitatory (glutamatergic, $VGLUT2^{+}$). Remarkably, the vast majority of leptin's anti-obesity effects are mediated by GABAergic neurons; glutamatergic neurons play only a minor role. Leptin, working directly on presynaptic GABAergic neurons, many of which appear not to express AgRP, reduces inhibitory tone to postsynaptic POMC neurons. As POMC neurons prevent obesity, their disinhibition by leptin action on presynaptic GABAergic neurons likely mediates, at least in part, leptin's anti-obesity effects.

INTRODUCTION

Leptin is secreted by adipocytes in proportion to fat stores providing feedback on the status of lipid reserves (Friedman, 2009). Leptin circulates and binds its receptor (LEPR) in the brain where it decreases food intake and promotes energy expenditure (Myers et al., 2010). When fat stores are reduced, such as by fasting, dieting, lipodystrophy or uncontrolled type 1 diabetes, blood leptin levels fall and this stimulates hunger while decreasing energy expenditure - physiologic adaptations aimed at returning fat stores to normal. Genetic deficiency of leptin or its receptor removes this adipostat signal, "misinforming" the organism about its state of energy balance and abundant fat stores. Consequently, extreme hyperphagia, reduced energy expenditure and massive obesity result. Thus, circulating leptin, by restraining food intake and maintaining energy expenditure, prevents obesity.

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SUPPLEMENTAL INFORMATION
Supplemental information includes five supplemental figures, a table, supplemental experimental procedures, supplemental text, and supplemental references.

The neurobiological mechanisms underlying these “anti-obesity” effects are unknown. Nevertheless, key components are likely to reside in the arcuate nucleus as suggested by the convergence of numerous lines of compelling research. First, the neuropeptides, αMSH (Smart et al., 2006; Yaswen et al., 1999) and AgRP (Ollmann et al., 1997; Shutter et al., 1997), and the neurons that express them (POMC and AgRP neurons which are located primarily in the arcuate) (Bewick et al., 2005; Gropp et al., 2005; Luquet et al., 2005; Xu et al., 2005), play key roles in regulating body weight. Second, POMC neurons and AgRP neurons project to brain regions likely to be important in regulating body weight (important examples include the paraventricular nucleus and the lateral parabrachial nucleus (Bagnol et al., 1999; Elias et al., 1998; Haskell-Luevano et al., 1999; Wu et al., 2009)). Third, αMSH and AgRP agonize and antagonize, respectively, melanocortin-4 receptors (MC4Rs) (Mountjoy et al., 1992; Ollmann et al., 1997), and importantly, MC4Rs mediate marked anti-obesity effects (Balthasar et al., 2005; Huszar et al., 1997). Since POMC and AgRP neurons are the sole sources of MC4R ligands (and since MC4Rs play a critical role in regulating energy balance), POMC and AgRP neurons must be playing a similarly important role. Fourth, LEPRs are expressed by most AgRP neurons and many POMC neurons (Baskin et al., 1999a; Elias et al., 1999; Williams et al., 2010; Wilson et al., 1999), and leptin, which promotes negative energy balance, inhibits AgRP neurons and excites POMC neurons (Cowley et al., 2001; Elias et al., 1999; Takahashi and Cone, 2005; van den Top et al., 2004). In addition, leptin decreases and increases, respectively, expression of the neuropeptide genes, *Agrp* and *Pomc* (Baskin et al., 1999a; Baskin et al., 1999b; Mizuno et al., 1998; Wilson et al., 1999). These effects of leptin on neuronal activity and neuropeptide gene expression are consistent with the catabolic effects of leptin, as well as the anabolic and catabolic nature, respectively, of AgRP and POMC neurons (Bewick et al., 2005; Gropp et al., 2005; Luquet et al., 2005; Xu et al., 2005) and their neuropeptides (Ollmann et al., 1997; Smart et al., 2006; Yaswen et al., 1999). Fifth, AgRP neurons, which also release NPY and GABA, send collaterals to POMC neurons, providing an additional means by which leptin can stimulate (via disinhibition) POMC neurons (Cowley et al., 2001; Horvath et al., 1992). The above findings suggest the following model: leptin binds directly to LEPRs on AgRP and POMC neurons, inhibiting AgRP neurons and activating POMC neurons, and this accounts for its anti-obesity actions.

If this model is correct, and if its the sole mechanism by which leptin regulates energy balance, then deletion of LEPRs on AgRP and POMC neurons should result in massive obesity – similar to that seen in mice with total lack of leptin action (i.e. $Lep^{ob/ob}$ mice and $Lepr^{db/db}$ mice). To investigate this, we generated mice that lack LEPRs on POMC neurons (i.e. *Pomc*-Cre, $Lepr^{lox/lox}$ mice), on AgRP neurons (i.e. *Agrp*-Cre, $Lepr^{lox/lox}$ mice), and on both POMC and AgRP neurons (i.e. *Pomc*-Cre, *Agrp*-Cre, $Lepr^{lox/lox}$ mice) (Balthasar et al., 2004; Hill et al., 2010; van de Wall et al., 2008). Of note, mice lacking LEPRs on either POMC neurons or on AgRP neurons developed very mild obesity (increase in body weight of ~ 5 g at 2–3 months of age) (Balthasar et al., 2004; van de Wall et al., 2008). This effect was much smaller than expected, especially when one compares this with the 26 g increase in body weight in 10 wk old mice with global deficiency of LEPRs (van de Wall et al., 2008)). One possibility for the smaller-than-expected effect is that deletion of LEPRs in one class of neurons (for example, the POMC neurons) might possibly be compensated by increased leptin action on the other class of neurons (for example, the AgRP neurons), or visa versa. However, this was not the case because an additive, and still much smaller-than-expected effect was observed in mice lacking LEPRs on both POMC and AgRP neurons (van de Wall et al., 2008). In total, the above findings suggest that direct leptin action on POMC and AgRP neurons plays a small role in controlling energy balance, and that there are likely to be other first-order, leptin responding neurons that contribute importantly to leptin’s anti-obesity actions.

Areas beyond the arcuate could mediate important actions of leptin. Of note, LEPRs are present in many sites outside the arcuate. Within the hypothalamus, LEPRs are found in the ventromedial hypothalamus (VMH), the dorsomedial hypothalamus (DMH), the lateral hypothalamus (LH), and the ventral premammillary nucleus (PMv) (in addition to the arcuate); within the midbrain, in the ventral tegmental area and raphe; and within the brainstem, in the parabrachial nucleus, periaquiductal gray, and dorsal vagal complex (Elias et al., 2000; Figlewicz et al., 2003; Fulton et al., 2006; Grill et al., 2002; Hommel et al., 2006; Leinninger et al., 2009; Leshan et al., 2009; Mercer et al., 1996; Mercer et al., 1998; Munzberg, 2008; Scott et al., 2009). Strong arguments have been made that neurons outside the arcuate are well positioned to play important roles in regulating appetite (Berthoud, 2002; Grill and Kaplan, 2002). Indeed, injection of leptin into brain sites distant from the arcuate inhibit food intake (examples include injections into the 4th ventricle (Grill et al., 2002) and the lateral hypothalamus (Leinninger et al., 2009)).

The VMH is a site of interest given that gene knockout of SF1 causes abnormal VMH development and obesity (Majdic et al., 2002; Zhao et al., 2004). To investigate SF1 neurons, we generated *Sf1*-Cre, *Lepr*$^{lox/lox}$ mice (Dhillon et al., 2006). These animals developed a small increase in body fat and body weight (~ 5 g increase in body weight at 2–3 months of age). Thus, as with LEPRs on POMC and AgRP neurons, the role of LEPRs on SF1 neurons is small. Another group has obtained qualitatively similar results regarding the role of LEPRs on SF1 neurons (Bingham et al., 2008).

Based on the above, the list of genetically verified, body weight-regulating, first order, leptin-responsive neurons includes POMC (Balthasar et al., 2004), AgRP (van de Wall et al., 2008) and SF1 neurons (Bingham et al., 2008; Dhillon et al., 2006). Given this, and the realization that these neurons account for only a portion of leptin's effects (thus other neurons must also be involved), it has been proposed that leptin action is mediated by a distributed network of leptin-responsive neurons (Leinninger and Myers, 2008; Myers et al., 2009; Scott et al., 2009). With such a distributed model in mind, it is of interest to determine if any deeper logic underlies first-order, leptin-responsive neurons and/or their mode of communication with energy balance-regulating neurocircuits.

Since the obvious, "first order" candidates have already been directly tested, a new approach is needed for narrowing in on these "unidentified" first order neurons. In the present study we evaluate if leptin's effects are mediated primarily by excitatory (glutamatergic, VGLUT2^{+}) or inhibitory (GABAergic, VGAT^{+}) neurons. This approach has two important features. First, it casts a wider net and provides insight into the neurotransmitter identity of the neurons mediating leptin's anti-obesity effects. Second, it provides information regarding the function of the leptin-responsive neurons (excitatory versus inhibitory). With this goal in mind, we have generated mice that express Cre-recombinase in either glutamatergic (*Vglut2*-ires-Cre knockin mice) or GABAergic neurons (*Vgat*-ires-Cre knockin mice). VGLUT2 is one of three synaptic vesicle glutamate transporters (Takamori, 2006). VGLUT1 is expressed primarily by neurons in the cortex while VGLUT3 is expressed by isolated, select groups of neurons, none of which are in the hypothalamus. Consequently, VGLUT2 is the transporter utilized by glutamatergic neurons in the hypothalamus, thalamus, midbrain, and hindbrain – thus it is relevant to our investigation of leptin-responsive neurons. VGAT, on the other hand, is the only transporter capable of importing GABA into synaptic vesicles; hence, VGAT is expressed by all GABAergic neurons (Wojcik et al., 2006). Through use of these Cre-expressing mice, we show that the majority of leptin's anti-obesity effects are mediated by inhibitory (GABAergic) neurons. We then build on this finding, and examine means by which leptin-responsive GABAergic neurons engage obesity-preventing POMC neurons.

RESULTS

Generation of *Vgat*-ires-Cre and *Vglut2*-ires-Cre Mice

To ensure eutopic expression of Cre recombinase by VGAT- and VGLUT2-expressing neurons, we inserted an ires-Cre cassette, by gene targeting, just downstream of the endogenous *Vgat* and *Vglut2* stop codons, respectively (Figure 1A). The alleles are used in the heterozygous state (i.e. $Vglut2^{ires\text{-}Cre/+}$, $Vgat^{ires\text{-}Cre/+}$), and do not have detectable effects on phenotype.

To confirm that Cre is eutopically expressed, *Vgat*-ires-Cre and *Vglut2*-ires-Cre mice were crossed with lox-GFP reporter mice (Novak et al., 2000), and brains were processed for immunohistochemical detection of GFP. As is evident from Figures 1B-G and S1A, Cre activity is detected in sites where it is expected (i.e. known to be composed primarily of GABAergic or glutamatergic $VGLUT2^+$ neurons) and is not seen in sites where it is unexpected. Brain areas known to be composed primarily of GABAergic cell bodies (see Figure S1B for *Vgat* mRNA *in situ* hybridization and supplemental text for detailed discussion and supporting references), which are depicted in Figure 1, include the caudate putamen (CPu), suprachiasmatic nucleus (SCh), central amygdaloid nucleus (CeA), and zona incerta (ZI). GABAergic areas depicted in Figure S1A include, in addition to those previously mentioned, the nucleus accumbens (ACB), lateral (LS) and medial septum (MS), reticular nucleus of the thalamus (Rt), substantia nigra pars reticulata (SNr), and Purkinje cell layer of the cerebellum. Brain areas known to be composed primarily of glutamatergic ($VGLUT2^+$) cell bodies (see Figure S1B for *Vglut2* mRNA *in situ* hybridization and supplemental text for detailed discussion and supporting references), which are depicted in Figure 1, include the thalamus (TH), paraventricular nucleus (PVN), nucleus of the lateral olfactory tract (LOT), basolateral nucleus of the amygdala (BLA), and ventromedial hypothalamus (VMH). Glutamatergic ($VGLUT2^+$) areas depicted in Figure S1A include, in addition to those previously mentioned, the piriform cortex (PIR), posterior hypothalamus (PH), ventral premammillary nucleus (PMv), subthalamic nucleus (STh), medial geniculate nucleus (MG), reticulotegmental nucleus (RTg), pontine grey (PG), external cuneate nucleus (ECu) and lateral reticular nucleus (LRt). Of note, the arcuate nucleus (ARC), dorsomedial nucleus of the hypothalamus (DMH) and lateral hypothalamus contain both glutamatergic and GABAergic neurons, with GABAergic neurons predominating. A striking feature of Figures 1 and S1A, in addition to what has previously been mentioned, is the lack of Cre activity where it should not be found – i.e. in areas where cell bodies of the opposing neurotransmitter predominate. For example, in sections from *Vglut2*-ires-Cre mice, there is an absence of Cre activity in known GABAergic sites (in Figure 1: CPu, SCh, CeA, and ZI; in Figure S1A: ACB, LS, MS, Rt and SNr) and in sections from *Vgat*-ires-Cre mice, an absence of Cre activity in known glutamatergic sites (in Figure 1: TH, PVN, LOT, BLA and VMH; in Figure S1A: PIR, PH, PMv, STh, MG, RTg, PG, ECu and LRt). In accordance with this, sections from *Vglut2*-ires-Cre mice versus *Vgat*-ires-Cre mice appear as "negative images" of each other. These results are consistent with Cre being expressed in all $VGLUT2^+$ or $VGAT^+$ neurons, and demonstrate that *Vglut2*-ires-Cre and *Vgat*-ires-Cre mice subdivide the brain into neurons that are either excitatory (glutamatergic, $VGLUT2^+$), inhibitory (GABAergic), or neither.

Energy Balance in *Vgat*-ires-Cre, $Lepr^{lox/lox}$ and *Vglut2*-ires-Cre, $Lepr^{lox/lox}$ mice

To generate study subjects, $Lepr^{lox/lox}$ mice were mated with either $Vgat^{ires\text{-}Cre/+}$, $Lepr^{lox/lox}$ mice or $Vglut2^{ires\text{-}Cre/+}$, $Lepr^{lox/lox}$ mice. From such matings, ~ 50% of all offspring are controls (i.e. $Lepr^{lox/lox}$ mice) and ~ 50% have deletion of LEPRs in either GABAergic ($Vgat^{ires\text{-}Cre/+}$, $Lepr^{lox/lox}$ mice) or glutamatergic (i.e. $Vglut2^{ires\text{-}Cre/+}$, $Lepr^{lox/lox}$ mice) neurons. Remarkably, deletion of LEPRs in GABAergic neurons of both male and female

mice resulted in a massive increase in body weight (Figure 2A) and fat mass (Figure 2B), which was associated with marked hyperphagia (Figure 2C). Deletion of LEPRs in glutamatergic neurons, on the other hand, produced minimal effects (Figures 2A-2C). These latter, small effects are likely to be due to deletion of LEPRs in the VMH as neurons in this site are glutamatergic (Figure 1 and (Tong et al., 2007)) and the magnitude of effect is similar to that seen in *Sf1*-Cre, $Lepr^{lox/lox}$ mice (Dhillon et al., 2006). As an additional comparison group, we generated mice that are global knockouts for a germline-deleted lox-*Lepr* allele (i.e. $Lep^{\Delta/\Delta}$ mice) (Figure S2). Of note, the weight gain seen in *Vgat*-ires-Cre, $Lepr^{lox/lox}$ mice is ~86% (in males) and ~83% (in females) of that seen in mice with total lack of LEPRs ($Lepr^{\Delta/\Delta}$ mice). The weight gain seen in *Vglut2*-ires-Cre, $Lepr^{lox/lox}$ mice, on the other hand, is only a small fraction of that seen in $Lepr^{\Delta/\Delta}$ mice. These results demonstrate that LEPRs on GABAergic neurons mediate the vast majority of leptin's anti-obesity effects; in comparison, LEPRs on glutamatergic ($VGLUT2^+$) neurons play only a small role.

To determine if deletion of LEPRs in GABAergic or glutamatergic ($VGLUT2^+$) neurons had effects on glucose homeostasis, blood glucose and insulin levels were measured (Supplemental Table I). *Vgat*-ires-Cre, $Lepr^{lox/lox}$ mice had significantly elevated fed and fasted blood glucose and serum insulin levels, which is consistent with the development of obesity-induced type 2 diabetes. In contrast, but consistent with the minimal increase in fat stores, fed and fasted blood glucose levels were unchanged, and serum insulin levels were only slightly increased (fed state only) in *Vglut2*-ires-Cre, $Lepr^{lox/lox}$ mice.

Of the Known First Order Neurons, Only AgRP Neurons are GABAergic

Previously established, functionally relevant, first order leptin-responsive neurons include POMC and AgRP neurons in the arcuate nucleus and SF1 neurons in the VMH (Balthasar et al., 2004; Dhillon et al., 2006; van de Wall et al., 2008), and possibly serotonin neurons in the raphe (Yadav et al., 2009). An important question is which, if any, of these neurons are GABAergic, as determined by Cre activity in *Vgat*-ires-Cre mice, and consequently contribute to the obesity phenotype of *Vgat*-ires-Cre, $Lepr^{lox/lox}$ mice.

As previously mentioned, SF1 neurons are glutamatergic (Figure 1 and (Tong et al., 2007)). In support of this, no GABAergic neurons were found in the VMH (Figure 1). To determine if POMC neurons are GABAergic or glutamatergic, and to confirm that AgRP neurons are GABAergic (Cowley et al., 2001; Tong et al., 2008), we used immunodetectable hrGFP, expressed from POMC-hrGFP (Parton et al., 2007) and NPY-hrGFP (van den Pol et al., 2009) BAC transgenes, to identify POMC and AgRP neurons, and colocalized this with Cre activity (tdTomato, as described below). Note that NPY and AgRP are coexpressed in the arcuate nucleus (van den Pol et al., 2009). GABAergic ($VGAT^+$) and glutamatergic ($VGLUT2^+$) neurons were identified by immunodetectable tdTomato in *Vgat*-ires-Cre, lox-tdTomato mice and *Vglut2*-ires-Cre, lox-tdTomato mice, respectively (lox-tdTomato, Ai9, (Madisen et al., 2010)). Of note, essentially no POMC neurons (< 1 %) were $VGAT^+$ (Figure 3A) and ~10% of POMC neurons were $VGLUT2^+$ (Figure 3B). AgRP neurons, as expected, were GABAergic (Figure 3C). It is important to note, however, that AgRP neurons represent only a subset of all GABAergic neurons in the arcuate (Figure 3C). Also of note, POMC neurons, which are not GABAergic, are situated in a dense background of GABAergic neurons (Figure 3A). Finally, serotonin neurons in the raphe (as identified by immunohistochemistry for TpH), do not express Cre activity in *Vgat*-ires-Cre mice (Figure S3). Thus, AgRP neurons, but not POMC or SF1 neurons, are GABAergic and therefore are the only previously established first order neurons that contribute, directly, to the obesity seen in *Vgat*-ires-Cre, $Lepr^{lox/lox}$ mice. However, as previously discussed, the contribution of LEPRs on AgRP neurons to regulation of energy balance is small (van de Wall et al.,

2008). Thus, the majority of leptin's anti-obesity must be mediated by previously uncharacterized first order leptin-responsive GABAergic neurons.

Colocalization of LEPRs with GABAergic and Glutamatergic Neurons

Given the above, a key question becomes the location of leptin-responisve GABAergic neurons. To address this, we colocalized LEPR activity, as assessed by leptin-inducible STAT3 phosphorylation, with Cre activity in *Vgat*-ires-Cre and *Vglut2*-ires-Cre, lox-GFP reporter mice, with or without neuron-specific deletion of LEPRs. Note, leptin-inducible P-STAT3 is a robust means of detecting LEPR activity (Munzberg et al., 2004). Leptin was injected (4 mg/kg BW i.p.) into mice that were fasted overnight, and 1 hour later brains were removed and assessed for P-STAT3 and GFP expression. With regards to GABAergic neurons, in control mice, P-STAT3 colocalized with GFP only in the arcuate (Figure 4A), the DMH (Figure 4B) and in the lateral hypothalamus (not shown). In the dorsal vagal complex (NTS/DMV), all P-STAT3 expression was detected in non-GABAergic neurons (Figure 4C). When LEPRs were deleted from GABAergic neurons, all colocalization disappeared; residual P-STAT3 was restricted to non-GABAergic neurons (Figures 4D, 4E and 4F). Thus, leptin-responsive GABAergic neurons are located in the arcuate, the DMH and the lateral hypothalamus.

With regards to glutamatergic ($VGLUT2^+$) neurons, in control mice, P-STAT3 colocalized with GFP only in the arcuate (small number of neurons, Figure 4G), the VMH (Figure 4H), the PMv (Figure 4I) and in the NTS/DMV (Figure 4J). When LEPRs were deleted from glutamatergic neurons, colocalization disappeared in the arcuate (Figure 4K), and in the VMH, PMv and NTS/DMV, all P-STAT3 signal was lost (Figures 4L, 4M and 4N). These findings indicate that leptin-responsive glutamatergic neurons are located primarily in the VMH, the PMv and the NTS/DMV (with a smaller number also found in the arcuate), and of note, in the VMH, PMv and NTS/DMV, 100% of LEPR-expressing neurons are glutamatergic.

Acute (*In Vitro*) Leptin Regulation of GABAergic Tone to POMC Neurons

POMC neurons play a critical role in preventing obesity as evidenced by massive weight gain in mice lacking αMSH (Smart et al., 2006; Yaswen et al., 1999), its receptor, MC4R (Balthasar et al., 2005; Huszar et al., 1997), and in mice with ablation of POMC neurons (Xu et al., 2005). Given this, we examined if POMC neurons are downstream of leptin-responsive GABAergic neurons. Specifically, we recorded inhibitory postsynaptic currents (IPSCs) in POMC neurons (visualized with the POMC-hrGFP BAC transgene) and assessed effects of leptin. Of interest, a prior study using 200-μm thick coronal slices found that leptin reduced IPSC frequency in POMC neurons by 25% in one third of POMC neurons, and this was attributed to AgRP/NPY GABAergic neurons (Cowley et al., 2001). In our studies, we prepared thicker slices (300-μm), positing that this might preserve more connections between the GABAergic and POMC neurons. In Figures 5, 6 and 7, we report effects on all neurons tested.

Addition of leptin decreased spontaneous IPSC (sIPSC) frequency in POMC neurons by 40% (Figures 5A and 5B). This effect was not dependent upon action potentials because, in the presence of tetrodotoxin (TTX), leptin reduced miniature-IPSC (mIPSC) frequency to a similar extent (Figure S4A). We and others (Cowley et al., 2001; Pinto et al., 2004) have observed that frequency and amplitude of sIPSCs in POMC neurons are minimally affected by the addition of TTX, demonstrating that most sIPSCs in POMC neurons, in the context of brain slice preparations, originate from spontaneous vesicle fusion events in presynaptic GABAergic neurons. Unlike the situation in POMC neurons, leptin failed to reduce sIPSC frequency in AgRP neurons (visualized with the NPY-hrGFP BAC transgene) (Figure S4B),

indicating that leptin-mediated suppression of IPSCs is not a generalized phenomenon. Of interest, leptin's inhibitory effect on IPSCs in POMC neurons was absent in *Vgat*-ires-Cre, $Lepr^{lox/lox}$ mice and persisted, unaffected, in *Pomc*-Cre, $Lepr^{lox/lox}$ mice (Figure 5B), demonstrating that LEPRs on presynaptic GABAergic neurons, and not postsynaptic POMC neurons, mediate this response. We next evaluated the role of LEPRs on AgRP neurons, which as mentioned above, are GABAergic and are thought to be in synaptic contact with POMC neurons (Cowley et al., 2001). Of note, in mice lacking LEPRs on AgRP neurons, leptin reduced IPSC frequency in POMC neurons by 31%, representing a small attenuation of the inhibitory response (40%) noted above in control mice. This suggests that the majority of leptin's effect is mediated by LEPRs on "non-AgRP" GABAergic neurons. To confirm a prominent role for non-AgRP GABAergic neurons, we utilized mice in which AgRP neurons are unable to release GABA (*Agrp*-ires-Cre, $Vgat^{lox/lox}$ mice (Tong et al., 2008)). In this case, all IPSCs originate exclusively from non-AgRP GABAergic neurons. Of note, leptin's inhibitory effect on IPSC frequency was still present (Figure 5B), establishing an important role for non-AgRP GABAergic neurons. To summarize, addition of leptin to brain slices reduces IPSC frequency in POMC neurons and this effect is mediated by LEPRs on presynaptic GABAergic neurons, the majority of which appear to be non-AgRP GABAergic neurons.

Long-term (*In Vivo*) Leptin Regulation of GABAergic Tone to POMC Neurons

We next evaluated if manipulation of LEPRs, *in vivo*, altered GABAergic tone to POMC neurons. Of note, a previous study reported that mice lacking leptin ($Lep^{ob/ob}$ mice) have a marked increase in IPSC frequency in POMC neurons (Pinto et al., 2004). In agreement with this, we observed that mice with global lack of LEPRs ($Lepr^{\Delta/\Delta}$ mice) have a large increase in sIPSC and mIPSC frequency in POMC neurons (Figure 6A), and also in amplitude. This latter effect on amplitude appears to occur in $Lep^{ob/ob}$ mice as well (Pinto et al., 2004). In addition, we observed an equally large increase in IPSC frequency and amplitude in POMC neurons from *Vgat*-ires-Cre, $Lepr^{lox/lox}$ mice, but found no increase in POMC neurons from *Pomc*-Cre, $Lepr^{lox/lox}$ mice (Figure 6A), demonstrating that deficient leptin signaling in presynaptic GABAergic neurons, but not postsynaptic POMC neurons, increases inhibitory tone in POMC neurons. This effect appears not to be caused by deletion of LEPRs from AgRP neurons since no increase in IPSC frequency or amplitude was observed in POMC neurons from *AgRP*-ires-Cre, $Lepr^{lox/lox}$ mice (Figure 6A).

Deletion of LEPRs in $VGAT^{+}$ neurons did not increase IPSC frequency or amplitude in AgRP neurons - while not statistically significant, IPSC frequency in AgRP neurons actually tended to decrease in *Vgat*-ires-Cre, $Lepr^{lox/lox}$ mice (Figure S5A). Furthermore, deletion of LEPRs in glutamatergic ($VGLUT2^{+}$) neurons did not affect excitatory postsynaptic currents (sEPSCs and mEPSCs) in POMC neurons (Figure S5B). In summary, deficiency of leptin signaling in presynaptic, non-AgRP GABAergic neurons, but not postsynaptic POMC neurons, selectively increases inhibitory tone in POMC neurons.

To determine if POMC neurons are affected by this increased GABAergic tone, we assessed their membrane potential and firing rate. POMC neurons from *Vgat*-ires-Cre, $Lepr^{lox/lox}$ mice, compared with neurons from control mice, tended to be hyperpolarized (−62.1 +/− 1.94 mV compared with −57.8 +/− 2.8 mV in control mice; Figure 6B, left panel) and, consistent with this, addition of the GABAA receptor blocker, picrotoxin (PTX) in *Vgat*-ires-Cre, $Lepr^{lox/lox}$ mice produced a greater degree of depolarization. PTX addition increased membrane potential by 6.4 +/− 0.97 mV in *Vgat*-ires-Cre, $Lepr^{lox/lox}$ mice compared with only 3.2 +/− 1.01 mV in control mice ($p<0.05$, t-test). In agreement with this, their firing rate was markedly reduced, 0.32 +/− 0.11 Hz in *Vgat*-ires-Cre, $Lepr^{lox/lox}$ mice compared with 1.81 Hz +/− 0.37 in control mice ($p=0.01$, t-test; Figure 6B, right panel), and this reduction was markedly attenuated by PTX. PTX addition increased firing

rate by 11.6 +/− 6.2 fold in *Vgat*-ires-Cre, *Lepr*$^{lox/lox}$ mice and by only 1.2 +/− 0.1 fold in control mice (p=0.01, Mann-Whitney test). These findings support the view that deficiency of leptin signaling in presynaptic GABAergic neurons inhibits the activity of POMC neurons.

Physiologic (*In Vivo*) Leptin Regulation of GABAergic Tone to POMC Neurons

We next evaluated whether a physiologic reduction in circulating leptin, as occurs with fasting (Ahima et al., 1996), also increases inhibitory input to POMC neurons. This is a key question because the marked effects observed in Figure 6, while suggestive of important regulation, might be seen only with “unphysiologic”, total absence of leptin signaling. Our studies, described below, were motivated by a prior study in which fasting markedly increased the firing rate of AgRP neurons (which are GABAergic) - an effect that was prevented by leptin treatment 3 hours prior to sacrifice (Takahashi and Cone, 2005). Of interest, we found that fasting for 24 hours produced a marked increase in sIPSC frequency and amplitude in POMC neurons (Figure 7A). Importantly, these fasting-mediated effects were completely prevented by injection of leptin (4 mg/kg), but not saline, 3 hrs prior to sacrifice. Complete prevention of the fasting-stimulated increase in IPSCs by leptin treatment is consistent with the view that increased inhibitory tone caused by fasting is, indeed, due to the fasting-mediated fall in leptin. We then assessed the effects of fasting in mice lacking LEPRs on GABAergic neurons (*Vgat*-ires-Cre, *Lepr*$^{lox/lox}$ mice). Of note, fasting in these mice failed to increase sIPSC frequency and amplitude (Figure 7B), which, as noted earlier, are increased in the fed state compared with control mice (Figure 6A). Amplitude not only failed to increase but actually decreased with fasting in *Vgat*-ires-Cre, *Lepr*$^{lox/lox}$ mice, which is a paradoxical response presumably unmasked by the absence of leptin signaling. To summarize, a physiologic fall in circulating leptin (induced by fasting in this case) causes a marked increase in inhibitory tone to POMC neurons, and this effect is likely mediated by decreased leptin action on presynaptic GABAergic neurons.

DISCUSSION

Despite intensive investigation, the neuronal, subcellular and molecular mechanisms responsible for leptin’s anti-obesity effects are incompletely understood. While strong evidence suggests a role for AgRP and POMC neurons in the arcuate, it is unclear to what degree, and by what means (directly or indirectly), leptin engages these functionally important neurons. Uncertainty about the direct neuronal targets of leptin (i.e. the first order, leptin-responsive neurons) has been a key obstacle. The elusive nature of these first order neurons, combined with other findings, suggests the possibility that leptin action occurs through a distributed network of leptin-responsive neurons. If this is so, it then becomes important to establish if any deeper organizing principles underlie leptin-responsive first order neurons and their integration with body weight-regulating neurocircuitry.

As part of a search for higher order, we have determined the inhibitory/excitatory nature of leptin-responsive, body weight-regulating neurons. Specifically, we used *Vgat*-ires-Cre and *Vglut2*-ires-Cre knock-in mice to manipulate LEPR expression on GABAergic (VGAT^{+}) and glutamatergic (VGLUT2^{+}) neurons, and then tested for effects on energy balance. Remarkably, we find that leptin’s anti-obesity effects are mediated predominantly by GABAergic (VGAT^{+}) neurons, and that glutamatergic (VGLUT2^{+}) neurons play only a small role. Importantly, this raises the likely possibility that modulation of GABAergic output is a key aspect of leptin action. Consistent with this, we find, using multiple approaches, that leptin action on presynaptic GABAergic neurons markedly decreases inhibitory tone to postsynaptic POMC neurons. This regulation was observed using the following paradigms: a) addition of leptin *in vitro* (Figure 5), b) removal of leptin signaling selectively from presynaptic GABAergic neurons *in vivo* (Figure 6), and c) importantly,

with physiologic reductions in circulating leptin brought about by fasting (Figure 7). Given the previously established role of POMC neurons in preventing obesity (Smart et al., 2006; Xu et al., 2005; Yaswen et al., 1999), these effects of leptin on presynaptic GABAergic neurons provide a basis for leptin's anti-obesity effects. Of note, indirect regulation of POMC neurons by leptin reconciles the known important role of POMC neurons in regulating body weight with the relatively unimportant role played by direct leptin action on POMC neurons (Balthasar et al., 2004; Hill et al., 2010; van de Wall et al., 2008). While in our studies we have focused on POMC neurons as the downstream target of leptin-responsive GABAergic neurons, it's certainly possible and perhaps likely that other anti-obesity neurons are similarly disinhibited by leptin action on presynaptic GABAergic neurons. This will need to be tested as other anti-obesity neurons are identified. Given the above-mentioned findings, GABAergic output appears to be an important, direct target of leptin action.

Identity and Location of Leptin-Responsive GABAergic Neurons

Using leptin-inducible P-STAT3 and GFP reporter expression (in *Vgat*-ires-Cre mice) to colocalize LEPRs and GABAergic neurons, we observed that LEPR-expressing GABAergic neurons are located in the arcuate, the DMH, and the lateral hypothalamus. Consequently, LEPR-expressing GABAergic neurons in one, two or all three of these sites mediate leptin's anti-obesity effects. At present, our results do not allow us to rule in or out any one, or any combination of these sites. Nevertheless, for reasons listed below, we favor an important role for neurons in the arcuate. First, LEPR-expressing arcuate neurons have unparalleled access to circulating leptin (Faouzi et al., 2007). Second, the arcuate has many GABAergic neurons, a small fraction of which are AgRP neurons (Figure 3C and (Acuna-Goycolea et al., 2005; Horvath et al., 1997; Ovesjo et al., 2001)). Third, POMC neurons, which are key targets of leptin-responsive GABAergic neurons (Figures 5–7 and (Cowley et al., 2001)), are located within the arcuate, surrounded by a dense population of GABAergic neurons (Figure 3A). Fourth, neurons in the arcuate make many local connections (Matsumoto and Arai, 1978; van den Pol and Cassidy, 1982), providing the apparatus for local regulation of POMC neurons. One previously defined local circuit, likely to be physiologically important, is that between AgRP neuron collaterals and POMC neurons (Cowley et al., 2001; Horvath et al., 1992). As discussed below, we postulate that this is just one of many local leptin-responsive GABAergic inputs to POMC neurons.

Role of AgRP GABAergic Neurons versus Other GABAergic Neurons

An earlier study (Cowley et al., 2001) established that leptin reduces the frequency of IPSCs in POMC neurons (25% reduction in one third of POMC neurons). The source of the reduced GABAergic input was attributed to AgRP neurons (which also express NPY). In the present study, we confirm leptin's inhibitory effect on IPSC frequency, but, of interest, note a larger effect (40% inhibition in all POMC neurons), perhaps due to our use of thicker brain slices (300 μm versus 200 μm). A key outcome of that prior study was the compelling proposal that leptin indirectly regulates POMC neurons via AgRP/NPY-GABAergic collaterals. The degree to which this accounts for leptin's anti-obesity effects, however, has been unclear, especially since deletion of LEPRs from AgRP neurons produces only a small disturbance in energy balance (van de Wall et al., 2008).

In the present study, we show that the above-mentioned effect (i.e. leptin-mediated reduction of IPSC frequency in POMC neurons) is mediated by LEPRs on presynaptic GABAergic neurons, and that LEPRs on postsynaptic POMC neurons play no role. Furthermore, our data indicates that LEPRs on "non-AgRP" GABAergic neurons are predominantly responsible for this effect. The following three findings support this view: a) leptin-mediated reduction of IPSC frequency is minimally affected when LEPRs are deleted from AgRP

neurons (*Agrp*-ires-Cre, *Lepr*$^{lox/lox}$ mice), but is totally abrogated when LEPRs are deleted from all GABAergic neurons (*Vgat*-ires-Cre, *Lepr*$^{lox/lox}$ mice, Figure 5B), b) this response is unimpaired in mice which cannot release GABA from AgRP neurons (*Agrp*-ires-Cre, *Vgat*$^{lox/lox}$ mice, Figure 5B), and c) deletion of LEPRs from GABAergic neurons (*Vgat*-ires-Cre, *Lepr*$^{lox/lox}$ mice) markedly increases IPSC frequency and amplitude in POMC neurons, while in contrast, no effect is seen when LEPRs are deleted from AgRP neurons (*Agrp*-ires-Cre, *Lepr*$^{lox/lox}$ mice, Figure 6A). These results clearly attest to the important role played by "non-AgRP" neurons in leptin-mediated disinhibition of POMC neurons, and, of interest, are congruent with the presence of massive obesity versus minimal obesity, respectively, in *Vgat*-ires-Cre, *Lepr*$^{lox/lox}$ mice (Figure 2) versus *Agrp*-ires-Cre, *Lepr*$^{lox/lox}$ mice (van de Wall et al., 2008). One notable caveat of the above analysis is the possibility of compensation, as was observed following diphtheria toxin-mediated ablation of AGRP neurons in neonates (Luquet et al., 2005). If such compensation were to occur following genetic deletion of LEPRs in AgRP neurons, then our approach could underestimate the contribution of AgRP GABAergic neurons. However, given that toxin ablation kills neurons, while LEPR deletion, on the other hand, leaves neurons largely intact, it is unclear if similar degrees or forms of compensation should be expected. To summarize, our results and those of others (Cowley et al., 2001) demonstrate that leptin reduces inhibitory tone to POMC neurons. This effect is mediated by LEPRs on presynaptic GABAergic neurons, some of which may express AgRP, many of which likely do not.

Leptin Action via GABAergic Neurons: Issues and Implications

It has previously been established that leptin's anti-obesity effects require Tyr_{1138} of the LEPR, which allows for phosphorylation-dependent docking and activation (via subsequent phosphorylation) of the latent transcription factor, STAT3 (Bates et al., 2003). Of note, marked obesity, similar in magnitude to that observed in mice totally lacking LEPRs, occurs in mice homozygous for the *Lepr*^{S1138} allele. This requirement for Tyr_{1138} strongly implicates STAT3-mediated gene expression in leptin's anti-obesity effects. The relevant downstream transcriptional targets, however, are not yet known but are of great interest. Prior studies have focused on the *Pomc* gene (Munzberg et al., 2003). However, given the important role of leptin-responsive GABAergic neurons in regulating body weight, most of which do not express AgRP, and none of which appear to express POMC (Figure 3 and (Ovesjo et al., 2001; Yee et al., 2009), and for a dissenting view - (Hentges et al., 2009)), it will be important to reexamine this issue. Perhaps the key leptin-responsive target genes do not encode neuropeptides, but instead, proteins involved in modulating GABA release, or possibly proteins that regulate plasticity of GABAergic synapses (Pinto et al., 2004).

Related to the above, it has been reported that leptin's ability to acutely decrease IPSC frequency in POMC neurons is unaltered in *Lepr*$^{S1138/S1138}$ mice (Munzberg et al., 2007). Specifically, intact inhibition was observed in 2 out of 5 POMC neurons (see Figure 3B of that prior study). Given that *Lepr*$^{S1138/S1138}$ mice are massively obese, this suggests that leptin-mediated acute suppression of IPSC frequency in POMC neurons, by itself, cannot prevent obesity, and, in addition, that it is not dependent on STAT3-mediated signaling. Alternatively, acute leptin suppression of IPSC frequency may still play an important role, as we hypothesize, but given the above, this role in regulating body weight may require intact STAT3 signaling as a necessary precondition. Resolution of these issues will require further investigation.

Finally, the subcellular site of action and molecular mechanism by which leptin modulates GABA release is unclear. Of interest, acute and chronic leptin modulation of GABA release, at least as detected *in vitro* in brain slices, is entirely independent of action potentials (i.e. occurs in the presence of TTX) (Figure S4A, Figure 6A and (Cowley et al., 2001; Pinto et al., 2004)). This, combined with our observation that leptin works on presynaptic

GABAergic neurons to produce its effects, raises the distinct possibility that GABAergic axon terminals are the ultimate subcellular site of action for leptin's effects. Whether this involves transcription/translation of proteins that subsequently affect the function of axon terminals or, alternatively, leptin receptor/signaling pathways that are self-contained within axon terminals, is presently unknown. Given the key role of leptin action on GABAergic neurons, further studies will be needed to address these interesting possibilities.

EXPERIMENTAL PROCEDURES

Animal Care

Care of all animals and procedures was approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Generation of *Vgat*-ires-Cre and *Vglut2*-ires-Cre mice

Vgat-ires-Cre and *Vglut2*-ires-Cre knockin mice were generated by gene targeting using the same approach as described previously (Tong et al., 2008). For details see Supplemental Information section.

Electrophysiological Studies

Slice preparation and whole-cell recordings—Brain slices were prepared from young adult mice (5–7 weeks old) as described previously (Dhillon et al., 2006) with the exception that 300 μM thick coronal sections were cut with a Leica VT1000S Vibratome.

Measuring inhibitory and excitatory post-synaptic currents—IPSCs and EPSCs were measured in whole-cell voltage clamp mode with a holding potential of -60 mV. The internal recording solution contained: 140 mM CsCl, 1 mM BAPTA, 10 mM HEPES, 5 mM $MgCl_2$, 2 mM Mg-ATP, 0.3 mM Na-GTP (pH 7.35 with NaOH). Currents were amplified, filtered at 1kHz, and digitized at 20 kHz. EPSCs were measured in the presence of picrotoxin (100μM). IPSCs were measured in the presence of NBQX (10 μM) + D, L-APV (50μM) or Kynurenate (1mM). Miniature EPSCs and IPSCs were recorded with 1 μm tetrodotoxin in aCSF recording solution. Frequency and peak amplitude were measured using the Mini Analysis program (Synaptosoft, Inc.). Cumulative probability distribution for mIPSC amplitudes (Figure 6A) was measured for 3 minute periods (Figure 6A).

Measuring membrane potential and firing rate—Membrane potential and firing rate were measured by whole-cell current clamp recordings from POMC neurons in brain slices from $Lepr^{lox/lox}$ mice and *Vgat*-ires-Cre, $Lepr^{lox/lox}$ mice. Recording electrodes had resistances of 2.5–4 MΩ when filled with the K-gluconate internal solution (128 mM K-gluconate, 10 mM HEPES, 1 mM EGTA, 10 mM KCL, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 2 mM Mg-ATP, 0.3 mM Na-GTP (pH 7.35 with KOH).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank members of the Lowell lab for helpful discussions, C. B. Saper, C. Bjorbaek and B. P. Bean for advice, J. K. Elmquist and D. P. Olson for giving comments on the manuscript, and M. Herman for help with statistics. This work was supported by grants from the National Institute of Health/National Institute of Diabetes and Digestive and Kidney Diseases (R01 DK089044, R01 DK075632, P30 DK046200, and P30 DK057521 to BBL; F32 DK078478 to LV).

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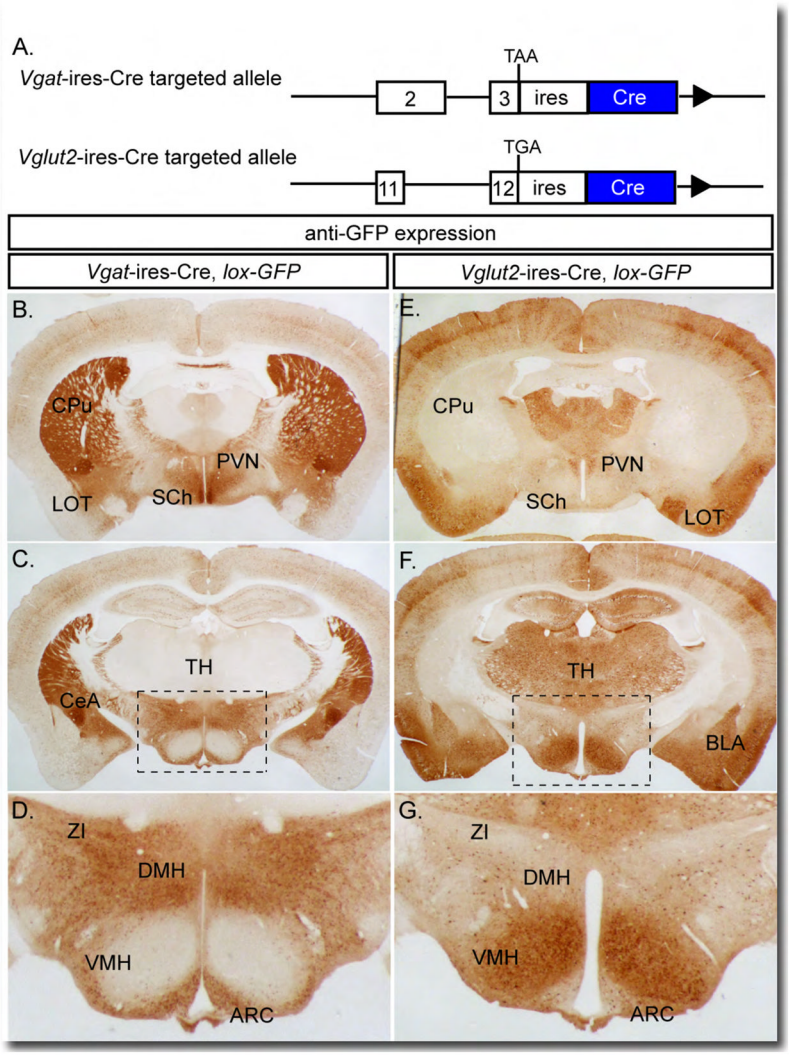

Figure 1. Generation of *Vgat*-ires-Cre and *Vglut2*-ires-Cre Mice

(A) Mice expressing Cre recombinase under the control of the endogenous *Vgat* and *Vglut2* genes were generated by inserting an ires-Cre cassette after the *Vgat* and *Vglut2* stop codons, respectively.

(B–D) Immunohistochemistry for eGFP expression in *Vgat*-ires-Cre, *lox-GFP* mice; brown-staining represents Cre activity. (D) Enlargement of boxed region in (C).

(E–G) Immunohistochemistry for eGFP expression in *Vglut2*-ires-Cre, *lox-GFP* mice; brown staining represents Cre activity. (G) Enlargement of boxed region in (F).

Arc=arcuate, BLA=basolateral amygdala, CeA=central amygdala, CPu=caudate putamen, DMH=dorsomedial hypothalamus, LOT=nucleus of the lateral olfactory tract, PVN=paraventricular nucleus, SCh=suprachiasmatic nucleus, TH=thalamus, VMH=ventromedial hypothalamus, ZI=zona incerta.

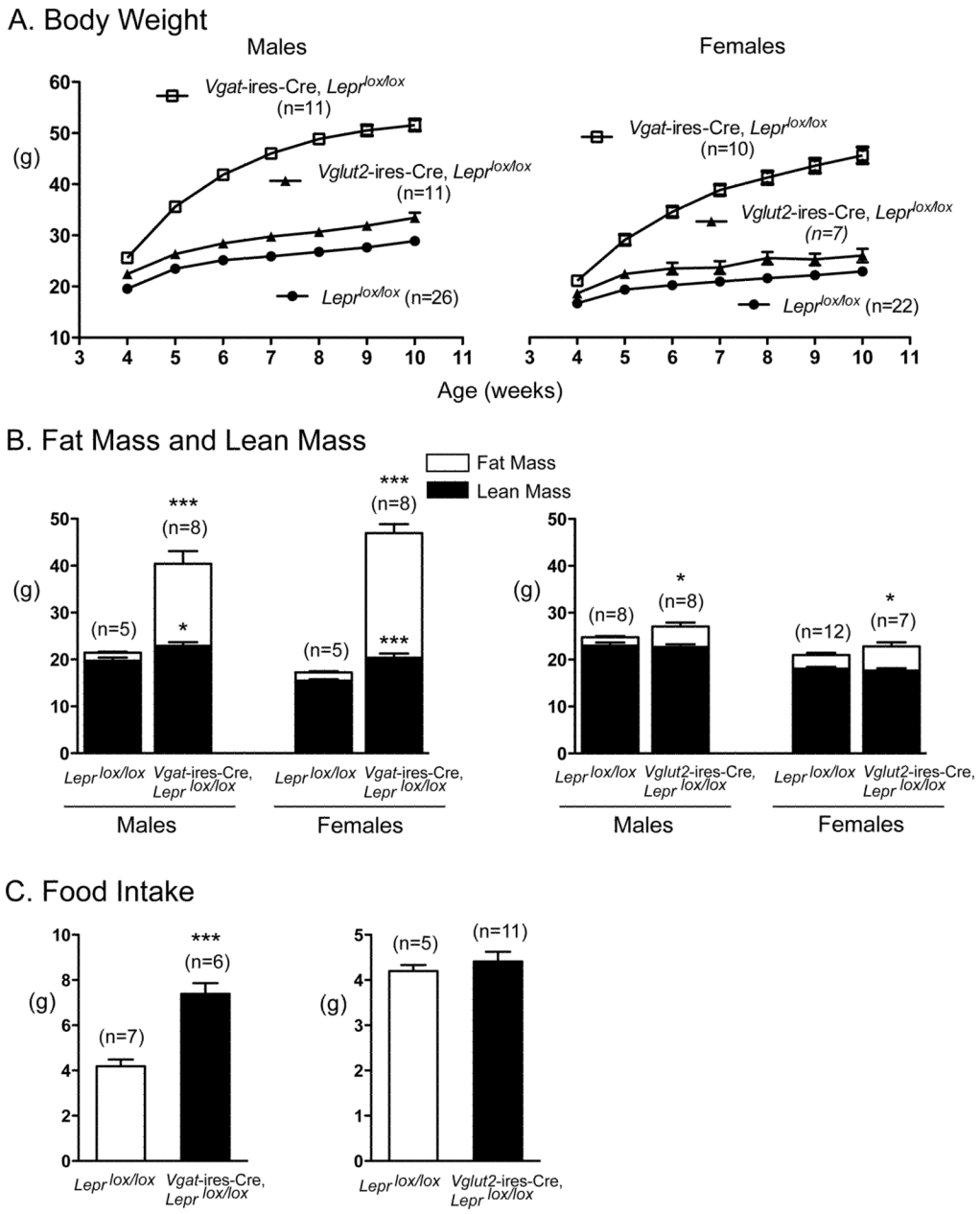

Figure 2. Effect of Deleting LEPRs from VGAT^{+} and VGLUT2^{+} Neurons on Body Weight, Body Composition, and Food Intake

(A) Body weight curves of male and female mice with LEPR deleted from VGAT^{+} neurons (open boxes), VGLUT2^{+} neurons (closed triangles) or controls (*Lepr*$^{lox/lox}$, closed circles). Data are presented as mean +/− SEM. Compared to the *Lepr*$^{lox/lox}$ controls, all values are significantly different except in week 4 and 7 for the *Vglut2*-ires-Cre, *Lepr*$^{lox/lox}$ females (assessed by one-way ANOVA with Dunnett's test of all groups compared to *Lepr*$^{lox/lox}$ mice).

(B) Fat mass and lean mass of male and female *Vgat*-ires-Cre, *Lepr*$^{lox/lox}$ mice (left) and *Vglut2*-ires-Cre, *Lepr*$^{lox/lox}$ mice (right) at 10 weeks of age were analyzed by EchoMRI. Data are presented as mean +/− SEM.*, p< 0.05; ***, p<0.001; unpaired t-test compared to *Lepr*$^{lox/lox}$ controls.

(C) Food intake of *Vgat*-ires-Cre, *Lepr*$^{lox/lox}$ mice (left) and *Vglut2*-ires-Cre, *Lepr*$^{lox/lox}$ mice (right). Data are presented as mean +/− SEM.***, $p<0.001$; unpaired t-test compared to *Lepr*$^{lox/lox}$ controls.

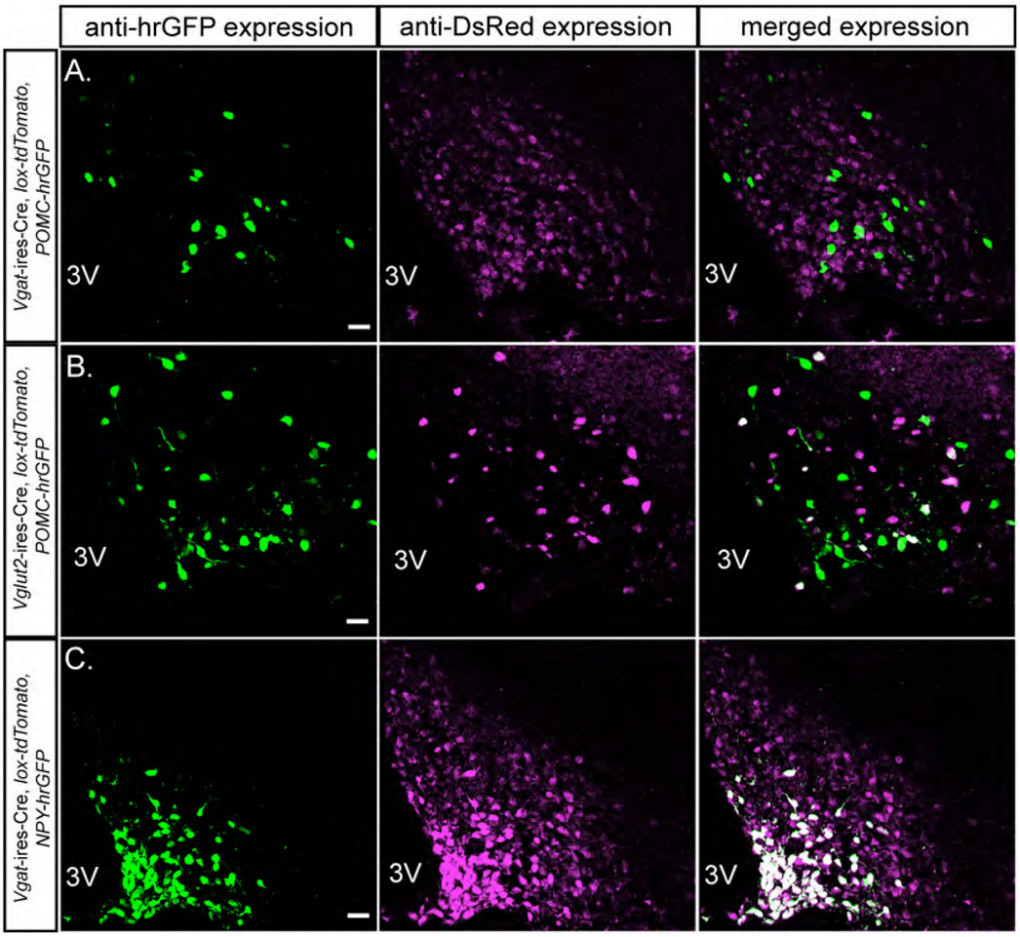

Figure 3. GABAergic (VGAT$^+$) and Glutamatergic (VGLUT2$^+$) Nature of POMC and AgRP Neurons

(A) Colocalization of hrGFP (POMC neurons) and DsRed (GABAergic neurons) in the arcuate in *Vgat*-ires-Cre, *lox-tdTomato, POMC-hrGFP* mice.

(B) Colocalization of the hrGFP (POMC neurons) and DsRed (glutamatergic VGLUT2$^+$ neurons) in the arcuate in *Vglut2*-ires-Cre, *lox-tdTomato, POMC-hrGFP* mice.

(C) Colocalization of the hrGFP (AgRP/NPY neurons) and DsRed (GABAergic neurons) in the arcuate in *Vgat*-ires-Cre, *lox-tdTomato, NPY-hrGFP* mice.

Green=anti-hrGFP, Magenta=anti-DsRed, white=hrGFP+DsRed. Scale bar=20 um.

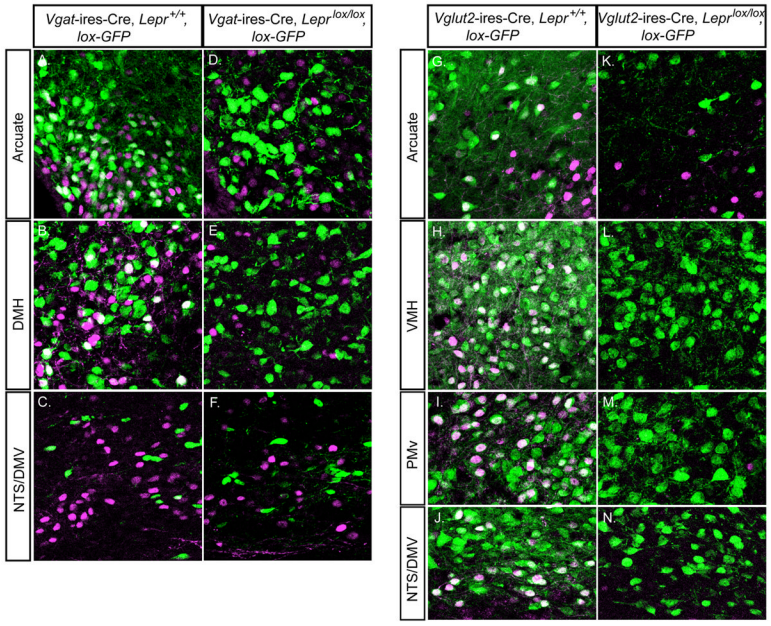

Figure 4. Leptin-induced pSTAT3 Expression in GABAergic and Glutamatergic (VGLUT2$^+$) Neurons With and Without LEPRs

(A–F) Double immunohistochemical staining for pSTAT3 and eGFP in the arcuate, DMH, and NTS/DMV from leptin-injected *Vgat*-ires-Cre, *Lepr*$^{+/+}$, *lox-GFP* mice (with LEPRs intact, A–C) and *Vgat*-ires-Cre, *Lepr*$^{lox/lox}$, *lox-GFP* mice (with LEPRs deleted from GABAergic neurons, D–F).

(G–N) Double immunohistochemical staining for pSTAT3 and eGFP in the arcuate, VMH, PMv, and NTS/DMV from leptin-injected *Vglut2*-ires-Cre, *Lepr*$^{+/+}$, *lox-GFP* mice (with LEPRs intact, G-J) and *Vglut2*-ires-Cre, *Lepr*$^{lox/lox}$, *lox-GFP* mice (with LEPRs deleted from glutamatergic VGLUT2$^+$ neurons, K-N).

Green=anti-eGFP, Magenta=anti-pSTAT3, white=eGFP+pSTAT3. Scale bar=20 um.

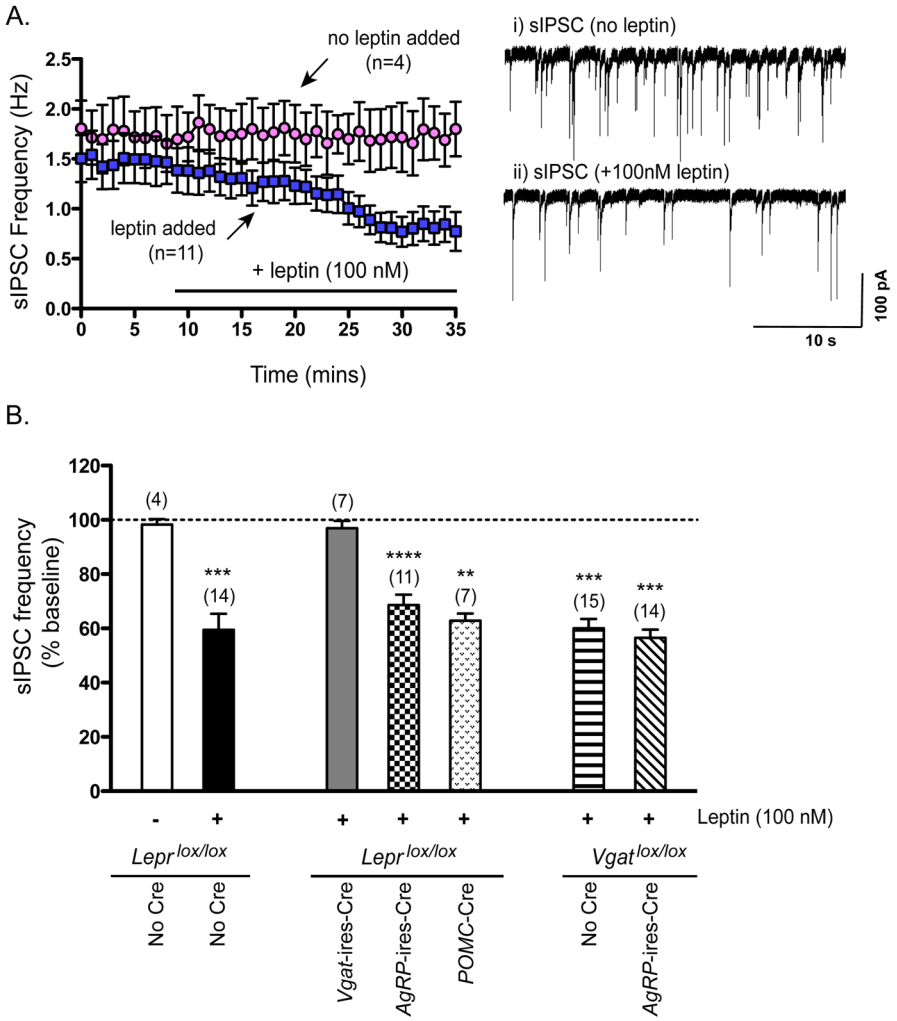

Figure 5. Effects of Leptin Addition on sIPSC Frequency in POMC Neurons

(A) Left–Time course of effects of leptin (100 nM) (blue squares) or of no additions (pink circles) on sIPSC frequency in POMC neurons from control (*Lepr*$^{lox/lox}$) mice. Right - Example traces of sIPSCs recorded from a POMC neuron i) just before and ii) after 25–30 minutes of leptin addition.

(B) Effects of 25–30 minutues of leptin treatment on sIPSC frequency, expressed as percent of baseline (prior to leptin addition), in POMC neurons from *Lepr*$^{lox/lox}$ (control) mice, *Vgat*-ires-Cre, *Lepr*$^{lox/lox}$ mice, *AgRP*-ires-Cre, *Lepr*$^{lox/lox}$ mice, and *POMC*-Cre, *Lepr*$^{lox/lox}$ mice, and also from *Vgat*$^{lox/lox}$ (control) mice and *AgRP*-ires-Cre, *Vgat*$^{lox/lox}$ mice (lacking VGAT in AGRP neurons). Data are presented as mean +/− SEM. **, p<0.01; ***, p<0.001; ****, p<0.0001, paired t-test for effect of leptin versus baseline (see “Supplemental Procedures” for details on how percent baseline was determined).

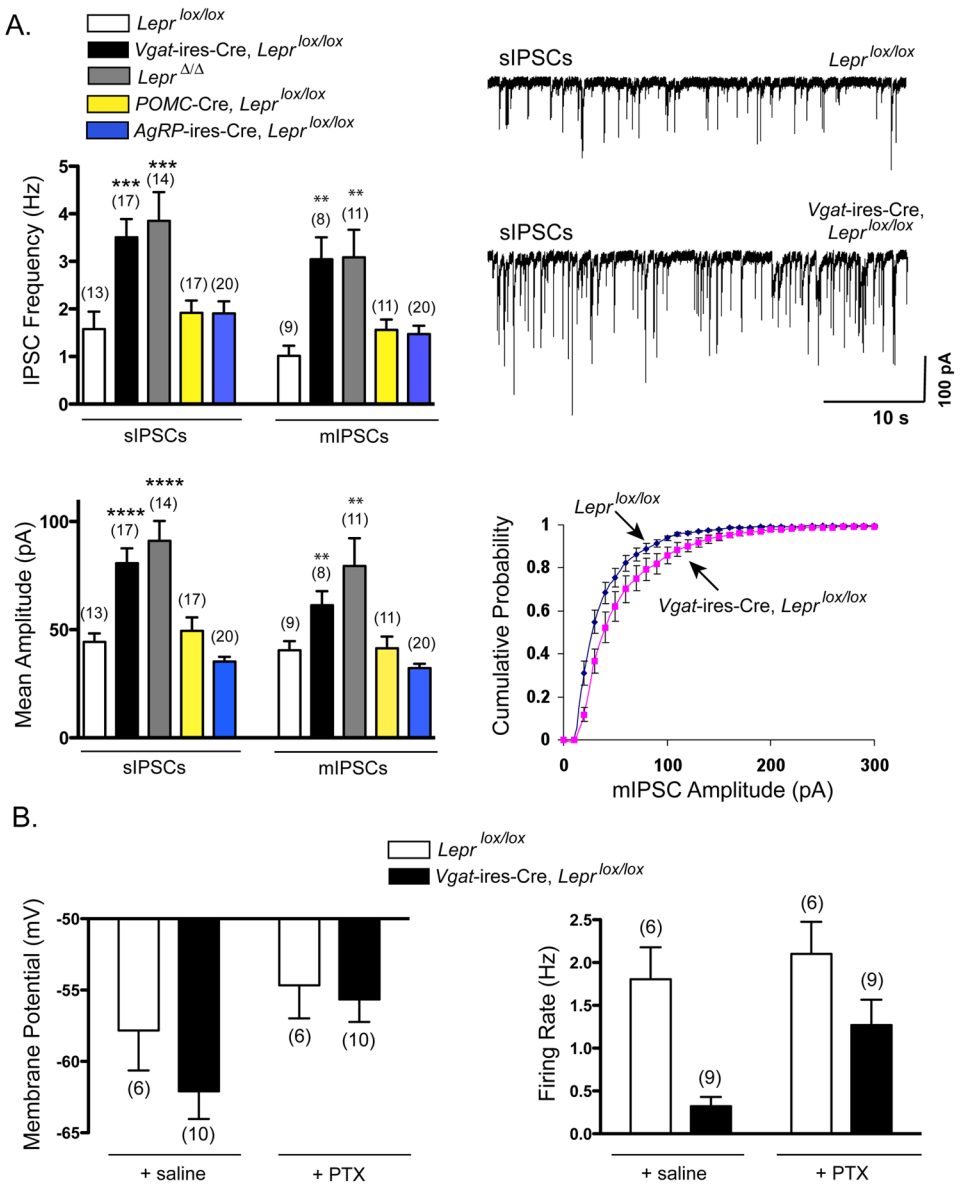

Figure 6. Effects of Deleting LEPRs Globally (*Lepr* $^{\Delta/\Delta}$ mice), from GABAergic Neurons, from AgRP neurons or from POMC Neurons on Inhibitory Tone to POMC Neurons

(A) Summary of sIPSC and mIPSC frequency (top left) and amplitude (bottom left) in POMC neurons from *Lepr*$^{lox/lox}$ (control) mice, *Vgat*-ires-Cre, *Lepr*$^{lox/lox}$ mice, *Lepr*$^{\Delta/\Delta}$ mice, *POMC*-Cre, *Lepr*$^{lox/lox}$, and *AgRP*-ires-Cre, *Lepr*$^{lox/lox}$ mice. Data are presented as mean +/− SEM. **, p<0.01; ***, p<0.001; ****, p<0.0001 one-way ANOVA with Dunnett's test of all groups compared to *Lepr*$^{lox/lox}$ mice. (Top right) Example traces of sIPSCs recorded from POMC neurons of a *Lepr*$^{lox/lox}$ (control) mouse and a *Vgat*-ires-Cre, *Lepr*$^{lox/lox}$ mouse. (Bottom right) Cumulative probability distribution of mIPSC amplitudes from POMC neurons showing a significant rightward shift in *Vgat*-ires-Cre, *Lepr*$^{lox/lox}$ mice compared to *Lepr*$^{lox/lox}$ (control) mice.

(B) Summary of membrane potential and firing rate of POMC neurons from *Lepr*$^{lox/lox}$ (control) mice and *Vgat*-ires-Cre, *Lepr*$^{lox/lox}$ mice before and after the addition of the GABAA receptor blocker, picrotoxin. Data are presented as mean +/− SEM.

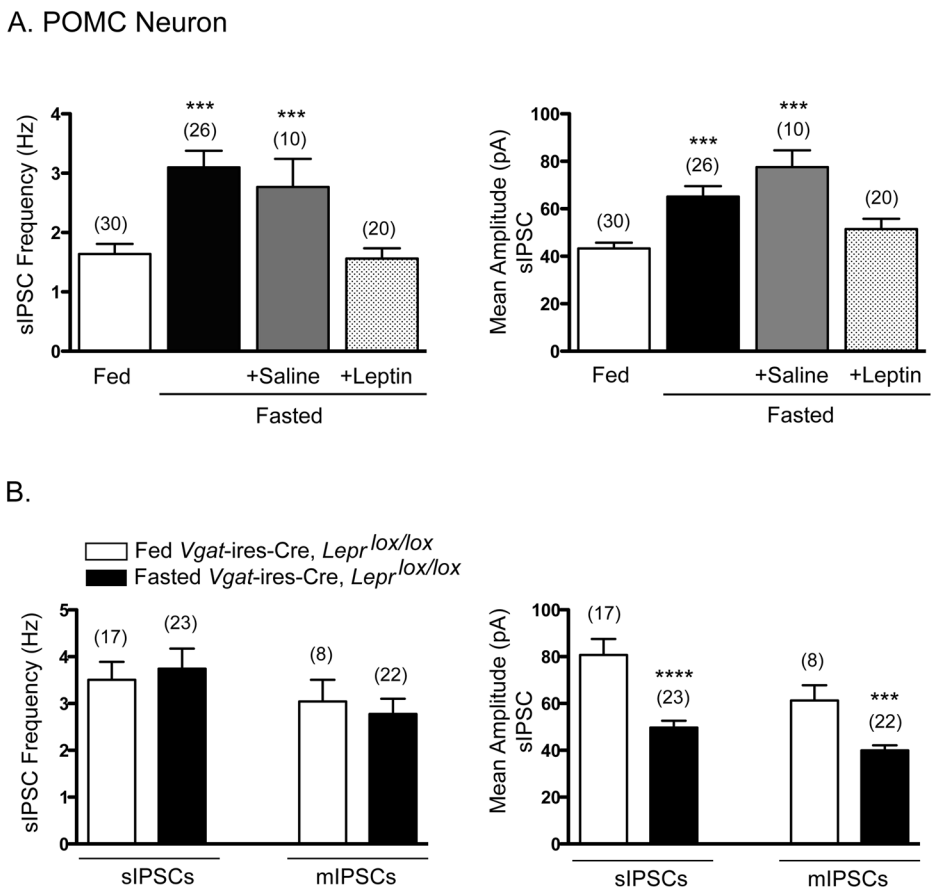

Figure 7. Effects of Fasting on sIPSC Frequency and Amplitude in POMC Neurons

(A) Summary of the effects of fasting on sIPSC frequency (left) and amplitude (right) in POMC neurons of fed *Lepr*$^{lox/lox}$ mice, fasted *Lepr*$^{lox/lox}$ mice, and fasted+saline- or fasted +leptin-injected *Lepr*$^{lox/lox}$ mice. Data are presented as mean +/− SEM. ***, p<0.001 one-way ANOVA with Dunnett's test of all groups compared to fed *Lepr*$^{lox/lox}$ mice.

(B) Summary of the effects of fasting on sIPSC frequency (left) and amplitude (right) in POMC neurons of fed and fasted *Vgat*-ires-Cre, *Lepr*$^{lox/lox}$ mice. Data are presented as mean +/− SEM. ***, p<0.001; ****, p<0.0001; t-test compared to fed *Vgat*-ires-Cre, *Lepr*$^{lox/lox}$ mice.