Leptin modulates nutrient reward via inhibitory galanin action on orexin neurons

Amanda Laque 1, Sangho Yu 1, Emily Qualls-Creekmore 1, Sarah Gettys 1, Candice Schwartzenburg 1, Kelly Bui 1, Christopher Rhodes 4, Hans-Rudolf Berthoud 3, Christopher D. Morrison 4, Brenda K. Richards 5, Heike Münzberg 1,*

ABSTRACT

Objective: Leptin modulates food reward via central leptin receptor (LepRb) expressing neurons. Food reward requires stimulation of midbrain dopamine neurons and is modulated by central leptin action, but the exact central mechanisms remain unclear. Stimulatory and inhibitory leptin actions on dopamine neurons have been reported, e.g. by indirect actions on orexin neurons or via direct innervation of dopamine neurons in the ventral tegmental area.

Methods: We showed earlier that LepRb neurons in the lateral hypothalamus (LHA) co-express the inhibitory acting neuropeptide galanin (GAL-LepRb neurons). We studied the involvement of GAL-LepRb neurons to regulate nutrient reward in mice with selective LepRb deletion from galanin neurons (GAL-LepRbKO mice).

Results: We found that the rewarding value and preference for sucrose over fat was increased in GAL-LepRbKO mice compared to controls. LHA GAL-LepRb neurons innervate orexin neurons, but not the VTA. Further, expression of galanin and its receptor GalR1 are decreased in the LHA of GAL-LepRbKO mice, resulting in increased activation of orexin neurons.

Conclusion: We suggest galanin as an important mediator of leptin action to modulate nutrient reward by inhibiting orexin neurons.

Keywords Sucrose; Intralipid; Incentive runway; Lateral hypothalamus; Locus coeruleus; Two-bottle choice

1. INTRODUCTION

Leptin acts centrally via leptin receptor (LepRb) expressing neurons that are found in the hypothalamus and many extra-hypothalamic sites. Recent research has focused on the identification of distinct LepRb populations to understand their contribution to diverse physiological leptin effects, including the ability of leptin to modulate food reward [16,23].

A key component of reward behavior is the mesolimbic dopamine system, which consists of dopaminergic (DA) neurons in the ventral tegmental area (VTA) and their projections to the nucleus accumbens (NAc) as well as other cortico-limbic areas [32,35]. The rewarding value of nutrients, e.g. sucrose or fat, requires activation of midbrain DA neurons and is enhanced by fasting and diminished by leptin [16,18,23], but the precise neuronal mechanisms employed by leptin to reduce nutrient reward are controversial. LepRb is expressed on some midbrain DA neurons, and leptin inhibits these LepRb-DA neurons [30]. Nutrient reward is encoded by NAc dopamine release, so that leptin-inhibited VTA DA neurons could explain a decreased nutrient reward and anorexia. Conversely, NAc DA levels are decreased in severely hyperphagic, leptin deficient ob/ob mice compared to control animals and leptin injections increase NAc DA levels [23]. Thus, NAc DA deficiency may increase motivated behavior, such as food intake, in an attempt to induce DA release and normalize NAc DA levels. This hypothesis is in line with the decreased NAc activity of obese compared to lean humans and shows striking resemblance to the low NAc activity observed in drug addicts [63].

Leptin action in the lateral hypothalamus (LHA) is sufficient to increase NAc DA levels [41] and involves indirect leptin action via orexin neurons [43]. LHA LepRb neurons are distinct from orexin/hypocretin neurons, but they directly innervate orexin/hypocretin neurons [40,43]. Orexin modulates the rewarding value and consumption of sucrose: Central orexin injections promote sucrose intake [24], while genetic or pharmacological blockade of orexin signaling decreases the rewarding value and intake of sucrose [44]. Orexin’s effect on reward is mediated via the VTA [27,70], where orexin stimulates DA neurons [47]. Leptin generally inhibits orexin neurons [66], so that leptin inhibition of orexin neurons would be consistent with an inhibitory effect on midbrain DA neurons.

We recently reported that a population of LepRb neurons in the LHA co-expresses the inhibitory acting neuropeptide galanin (GAL-LepRb KO mice).
neurons) and that LHA galanin mRNA (Gal) expression is stimulated by leptin [40]. Central galanin peptide (GAL) injections selectively increase fat intake [33,38], and deficiency in Gal or galanin-receptor-1 (GalR1) results in decreased dietary fat intake [1,34,71]. GAL also modulates reward circuits by inhibition of mesolimbic DA neurotransmission [60], inhibition of noradrenergic LC neurons [54] and counteracts opiate withdrawal behavior [29,52].

We thus hypothesized that GAL-LepRb neurons play a role in nutrient reward and selection, which could lead to changes in body weight. To test this, we studied mice with conditional deletion of LepRb from GAL neurons (GAL-LepRbKO mice). Our data strongly suggest that GAL via GalR1 mediates inhibitory leptin action onto orexin neurons, which could mediate the differential reward modulation of sweet and fatty stimuli.

2. RESULTS

2.1. Generation and validation of GAL-LepRbKO mice

We studied the physiological importance of leptin action in GAL neurons by generating mice with conditional LepRb deletion from GAL neurons (GAL-LepRbKO mice). Our data strongly suggest that GAL via GalR1 mediates inhibitory leptin action onto orexin neurons, which could mediate the differential reward modulation of sweet and fatty stimuli.

Figure 1: Increased weight gain and late-onset obesity in KO mice. A. Schematic drawing for the generation of GalCre and KO mice. B–D. Verification of functional LepRb deletion with leptin induced pSTAT3 by immunohistochemistry in the LHA of WT (left) and KO mice (right) and cell counts for pSTAT3 positive nuclei in the LHA (C.) and NTS (D.) (n = 4–8, *pLHA < 0.01, *pNTS < 0.05). E. Body weight in WT and KO mice (n = 8–11, *p-test < 0.05). F. Body weight gain over 8 weeks. G. Increased body weight in aged KO and WT mice (n = 8–11). H. Cumulative food intake in WT and KO mice (n = 8–11). I. Locomotor activity in WT and KO mice (n = 8–11). Gal = galanin, KO = knock out, LepRb = long form leptin receptor, LHA = lateral hypothalamic area, WT = wildtype, pSTAT3 = phosphorylated signal transducer and activator of transcription-3, NTS = nucleus of the solitary tract, fx = fornix.
2.2. Increased body weight gain in GAL-LepRbKO mice

The metabolic phenotype of KO mice was assessed from 4 to 12 weeks of age (n = 8–11). At 4 weeks of age, KO mice showed a significantly lower body weight compared to WT mice (Figure 1E, p<0.05), but at 12 weeks of age body weights were undistinguishable between groups (Figure 1E). However, body weight gain over 8 weeks was significantly increased in KO mice compared to WT mice (Figure 1F, p<0.02). Furthermore, we found that body weight of KO mice was significantly increased compared to WT mice in a separate cohort of older (7–9 month old) mice (Figure 1G, n = 7, p<0.05), consistent with an increase in body weight gain. Food intake (Figure 1H), energy expenditure (Figure 1I) or locomotor activity (Figure 1J), measured between 4 and 12 weeks of age, showed no significant difference between groups. At 12 weeks of age we also evaluated fed and fasted blood glucose levels, rectal body temperature and body composition in WT and KO mice with no significant differences between genotypes (Figure S5).

2.3. Decreased LHA galanin signaling is associated with decreased fat intake

Earlier data indicated that leptin induced LHA Gal gene expression [40]. Consistent with this, KO mice showed a 60% decrease in LHA Gal gene expression (Figure 2A, p<0.01). Furthermore, GalR1 expression, was significantly decreased by 30% (Figure 2B, p<0.05), while GalR2 and GalR3 expression remained unchanged within the LHA (Figure 2B). GAL mainly acts as an inhibitory neuropeptide via GalR1 and selectively modulates the ingestion of fat [59], suggesting that decreased Gal and GalR1 mRNA can affect fat intake. To test this, naïve KO and WT mice had access to a 10% Intralipid solution (1 kcal/ml) for 1 h per day over 10 days. Intralipid solution is highly palatable for mice and the amount ingested correlates with the rewarding value of the solution [18,53]. As expected, both WT and KO mice increased their Intralipid consumption over 10 consecutive 1 h sessions. However, over 10 days KO mice consumed significantly less Intralipid solution compared to WT mice.

![Figure 2](image_url)
In a separate cohort of LepRbCre (n = 7) or GalR1Cre (n = 4) mice, we injected another adenoviral construct, Ad-in/WED [43], that resulted in c-fos-dependent expression of wheat germ agglutinin (WGA) and the fluorescent protein DsRed. WGA can be transported anterogradely and transsynaptically into 2nd order neurons, while DsRed remains in 1st order neurons (here LepRb or GAL neurons) (Figure 4G–L).

LHA Ad-in/WED injections in LepRbCre or GalR1Cre mice, resulted in WGA labeling within the LHA, where 1st order neurons are identified by WGA/DsRed co-expression (= yellow neurons) and 2nd order neurons by single WGA labeling (= green neurons) (Figure 4G, J). Several 2nd order neurons were found in the LHA indicating that GAL-LepRb neurons project locally onto LHA neurons. We further observed many 2nd order neurons in the LC labeled by GAL-LepRb neurons (Figure 4L), while 2nd order labeling from LHA LepRb neurons (Figure 4I) was less strong and not observed in all cases of injected animals. LC neurons do not express LepRb as shown in a LepRbCre reporter mouse with prominent GFP labeling in the hypothalamus, but complete absence of GFP labeling within the LC of the same animal (Figure S6A, B, respectively). We further confirmed the projections of GAL-LepRb neurons to the LC with injections of the retrograde tracer fluorogold (FG) into the LC of GalR1Cre mice. GAL is densely co-expressed with noradrenergic LC neurons as indicated by co-labeling of GalR1Cre with tyrosine hydroxylase (TH) (Figure S6C), so that the GalR1Cre signal (green label) served as an excellent visual guide for LC neurons to verify the accuracy of FG injections (red label) (Figure S6A). We found many triple labeled FG/pSTAT3/GalR1Cre neurons in the LHA surrounding the fornix (Figure S6B), which showed that many GAL-LepRb neurons innervate the LC. Furthermore, GAL neurons derived WGA labeled LC neurons indeed represent noradrenergic TH-positive neurons (Figure 5A).

LepRb neurons in the LHA innervate local orexin/hypocretin neurons [43]. Similarly, we found that WGA labeled 2nd order neurons from LHA GAL and LepRb neurons co-labeled with orexin/hypocretin (Figure 5B, C, respectively). Importantly, neither LepRb nor GAL neurons co-localize with orexin/hypocretin [40], thus further supporting that GAL-LepRb neurons innervate orexin/hypocretin neurons.

2.6. Galanin mediated inhibition of orexin neurons

LHA Gal and GalR1 expression was decreased in KO mice (Figure 2A, B) and GAL acts via GalR1 to potently inhibit neuronal excitation in many neuronal systems, including the hypothalamus [4,19,20]. We hypothesized that GAL may mediate the inhibitory actions of leptin on orexin neurons. Therefore, we predicted an increased activation of orexin neurons in KO mice. We analyzed KO and WT brains for basal levels of cFos (as a surrogate for neuronal activation) in orexin neurons and indeed found increased cFos/ox co-localization, which was most prominent in the rostral portion of orexin/hypocretin neurons (Figure 6A–E, n = 5, pANNOVA < 0.003, pHolm-Sidak < 0.05). This was due to an increased number of cFos/ox neurons (Figure 6D), while the total number of orexin neurons was unchanged between groups (Figure 6E). Enhanced cFos expression was restricted to the LHA, while other adjacent sites, e.g. the DMH, showed similar cFos expression (Figure S8). Further, orexin gene expression was similar in KO and WT mice (Figure 6F). However, the gene expression of LHA orexin receptor OX2, but not OX1, was significantly decreased (Figure 6F, pHolm-Sidak < 0.02), indicating that within the LHA OX1 signaling would be preferentially enhanced. We further determined that orexin neurons were co-labeled with GalR1 (Figure S6G), demonstrating that orexin neurons have the molecular capability to respond to GAL (Figure S6H).
3. DISCUSSION

Our data demonstrate that leptin action via GAL neurons regulates nutrient reward. Dysregulation of this system by deleting LepRb from GAL neurons caused increased reward value and consumption for sucrose, while fat consumption was decreased.

Our data uncover a novel neuronal circuit where LHA GAL-LepRb neurons directly innervate orexin neurons as well as noradrenergic locus coeruleus neurons. In contrast to LHA LepRb neurons, LHA galanin neurons and therefore GAL-LepRb neurons do not innervate the VTA directly.

Leptin stimulates LHA Gal gene expression, while lack of LepRb in GAL neurons decreased LHA Gal gene expression. GAL is an inhibitory neuropeptide and we further speculate that leptin inhibits orexin neurons via GAL→GalR1 signaling to modulate nutrient reward and body weight.

3.1. Leptin and regulation of VTA DA neurons

Central leptin controls nutrient reward via interactions with VTA dopaminergic neurons [18,22,23], but the exact circuits involved are unclear. There is an ongoing controversy regarding the stimulatory or inhibitory effect of leptin on DA neurons. Some VTA dopamine neurons express LepRb and leptin inhibits these DA-LepRb neurons [30], suggesting that leptin decreases food reward to mediate its anorexigenic effects.

In contrast, the lack of leptin in ob/ob mice results in decreased DA content within the NAc. Peripheral, central and intra-LHA leptin
Injections increase NAc DA content and increases tyrosine-hydroxylase expression in DA neurons, indicating a stimulatory leptin effect on DA neurons [23,41]. This is consistent with the DA deficiency theory, which argues that a low DA content would enhance behaviors like feeding, aimed at increasing NAc DA content and avoiding potentially unpleasant effects of low NAc DA levels [8]. Thus, inhibitory and stimulatory leptin effects on DA neurons are plausible and may both contribute to the modulation of food reward.

The presented data demonstrate that sucrose preference further translates into enhanced work for a sweet treat in KO mice. KO mice demonstrate increased reward value for sucrose and sugar, indicated by increased sucrose consumption and incentive runway performance. Orexin action is well known to modulate the rewarding value of food, by inducing NAc DA release via activation of VTA DA neurons [61,62]. This enhances the motivation to work for sucrose reward, which is blocked by orexin receptor inhibitors [5,12,27,44]. Thus, the increased sucrose reward value in KO mice can be explained by the observed increase in baseline activation of orexin neurons.

Leptin inhibits orexin neurons, possibly via inhibitory GABAergic LHA LepRb neurons [43]. In addition, our data strongly support a role of GAL to mediate inhibitory leptin effects onto orexin neurons: 1) LHA GAL and LepRb neurons innervate local orexin neurons 2) Leptin regulates LHA Gal mRNA [40] and LepRb expression on GAL neurons being necessary to

**Figure 4:** GAL-LepRb neurons project to the LC, but not the VTA. A. Overview of LHA Ad-iZ/EGFPf injections into the LHA of LepRbCre mice, the spread of infected neurons is shown in red (β-gal expression), virally infected neurons with cre-recombination are shown in green (representing LepRb neurons). B/C. EGFPf projections from LHA LepRb-cre neuron to the VTA (B.) or LC (C.). D. Overview of LHA Ad-iZ/EGFPf injections into the LHA of GalCre mice, the spread of infected neurons is shown in red (β-gal expression), virally infected neurons with cre-recombination are shown in green (representing GAL neurons). E/F. LHA GAL neurons do not project into the VTA (E.), but strongly innervate the LC (F.). G. Injection of Ad-ΔN/WED into the LHA of LepRbCre mice (n = 7) with infected first order neurons in yellow, co-localization of DsRed (red) and wheat germ agglutinin (WGA, green) and second order neurons in green (WGA). H/I. Second order neurons with single WGA labeling (green) in the VTA (H.) and LC (I.). J–L. Injection of Ad-ΔN/WED into the LHA of GalCre mice (n = 6) shows local first order neurons (yellow) and second order neurons (green) in the LHA (J.) and second order neurons in the LC (K.), but not the VTA (L.). Areas of interest are highlighted with white boxes. Gal = galanin; LepRb = long form leptin receptor; LC = locus coeruleus; VTA = ventral tegmental area; LHA = lateral hypothalamic area; Ad = adenovirus; EGFPf = farnesylated enhanced green fluorescent protein; β-gal = β-galactosidase.
maintain normal LHA Gal mRNA expression. 3) GalR1 mRNA, the predominant receptor responsible for inhibitory GAL actions [4,20], is decreased in the LHA of KO mice. 4) GalR1 is co-expressed with orexin neurons. 5) KO mice show an increased percentage of activated orexin neurons. Indeed, a recent study confirmed in slice preparations that GAL inhibits orexin neurons [26]. Thus, our study is the first to highlight a direct interaction of LHA galanin neurons with orexin neurons and we speculate that the inhibitory acting neuropeptide GAL could modulate food reward by inhibiting orexin neurons.

LHA LepRb neurons also express the inhibitory neurotransmitter GABA and innervate the VTA and local orexin neurons [41,43]. Our study confirms LHA LepRb → VTA projections, but LHA GAL neurons do not innervate the VTA. Therefore, we conclude that LHA GAL-LepRb neurons are distinct from LHA LepRb → VTA projecting neurons.

A subset of LHA LepRb neurons and LHA GAL neurons co-express the neuropeptide neurotensin (Nts) [40]. LHA neurotensin neurons, but not LHA GAL neurons, project directly to the VTA, thus we conclude that LHA Nts-LepRb → VTA projecting neurons are distinct from GAL-LepRb neurons.

LHA Nts-LepRb neurons [42] and GAL-LepRb neurons both innervate orexin neurons, further suggesting that LHA LepRb → orexin projecting neurons may indeed co-express GAL and neurotensin. However, orexin neurons do not express neurotensin receptors [49], while we found GalR1 co-expressed with orexin neurons and GalR1 mRNA down-regulation in KO mice. Thus, we further suggest that GAL, not neurotensin, regulates orexin neurons and that this involves inhibitory GAL actions via GalR1.

We found a strong projection of GAL-LepRb neurons to the LC. LHA Nts neurons do not project to the LC [42]; and personal communication with Dr. MG Myers); thus, we further conclude that GAL-LepRb → LC projecting neurons are distinct from LHA Nts neurons. We have further clarified this classification of LHA LepRb populations in Figure 6H.

3.2. Fat intake and galanin

Selective deletion of LepRb in GAL neurons caused a decrease in fat intake, which cannot be explained by anorexigenic leptin action. The concept that GAL controls macronutrient selection was first introduced by Leibowitz and colleagues, who found that central GAL injections selectively increased fat intake, while carbohydrate and protein intake remained unchanged [59]. The reported effects of GAL on macronutrient selection were small and varied dependent on the initial nutrient preferences [56,57]. More recently, GAL’s effect on fat intake was further supported in GAL deficient mice, which ate less of a high fat diet compared to WT mice [1,34]. Similarly, GalR1 null mice consume less high fat diet [71], therefore low Gal and GalR1 mRNA expression in the LHA of GAL-LepRb KO mice could sufficiently explain the observed decrease in fat consumption. Furthermore, in a macronutrient choice paradigm Gal-KO mice preferred carbohydrates over fat [1]. This effect is very similar to the observed sucrose preference over Intralipid in GAL-LepRb KO mice and strongly suggests that GAL indeed mediates the differential effects on sucrose and fat intake in GAL-LepRb KO mice. Similar to sucrose, fat rich solutions like Intralipid are highly rewarding for rodents and similarly involve reward circuits that are associated with DA release [36,58]. Thus, a differential regulation of sucrose and fat reward value cannot be explained exclusively by a regulation of DA neurons and suggests that other reward circuits are modulated in KO mice.

Nutrient reward is also modulated by the opioid system, a pathway that interacts with the dopaminergic system as well as other central sites. Importantly, activation or inhibition of μ-opioid receptors (MOR) in the NAc of rats robustly modulates fat intake, while chow intake remains unchanged and sucrose intake is only minimally affected [64,65,67,68]. Indeed, several studies found that fat intake stimulates the endogenous opioid enkephalin and Gal expression simultaneously [6], and GAL induced fat intake is prevented by selective blockade of

Figure 5: GAL-LepRb neurons innervate orexin neurons and noradrenergic LC neurons. A/B. GalCre mice with LHA Ad-iN/WED injections show that within the LC WGA neurons co-express tyrosine hydroxylase (TH), depicting noradrenergic neurons (A) and within the LHA many WGA neurons are co-expressed with orexin (B). C. LepRbCre mice with LHA Ad-iN/WED injections show that many LHA WGA neurons are co-expressed with orexin. Bar size is 1 mm (Figure 5B and C) and 500 μm (Figure 5A). Gal = galanin; LepRb = long form leptin receptor; LC = locus coeruleus; LHA = lateral hypothalamic area; Ad = adenovirus; WGA = wheat germ agglutinin; ORX = orexin.
Thus, it may be possible that sucrose intake can be regulated by the enhanced orexin release in the VTA, while fat intake remains suppressed due to the blunted GAL action on MOR. Further studies will need to address these distinct aspects of nutrient reward.

3.3. Leptin and galanin interaction with the locus coeruleus

GAL-LepRb neurons strongly innervate noradrenergic neurons in the LC. The LC is the major projection sites of orexin neurons and orexin and LC neurons both control arousal and reward [9–11]. The LC is the sole NE source for many central sites (e.g. prefrontal cortex, PFC), while other sites (e.g. hypothalamus) receive additional NE input from the brainstem (NTS). Noradrenergic LC neurons do not express LepRb, and whether leptin regulates LC function has not been investigated to our knowledge. However, leptin injections decrease and leptin deficiency increases hypothalamic NE levels (even though the source of NE was unclear in these studies), while central blockade of NE signaling

Figure 6: Increased activation of orexin neurons in KO mice. A–B. Immunohistochemical staining for cFos (red) and orexin (green). The fornix (fx) is shown as a landmark for the location within the LHA. C. Percentage of orexin neurons that co-express cFos, in comparison to the total number of cFos/orexin neurons (D.) and total number of orexin neurons (E.). F. Quantification of LHA orexin mRNA and its receptors OX1 and OX2 (n = 10–13; *p<0.02). G. Immunohistochemical staining for orexin (red) and GalR1 (green). H. Schematic summary of distinct LHA LepRb neurons and their projections. KO = knock out; GalR1 = galanin receptor 1; LHA = lateral hypothalamic area; OX1 and OX2 = orexin receptor 1 and 2.
substantially decreases hyperphagia in leptin deficient mice [13–15,37].

In line with this, chemical deletion of noradrenergic LC fibers, but not noradrenergic NTS fibers, results in decreased food and sucrose consumption [2] as well as impaired incentive runway behavior [3], further suggesting that LC neurons could at least contribute to the observed sucrose preference and rewarding value of sweet treats in KO mice. The functional connectivity of orexin and LC neurons has been convincingly shown for arousal behavior and may be reflected in the improved runway behavior, where KO mice are less distracted during trials. The LC is clearly an important mediator of opioid signaling and best known for its role in physical dependence and opiate withdrawal behavior (recently reviewed in [45]). Importantly, orexin and GAL have reciprocal effects on opiate withdrawal, which further supports an inhibitory role of GAL for orexin neurons. GAL decreases opiate withdrawal behavior via GaR1 [28,29], while orexin increases opiate withdrawal behavior [25]. Thus, future experiments will have to further depict if the LC indeed plays a role in GAL-LepRb mediated nutrient reward.

In summary, our data highlight an important role of GAL-LepRb neurons in nutrient reward. We show evidence that galanin mediates the inhibitory actions of leptin on orexin neurons via GaR1 and suggest that this contributes to the differential regulation in the rewarding value of sucrose and Intralipid.

4. MATERIAL AND METHODS

4.1. Animals

Mice were bred and housed at 22 °C on a 12-hour light/dark cycle. Food and water were available ad libitum unless otherwise specified. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center. Hemizygous BAC transgenic GalGF mice with green fluorescent protein (GFP) expression under the control of the Gal promoter (GalGF, Stock Tg(Gal-EGFP)109Gsat, [0163420UCD]) were obtained from the Mutant Mouse Regional Resource Center (MMRRC, http://www.mmrrc.org), a NCRR-NIH funded strain repository, and was donated to the MMRRC by the NINDS funded GENSAT BAC transgenic project (http://www.gensat.org).

4.2. Generation of GalCre mice

An IRES-cre sequence was inserted into the murine Gal gene between the Stop codon and polyadenylation site; an floxed neo cassette was inserted downstream of the polyadenylation sites. The construct was electroporated into mouse ES cells (albino C57/B6 background), screened for correct insertion and injection into blastocysts to generate chimeras. Chimeras were bred to C57/B6 and germ line transmission was determined by coat color. Correct genotypes and 5'–recombination was confirmed by PCR with transgene spanning amplicons (fwd: 5’-TTG AAA CCT GCC CTG ACT CTC AGC A, reverse: 5’-AGG GAA ACC GTT GTG GTC TGA CTA) and 3'-recombination (fwd: 5’-CCA TCA GAA GCT GAC TCT AGC TCA, rev: 5’-CTT GCT AGC TCT TCC CCA ACT CTA).

4.3. Experimental mice

GalGF mice were crossed with LepRbfl/fl mice [46] and kindly provided by Dr. Streamson Chua to generate LepRbfl/fl mice (referred to as WT mice) and GalGF/+. LepRbfl/fl mice (referred to as Gal-LepRbKO or KO mice). GalGF reporter mice were generated by breeding with B6.129X1-Chr1(Tg(Rosa26Sor-tdTomato-cre-loxP-flxP-cre)ZJ) (stock#006148) and compared to transgenic GalGF mice, which we used earlier to identify and characterize GAL-LepRb neurons in the LHA [40]. In some mice the flox-flxed neo cassette was removed from the genome by crossing GalGF mice with 129S4/SvJaeSor-Gt(Rosa26Sor-cre-loxP-flxP-cre)ZJ (stock#003948) (GalGFneoFF reporter mice) to prove that reporter expression pattern was not compromised by the presence of the neo cassette.

Genotyping of experimental animals was performed by PCR: GalCre (cre primers: 5’-CTC TTC CCA AGC AGC CCG AGA ACC (fwd), 5’-CGG CCT CCG TTT TTT GCC GGG CCC TTC GGC GGG (rev); wt-cre primers: 5’-TCC TGA GAC CAT GTC CAC TG (fwd), 5’-CTG CCA CTC CTG TGA TCT GA (rev); Leprko (mLepr106: 5’-GTC TGA TTT GAT AGA TGG TCT T (fwd), mLepr105: 5’-ACA GGC TTG AGA TCA TGA ACA (fwd), mLepr65A: 5’-AGA ATG AAA AAG GTG CCA TTG (rev)). We used the latter primers also to screen for potential germline Lepr excision as described elsewhere [39]; such animals were excluded from breeding and experiments.

4.4. Metabolic phenotyping

Male KO and WT littermates (n = 11–15) were individually housed at 4 weeks of age; food intake and body weight was measured weekly until twelve weeks. At 12 weeks of age body composition was determined by NMR (minispec-mq series Bruker Bilerica), fed and over-night fasted blood glucose was evaluated with a glucometer (One Touch Ultra Mini). After that mice were acclimated to the Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments) for 3 days and energy expenditure and locomotor activity was recorded continuously over 4 days. In another cohort of 7–9 month old, male KO and WT littermates (n = 7) body weight was again evaluated. Differences between groups were statistically analyzed with a repeated measure 2-way ANOVA or a Student’s t-test.

4.5. Microdissection and qPCR

Microdissection was performed as described earlier [21] and visual landmarks, such as the mammillothalamic tract, fornix and the third ventricle were used to strategically dissect the LHA. RNA was prepared following manufacturers recommendations (ToTALLY RNA kit, Ambion) and 350 ng RNA was converted to cDNA (RETrOscript Kit, Ambion). For qPCR 10.5 ng cDNA was used in triplicates to analyze multiple TaqMan assays (Applied Biosystems): Gapdh (housekeeping gene; Mm99999915_g1), Galanin (Mm01023508_g1), Galanin (Mm01236508_m1), GalR1 (Mm00433515_m1), GalR2 (Mm00726392_s1), and GalR3 (Mm00443817_m1), orexin1 (Mm04210469_m1), OX1 (Mm01185776_m1), OX2 (Mm01179312_m1). Reactions were performed in a robotic liquid handling system (Perkin Elmer MultiIRCLE II E) and run on the 7500 HT Fast-Real-Time PCR System (Applied Biosystems). Fold induction of gene expression was calculated using the ΔΔCT method as recommended for TaqMan assays.

4.6. Nutrient preference and reward

To test the consumption of palatable fat we provided a fat emulsion (10% Intralipid, Baxter) in a sipper bottle for 1 h per day over 10 days to naive WT and KO mice (n = 4–5) and compare their cumulative fat intake using a repeated measure 2-way ANOVA. Similarly, we tested their preferences for isocaloric solutions (1 kcal/ml) of sucrose (25% sucrose) and Intralipid (10%) in a two-bottle-choice test in naive WT and KO mice (n = 5–9), again with 1 h daily access over 10 days; bottle positions were alternated every other day to prevent side preferences. Statistical differences were analyzed via 3-way repeated-measure ANOVA and post-hoc analysis. WT and KO mice were further tested in an incentive runway [51]. Briefly, mice (n = 4) were food restricted (ad lib food restriction from

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12:00—4:00 p.m.) and allowed overnight access to the sugar treat (Froot Loops®, Kellogg’s) to avoid neophobic responses. Incentive runway training was performed every other day for a total of 13 training sessions. Each session consisted of 5 runway trials (between 8:00a.m. and 12:00 p.m.), sessions 1—3 were strictly acclimation trials with access to thegoal box + treat. Session 4 included a 15 cm runway distance from the start to the goal box. With each subsequent session the runway length was increased in 15 cm increments until reaching a maximal distance of 75 cm and mice could consume the treat for 30 s after fulfilling their task. Session were recorded and analyzed in slow motion to determine completion speed (time from leaving the start box to reaching the goal box [sec/runway length [cm]], running speed (=completion speed of direct runs in seconds/cm), number of direct and distracted runs (distractions = pauses, falters and reversals)). Differences between groups were analyzed by repeated measure 3-way ANOVA (completion speed over time) or Student’s t-test for two-group comparison.

4.7. Viral tracing studies
Stereotoxic surgeries were performed as reported earlier [40]. The LHA was targeted at coordinates: X = −0.9 mm, Y = −1.3 mm, Z = −5.2 mm relative to Bregma according to the Paxinos Mouse Brain Atlas [50]. Two adenoaviral constructs (Ad-iZ/EGFP and Ad-iIN/WED; described in detail earlier [41,43]) were acutely injected into the LHA of Gal[2e] or LepRb[2e] mice. Ad-iZ/EGFP results in cre-inducible expression of farnesylated EGFP and visualizes cell bodies and processes of cre-expressing neurons, the viral injection spread can be monitored by β-galactosidase expression in non-cre expressing neurons. Ad-iN/WED results in cre-inducible expression of DsRed and wheat germ agglutinin (WGA). WGA is anterogradely and transsynaptically transported into second order neurons, while DsRed remain in anatomically in a rostral to caudal manner and the total number of orexin neurons and the number of cFos expressing orexin neurons were counted (similar as described previously [40]). Differences between groups were evaluated with a 2-way ANOVA and posthoc-test.

4.10. Statistic
Statistical analysis was done with SPSS Statistics (repeated measure 3-way ANOVA) (IBM, Armonk, NY) or SigmaPlot 11.2 (all other statistics) (Systat Software Inc, San Jose, CA). The individual tests used are noted with the according experiments. Significant differences were accepted at a p-value < 0.05.

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CONFLICT OF INTEREST
All authors declare no conflict of interest.

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APPENDIX A. SUPPLEMENTARY DATA
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Original article

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