Original Article

MOLECULAR METABOLISM

Specific subpopulations of hypothalamic leptin receptor-expressing neurons mediate the effects of early developmental leptin receptor deletion on energy balance

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ABSTRACT

Objective: To date, early developmental ablation of leptin receptor (LepRb) expression from circumscribed populations of hypothalamic neurons (e.g., arcuate nucleus (ARC) *Pomc*- or *Agrp*-expressing cells) has only minimally affected energy balance. In contrast, removal of LepRb from at least two large populations (expressing *vGat* or *Nos1*) spanning multiple hypothalamic regions produced profound obesity and metabolic dysfunction. Thus, we tested the notion that the total number of leptin-responsive hypothalamic neurons (rather than specific subsets of cells with a particular molecular or anatomical signature) subjected to early LepRb deletion might determine energy balance.

Methods: We generated new mouse lines deleted for LepRb in ARC $Ghrh^{Cre}$ neurons or in $Htr2c^{Cre}$ neurons (representing roughly half of all hypothalamic LepRb neurons, distributed across many nuclei). We compared the phenotypes of these mice to previously-reported models lacking LepRb in *Pomc*, *Agrp*, *vGat* or *Nos1* cells.

Results: The early developmental deletion of LepRb from *vGat* or *Nos1* neurons produced dramatic obesity, but deletion of LepRb from *Pomc*, *Agrp*, *Ghrh*, or *Htr2c* neurons minimally altered energy balance.

Conclusions: Although early developmental deletion of LepRb from known populations of ARC neurons fails to substantially alter body weight, the minimal phenotype of mice lacking LepRb in *Htr2c* cells suggests that the phenotype that results from early developmental LepRb deficiency depends not simply upon the total number of leptin-responsive hypothalamic LepRb cells. Rather, specific populations of LepRb neurons must play particularly important roles in body energy homeostasis; these as yet unidentified LepRb cells likely reside in the DMH.

Keywords leptin receptor; arcuate nucleus; DMH; obesity; cre recombinase; ghrh; htr2c

1. INTRODUCTION

Obesity, which affects more than 1/3 of people in developed countries, predisposes to diabetes, cardiovascular disease, and other serious comorbidities [1]. To design effective treatments for obesity, we must first understand the systems that control energy balance and which represent potential therapeutic targets. The hormone leptin, which is produced by adipose tissue to signal the repletion of fat stores, acts via its receptor (LepRb) on hypothalamic neurons to suppress food intake and permit energy expenditure [2]. Leptin- or LepRb-deficient humans and rodent models display dramatic hyperphagia and reduced energy expenditure, leading to severe obesity [3–5]. Thus, the hypothalamic neurons by which leptin mediates the control of energy balance represent important controllers of energy balance.

1.1. Hypothalamic LepRb neurons

Within the hypothalamus, the arcuate nucleus (ARC), ventromedial hypothalamic nucleus (VMN), dorsomedial hypothalamus (DMH), lateral hypothalamic area (LHA), and ventral premammillary nucleus (PMv) contain substantial numbers of LepRb neurons [6]. While roles for many molecularly-defined and anatomically-circumscribed populations of LepRb have been examined, the early developmental deletion of LepRb from these previously-studies populations has not resulted in obesity similar to the severe obesity observed in entirely LepRb-deficient *db/db* mice [7–11]. Indeed, while orexigenic ARC neurons that contain agouti-

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related peptide (AgRP), neuropeptide Y (NPY) and gamma amino butyric acid (GABA) (AgRP neurons) and anorexigenic ARC proopiomelanocortin (POMC)-containing neurons each express LepRb and play crucial roles in energy balance, early developmental ablation of LepRb from AgRP and/or POMC neurons minimally alters energy balance [7,8].

Similarly, manipulation of LepRb expression in other circumscribed sets of LepRb neurons examined to date (e.g., neurotensin (*Nts*) neurons of the LHA, steroidogenic factor-1 (*Sf1*) neurons in the VMN) minimally impacts energy balance [10,11]. In contrast, LepRb deletion from some large, widely-distributed hypothalamic populations results in profound obesity and hyperphagia. Indeed, deletion of LepRb in *vGat* (*Slc32a1*)-expressing GABAergic neurons (representing ~50% of hypothalamic LepRb neurons, including the majority of LepRb neurons in the DMH and LHA, along with AgRP cells and other ARC neurons) or *Nos1* neurons (representing ~25% of hypothalamic LepRb neurons, including the majority of PMv neurons plus smaller numbers of LepRb cells in the ARC, DMH, VMN and LHA) promotes dramatic obesity [12,13].

1.2. Populations of LepRb neurons that are or are not subject to compensation following early developmental deletion

Early alterations in AgRP neurons (e.g., neuron ablation or LepRb deletion) are compensated during postnatal development, while alterations in mature AgRP neurons in adults have profound effects on leptin action and energy balance [7,14–16]. Indeed, while the deletion of LepRb neurons from adult AgRP neurons provokes dramatic obesity, the early developmental deletion of LepRb from AgRP neurons produces little metabolic derangement. Thus, while leptin action on AgRP neurons in adults plays important roles in energy balance, the lack of direct leptin action on AgRP neurons is unlikely to contribute substantially to the phenotype of entirely LepRb-null *db/db* mice. Hence, additional (non-AgRP) LepRb neurons that are not subject to developmental compensation must underlie the majority of the *db/db* phenotype and play important roles in leptin action. In this manuscript, we employ multiple models that mediate the early developmental deletion of LepRb in subpopulations of hypothalamic neurons to define sets of LepRb neurons that mediate leptin action and in which the loss of LepRb is not developmentally compensated (unlike AgRP neurons).

1.3. Potential types of LepRb neurons that control energy balance

This non-developmentally compensated control of energy balance might be distributed across multiple hypothalamic nuclei and cell types, with many types of LepRb neurons contributing similarly to the control of energy balance, such that the ablation of leptin action in a threshold number of LepRb neurons (without regard to their location or identity) disrupts the control of energy balance. It is also possible that small, but currently unidentified, populations of LepRb neurons mediate the main effect of leptin/LepRb on energy balance that is not subject to developmental compensation.

Here, we test these possibilities by studying mice deleted for LepRb in previously unexamined sets of growth hormone-releasing hormone-Cre (*Ghrh*^{Cre}) LepRb neurons of the ARC (LepRb^{Ghrh} cells) as well as serotonin receptor 2c (*Htr2c*)-expressing LepRb (LepRb^{Htr2c}) neurons that lie mainly in the PMv, VMH, and LHA (a few also lie in the ARC and DMH). We examined these mouse strains together with mice subjected to early developmental deletion of LepRb in *Agrp*, *Pomc*, *vGat*, and *Nos1* neurons. Our results and analysis suggest that small (and as yet unidentified) populations of DMH LepRb neurons that are not subject to developmental compensation likely play crucial roles in the control of energy balance by leptin.

2. RESULTS

2.1. The role for *Ghrh*^{Cre} neurons in leptin action

To identify novel subpopulations of hypothalamic LepRb neurons, we previously performed translational profiling of their transcriptome using translating ribosome affinity purification (TRAP) followed by RNA-seq (TRAP-seq) [17]. This study revealed the enrichment of *Ghrh* mRNA in hypothalamic LepRb neurons [17]. Leptin modulates food intake, glucose homeostasis, and linear growth [2], and *Ghrh* neurons also likely participate in the control of these parameters [18–20]. We therefore postulated that direct leptin action on LepRb^{Ghrh} neurons might mediate these effects.

To test this notion, we generated a knock-in mouse line to cotranslationally express Cre recombinase with *Ghrh* mRNA (*Ghrh*^{Cre} mice) (Figure 1A). Breeding *Ghrh*^{Cre} onto the Cre-dependent *Rosa26*$^{eGFP\text{-}L10a}$ reporter background (Ghrh$^{eGFP-L10a}$ mice) revealed the presence of Cre-expressing neurons in the expected areas of the hypothalamus, including the ARC (Figure 1B–D) [21], as well as in a few regions in the midbrain and hindbrain (Fig. S1). While the hypothalamic distribution of eGFP-L10a neurons mirrored the known adult expression pattern of *Ghrh* [21], the adult midbrain and hindbrain express little detectable *Ghrh*, suggesting either early developmental *Ghrh* expression in these regions or low-level expression in adults.

Single-cell sequencing of ARC neurons identified *Ghrh* neurons as distinct from AgRP and POMC neurons [22]. Similarly, *Ghrh*^{Cre} neurons are more lateral to the third ventricle than AgRP neurons [23] and do not colocalize with the laterally localized POMC neurons (Figs. S1E and F). Thus, our manipulation of *Ghrh*^{Cre} neurons will not directly impact AgRP or POMC neurons.

To identify LepRb^{Ghrh} neurons, we examined the detection of leptin-stimulated phosphorylated STAT3 (pSTAT3)-immunoreactivity (IR) and eGFP in Ghrh$^{eGFP-L10a}$ mice by immunohistochemistry (IHC) (Figure 1C–D). The IHC detection of pSTAT3-IR reveals cell-autonomous leptin action on LepRb-expressing neurons [24]. As previously reported, no pSTAT3-immunoreactivity is detected in the brain in the absence of leptin action in *ob/ob* or *db/db* mice, and exogenous leptin promotes pSTAT3 in many hypothalamic cells in a distribution consistent with LepRb neurons (Fig. S2); previous results have demonstrated the colocalization of leptin-stimulated pSTAT3 with LepRb neurons [17]. Our analysis in Ghrh$^{eGFP-L10a}$ mice revealed extensive colocalization of pSTAT3-IR and eGFP in the ARC (~45% of ARC eGFP neurons were pSTAT3 positive). LepRb^{Ghrh} neurons represent a minority of total pSTAT3 neurons in the ARC, however. We also observed sparse colocalization in the DMH, but none in other areas, including the midbrain and hindbrain (data not shown).

To determine the importance of LepRb^{Ghrh} neurons to leptin action, we crossed *Ghrh*^{Cre} onto the *Lepr*^{flox} background to generate *Ghrh*$^{Cre/+}$;*Lepr*$^{fl/fl}$ (LepRb^{Ghrh}KO) and littermate control (*Lepr*$^{fl/fl}$) mice for study. Leptin treatment failed to promote the accumulation of pSTAT3 in eGFP-containing neurons in LepRb^{Ghrh}KO mice on the *Rosa26*$^{eGFP\text{-}L10a}$ background (Figure 1E,F), consistent with the ablation of LepRb from *Ghrh*^{Cre} neurons in these animals.

We examined the body weight and composition of LepRb^{Ghrh}KO mice, as well as of mice with LepRb deleted in ARC POMC or AgRP neurons (LepRb^{Pomc}KO and LepRb^{Agrp}KO mice, respectively) (Figure 2). This analysis recapitulated the small (2–3 g) increase in body weight previously observed [7,8] in both male and female LepRb^{Pomc}KO and LepRb^{Agrp}KO mice (Figure 2C,E). The increase in body weight in these animals reflected a tendency toward increased adiposity, except in the case of male LepRb^{Pomc}KO mice, in which the increase in body weight reflected increased lean mass (Figure 2D,F). In contrast to the

