Original article

MOLECULAR METABOLISM

Deletion of leptin signaling in vagal afferent neurons results in hyperphagia and obesity

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ABSTRACT

The vagal afferent pathway senses hormones released from the gut in response to nutritional cues and relays these signals to the brain. We tested the hypothesis that leptin resistance in vagal afferent neurons (VAN) is responsible for the onset of hyperphagia by developing a novel conditional knockout mouse to delete leptin receptor selectively in sensory neurons (*Nav1.8/LepR*$^{fl/fl}$ mice). Chow fed *Nav1.8/LepR*$^{fl/fl}$ mice weighed significantly more and had increased adiposity compared with wildtype mice. Cumulative food intake, meal size, and meal duration in the dark phase were increased in *Nav1.8/LepR*$^{fl/fl}$ mice; energy expenditure was unaltered. Reduced satiation in *Nav1.8/LepR*$^{fl/fl}$ mice is in part due to reduced sensitivity of VAN to CCK and the subsequent loss of VAN plasticity. Crucially *Nav1.8/LepR*$^{l/fl}$ mice did not gain further weight in response to a high fat diet. We conclude that disruption of leptin signaling in VAN is sufficient and necessary to promote hyperphagia and obesity.

Keywords Leptin; Vagus nerve; Obesity; Hyperphagy; High fat diet; Meal pattern

1. INTRODUCTION

Obesity has become recognized as a worldwide health threat and a major public health challenge. There is currently a lack of simple and effective therapies or preventative treatments against obesity, and the mechanisms involved in the onset of diet-induced obesity remain unknown. There is growing evidence that cellular leptin resistance in the hypothalamus is important in maintenance of obesity but is unlikely to have a causative role in the onset of obesity [1]. There is growing evidence that altering the strength or sensitivity to the hedonic attractiveness of food [2], availability of food [3], learned preferences [4], or signaling from the gut [5]may be involved in initiating diet-induced obesity.

Since its identification in 1994, leptin has attracted much attention as a key central and peripheral signal involved in energy homeostasis [6–8]. Global deficiency in leptin or leptin receptor (LepR) results in an increase in appetite, hyperphagia, and morbid obesity in both humans and rodents [9–11]. Few cases of obesity have been attributed to leptin deficiency [12,13]; rather hyperphagia and obesity are associated with cellular resistance to leptin and the consequent lack of anorexigenic action of leptin [14]. Considerable attention has focused on leptin resistance in arcuate neurons of the hypothalamus as a key event in development of hyperphagia and obesity [15]. However, in rodent models of diet-induced obesity, leptin resistance in arcuate neurons does not develop until after food intake, body weight and adiposity increase, calling into question whether leptin resistance in hypothalamic neurons drives the initial hyperphagia and obesity [1]. Other populations of neurons important in regulation of food intake express the leptin receptor, including vagal afferent neurons (VAN) [16,17] and neurons in the nucleus of the solitary tract [18], the site of central termination of VAN. We have shown that within 6 weeks of feeding a high-fat diet in rats, VAN become leptin-resistant; this leptin resistance coincides with the development of hyperphagia without any measurable change in leptin signaling in the hypothalamus [19].

Leptin is a gut and adipose tissue-derived hormone that regulates a range of biological functions and processes, including energy intake and expenditure, body fat, neuroendocrine systems, autonomic function, and insulin and glucose balance [20]. Multiple splice variants of the LepR (LepRa-f) have been identified with identical extracellular, transmembrane, and proximal intracellular domains [11,21]. Only LepRb, the long isoform containing a 300 amino acid intracellular tail can mediate the physiological effects of leptin [22]. Binding of leptin to LepRb results in the activation of Janus tyrosine kinase 2 and leads to the phosphorylation of signal transducer and activator of transcription 3 (STAT3) [22]. Mice with a neuron-specific disruption of neuronal STAT3 are hyperphagic, obese, diabetic, and infertile [23].

VAN express a plethora of receptors and carry the bulk of the information about the nutritional content of a meal from the gastrointestinal (GI) tract to the brain [24]. VAN have been implicated in short term control of meal size and duration [25,26], but whether inputs from the gut via VAN play a role in the long term regulation of food intake and body weight is not clear. In the current study, we test the hypothesis that leptin resistance in VAN is an initiating factor in the development of hyperphagia and obesity. Using a *Nav1.8*cre-LoxP system we

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developed a conditional knockout mouse that lacks leptin receptor only in primary afferent neurons.

2. RESULTS

2.1. *Nav1.8* cre selective deletion of LepR in VAN

The conditional leptin receptor allele has been used previously to generate liver- and brain-specific KO mice [27]. Lox P sites flank either side of the first coding exon of *LepR* (LepRlox), which includes the signal sequence; thus cre-mediated recombination deletes all splice variants. LepRlox mice were bred with mice expressing cre driven by the Nav1.8 promoter [28] to generate selective deletion of leptin receptor in primary afferent neurons (Figure 1A). Nav1.8/LepR$^{fl/wt}$ offspring were subsequently crossed with *LepR*$^{fl/fl}$ mice to generate *Nav1.8/LepR*$^{fl/fl}$ mice. Cre-negative, *LepR*$^{fl/fl}$, and *LepR*$^{fl/wt}$ littermates (WT) were used as controls in all studies.

Both WT and *Nav1.8/LepR*$^{fl/fl}$ mice were born at the expected Mendelian frequency, survived to adulthood, and were fertile. The average litter size was 6 for both genotypes and ranged from 2 to 13/litter in *Nav1.8/LepR*$^{fl/fl}$ mice and 1–13/litter in WT mice. Nav1.8 has previously been demonstrated to be exclusively expressed in sensory neurons, and was actively found to be absent from the cortex, cerebellum, and hippocampus in the brain [29]. Here we report that *LepR* expression was unchanged in both hypothalamus and whole brain extracts of WT and *Nav1.8/LepR*$^{fl/fl}$ mice by real-time quantitative PCR analysis (Figure 1B). We confirmed by immunohistochemistry that there was no ectopic cre recombinase in discreet neurons of the arcuate nucleus or nucleus of the solitary tract (Sup Figure 1). In addition we demonstrated that other organs that do not express Nav1.8, including liver, spleen, muscle, white adipose, heart, lung, and kidney had similar *LepR* expression in both *Nav1.8/LepR*$^{fl/fl}$ mice or WT mice (Sup Figure 1).

We did observe a significant decrease (93%) in *LepR* mRNA in neurons of the nodose ganglia in *Nav1.8/LepR*$^{fl/fl}$ mice compared with WT mice (Figure 1C). In contrast, there was no significant decrease in *LepR* expression in populations of other primary afferent neurons that express Nav1.8 including the trigeminal ganglia (TG), dorsal root ganglia (DRG), spinal cord, and superior cervical ganglia (SCG) in *Nav1.8/LepR*$^{fl/fl}$ mice compared with WT mice. We suggest that this was at least in part due to the overlap between *LepR* and *Nav1.8* expression within subsets of sensory neurons. Approximately 70% of VAN express leptin receptor (LepR) [16,17] and a similar percentage of these neurons express Nav1.8 [28]. The large reduction in LepR expression in nodose ganglia suggests that there is significant overlap between *LepR* and *Nav1.8* expression in these neurons. Around 70% of DRG neurons are positive for Nav1.8 [28], but only a small population of DRG neurons express *LepR* [30]. There was a small decrease in *LepR* expression in DRG of *Nav1.8/LepR*$^{fl/fl}$ mice that did not reach statistical significance, suggesting that spinal afferent neurons expressing *Nav1.8* are a different subpopulation to those expressing *LepR*. Although TG neurons express high levels of *LepR* protein [31], very few are *Nav1.8* positive [28]; we found no difference in *LepR* expression in the TG. There are currently no reports in the literature demonstrating that SCG neurons express *LepR*; although it has been proposed that cultured SCG neurons may be responsive to leptin [32], suggesting that at least a proportion of these neurons may express the *LepR* gene. Here we report that SCG neurons do express *LepR*, although in lower concentrations than in NG, DRG, and TG (Figure 1C); no change in *LepR* expression was found, presumably as a result of low *Nav1.8* expression in these neurons [28].

To demonstrate that the lack of *LepR* mRNA results in loss of LepR protein we stained nodose ganglia with a LepR antibody. WT mice express LepR on the plasma membrane, while *Nav1.8/LepR*$^{fl/fl}$ mice little to no LepR staining (Figure 1F). To confirm that the absence of LepR results in the absence of functional responsiveness to leptin in VAN, we measured the ability of an intraperitoneal administration of leptin to induce nuclear translocation of phosphorylated STAT3 (pSTAT3), a known mediator of leptin signaling downstream of LepRb [33,34] (Figure 1D, E). In WT mice, intraperitoneal leptin (80 μg/kg) increased nuclear pSTAT3 in VAN compared with saline (27.6 ± 3.3 vs. 8.4 ± 1.8%; $p < 0.001$). Leptin failed to increase nuclear pSTAT in VAN of *Nav1.8/LepR*$^{fl/fl}$ mice compared with saline (5.6 ± 0.8 vs. 4.0 ± 1.2%; $p > 0.05$). The 93% reduction in LepR expression results in an 80% reduction in pSTAT3 nuclear expression in VAN. These data confirm that the *LepR* deletion was specific to vagal afferent neurons and we conclude that any phenotypic alteration observed in *Nav1.8/LepR*$^{fl/fl}$ mice is due to the loss of LepR in VAN.

2.2. Deletion of LepR in VAN leads to obesity

To determine the importance of endogenous leptin signaling in VAN on the regulation of energy homeostasis, we monitored the body weight of WT and *Nav1.8/LepR*$^{fl/fl}$ mice. Deletion of *LepR* in VAN of chow-fed mice led to a small but significant increase in body weight at 10 weeks ($p < 0.05$) that increased further by 12 weeks ($p < 0.001$; Figure 2A). The increase in body weight is less pronounced than seen in whole body [35] or neuronal *Nav1.8/LepR*$^{fl/fl}$ mice [27]; however, it more closely resembles weight gain of WT mice fed a high fat diet post-weaning for 12 weeks [36]. Importantly this increase in body weight was a result of increased fat mass (Figure 2B and Figure 3A–D). In 12-week-old mice, the naso-anal length was not significantly different between the groups (9.8 ± 0.1 vs. 9.9 ± 0.3 cm; $p > 0.05$); however, adiposity increased 40% in *Nav1.8/LepR*$^{fl/fl}$ mice compared to WT mice (Figures 2B and 3B). The weight of subcutaneous, retroperitoneal, mesenteric, and epididymal fat pads were increased in *Nav1.8/LepR*$^{fl/fl}$ mice compared with WT mice (Figure 2D) as a result of increased adipocyte cell size (Figure 2F–I). When the fat mass was adjusted for body weight we determined that there was a redistribution of fat pad mass to mesenteric and retroperitoneal depots in the *Nav1.8/LepR*$^{fl/fl}$ mice compared with WT mice (Figure 2E). This is consistent with previous studies in which disrupting vagal afferent signaling altered visceral fat depots [37–39]. However, the mechanism remains unclear since there appears to be little parasympathetic supply to white adipose tissue [40]. Interestingly, despite the very significant increase in adiposity, circulating plasma leptin concentrations were indistinguishable between genotypes at 6 or at 12 weeks (Figure 2C). Dissociation between circulating leptin and adiposity has been reported in female Wistar rats fed a moderately high-fat diet and was suggested to contribute to weight gain [41]. It is possible that the lack of feedback from the adipose tissue in the *Nav1.8/LepR*$^{fl/fl}$ mice contributes to the weight gain although this needs further investigation.

2.3. Deletion of LepR in VAN increases food intake in the dark phase

To determine the mechanism by which LepR knockout in VAN increases body weight, WT and *Nav1.8/LepR*$^{fl/fl}$ mice (12 weeks old, $n = 8$) were randomly selected to be placed in metabolic cages to measure food intake, meal patterns, indirect calorimetry, and locomotor activity; based on their body weight these mice were representative of the whole population. Whole body composition analysis revealed that *Nav1.8/LepR*$^{fl/fl}$ mice weighed significantly more than WT mice as a result of increased fat mass, with no change in lean mass

