Original article

MOLECULAR METABOLISM

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

Guillaume de Lartigue, Charlotte C. Ronveaux, Helen E. Raybould*

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

Department of Anatomy, Physiology and Cell Biology, UC Davis School of Veterinary Medicine, 1 Shields Ave, Davis, CA 95616, USA

*Corresponding author. Vet Med: APC, Vet Med 3B, UC Davis, 1 Shields Ave, Davis, CA 95616, USA. Tel.: +1 530 754 6555; fax: +1530 754 7690. E-mail: heraybould@ucdavis.edu (H.E. Raybould).

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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

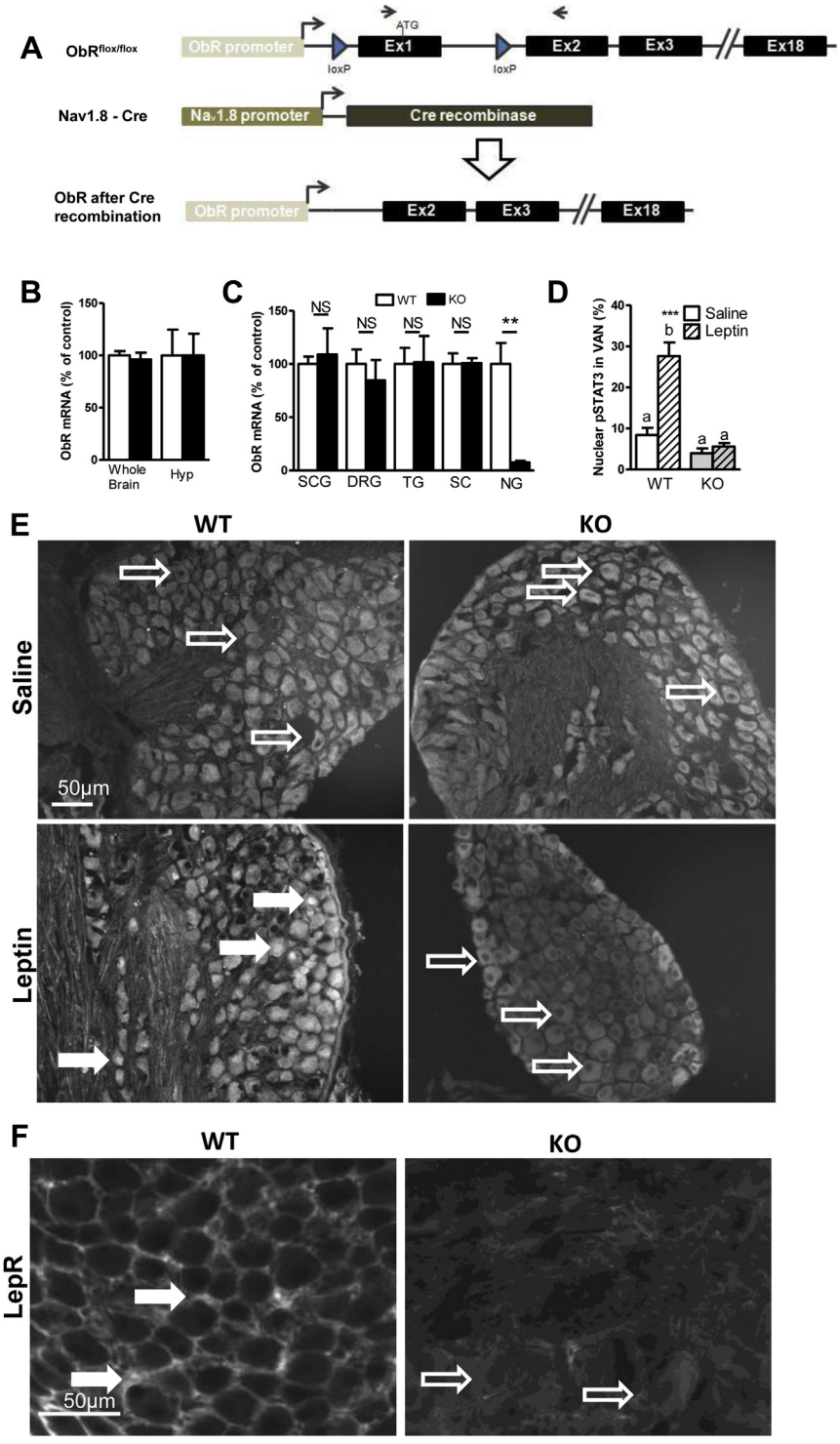

Figure 1: Generation and verification of conditional sensory-neuron-LepR KOby Cre-loxP system. (A) Gene targeting was used to insert loxP on either side of the *LepR* coding exon. Arrows denote target site of primers. (B) Percent change in *LepR* mRNA compared with WT in sensory neurons: superior cervical ganglia (SCG), dorsal root ganglia (DRG), trigeminal ganglia (TG), spinal cord (SC) and nodose ganglion (NG). (C) Ct values for sensory neurons. ($N = 6$; NG and SCG from 12 animals were used and ganglia from two animals were pooled to get sufficient cDNA) (D) *LepR* mRNA of non-sensory neurons: whole brain and hypothalamus tissue samples for *Nav1.8/LepR*$^{fl/fl}$ mice and WT controls. (E) Immunoreactivity phosphoSTAT-3 in 18 h food deprived *Nav1.8/LepR*$^{fl/fl}$ mice and WT mice administered saline or 120 µg/kg leptin. (F) Immunoreactivity of LepR in nodose ganglia of *Nav1.8/LepR*$^{fl/fl}$ mice and WT mice. Representative image from 6 animals.

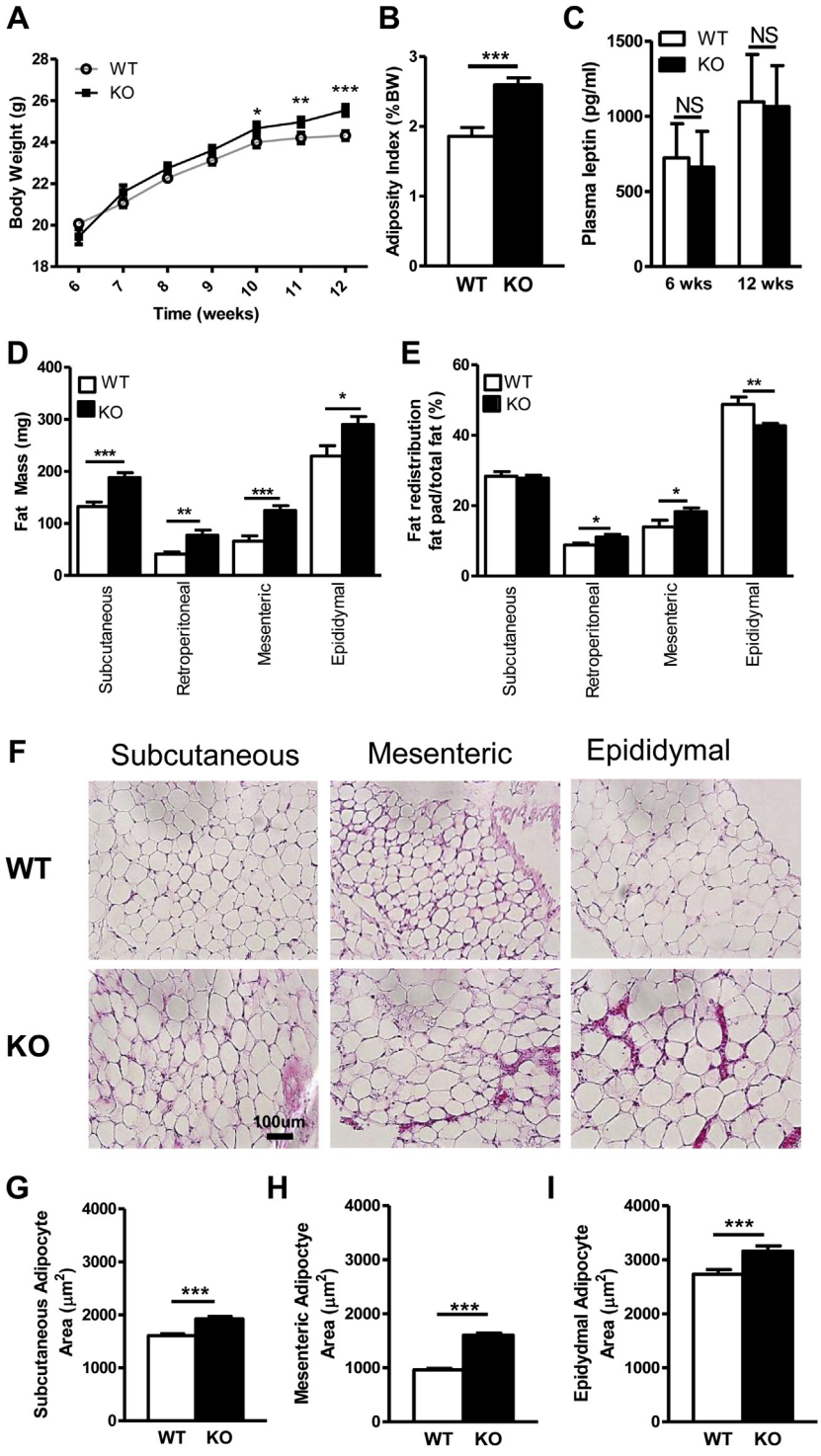

Figure 2: Analysis of energy homeostatic parameters. (A) Weekly body weight was significantly increased in chow-fed *Nav1.8/LepR*$^{fl/fl}$ mice compared with WT mice ($n = 52$). (B) Adiposity was significantly increased in *Nav1.8/LepR*$^{fl/fl}$ mice compared with WT mice. (C) No change in plasma leptin concentrations were found between *Nav1.8/LepR*$^{fl/fl}$ mice and WT mice at 6 or at 12 weeks of age. (D) Fat mass of subcutaneous, retroperitoneal, epidydmal and mesenteric fat were all significantly increased in *Nav1.8/LepR*$^{fl/fl}$ mice compared with WT mice at 12 weeks, but fat was different redistributed based on body weight (E). (F) H&E stained section of fat pads. Quantification of subcutaneous (G), mesenteric (H) and epididymal (I) fat showing increased adipocyte area ($N = 8$).

(Figure 3A–D). Energy expenditure and meal patterns were evaluated using a comprehensive lab animal monitoring system in which animals were fed powdered LabDiet 5058. There were no changes in energy expenditure (Figure 3E–G), activity, or dietary fuel oxidation between the groups (Figure 3H–I). There was a modest increase in energy expenditure, activity (data not shown) and respiratory quotient in all animals during the dark cycle, reflecting their nocturnal behavior.

Overall cumulative daily food intake trended to increase in *Nav1.8/LepR*$^{fl/fl}$ mice but this did not reach statistical significance ($p = 0.07$); however, *Nav1.8/LepR*$^{fl/fl}$ mice ate significantly more than WT during

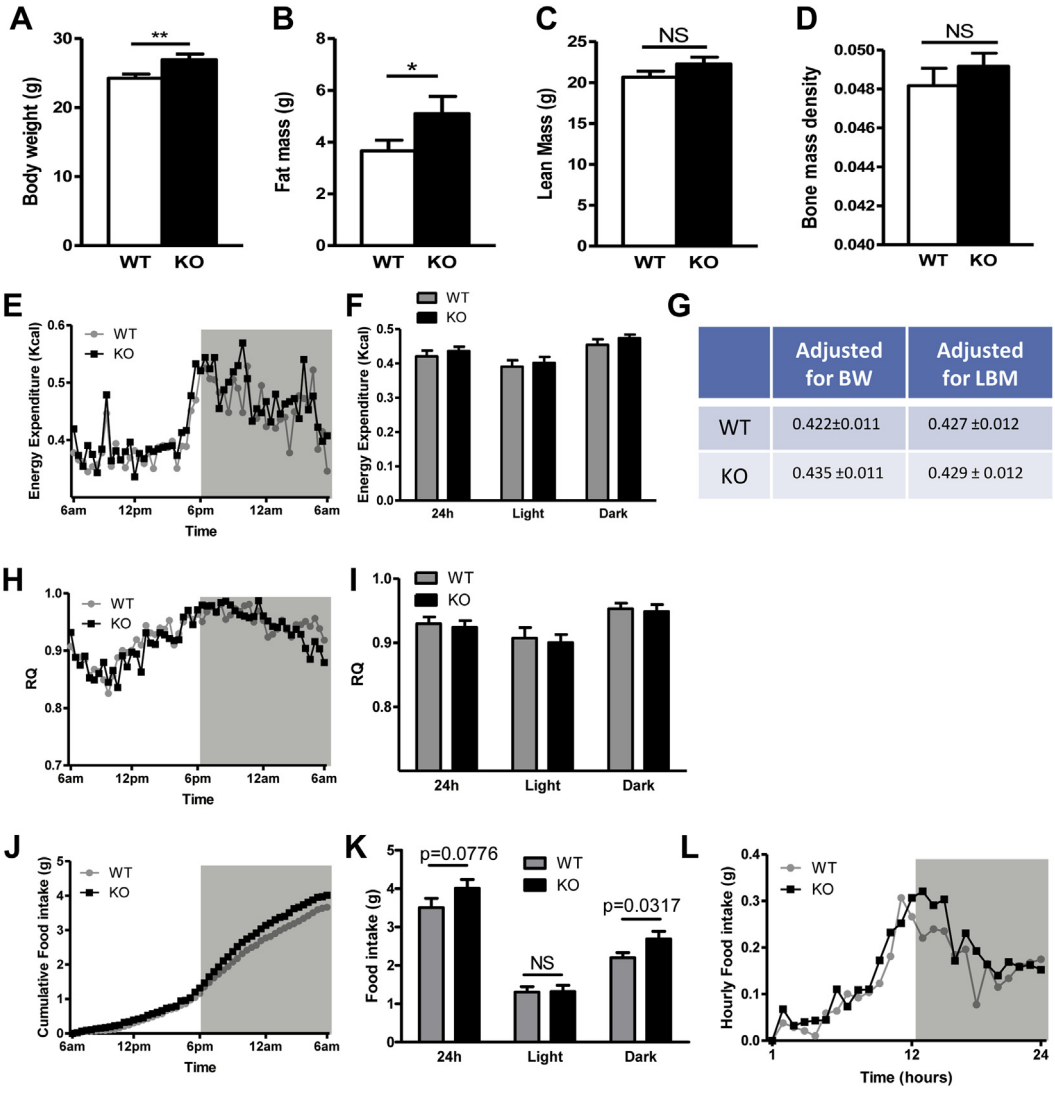

Figure 3: Analysis of metabolic parameters. Total body composition analysis was conducted by dual energy X-ray absorptiometry (DEXA) in *Nav1.8/LepR*$^{fl/fl}$ mice and WT controls used for metabolic studies ($N = 8$). (A) Body weight and (B) fat mass were increased in *Nav1.8/LepR*$^{fl/fl}$ mice compared with controls, with no change in (C) lean mass or (D) bone mass density. (E–L) *Nav1.8/LepR*$^{fl/fl}$ mice and WT mice were placed in metabolic cages ($N = 8$) and energy expenditure, respiratory quotient and food intake were recorded over 48 h. (E) Average hourly energy expenditure over 24 h, (F) average 24 h, light and dark energy expenditure, and (G) table of average energy expenditure adjusted for body weight and lean body mass was unchanged between *Nav1.8/LepR*$^{fl/fl}$ mice and WT mice. (H) Average hourly respiratory quotient (RQ) over 24 h and (I) total, light and dark RQ were unchanged between *Nav1.8/LepR*$^{fl/fl}$ mice and WT mice. (J) Cumulative food intake over 24 h was increased in *Nav1.8/LepR*$^{fl/fl}$ mice compared with WT mice. (K) Average food intake in *Nav1.8/LepR*$^{fl/fl}$ mice was significantly increased in the dark phase with no change in the light phase, resulting in a trend towards increased 24 h food intake compared with WT mice. (L) Average hourly food intake over 24 h demonstrating increased food intake in *Nav1.8/LepR*$^{fl/fl}$ mice in the first half of the dark phase.

the dark cycle (+22%; Figure 3J–L; $p < 0.05$). The increase in food intake occurred predominantly in a 3 h window at the onset of the dark cycle (Figure 3L). Meal patterns over 24 h and in the light phase were not significantly different between groups, but we found prolonged meal duration and increased meal size in the dark phase, especially in the first few hours of the dark phase. Meal pattern analysis revealed that *Nav1.8/LepR*$^{fl/fl}$ mice ate longer meals (+26%) compared to WT mice in the early and total dark cycle ($p < 0.05$; Figure 4C, D). The increased meal duration in the dark, but not the light phase, led to a trend towards increased 24 h meal duration ($p = 0.06$; Figure 4A,B). The quantity of food ingested in each meal during the total dark phase trended to increase (+16%) in *Nav1.8/LepR*$^{fl/fl}$ mice compared with WT ($p = 0.05$; Figure 4G), and was significant in the early part of the dark phase ($p < 0.05$; Figure 4H). *Nav1.8/LepR*$^{fl/fl}$ mice had a smaller satiety ratio than WT mice in the early dark phase ($p < 0.05$; Figure 4L). The satiety ratio correlates meal size with the time to the subsequent meal; the smaller satiety ratio indicates that *Nav1.8/LepR*$^{fl/fl}$ mice are less satiated by a meal than WT mice. No difference in meal number, intermeal interval, rate of ingestion was observed between genotypes (Sup Table 1). Thus, deletion of the leptin receptor in VAN produces a significant effect on dark phase calorie consumption and meal patterns independent of energy expenditure. These findings indicate that disruption of leptin signaling in VAN ablates a physiological satiety mechanism that controls meal termination. Notably, this mechanism primarily operates during the first hours of nocturnal feeding when rodents eat the first and largest of their daily meals.

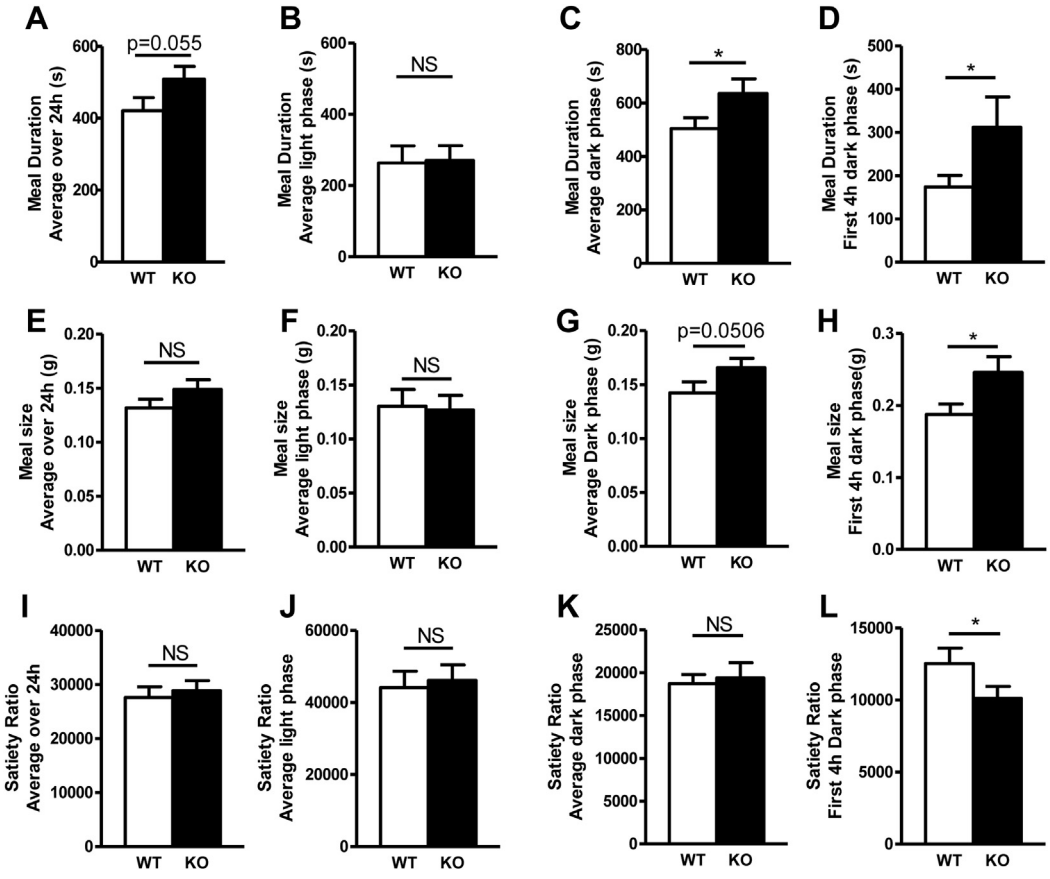

Figure 4: Meal pattern analysis. Meal duration (A—D) was significantly increased in the early dark phase (D), and total dark phase (C), but not the light phase (B), in *Nav1.8/LepR*$^{fl/fl}$ mice; with a trend towards 24 h increase in meal duration (A). Meal size (E—H) was significantly increased in the early dark phase and trended towards increasing in total dark phase(F), but there was no change in the light phase (E) or total 24 h (D) in *Nav1.8/LepR*$^{fl/fl}$ mice compared with WT mice. Satiety ratio (I—L), a measure of fullness, was decreased in the early dark phase of the *Nav1.8/LepR*$^{fl/fl}$ mice compared with WT (L), but no changes were observed in total dark phase (K), light phase (J), or total 24 h (I) ($N = 8$).

2.4. Deletion of LepR in VAN reduces CCK- and leptin-induced satiation

Intestinal feedback inhibition of food intake is mediated by CCK-induced activation of the vagal afferent pathway and comprises of a decrease in meal size and duration [42]. We hypothesized that a reduced sensitivity of VAN to CCK may be responsible for increasing meal size and duration in the *Nav1.8/LepR*$^{fl/fl}$ mice. Leptin and CCK synergism is well established [43—45] although the site of synergism remains unclear. CCK predominantly mediates its effects on food intake by activating CCK1 receptors on vagal afferent terminals innervating the gut. There is evidence that leptin is required for CCK to signal in VAN: in cultured VAN, leptin increases CCK signaling and leptin resistance in VAN reduces CCK-induced satiation [44]. To test whether the absence of leptin signaling in VAN could inhibit CCK-induced satiation, we compared feeding responses to peripheral injections of exogenous CCK in WT and *Nav1.8/LepR*$^{fl/fl}$ mice. CCK (0.3 μg/kg or 3 μg/kg, IP) inhibited 2 h food intake in WT mice (Figure 5A), but failed to have any effect in *Nav1.8/LepR*$^{fl/fl}$ mice (Figure 5B). A higher dose of CCK (30 μg/kg, IP) significantly reduced food intake in both WT and *Nav1.8/LepR*$^{fl/fl}$ mice (Figure 5A, B). Thus, the absence of leptin signaling in VAN significantly reduces the ability of CCK to inhibit food intake. This deficit in CCK-induced signaling in the *Nav1.8/LepR*$^{fl/fl}$ mice reduces vagal afferent signaling of intestinal feedback inhibition of food intake, leading to hyperphagia, increase in body weight and a redistribution of fat to visceral depots.

In a previous study [45], leptin (120 μg/kg; IP) was demonstrated to significantly reduce food intake over 7 h in fasted C57BL/6J mice compared with saline. We confirmed previous reports that peripheral leptin significantly reduces food intake after 7 h in WT mice (Figure 5C, D). In the *Nav1.8/LepR*$^{fl/fl}$ mice, leptin failed to significantly reduce food intake compared to saline (Figure 5C, D). At least at this dose, peripheral administration of leptin appears to mediate feeding behavior predominantly via a vagal afferent pathway. It should be noted that the trend in reduced rate of food intake observed over the course of the 7 h in the *Nav1.8/LepR*$^{fl/fl}$ mice may be a result of exogenous leptin crossing the BBB and acting on leptin receptors in the NTS or hypothalamus, since LepR expression remains intact in the CNS.

2.5. Deletion of LepR prevents CCK-induced plasticity in VAN

Plasticity of VAN is a well-established concept. Nerve damage or inflammation alters gene expression, changing sensitivity and excitability of VAN [46—48]. More recently, nutrient availability in the gut has been association with changes in expression of GPCRs and neuropeptide transmitters in VAN of rodents and humans [49]. The neurochemical phenotype of VAN reversibly switches from an anorectic phenotype post-prandially to an orexigenic phenotype under fasting conditions. Since CCK is a predominant mediator of this switch in phenotype and the absence of the leptin receptor expressed by VAN markedly compromises CCK-induced activation of VAN, we hypothesized that changes in VAN phenotype between fasted and fed

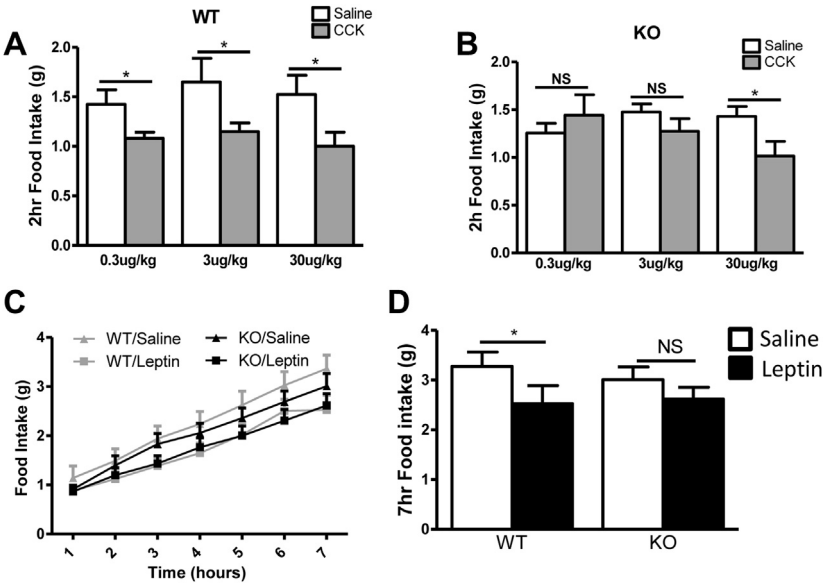

Figure 5: Satiating effects of CCK and leptin. *Nav1.8/LepR*$^{fl/fl}$ and WT mice were deprived of food for 18 h and administered IP saline or CCK (A–B), and IP saline or leptin (C–D) and food intake was recorded ($N = 8$). (A) CCK reduced 2 h food intake in WT mice at doses of 0.3 μg/kg, 3 μg/kg of CCK and 30 μg/kg; (B) satiating effects of CCK were only observed at highest dose in *Nav1.8/LepR*$^{fl/fl}$ mice. (C) Food intake was measured every hour for 7 h following IP administration of 120 μg/kg of leptin (D) Leptin reduced 7 h food intake in WT mice but did not significantly inhibit food intake in *Nav1.8/LepR*$^{fl/fl}$ mice ($N = 8$).

conditions would be attenuated or abolished in the *Nav1.8/LepR*$^{fl/fl}$ mice.

Expression of the neuropeptide transmitter cocaine and amphetamine regulated transcript (CART; Figure 6A) and peptide YY receptor type 2 (Y2R; Figure 6C) were high, while the transmitter melanin concentrating hormone (MCH; Figure 6B) and cannabinoid receptor type 1 (CB1R; Figure 6D) expression were low in 2 h refed compared with fasted WT mice, as previously described in lean rats [50–52]. Conversely, food withdrawal decreased Y2R and CART expression and increased CB1R and MCH expression. However, this phenotypic switch was markedly attenuated or absent in *Nav1.8/LepR*$^{fl/fl}$ mice. Y2R and CART expression was constitutively low in VAN of KO mice and the expression of the CB1R and MCH in VAN was high. Thus, the inability of VAN to respond to leptin results in a loss of CCK-induced neuronal plasticity.

2.6. Leptin resistance in VAN is necessary for the development of obesity

To determine whether leptin resistance in VAN is necessary for the onset of obesity we chronically fed *Nav1.8/LepR*$^{fl/fl}$ and WT mice with high fat diet. At 9 weeks, when all the animals still weighed the same (Figure 7A), mice were either kept on a chow diet or switched to a 45% high fat diet. As expected WT mice gained more weight on a high fat diet than on a chow diet (Figure 7B). After 21 weeks on chow *Nav1.8/LepR*$^{fl/fl}$ mice weigh more than WT mice (Figure 7B, C). Crucially, *Nav1.8/LepR*$^{fl/fl}$ mice failed to gain additional weight despite chronic ingestion of an HFD for 12 weeks (Figure 7C), and weighed less than high fat fed WT mice (Figure 7B, C). Many factors are involved in weight gain following consumption of a high fat diet. We demonstrate that leptin resistance in VAN is sufficient to promote weight gain in the absence of a high fat diet, and that consumption of a high fat diet fails to increase weight gain in *Nav1.8/LepR*$^{fl/fl}$ mice. We infer from this data that other factors are involved in high fat diet-induced weight gain and that they are downstream of leptin resistance onset in VAN. We postulate that KO mice acquire compensatory mechanisms to deal with the loss of lepR in VAN during development which prevents them from gaining further weight on a high fat diet.

Adiposity comparisons in 21-week-old mice fed their respective diets for 12 weeks, revealed that *Nav1.8/LepR*$^{fl/fl}$ mice gained significantly less fat than WT mice when fed a high fat diet (Figure 7D). As expected adiposity was significantly increased in WT mice fed a high fat diet compared with chow fed WT mice, however, despite a large trend there was no statistical difference in adiposity between *Nav1.8/LepR*$^{fl/fl}$ mice fed chow or a high fat diet (Figure 7D). When studied at the individual fat pad level, we observed significant increases in epididymal and retroperitoneal fat pads of high fat fed *Nav1.8/LepR*$^{fl/fl}$ mice compared with chow fed *Nav1.8/LepR*$^{fl/fl}$ mice (Figure 7E).

3. DISCUSSION

Considerable evidence has accumulated to suggest that the inability of leptin receptor-bearing neurons to respond to leptin plays a pivotal role in the development and/or persistence of an obese phenotype [7]. We have developed and utilized a powerful new tool which allows the first targeted approach to determine the functional role of specific proteins in gut-brain signaling. Using the cre-lox method, we conclusively demonstrate that knocking out LepR in vagal afferent neurons is sufficient and necessary to increase food intake, weight gain and adiposity. There is evidence that LepRa, LepRb and LepRe splice variants are expressed in vagal afferent neurons [16,17,53]. In this study we have deleted all isoforms of LepR; however, based on the fact that LepRb is currently the only isotype found to be involved in the control of food intake [22], and that LepRb signaling (i.e. STAT3 activation) is severely blunted in *Nav1.8/LepR*$^{fl/fl}$ mice, our data suggests that deletion of LepRb is responsible for the phenotype of the *Nav1.8/LepR*$^{fl/fl}$ mice. The data also show that the lack of LepR expression in vagal afferent neurons leads to hyperphagia via a mechanism involving the reduction in sensitivity to gut hormones. Taken together with our previous findings that leptin resistance in vagal afferent neurons develops early in diet-induced obesity and

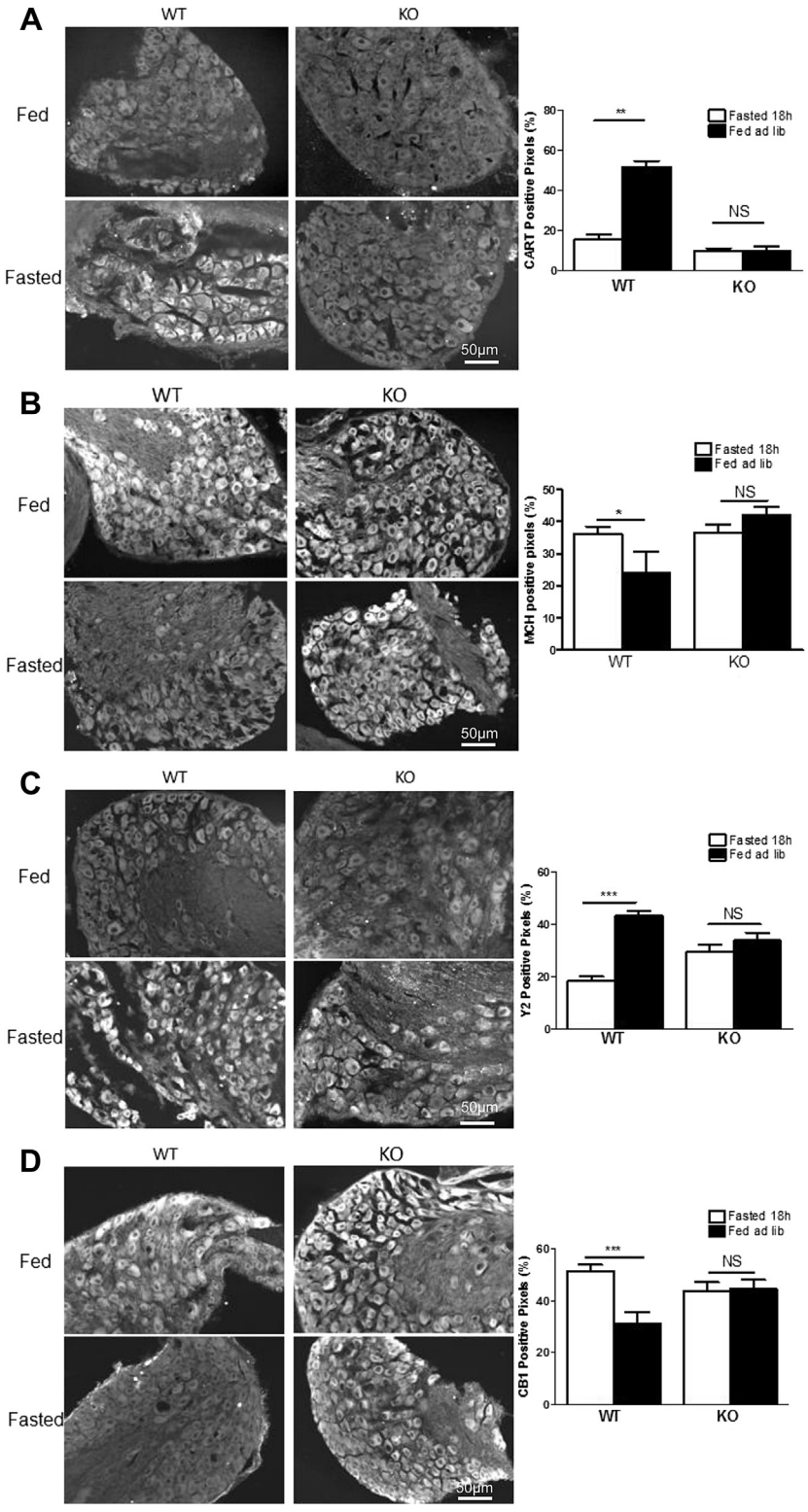

Figure 6: Analysis of VAN plasticity. WT and *Nav1.8/LepR*$^{fl/fl}$ mice were either food deprived for 18 h or fed ad libitum. (A) Immunoreactivity of CART protein in nodose ganglia. Percent of CART positive pixels was higher in fed compared with fasted conditions in WT mice, but constitutively low in *Nav1.8/LepR*$^{fl/fl}$ mice. (B) Immunoreactivity of MCH protein in nodose ganglia. Percent of MCH positive pixels was higher in fasted compared with fed conditions in WT mice, but constitutively high in *Nav1.8/LepR*$^{fl/fl}$ mice. (C) Immunoreactivity of Y2 receptor in nodose ganglia. Percent of Y2 positive pixels was higher in fed compared with fasted conditions in WT mice. Feeding status had no effect on Y2 positive pixels in *Nav1.8/LepR*$^{fl/fl}$ mice. (D) Immunoreactivity of CB1 receptor in nodose ganglia. Percent of CB1 positive pixels was higher in fasted compared with fed conditions in WT mice, but constitutively high in *Nav1.8/LepR*$^{fl/fl}$ mice ($N = 4$).

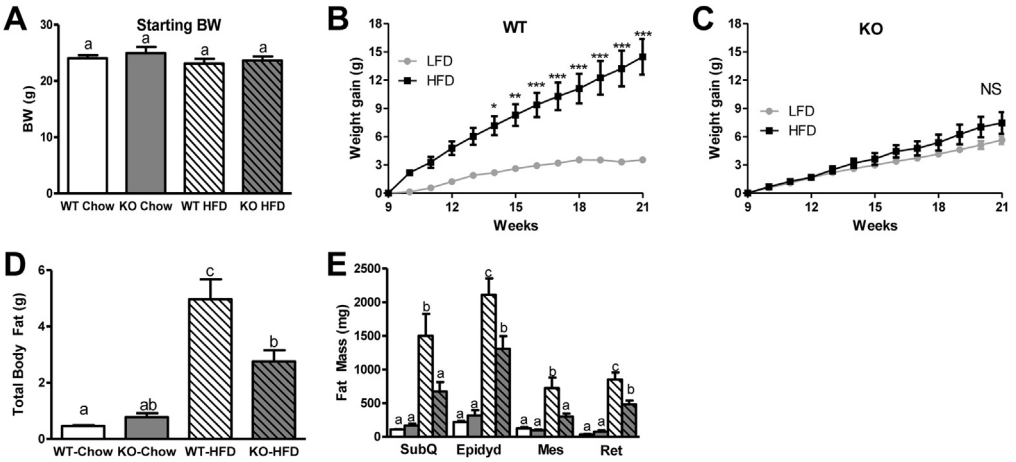

Figure 7: Analysis of weight gain and adiposity in response to a high fat diet. WT and *Nav1.8/LepR*$^{fl/fl}$ mice were fed chow or high fat diet for 12 weeks ($N = 6–9$). (A) The starting body weight of all the mice was not significantly different. (B) WT mice fed a high fat diet gained significantly more weight from 5 weeks of high fat feeding onwards. (C) *Nav1.8/LepR*$^{fl/fl}$ mice did not gain further weight in response to a high fat diet. (D) At the end of the study, high fat diet significantly increased adiposity in WT mice, but not in *Nav1.8/LepR*$^{fl/fl}$. (D) Chronic ingestion of a high fat diet for 12 weeks increased epididymal, subcutaneous, mesenteric and retroperitoneal fat pads in WT mice. *Nav1.8/LepR*$^{fl/fl}$ mice fed a high fat diet had elevated epididymal and retroperitoneal fat pads, but not mesenteric and subcutaneous fat pads.

coincides with hyperphagia, these findings demonstrate that the leptin receptor signaling in VAN mediates the hyperphagic response to chronic ingestion of a high fat diet.

The finding that VAN are important in the pathophysiology of diet-induced obesity by initiating overconsumption of food is particularly significant given that the vagal afferent pathway has largely been discounted as a putative mechanism for the onset of obesity and has only been thought to be involved in short term, meal-to-meal regulation of food intake. VAN are well known to carry the bulk of the information about the nutritional content of a meal from the gastrointestinal tract to the brain, and lead to meal termination [24]. Although we did not specifically study LepR expression in VAN innervating the gastrointestinal tract, Nav1.8-cre mice have been demonstrated to have extensive vagal innervation of the gastrointestinal tract [54]. Furthermore, retrograde tracing experiments have established that VAN innervating the gut are located in the caudal portion [55] and express CCK1 receptor [56]. LepR notably colocalizes with CCK1 receptor in this population of VAN [16]. We report here that LepR immunostaining is lost in the caudal region of the nodose ganglia and that CCK signaling is blunted in the *Nav1.8/LepR*$^{fl/fl}$ mice. Therefore, this is the first conclusive evidence that chronic disruption of gut-brain signaling via a vagal pathway reduces satiation over multiple meals leading to hyperphagia and obesity.

We propose that the absence of weight gain in *Nav1.8/LepR*$^{fl/fl}$ mice fed a high fat diet suggests that leptin resistance in VAN is a necessary initiating step in the development of diet-induced obesity. We demonstrate that ingestion of a high fat diet leads to weight gain in WT mice, and that deletion of LepR in VAN leads to weight gain, but that adding high fat diet to *Nav1.8/LepR*$^{fl/fl}$ mice does not cause additional weight gain. Since we know that leptin resistance in VAN develops as a result of chronic ingestion of a high fat diet [19], we conclude that leptin resistance in VAN is a necessary initial step in diet-induced obesity. We suggest that the *Nav1.8/LepR*$^{fl/fl}$ mice have acquired compensatory mechanisms to deal with the loss of leptin receptor in VAN during development which prevents them from gaining further weight on a high fat diet. WT mice fed a high fat diet develop leptin resistance which initiates weight gain, and secondary mechanisms promote further weight gain. The compensatory mechanisms acquired by the *Nav1.8/LepR*$^{fl/fl}$ mice prevent the secondary mechanisms from promoting further weight gain in response to a high fat diet. We suggest that preventing acquisition of compensatory mechanisms by knocking out LepR in VAN during adulthood would result in more pronounced weight gain on a chow diet and increased susceptible to additional weight gain in response to a high fat diet.

The data show that the hyperphagia observed in the conditional knockout mice occurs as a result of reduced meal termination rather than meal initiation. The leptin receptor knockout in VAN increases intake in the early dark phase when the animals consume the majority of food. We observed prolonged meals (reduced meal termination) with no increase in meal numbers or a reduction in the intermeal interval (meal initiation). This suggests that leptin signaling in VAN is involved in meal termination, and that knocking out leptin receptor reduces satiation. The data is consistent with previous findings that VAN are involved in meal termination [57] and that gastrointestinal hormones released post-prandially (i.e. CCK, PYY, GLP-1) activate VAN to mediate satiation. There is substantial evidence in the literature that leptin potentiates CCK signaling [44,45,58]. Here we report that leptin signaling in VAN is required for CCK-induced satiation.

We investigated the possible mechanism by which leptin receptor knockout in VAN initiates hyperphagia. We have previously demonstrated that during fasting, when there is little nutrient content in the proximal gut, the neurochemical phenotype of VAN is to express orexigenic peptides (e.g. MCH) and receptors (e.g. CB1R) [49]. Postprandial release of CCK induces a "switch" in the phenotype of VAN shown by an increase in expression of anorectic peptide (e.g. CART) and receptors (e.g. Y2R) [49]. The reduced sensitivity of VAN to CCK in mice lacking leptin receptor in VAN results in loss of this plasticity; expression of the peptide transmitters, CART and MCH, and expression of the receptors, Y2R and CB1R, fail to change in response to feeding or fasting in the *Nav1.8/LepR*$^{fl/fl}$ mice. There is some evidence that at least CART is released from cultured VAN and that CART can prolong satiation *in vivo* [59]. Furthermore, knocking down CART expression in VAN of freely behaving rats has been shown to increase food intake in short term studies [60]. Together these data suggest that CART may act as a neuropeptide transmitter involved in inhibiting food intake. Thus, the reduction in CART expression in VAN of *Nav1.8/LepR*$^{fl/fl}$ mice may account for the reduced satiation and consequently hyperphagia.

We have developed and utilized a novel tool, namely a mouse with a conditional knockout of the leptin receptor, which allows the first targeted approach to determine the functional role of vagal afferent neurons. Using this method we have been able to show for the first time that the vagal afferent pathway influences food intake, adiposity, and body weight over the long term. This result contrasts to surgical and chemical ablation studies in which long term effects on body weight or adiposity have not been reported. Prior ablation studies have lacked specificity in targeting the vagal afferent pathway. Total subdiaphragmatic vagotomy ablates both afferent and efferent pathways; the more selective subdiaphragmatic deafferentation ablates 50% of the efferent fibers, in addition to afferent fibers. Perivagal application of capsaicin, thought to cause degeneration of afferent C fibers may also damage efferent neurons [61]. More recently a *Phox2b*-Cre mouse has been used to target vagal neurons [62,63]. However, this cre-lox system does not discriminate between vagal afferent and vagal efferent neurons, and is also expressed in enteric neurons of the intestine [64], central noradrenergic neurons of the nucleus of the solitary tract, neurons of the area postrema, in most of the rhombencephalon (caudal to r1) at least during development, in a subset of sympathetic neurons, and extensively in neurons of the IIIrd, IVth, VIIth, IXth, Xth and XIth cranial ganglia [65]. Thus, the current study is the first to selectively target vagal afferent signaling to determine their role in food intake and body weight. It should be noted that Nav1.8 cre targets DRG and SCG neurons in addition to VAN; however, because few DRG and SCG neurons express *LepR*, the method provides knock down of leptin receptor specifically in VAN.

It is interesting to note that deleting all the splice variants of LepR using Phox2b-cre [66] or Nav1.8-cre produces similar phenotypes. Both mice lines increase weight gain on a chow diet, increase food intake at 12 weeks, had no change in respiratory quotient, and were satiated in response to a high dose of CCK. Finally, similarly to our *Nav1.8/LepR*$^{fl/fl}$ mice the Phox2b-cre LepR$^{flox/flox}$ mice failed to gain weight on an HFD [66]. The similarity in phenotype suggests that the Phox2b-cre LepR$^{flox/flox}$ is more likely a result of LepR deletion from VAN rather than NTS neurons.

4. CONCLUSION

Vagal afferent neurons convey information about the availability of nutrients in the gut to the brain [24]. In the post-prandial period, the vagal afferent pathway plays a pivotal role in regulation of gastrointestinal and pancreatic function and also plays a role in the control of meal size and duration [67]. Here we demonstrate that knocking out leptin receptor expression in vagal afferent neurons prevents appropriate post-prandial gut-brain signaling, resulting in increased food intake, weight gain, and adiposity. Taken together with our previous data showing that leptin resistance is an early event in high-fat diet induced hyperphagia and weight gain, the current data strongly suggest that defects in leptin signaling in the vagal afferent pathway is a novel mechanism for the initiation of obesity. This novel approach may provide insight into the role of other factors (i.e. hormones, cytokines, microbial products, mechanosensitivity, and nutrients) involved in gut-brain signaling as they relate to food intake, inflammation, microbiota-brain signaling, and neurodegenerative diseases.

5. METHODS

5.1. Animals

All experiments were approved by the UC Davis Institutional Animal Care and Use Committee (protocol #16793) and PHS animal welfare assurance to UC Davis (#A3433-01). Cre mice were generously donated by Dr. John Woods at UCL [28]. LepR flox mice were purchased from Jax [27]. Mice were bred to generate selective deletion of leptin receptor in primary afferent neurons. 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. Mice were individually housed after weaning under a 12 h light:12 h dark schedule and allowed ad libitum access to food (Purina5008) and water unless specified otherwise. All experiments were performed in 12-week-old mice except for the high fat diet experiment in which 9-week-old chow fed mice were either kept on chow or given ad libitum access to high fat diet (45% kcal/g fat; Research diets D12451) for 12 weeks.

5.2. Tissue collection

Mice were fasted overnight, or fasted and refed 2 h. Tissue and cardiac blood were collected immediately. The quantity of food ingested during the last meal and the time of the last meal before blood collection are important confounding variables since leptin is known to be released from the gut in response to a meal and can account for as much as 20% of circulating leptin [68]. Therefore we minimized the variability in circulating leptin levels between animals by collecting blood, and measuring leptin levels, from fasted animals. Leptin was measured by ELISA according to the manufacturer's protocol (Alpco Diagnostics, Salem, NH). For qPCR tissue was snap frozen. For immunohistochemistry tissue was fixed in 4% paraformaldehyde and left in 25% sucrose overnight prior to sectioning.

5.3. PCR

Flox mice were genotyped according to prior reports [27].

To quantify *LepR* knockdown in tissue we used real-time PCR. All samples were repeated in triplicate to assure reproducibility of results. *LepR* was expression was quantified using validated TaqMan primer/probe sets (Mm01262072_m1) and conditions for the real-time RT-PCR detection of mouse leptin receptor. Specific PCR products were confirmed by demonstrating the presence on an agarose gel by electrophoresis. All samples were compared with a reference gene 18S (Mm03928990_g1). Quantification was then performed using the comparative Ct method. Nodose ganglia and superior cervical ganglia samples from 2 mice were pooled together to get sufficient cDNA, a total of 12 animals were used for these tissue.

5.4. Immunohistochemistry

As previously described in Ref. [44]. Primary antibodies raised against CART H-003-63 and MCH H-070-47 (1:200; Phoenix Peptides, Buringlame, CA), CB1 sc20754, Y2 sc14736, and LepR sc8391 (1:100; Santa Cruz Biotechnology Inc., Dallas, TX), pSTAT3 9145 (1:100; Cell Signaling Technology, Beverly, MA), and CRE recombinase MMS-106P (1:200, Covance Inc, Emeryville, CA) were used. Secondary antibodies were used as appropriate and included donkey anti-rabbit immunoglobulin and donkey anti-goat immunoglobulin conjugated with Alexa Fluor 488 or 555 (1:400; Molecular Probes, Eugene, OR). Percentage of positive pixels and positive neurons were quantified using Scion software as previously described.

5.5. Body compostion

Body fat and lean mass of mice were assessed by DEXA.

5.6. Metabolic analysis

Energy expenditure was evaluated in two separate cohorts of age matched (±2 days) WT and *Nav1.8/LepR*$^{fl/fl}$ mice fed powdered chow diet (LabDiet 5058) using a comprehensive lab animal monitoring

system (CLAMS, Columbus Instruments, Comlubus, OH). Data was combined since no statistical difference between runs was identified (data not shown). Mice were fed powdered diet for one week and acclimated to monitoring chambers for 2 days prior to 48 h data collection, data is presented as an average of both days. Energy expenditure was calculated from the oxygen intake. Activity levels were determined by counting laser breaks along a *x*, *y*, and *z* axis. The food bout was defined as an episode of uninterrupted feeding of at least 0.02 g, and meal termination was when a bout of feeding was followed by 10 min with no measurable intake.

5.7. Feeding studies

Mice were fasted 12 h prior to feeding tests. Cholecystokinin (octapeptide, sulfated) was purchased from Bachem (Torrance, CA) and leptin (rat) from Sigma Aldrich (St. Louis, MO). CCK (0.3–30 μg/kg; ip), leptin (120 μg/kg; ip), or saline (100 μl; ip) were administered and food was immediately returned to the cage; food intake was recorded every 20 min over 2 h for CCK studies and every hour for 7 h in leptin studies.

5.8. Statistics

Statistical analysis was performed using Prism software (Prism 5.0; GraphPad Software, La Jolla, CA). Unpaired *t*-test was used to make direct comparisons between WT and *Nav1.8/LepR*$^{fl/fl}$ mice. In feeding experiments paired *t*-test was used to compare saline with either CCK or leptin. Two-way anova with Bonferroni post hoc test was used to compare the effects of leptin and saline over time in the WT and *Nav1.8/LepR*$^{fl/fl}$ mice; to compare circulating leptin concentrations in WT and *Nav1.8/LepR*$^{fl/fl}$ mice at 6 and 12 weeks; to compare the expression of CART, MCH, Y2, and CB1 expression in nodose ganglia of WT and *Nav1.8/LepR*$^{fl/fl}$ mice in response to feeding or fasting; and to compare weight gain over time in chow and high fat fed animals. One way ANOVA with Bonferroni post hoc test was used to compare adiposity between *Nav1.8/LepR*$^{fl/fl}$ mice fed high fat or chow diets, and their starting weights before going on their respective diets. Differences were considered significant if $p < 0.05$. Data are means ± SEM. * represents $p < 0.05$; ** represents $p < 0.01$; and $p < 0.001$. For all experiments in which one way ANOVA was performed different letters ^{a,b,c} denote significant differences between groups.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2014.06.003.

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