ORIGINAL ARTICLE

Intranasal Leptin Relieves Sleep-disordered Breathing in Mice with Diet-induced Obesity

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Abstract

Rationale: Leptin treats upper airway obstruction and alveolar hypoventilation in leptin-deficient *ob/ob* mice. However, obese humans and mice with diet-induced obesity (DIO) are resistant to leptin because of poor permeability of the blood–brain barrier. We propose that intranasal leptin will bypass leptin resistance and treat sleep-disordered breathing in obesity.

Objectives: To assess if intranasal leptin can treat obesity hypoventilation and upper airway obstruction during sleep in mice with DIO.

Methods: Male C57BL/6J mice were fed with a high-fat diet for 16 weeks. A single dose of leptin (0.4 mg/kg) or BSA (vehicle) were administered intranasally or intraperitoneally, followed by either sleep studies ($n = 10$) or energy expenditure measurements ($n = 10$). A subset of mice was treated with leptin daily for 14 days for metabolic outcomes ($n = 20$). In a separate experiment, retrograde viral tracers were used to examine connections between leptin receptors and respiratory motoneurons.

Measurements and Main Results: Acute intranasal, but not intraperitoneal, leptin decreased the number of oxygen desaturation events in REM sleep, and increased ventilation in non-REM and REM sleep, independently of metabolic effects. Chronic intranasal leptin decreased food intake and body weight, whereas intraperitoneal leptin had no effect. Intranasal leptin induced signal transducer and activator of transcription 3 phosphorylation in hypothalamic and medullary centers, whereas intraperitoneal leptin had no effect. Leptin receptor–positive cells were synaptically connected to respiratory motoneurons.

Conclusions: In mice with DIO, intranasal leptin bypassed leptin resistance and significantly attenuated sleep-disordered breathing independently of body weight.

Keywords: leptin; sleep apnea syndromes; hypoventilation; respiration

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At a Glance Commentary

Scientific Knowledge on the Subject: Obesity-related sleep-disordered breathing (SDB) syndromes, including obstructive sleep apnea and obesity hypoventilation, are common diseases without effective pharmacotherapy. An adipocyte-produced hormone, leptin, relieves upper airway obstruction and stimulates respiratory control centers in the brain in the leptin-deficient state. However, humans and rodents with diet-induced obesity (DIO) are leptin resistant owing, at least in part, to limited permeability of the blood–brain barrier (BBB) to leptin. Intranasal leptin delivery bypasses the BBB and activates leptin signaling in the brain, leading to weight loss, yet the therapeutic effect of intranasal leptin in SDB has not been examined.

What This Study Adds to the Field: This translational study is the first demonstration that, in leptin-resistant mice with DIO, a single dose of intranasal leptin successfully treats upper airway obstruction and hypoventilation during sleep, independent of metabolism, whereas intraperitoneal leptin has no respiratory effect. Intranasal leptin, but not intraperitoneal leptin, induced leptin receptor signaling in hypothalamic and medullary centers. Respiratory motoneurons innervating the diaphragm and the genioglossus muscle did not express leptin receptors but were synaptically connected to leptin receptor–expressing cells. Our study suggests that intranasal leptin can be tested as a potential therapy in patients with SDB.

Obesity is associated with a spectrum of sleep-disordered breathing (SDB), including obstructive sleep apnea (OSA) and obesity hypoventilation syndrome (OHS). OSA, recurrent closure of upper airway during sleep, is a highly prevalent disease, affecting 30% of the adult population and over 50% of obese individuals (1, 2). OHS is defined as daytime hypercapnia and hypoventilation during sleep in obese individuals, attributed to abnormal ventilatory control (3). There is no effective pharmacotherapy for SDB. The pathogenesis of SDB in obesity has been linked to an adipocyte-produced hormone leptin (4, 5), yet leptin has not been identified as a therapeutic target.

Leptin is produced by adipose tissue, and is transported across the blood–brain barrier (BBB) to the hypothalamus and medulla, where it suppresses appetite and increases energy expenditure (6–10). Leptin signals via the long isoform of leptin receptor (ObRb) and downstream intracellular pathways, including STAT3 (signal transducer and activator of transcription 3) (11). However, obese humans and rodents with diet-induced obesity (DIO) have high circulating leptin levels, which are directly proportional to the adipose mass (9, 10). This phenomenon is called leptin resistance, and is defined as a failure of high circulating levels of leptin to promote metabolic responses (12). Evidence from animal models and humans strongly suggests that the main mechanism mediating leptin resistance in obesity is an impaired transport of leptin across the BBB (13–16).

Previous investigations in humans and animals showed that leptin is involved in the pathogenesis of SDB through central regulation of respiratory pump muscles and upper airway patency (17–19). Leptin-deficient obese *ob/ob* mice have suppressed hypercapnic ventilatory response (HCVR) and are hypercapnic while awake, mimicking human OHS (20). Leptin administration corrected a defect in control of breathing normalizing HCVR (20). Intracerebroventricular administration of leptin in rodents, as well as direct microinjections of leptin in brain CO_2-sensing areas, acutely increased ventilation (21, 22). Our laboratory established that, in *ob/ob* mice, leptin stimulates upper airway reflexes and decreases inspiratory flow limitation

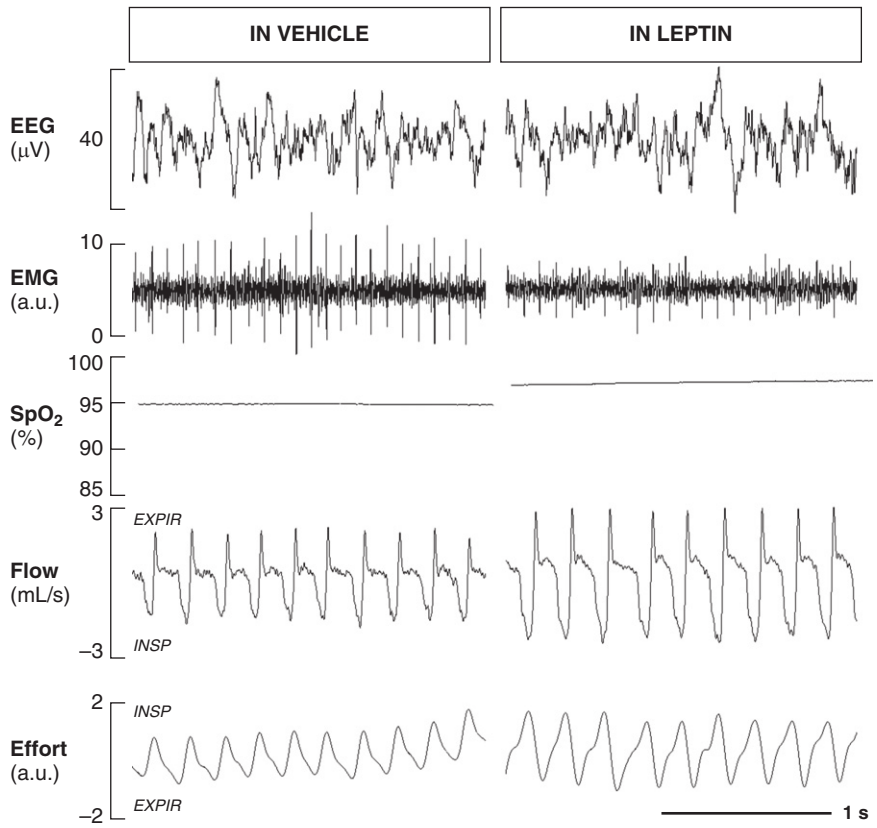

Figure 1. A representative sleep recording in the diet-induced obese mouse during non-REM (NREM) sleep treated with intranasal BSA alone (left panel) or intranasal leptin dissolved in BSA (right panel). EEG, nuchal electromyogram (EMG; arbitrary units [a.u.]), oxygen saturation as measured by pulse oximetry (Sp_{O_2}), respiratory flow, and breathing effort were recorded simultaneously. *EXPIR* = expiration; *INSP* = inspiration.

(IFL) during sleep, a cardinal feature of OSA (18, 19). Thus, leptin may act in the brain stimulating both pharyngeal muscles and the diaphragm. However, human studies have shown that SDB is highly prevalent in obese patients, who have elevated circulating levels of leptin (23–26). We propose that SDB in obesity is caused by inadequate leptin receptor activation in the brain, mediated by reduced transport through the BBB. One attractive option to increase leptin receptor activity is to administer leptin intranasally bypassing the BBB (27).

The major goal of this translational study was to examine effects of intranasal leptin on SDB. We studied DIO mice that develop leptin resistance and SDB, similarly to obese humans (28). We assessed effects of leptin on upper airway by examining IFL breathing (29) and on respiratory control and respiratory pump muscles by examining non–flow-limited breathing (18).

We hypothesized that intranasal leptin will bypass leptin resistance and treat hypoventilation and upper airway obstruction during sleep. To test this hypothesis, we: *1*) performed polysomnography in DIO mice treated with a single-dose intranasal versus intraperitoneal leptin or vehicle; *2*) determined effects of intranasal versus intraperitoneal leptin on metabolism; and *3*) identified synaptic connections between respiratory motoneurons and ObRb-expressing cells. Some of the results of these studies have been previously reported in the form of an abstract (30).

Methods

In total, 48 adult male C57BL/6J mice with DIO, six adult male *ObRb-Cre* mice (B6.129(Cg)-Leprtm2(cre)Rck/J), expressing Cre recombinase in $ObRb^+$ cells and 12 *ObRb-GFP* mice expressing GFP in $ObRb^+$ cells were used in the experiments. *ObRb-GFP* mice were generated by crossing *ObRb-Cre* with *GFP*-floxed mice (B6.129-Gt(ROSA)26Sortm2Sho/J). Food and water were provided *ad libitum*, except during sleep recording. Mice were housed in a standard laboratory environment at 24–26°C in a 12-hour light/dark cycle (9 A.M.–9 P.M. lights on). All surgical procedures were performed under 1–2% isoflurane anesthesia. The study was approved by the Johns Hopkins University Animal Use and Care Committee, and complied with the American Physiological Society Guidelines for Animal Studies. The study included seven experimental protocols described in the online supplement. Briefly, to study effects of intranasal leptin on SDB and metabolism, DIO mice were treated with a single dose of intranasal or intraperitoneal leptin or vehicle in a randomized cross-over study. Leptin signaling in the hypothalamus and medulla was assessed by phosphorylated STAT3 (pSTAT3) immunofluorescence. To examine effects of chronic intranasal leptin on food intake and body weight, DIO mice were treated daily for 14 days. Synaptic connections between respiratory motoneurons and ObRb-expressing cells were identified using a retrograde transneuronal tracer PRV263 carrying a cre-dependent Brainbow cassette. For statistical analyses, effects of leptin were analyzed separately for the intranasal and intraperitoneal routes using the Kruskal-Wallis test for respiratory outcomes and mixed effects linear regression for metabolic measurements.

Results

Effects of a Single Dose of Intranasal and Intraperitoneal Leptin on Basic Characteristics and Sleep Architecture

DIO mice were of similar age and body weight in all treatment groups, intranasal leptin, intranasal vehicle, intraperitoneal leptin, and intraperitoneal vehicle (*see* Table E1 in the online supplement). High baseline plasma leptin levels (48.7 ± 8.6 ng/ml) suggested resistance to leptin's metabolic effects. Acute intranasal or intraperitoneal leptin administration had no effect on body temperature compared with the vehicle (Table E1). Sleep recordings were performed from 11 A.M. to 5 P.M. The sleep architecture was similar to that previously described in C57BL/6J mice (31), and there was no difference between any of the four groups of mice (Figure E1 and Table E2).

Effects of a Single Dose of Intranasal Leptin on Breathing in Sleeping Mice

IFL breathing is a cardinal feature of OSA in humans and mice alike (18, 32). We

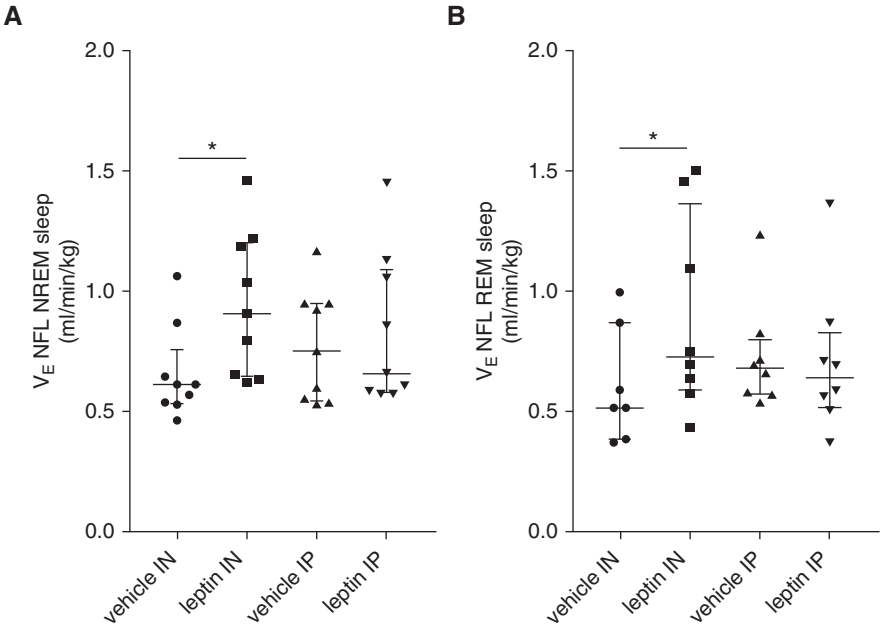

Figure 2. Effect of a single dose of intranasal (IN) and intraperitoneal (IP) leptin on non–flow-limited breathing in diet-induced obese C57BL/6J mice. The dot plots show values for $\dot{V}_E$ (per kilogram of body weight) in individual mice during unobstructed breaths (ventilatory control, non–flow-limited breaths) in (*A*) non-REM (NREM) and (*B*) REM sleep. The whiskers denote the first and third quartiles divided by the median. $^*P < 0.05$ for the difference between IN leptin and IN vehicle (BSA) treatments. Number of animals in each analysis is represented by dots. One mouse was excluded owing to poor quality of the flow signal, resulting in $n = 9$ for the NREM sleep analyses. REM sleep was not achieved during several sleep studies, resulting in $n = 7$ in the IN vehicle group and $n = 8$ in all other groups. $\dot{V}_E$NFL = $\dot{V}_E$ during non–flow-limited.

measured IFL breathing to assess the upper airway function. In contrast, non–flow-limited breathing characterized the respiratory pump and ventilatory control. The analysis of non–flow-limited breathing during non-REM (NREM) sleep showed that, compared with the vehicle, intranasal leptin increased $\dot{V}_E$ from 0.61 to 0.86 ml/min/kg during NREM sleep ($P < 0.05$) and from 0.51 to 0.72 ml/min/kg in REM sleep ($P < 0.05$) (Figures 1 and 2). V_T tended to be higher after intranasal leptin, whereas respiratory rate was unchanged (data not shown). Intraperitoneal leptin had no effect on non–flow-limited breathing (Figure 2).

IFL was observed in 13.3% and 35.7% of all breaths in NREM and REM sleep, respectively (Figures 3 and 4A). IFL breathing was associated with recurrent severe oxyhemoglobin desaturations in REM sleep (Figures 3 and 4B). Neither intranasal nor intraperitoneal leptin treatments affected IFL prevalence (Figure 4A). However, intranasal leptin treatment significantly decreased—oxygen desaturation index of 4% (ODI 4%) in REM sleep—from 40 to 17 events/h (Figure 4B). No effect was observed in NREM sleep where hypoxemic events were uncommon (Figure 4B). A representative polysomnography (Figure 3) demonstrates SDB in a DIO mouse during REM sleep characterized by IFL (note early plateaus in inspiratory airflow), reductions in V_T, and increased respiratory effort. These abnormalities improved after intranasal leptin treatment. Compared with intranasal vehicle (BSA), intranasal leptin significantly alleviated IFL severity, by increasing maximal inspiratory flow ($\dot{V}_I$max) at the onset of flow limitation, from 2.2 to 2.7 ml/min in NREM sleep and from 1.6 to 2.1 ml/min in REM sleep (Figure 4C). Intranasal leptin also increased $\dot{V}_E$ during IFL breathing in NREM and REM sleep (Figure 4D). The effect of intranasal leptin on ventilation during flow-limited breathing was entirely related to significant increases in V_T, but not in respiratory rate (Figure E2). Intraperitoneal leptin had no effect on the ODI, $\dot{V}_I$max, or $\dot{V}_E$ (Figure 4).

Effect of a Single Dose of Intranasal Leptin on Metabolism in DIO Mice

Oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were measured for 24 hours. As expected, $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were higher during the dark period, when mice are mostly awake, compared with the light period, when animals are mostly asleep (Figures 5A and 5B). Intranasal leptin induced modest increases in $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ during both light and dark periods; 1.03- and 1.04-fold increases, respectively, for $\dot{V}_{O_2}$, and 1.05 and

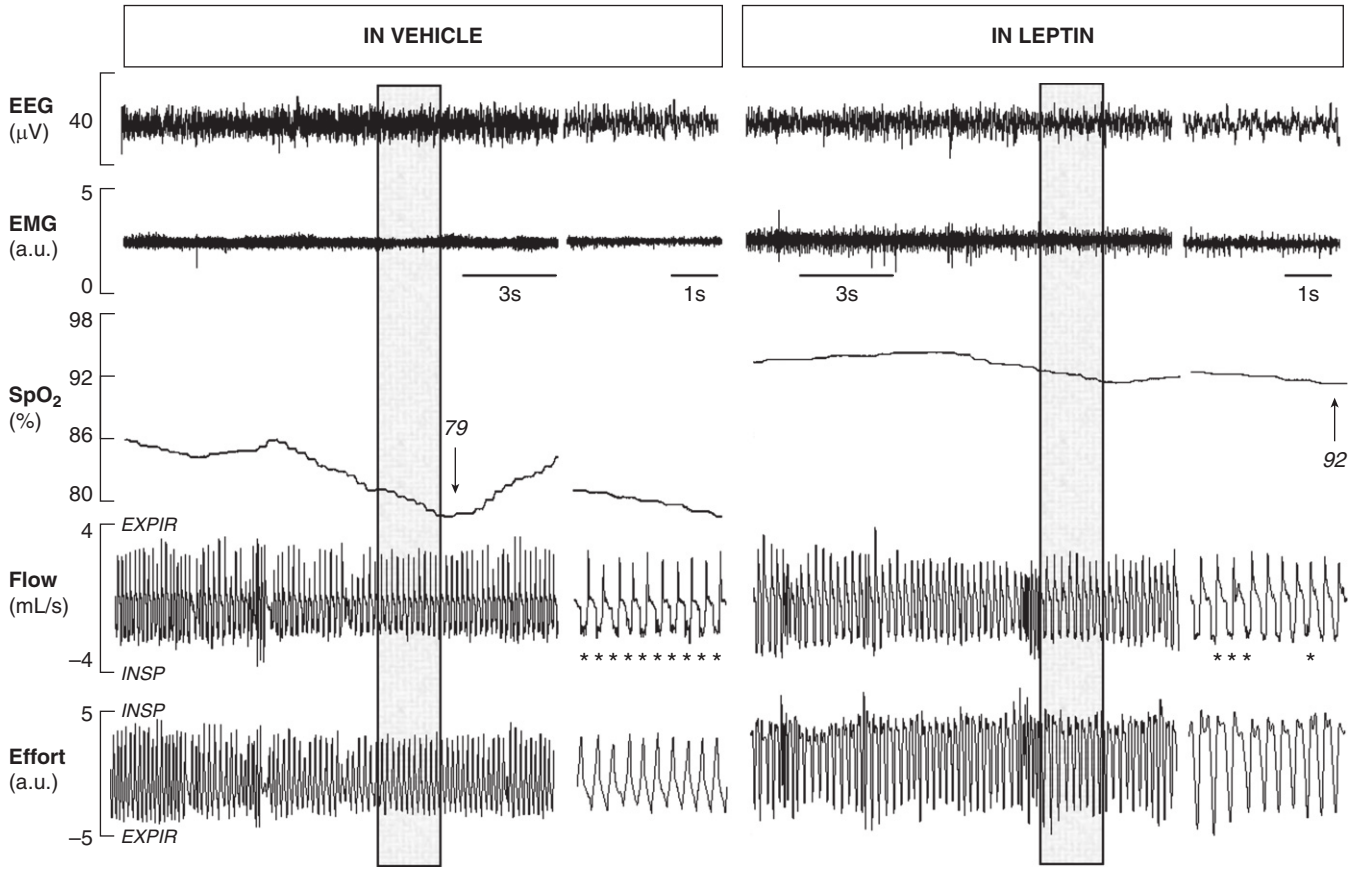

Figure 3. A representative sleep recording in the diet-induced obese mouse during REM sleep treated with intranasal (IN) BSA alone (left panel) or IN leptin dissolved in BSA (right panel). Compressed recordings of EEG, nuchal electromyogram (EMG), oxygen saturation as measured by pulse oximetry (Sp_{O_2}), respiratory flow, and effort are presented. The shaded areas are decompressed on the right side of each panel. Obstructive hypopneas characterized by inspiratory flow limitation with increases in respiratory effort terminated by oxygen desaturations (*see* the arrows). The asterisks indicate flow-limited breaths. a.u. = arbitrary units; *EXPIR* = expiration; *INSP* = inspiration.

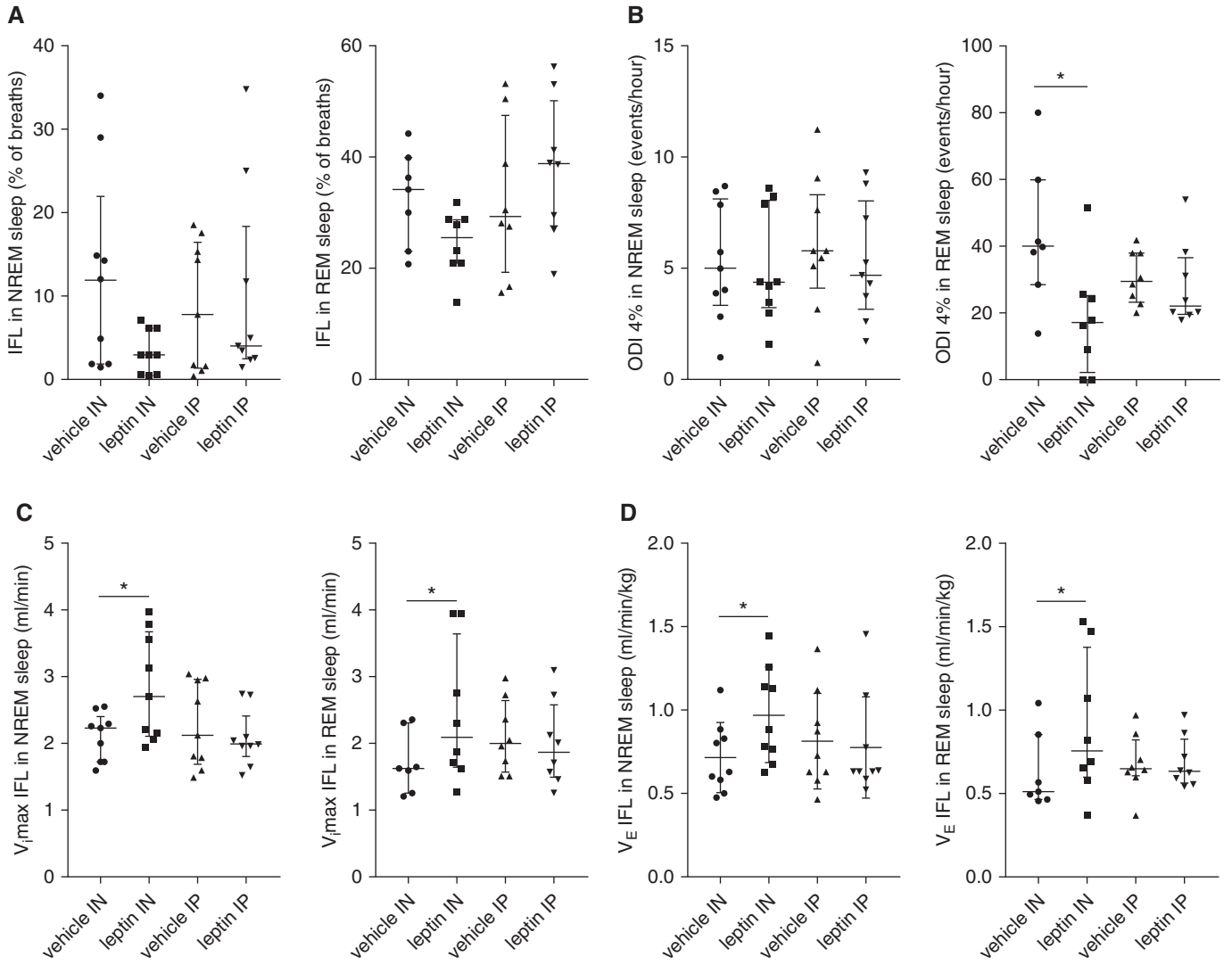

Figure 4. Effect of a single dose of intranasal (IN) and intraperitoneal (IP) leptin on flow-limited breathing in diet-induced obese C57BL/6J mice during non-REM (NREM) and REM sleep. (*A*) Prevalence of inspiratory flow–limited (IFL; or obstructed) breaths was presented as the percentage of all breaths. (*B*) A number of Sp_{O_2} desaturations of 4% or greater from the baseline per hour of sleep (oxygen desaturation index of 4% [ODI 4%]) was measured. (*C*) The severity of upper airway obstruction was characterized as the maximal inspiratory flow ($\dot{V}$imax IFL). (*D*) $\dot{V}_E$ (per kilogram of body weight) during flow-limited (obstructive) breathing was measured. Each dot represents data per one animal. The whiskers denote the first and third quartiles divided by the median. *$P < 0.05$ for the difference between IN leptin and IN vehicle (BSA) treatments. One mouse was excluded owing to poor quality of the flow signal, resulting in $n = 9$ for the NREM sleep analyses. REM sleep was not achieved during several sleep studies, resulting in $n = 7$ in the IN vehicle group and $n = 8$ in all other groups.

1.06 increases, respectively, for $\dot{V}_{CO_2}$. Intraperitoneal leptin demonstrated a delayed effect on $\dot{V}_{O_2}$, stimulating it only during the dark phase, whereas $\dot{V}_{CO_2}$ was slightly increased during both light and dark phases (Figures 5A and 5B). Small increases of the respiratory exchange ratio (RER) were noted after intranasal leptin during the light period (from 0.73 to 0.75) and intraperitoneal leptin during both light and dark periods (from 0.72 to 0.74 and from 0.73 to 0.75, respectively; Figures 5C).

Intranasal Leptin Induces STAT3 Signaling in the Brain

We used *ObRb-GFP* mice that express GFP in $ObRb^+$ cells to examine which cellular population expresses leptin receptors. We found that the ObRb isoform was expressed in neurons, but not in astrocytes or microglia (Figure E3). To determine whether intranasal leptin bypasses leptin resistance and activates the ObRb receptor, brain slices of intranasal/intraperitoneal leptin or vehicle-treated mice were examined by pSTAT3immunostaining 1 hour after each treatment (Figure 6). Intranasal leptin induced STAT3 phosphorylation in the dorsomedial hypothalamus, ventromedial hypothalamus, and arcuate nucleus, which was not observed after vehicle or intraperitoneal leptin (Figures 6A and 6C). Intranasal leptin also induced STAT3 phosphorylation in the dorsal medulla, including dorsal motor nucleus of the vagus (DMV) and in the hypoglossal nucleus

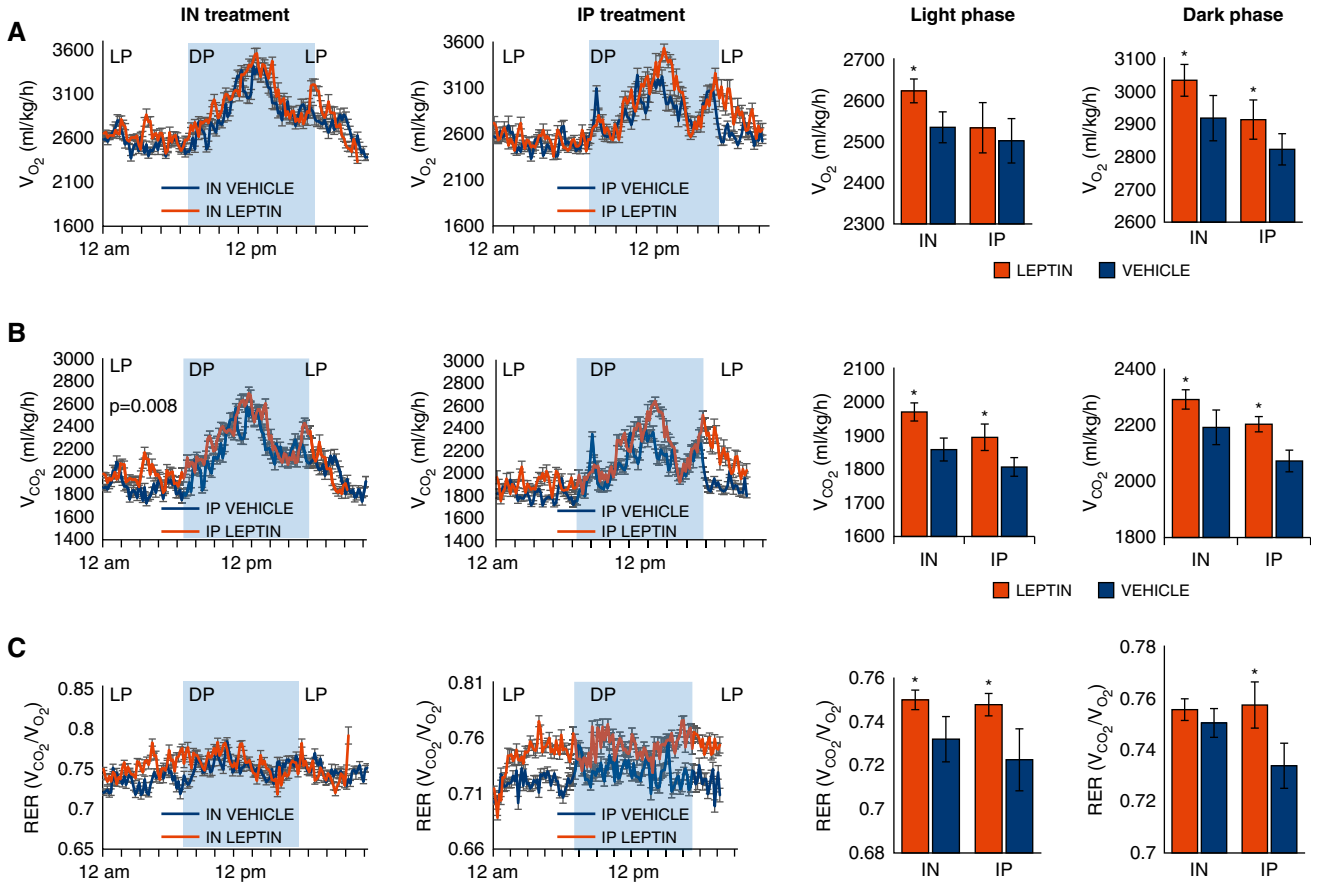

Figure 5. Effect of a single dose of intranasal (IN) and intraperitoneal (IP) leptin on metabolism in diet-induced obese (DIO) C57BL/6J mice. Determination of (*A*) oxygen consumption ($\dot{V}O_2$), (*B*) carbon dioxide production ($\dot{V}CO_2$), and (*C*) respiratory exchange ratio (RER) in DIO mice treated with IN or IP leptin versus vehicle controls over a 24-hour period, which included the 12-hour light and dark phases (mean ± SEM; $n = 10$ per group). The time course is presented on the left, whereas mean ± SEM during the light and dark phases are presented on the right. DP = dark phase; LP = light phase. *$P < 0.05$, between leptin and vehicle treatment.

(XII), and in the rostral ventrolateral medulla (RVLM; Figures 6B and 6C). Overall, intranasal leptin induced STAT3 phosphorylation throughout ObRb-expressing areas of the brain (Figures E4A–E4D), but not in all ObRb$^+$ cells (Figures E4E and E4F). In contrast, consistent with previous studies (33, 34), intraperitoneal leptin administration did not induce STAT3 phosphorylation in the hypothalamus and medulla (Figures 6A and 6B), despite very high plasma levels of leptin 1 hour after the intraperitoneal injection (Figure 6D). Intranasal leptin did not affect plasma leptin levels throughout the time course (Figure 6D).

Chronic Intranasal Leptin Reduces Food Intake and Body Weight in DIO Mice

We compared the effects of chronic intranasal and intraperitoneal leptin on food intake and body weight progression. DIO mice (age, 15 wk; weight, 36.4 ± 2.8 g, on high-fat diet) were treated with intranasal or intraperitoneal leptin (0.4 mg/kg) or BSA for 14 days ($n = 5$/group). Compared with the vehicle control groups, intranasal leptin treatment caused significant reductions in food intake and weight ($P = 0.001$), whereas intraperitoneal leptin had no effect (Figure 7). The intranasal vehicle group showed slower weight gain than the intraperitoneal vehicle group ($P < 0.05$), probably due to daily use of anesthesia.

Leptin Signaling in the Brain and Respiratory Motoneurons

To localize leptin signaling in the brain, a retrograde transneuronal tracer PRV263 was applied to either the diaphragm or genioglossus muscle of *ObRb-Cre* mice killed 96 hours later. In the medulla, motoneuron projections stained with dTomato reporter (red) changed color to mCerulean or EYFP reporters (cyan) in the presence of *Cre*-recombinase, indicating synaptic projections to ObRb$^+$ neurons (Figure 8A). Genioglossus infection resulted in numerous PRV263-positive neurons in the XII nerve, the nucleus ambiguus, DMV, and RVLM. Diaphragm infection with PRV263 also yielded positive red staining in the DMV, nucleus ambiguus, and RVLM, but not in the XII nucleus. Additional analysis using EYFP and mCerulean fluorescent filters demonstrated *Cre*-mediated recombination of the PRV263 genome (cyan staining) in the DMV and RVLM after both treatments and in the XII nucleus after genioglossus infection, indicating the presence of synaptic connections between respiratory motoneurons and ObRb$^+$ cells in the medulla (Figure 8A). In contrast, there was no *Cre*-mediated recombination of the

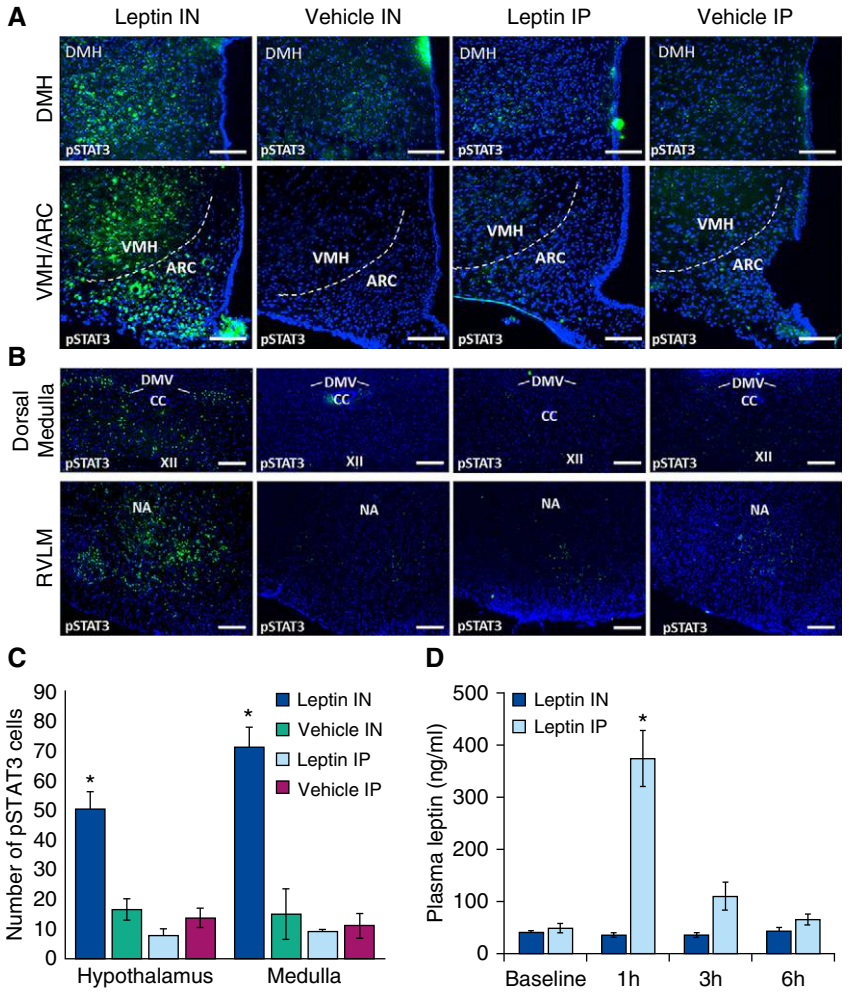

Figure 6. Effect of intranasal (IN) and intraperitoneal (IP) leptin on the leptin ObRb receptor signaling in the brain in diet-induced obese C57BL/6J mice. Representative photomicrographs of phosphorylated signal transducer and activator of transcription 3 (pSTAT3) (green) staining in the hypothalamus (*A*) and medulla (*B*) from mice 1 hour after IN or IP leptin injection (0.4 mg/kg) versus vehicle (BSA) control. Cell nuclei were stained with DAPI (blue). Scale bars = 100 μm; $n = 4$ for each treatment. (*C*) Quantification of pSTAT3-positive cells in the hypothalamus and medulla. Multiple sections from four mice were counted blindly, averaged, and presented as cells per section. (*D*) Plasma leptin levels 1, 3, and 6 hours after IN and IP leptin administrations, as compared with baseline; $n = 4$ for each treatment. $^*P < 0.05$. ARC = arcuate nucleu; CC = central canals; DMH = dorsomedial hypothalamus; DMV = dorsal motor nucleus of vagus; NA = nucleus ambiguus; RVLM = rostral lateral ventral medulla; VMH = ventromedial hypothalamus; XII = hypoglossal nucleus.

PRV263 genome in the medulla of wild-type mice (Figure 8C). PRV263 is a trans-synaptic tracer; therefore, synaptic connections between respiratory motoneurons and ObRb$^+$ cells may not be direct. To identify whether respiratory motoneurons express the leptin ObRb receptor, *ObRb-GFP* mice were infected with PRV263 and brain slices were analyzed after 96 hours, as described previously here (Figures 8C and 8D). As expected, infection of the genioglossus and diaphragm resulted in PRV263 projections (red) in DMV and RVLM. The ObRb$^+$ (GFP, green) cells were observed in immediate proximity to PRV263$^+$ cells. However, no colocalization of the PRV263 reporter (dTomato) and EGFP (enhanced green fluorescent protein) was detected.

Relationships between ObRb signaling and phrenic motoneurons were also examined. In the cervical spine of *ObRb-Cre* mice, in which the diaphragm was infected with PRV263 (Figure E5), dTomato-positive motoneurons were observed unilaterally on the side of diaphragm injection, but not counter-laterally (Figure E5). *ObRb-GFP* mouse showed very few ObRb$^+$ cells in the cervical spine sections (Figure E5C). However, phrenic motoneurons were not synaptically connected to ObRb$^+$ cells, because mCerulean or EYFP reporters (cyan) colors were not seen (Figure E5B).

Discussion

The main novel findings of the current study were that, in leptin-resistant DIO mice, acute intranasal leptin (1) successfully treated upper airway obstruction during sleep, increasing $\dot{V}_{I}$max during flow-limited breathing, decreasing the number of oxygen desaturation events in REM sleep and increasing $\dot{V}_E$ during flow-limited breathing (2); successfully treated sleep-related hypoventilation by increasing $\dot{V}_E$ during non–flow-limited breathing, suggesting a direct effect on control of breathing and respiratory pump muscles. These effects were associated with augmentation of the leptin ObRb receptor signaling in hypothalamic and medullary centers, suggesting that intranasal leptin bypassed the BBB. In contrast, intraperitoneal leptin had no impact on breathing, and did not induce leptin receptor signaling. In addition, we showed that respiratory motoneurons innervating the diaphragm and the genioglossus muscle did not express leptin receptors, but were synaptically connected to leptin receptor–expressing cells. In terms of metabolism, acute intranasal and intraperitoneal leptin both modestly increased $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$, suggesting that resistance to respiratory and metabolic effects of leptin has distinct patterns. Chronic intranasal leptin significantly decreased food intake and body weight in DIO mice, whereas intraperitoneal leptin had no effect.

Leptin Resistance, Intranasal Leptin, and OHS

In the absence of IFL, $\dot{V}_E$ is governed by basal metabolic rate according to the alveolar ventilation equation: alveolar gas or plasma partial pressure of $CO_2 \sim \dot{V}_{CO_2}/\dot{V}_E$. Our data show that intranasal leptin had a modest impact on $\dot{V}_{CO_2}$, increasing it by 5–6% (Figure 5), similar to intraperitoneal leptin. In contrast, intranasal leptin, but not intraperitoneal leptin, had a striking effect on NREM sleep

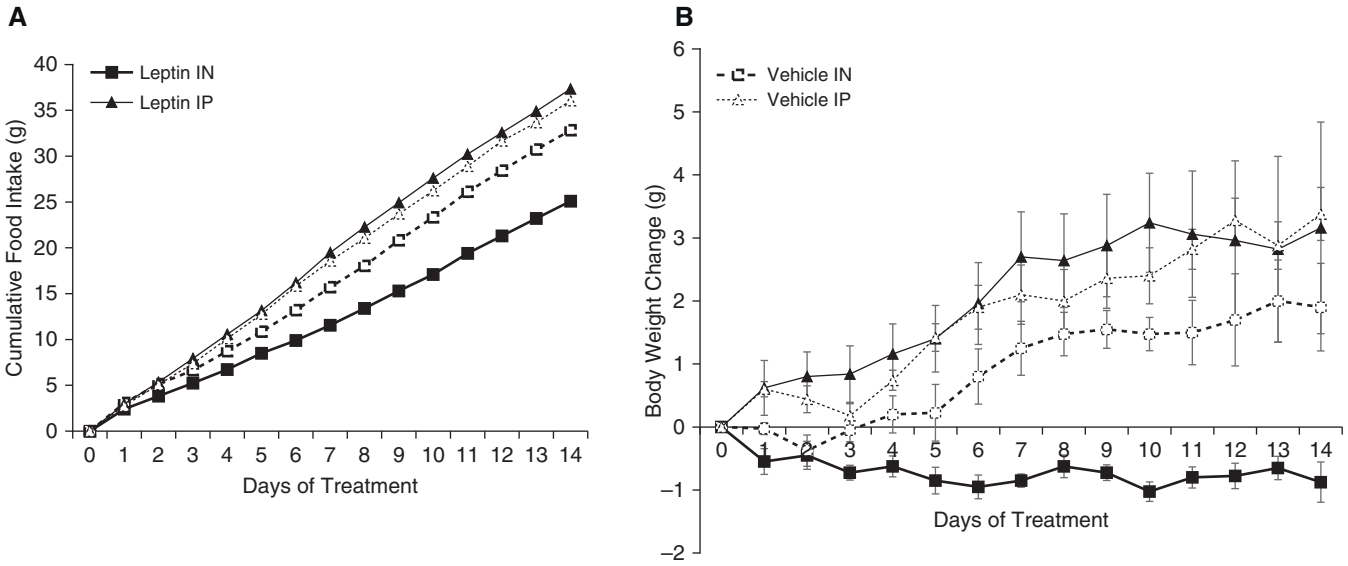

Figure 7. Effect of chronic intranasal (IN) and intraperitoneal (IP) leptin on food intake and body weight in diet-induced obese (DIO) C57BL/6J mice. Change in cumulative food intake (*A*) and body weight (*B*) of DIO mice treated daily for 14 days with IN or IP leptin versus vehicle (BSA) controls. Mice treated with IN leptin consumed less food and weighed significantly less at the end of the study ($P < 0.001$) compared with mice treated with IP leptin or BSA controls. IP leptin had no effect on food intake and body weight as compared with BSA control. Mice treated with IN vehicle had lower food intake and body weight as compared with IP vehicle ($P < 0.05$). (*A*) Data are presented as cumulative pooled food intake per cage, because each treatment group ($n = 5$ per group) was caged together. (*B*) Data from individual mice are presented as mean ± SEM; $n = 5$ in each group.

$\dot{V}_E$, increasing it by 40.1% (Figure 2). This increase in $\dot{V}_E$, out of proportion to $\dot{V}_{CO_2}$, strongly suggests that intranasal leptin, but not intraperitoneal leptin, acted as a powerful respiratory stimulant. The stimulating effect of leptin on respiratory drive was first demonstrated in leptin-deficient *ob/ob* mice, which have depressed HCVR and retain CO_2 during wakefulness, like patients with OHS (20, 35). Systemic and central leptin replacement in these animals increased HCVR and stimulated breathing in NREM and REM sleep (18, 19, 36).

In contrast to leptin-deficient mice, obese subjects with OHS and OSA have high circulating leptin levels (23, 24). Moreover, high fasting leptin levels were strongly associated with the presence of daytime hypercapnia, a defining criterion for OHS (26). We have recently shown that DIO mice with high circulating levels of leptin hypoventilate during sleep and wakefulness, showing significantly higher awake CO_2 levels than lean mice (28). Taken together, these data suggest that leptin resistance is implicated in the pathogenesis of OHS and that leptin resistance can be overcome by intranasal leptin.

Leptin transport from the bloodstream to the brain normally occurs via a saturable mechanism in the choroid plexus. Limited permeability of the BBB for leptin has been previously identified as a key mechanism of resistance to metabolic effects of leptin (14–16). The intranasal route leads to perineural and perivascular transport, which allows direct transfer of macromolecules from nasal passages to the cerebrospinal fluid, avoiding the BBB (37–39). We did not measure leptin levels in the brain. Instead, we examined leptin signaling via the ObRb receptor by determining leptin-induced STAT3 phosphorylation (11). STAT3 phosphorylation is a universally accepted indicator of leptin activity (40). Intranasal leptin dramatically increased STAT3 phosphorylation in the brain, which did not occur with intraperitoneal leptin (Figure 6). Our data are consistent with the previous reports that intranasal leptin is directly transported to the brain, circumventing bloodstream and the BBB (41).

In our study, intranasal leptin, but not intraperitoneal leptin, increased $\dot{V}_E$, whereas $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were augmented by both routes of administration. Experiments with intranasal administration of iodinated radioactive leptin showed that the half-life of leptin in the hypothalamus and medulla is between 60 minutes and 2 hours (41). It is conceivable that intraperitoneal leptin's penetration into the brain is slower than intranasal leptin's, which would explain the lack of respiratory effects measured 6 hours after injection and pSTAT3 effects measured 1 hour after injection, whereas metabolic measurements were performed for 24 hours. Regardless, our findings suggest that metabolic and respiratory effects of leptin are differentially regulated. Thus, intranasal leptin may circumvent resistance to respiratory central effects of leptin and treat sleep-related hypoventilation in obesity, and that respiratory effects of leptin are independent of metabolic effects of this hormone.

Leptin Resistance, Intranasal Leptin, and OSA

DIO mice showed significant upper airway obstruction and recurrent oxyhemoglobin desaturation during REM sleep, indicating sleep apnea (28). Our laboratory previously demonstrated that mouse obesity and leptin deficiency induced a defect in upper airway neuromuscular control and recurrent obstructive hypopneas during REM sleep, which were reversed by leptin infusion (17, 18). However, DIO mice showed obstructive REM sleep hypopneas (28), similar to obese humans, despite high levels of circulating leptin (23). These findings may suggest that leptin resistance plays a role in the pathogenesis of OSA (4, 5). Intranasal leptin reversed upper airway obstruction during sleep in DIO mice by increasing $\dot{V}_{I}$max, whereas intraperitoneal

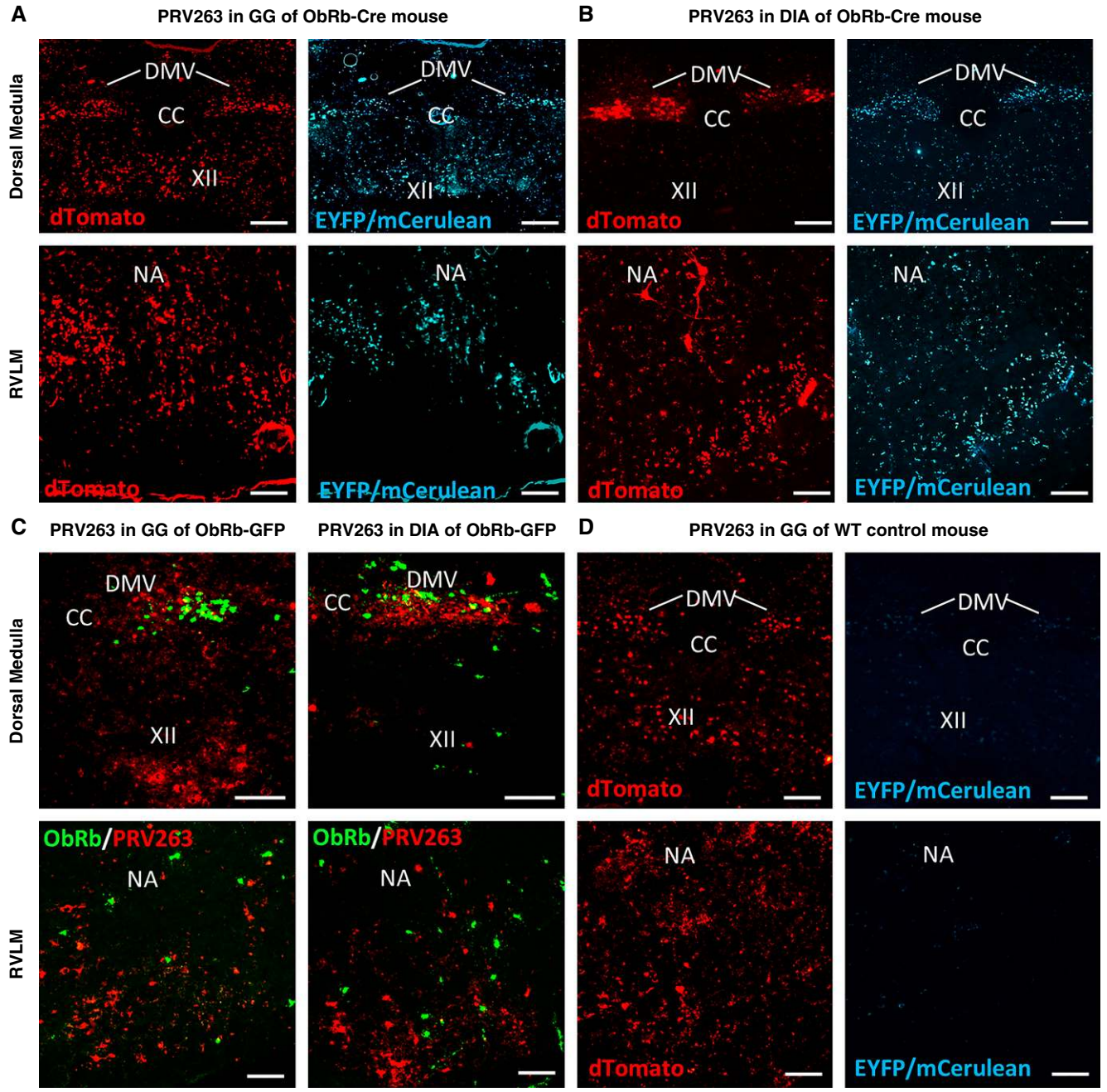

Figure 8. Relationships between motoneurons innervating genioglossus (GG) or diaphragm (DIA) and the leptin ObRb receptor. To establish whether ObRb-positive cells have synaptic connections with respiratory motoneurons, the Bartha strain of pseudorabies virus (PRV263) containing Brainbow cassette was injected in the (*A*) GG muscles ($n = 3$) or topically applied to the inferior surface of the (*B*) DIA ($n = 3$) of *ObRb-Cre* mice. Neurons involved in the upper airway function or respiratory control were detected in various nuclei of dorsal and rostral lateral ventral medulla (RVLM) by expression of dTomato reporter (red). Synaptic connection to ObRb signaling cells was observed by detection of *Cre*-dependent recombination of the viral genome and expression of EYFP/mCerulean (cyan) reporter in the areas of PRV263 infection. (*C*) To establish whether respiratory motoneurons express the leptin ObRb receptor, PRV263 was injected in the GG muscles ($n = 3$) or topically applied to the inferior surface of the DIA ($n = 3$) of *ObRb-GFP* mice. ObRb-positive cells (green) and PRV263-infected cells (red) were detected in dorsal medulla and RVLM. However, no significant colocalization was observed. (*D*) In the control experiment, PRV263 was injected into genioglossal muscles of wild-type C57BL/6J mice ($n = 3$). dTomato reporter (red)–expressing neurons were detected in the dorsal medulla and RVLM, as expected. However, no Cre-dependent recombination of the viral genome or expression of EYFP/mCerulean (cyan) reporter was observed. CC = central canal; DMV = dorsal motor nucleus of the vagus; NA = nucleus ambiguus; WT = wild-type; XII = hypoglossal nucleus. Scale bars = 100 μm.

leptin had no effect. Taking together with pSTAT3 data showing ObRb signaling in the brain with intranasal, but not intraperitoneal, treatment, our findings indicate that leptin resistance at the BBB may be implicated in the pathogenesis of OSA and that the effect of leptin resistance on upper airway function is reversed by intranasal administration of the hormone.

Intranasal leptin relieved upper airway obstruction during sleep by increasing $\dot{V}$Imax without an effect on IFL prevalence. This apparent discrepancy is likely related to complexity of respiratory effects of leptin. As we discussed previously here, intranasal leptin increased ventilatory drive. As ventilatory drive increases, the upper airway is exposed to increasingly negative tracheal pressures. The latter could actually increase the prevalence of IFL. This increase in IFL prevalence, however, was mitigated by concomitant increases in $\dot{V}$Imax, indicating reductions in pharyngeal collapsibility. Thus, our study characterizes responses to leptin with primary effects on ventilatory drive and upper airway collapsibility and an integrated effect on IFL prevalence, resulting in an apparent discrepancy between increases in $\dot{V}$Imax and $\dot{V}$E and IFL prevalence.

Putative Mechanisms of Respiratory Effects of Leptin

Previous experiments in anesthetized rodents showed that leptin activates neurons in brain sites essential for breathing, including the nucleus of the solitary tract, RVLM, and possibly the retrotrapezoid nucleus, key central, chemosensitive areas (21, 22, 42). The melanocortin pathway has been identified as a putative downstream mechanism (43). However, it is not clear how leptin may affect respiratory motoneurons. Our experiment with the transneuronal tracer harboring a Brainbow cassette demonstrated synaptic connections between hypoglossal and phrenic motoneurons and ObRb-expressing cells. However, a complementary experiment in *ObRb-GFP* mice showed that respiratory motoneurons lack ObRb receptors. All in all, these data indicate that both diaphragm and upper airway respiratory neurons do not express the ObRb receptor, but, rather, interact with $ObRb^+$ cells.

Study Limitations

Our study had several limitations. First, it is possible that intranasal leptin will be less effective in humans due to differences in the anatomy of the cribriform plate and the olfactory bulb. Second, intranasal administration of leptin required anesthesia, which may have had an impact on respiration, food intake, and weight gain. We used the intranasal vehicle control to mitigate this limitation. Third, intranasal leptin decreased severity of upper airway obstruction during sleep and REM ODI, but IFL prevalence was not affected. This finding does not diminish the significance of our data. By analogy, in humans, it may mean converting apneas to hypopneas, hypopneas to simple snoring, or snoring to normal breathing. Fourth, although we provide evidence that intranasal leptin induced ObRb signaling in the hypothalamus and in the respiratory nuclei of medulla that synaptically connected to respiratory motoneurons, specific mechanisms and sites of intranasal leptin's effect were not identified. Fifth, both intranasal and intraperitoneal leptin increased energy expenditure, but only intranasal leptin induced weight loss. However, intranasal leptin, but not intraperitoneal leptin, decreased food intake. In DIO mice, chronic intraperitoneal leptin may have differential effects on food intake and energy expenditure (44). Sixth, leptin inexplicably increased RER. Although statistically significant, these increases were minimal, and probably physiologically irrelevant, given that RER remained in the low range, reflecting preferential utilization of fat. Finally, in the current study, we treated mice with a single dose of leptin chosen based on our experience with intracebroventricular injections (18). Further studies are needed to examine the dose response and effects of chronic intranasal treatment on sleep-disordered breathing.

Conclusions and Clinical Implications

In summary, our data showed that intranasal leptin attenuated sleep-disordered breathing in DIO mice, independent of metabolism. This study provides a proof of principle for novel human pharmacotherapy for obesity-associated sleep-disordered breathing. Clinical trials are needed to examine the efficacy of intranasal leptin delivery in humans. All in all, our study suggests that intranasal leptin should be tested as a potential therapy in patients with OSA and OHS. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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