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Intranasal leptin relieves sleep disordered breathing in mice with diet induced obesity

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14.5 Pediatric Sleep and Control of Breathing

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15.7 Sleep Disordered Breathing: Epidemiology

15.8 Sleep Disordered Breathing: Management

15.9 Sleep Disordered Breathing: Outcomes

15.10 Sleep: Other

8.28 Upper Airway: Sleep

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 are leptin resistant due to, at least in part, limited permeability of the blood-brain barrier (BBB) to leptin. Intranasal (IN) leptin delivery bypasses the BBB and activates leptin signaling in the brain leading to weight loss, yet the therapeutic effect of IN 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 diet-induced obesity, a single dose of IN leptin successfully treats upper airway obstruction and hypoventilation during sleep, independent of metabolism, whereas intraperitoneal (IP) leptin has no respiratory effect. IN leptin, but not IP 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 IN leptin can be tested as a potential therapy in patients with SDB

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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 are resistant to leptin due to 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 diet-induced obesity. *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 bovine serum albumin (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 NREM and REM sleep, independently of metabolic effects. Chronic intranasal leptin decreased food intake and body weight, while 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 diet-induced obesity, intranasal leptin bypassed leptin resistance and significantly attenuated sleep-disordered breathing, independently of body weight.

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Introduction

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 >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 signal transducer and activator of transcription 3 (STAT3) (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 suggest 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

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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 (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 showed 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 (IN) bypassing the BBB (27).

The major goal of this translational study was to examine effects of IN 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 IN 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 IN *vs* intraperitoneal (IP) leptin or vehicle; 2) determined effects of IN *vs* IP 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 (33).

Materials and Methods

In total, forty-eight 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 twelve *ObRb-GFP* mice expressing green fluorescent protein 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 the 12h-light/dark cycle (9 am–9 pm 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 Online Supplement. Briefly, to study effects of IN leptin on SDB and metabolism, DIO mice were treated with a single dose of IN or IP leptin or vehicle in a randomized crossover study. Leptin signaling in the hypothalamus and medulla was assessed by pSTAT3 immunofluorescence. To examine effects of chronic IN 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 IN and IP routes using the Kruskal-Wallis test for respiratory outcomes and mixed effects linear regression for metabolic measurements.

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Results

Effects of a Single Dose of IN and IP Leptin on Basic Characteristics and Sleep Architecture

DIO mice were of similar age and body weight in all treatment groups, IN leptin, IN vehicle, IP leptin, and IP vehicle (Table E1). High baseline plasma leptin levels (48.7±8.6 ng/ml) suggested resistance to leptin's metabolic effects. Acute IN or IP leptin administration had no effect on body temperature compared to the vehicle (Table E1). Sleep recordings were performed from 11 am to 5 pm. The sleep architecture was similar to previously described in C57BL/6J mice (34) and there was no difference between all four groups of mice (Figure E1, Table E2).

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

IFL breathing is a cardinal feature of OSA in humans and mice alike (18,35). We 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 NREM sleep showed that, compared to the vehicle, IN leptin increased minute ventilation (V_E) from 0.61 to 0.86 ml/min/g during NREM sleep ($p<0.05$) and from 0.51 to 0.72 ml/min/g in REM sleep ($p<0.05$) (Figures 1,2). Tidal volumes trended to be higher after IN leptin, whereas respiratory rate was unchanged (not shown). IP 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 IN nor IP leptin treatments affected IFL prevalence (Figure 4A). However, IN leptin treatment significantly decreased ODI4% 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 tidal volume, and increased respiratory effort. These abnormalities improved after IN leptin treatment. Compared to IN vehicle (BSA), IN leptin significantly alleviated IFL severity, by increasing maximal inspiratory flow (V_{Imax}) 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). IN leptin also increased minute ventilation during IFL breathing in NREM and REM sleep (Figure 4D). The effect of IN leptin on ventilation during flow-limited breathing was entirely related to significant increases in tidal volume but not in respiratory rate (Figure E2). IP leptin had no effect on the ODI, V_{Imax}, or V_E (Figure 4).

Effect of a Single Dose of IN Leptin on Metabolism in DIO mice

$\dot{V}_{O2}$ and $\dot{V}_{CO2}$ were measured for 24hs. As expected, $\dot{V}_{O2}$ and $\dot{V}_{CO2}$ were higher during the dark period, when mice are mostly awake, compared to the light period, when animals are mostly asleep (Figures 5A,B). IN leptin induced modest increases in $\dot{V}_{O2}$ and $\dot{V}_{CO2}$ during both light and dark periods; 1.03 and 1.04-fold increases respectively for $\dot{V}_{O2}$, and 1.05 and 1.06 increases respectively for $\dot{V}_{CO2}$. IP leptin demonstrated a delayed effect on $\dot{V}_{O2}$ stimulating it only during the dark phase, while $\dot{V}_{CO2}$ was slightly increased both during light and dark phases (Figures 5A and B). Small increases of the respiratory exchange ratio (RER) were noted after IN leptin during

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the light period (from 0.73 to 0.75) and IP leptin during both light and dark period (from 0.72 to 0.74 and from 0.73 to 0.75 respectively, Figures 5C).

IN 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 IN leptin bypasses leptin resistance and activates the ObRb receptor, brain slices of IN/IP leptin or vehicle-treated mice were examined by pSTAT3immunostaining 1 h after each treatment (Figure 6). IN leptin induced STAT3 phosphorylation in the dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), and arcuate nucleus (ARC), which was not observed after vehicle or IP leptin (Figure 6A,C). IN leptin also induced STAT3 phosphorylation in the dorsal medulla, including dorsal motor-nucleus of the vagus (DMV) and in the hypoglossal nucleus (XII), and in the rostral ventrolateral medulla (RVLM, Figure 6B,C). Overall, IN leptin induced STAT3 phosphorylation throughout ObRb expressing areas of the brain (Figure E4, A-D), but not in all $ObRb^+$ cells (Figure E4 E,F). In contrast, consistent with previous studies (36,37), IP leptin administration did not induce STAT3 phosphorylation in the hypothalamus and medulla (Figure 6A,B), despite very high plasma levels of leptin 1 h after the IP injection (Figure 6D). IN leptin did not affect plasma leptin levels throughout the time course (Figure 6D).

Chronic IN Leptin Reduces Food Intake and Body Weight in DIO mice

We compared the effects of chronic IN and IP leptin on food intake and body weight progression. DIO mice (age 15 weeks, weight 36.4±2.8g, on HFD) were treated with IN or IP leptin (0.4 mg/kg) or BSA or 14 days (n=5/group). Compared to the vehicle control groups, IN leptin treatment caused significant reductions in food intake and weight ($p=0.001$), while IP leptin had no effect (Figure 7). The IN vehicle group showed slower weight gain than the IP 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 sacrificed 96 hs 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 hypoglossal nucleus (XII nerve), the nucleus ambiguous (NA), DMV, and RVLM. Diaphragm infection with PRV263 also yielded positive red staining in the DMV, NA, 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 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 PRV263 genome in the medulla of wild-type mouse (Figure 8C). PRV263 is a transsynaptic tracer, therefore synaptic connections between respiratory motoneurons and $ObRb^+$ cells may not

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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 above (Figure 8C,D). 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 the immediate proximity to $PRV263^+$ cells. However, no co-localization of the PRV263 reporter (dTomato) and EGFP was detected.

Relationships between ObRb signaling and phrenic motoneurons were also examined. In the cervical spine of *ObRb-Cre* mice, in whom 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, in leptin resistant DIO mice, acute IN leptin (1) successfully treated upper airway obstruction during sleep increasing maximal inspiratory flow ($\dot{V}_{Imax}$) during flow limited breathing, decreasing the number of oxygen desaturation events in REM sleep and increasing minute ventilation during flow limited breathing; (2) successfully treated sleep-related hypoventilation by increasing minute ventilation 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 IN leptin bypassed the BBB. In contrast, IP 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 IN and IP leptin both modestly increased $\dot{V}_{O2}$ and $\dot{V}_{CO2}$, suggesting that resistance to respiratory and metabolic effects of leptin has distinct patterns. Chronic IN leptin significantly decreased food intake and body weight in DIO mice, while IP leptin had no effect.

Leptin Resistance, IN Leptin and OHS

In the absence of IFL, V_E is governed by basal metabolic rate according to the alveolar ventilation equation, alveolar gas or plasma partial pressure of CO_2 ($PaCO_2$) ~ $\dot{V}_{CO2}/V_E$. Our data showed that IN leptin had a modest impact on $\dot{V}_{CO2}$ increasing it by 5-6% (Figure 5), similar to IP leptin. In contrast, IN leptin, but not IP leptin, had a striking effect on NREM sleep V_E increasing it by 40.1% (Figure 2). This out of proportion to $\dot{V}_{CO2}$ increase in V_E strongly suggests that IN leptin, but not IP 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 (20,38). Systemic and central leptin replacement in these animals increased HCVR and stimulated breathing in NREM and REM sleep (18,19,39).

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

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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, this data suggest that leptin resistance is implicated in the pathogenesis of OHS and that leptin resistance can be overcome by IN 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 (14,16,40). The IN route leads to perineural and perivascular transport, which allows direct transfer of macromolecules from nasal passages to the CSF, avoiding the BBB (41-43). 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 (44). IN leptin dramatically increased STAT3 phosphorylation in the brain, which did not occur with IP leptin (Figure 6). Our data are consistent with the previous reports that IN leptin is directly transported to the brain circumventing bloodstream and the BBB (45).

In our study, IN leptin, but not IP leptin, increased minute ventilation, whereas $\dot{V}_{O2}$ and $\dot{V}_{CO2}$ were augmented by both routes of administration. Experiments with IN administration of iodinated radioactive leptin showed that the half-life of leptin in the hypothalamus and medulla is between 60 min and 2 hrs (42). It is conceivable that IP leptin's penetration into the brain is slower than IN leptin's, which would explain the lack of respiratory effects measured 6 hs after injection and pSTAT3 effects measured 1h after injection, whereas metabolic measurements were performed for 24 hs. Regardless, our findings suggest that metabolic and respiratory effects

of leptin are differentially regulated. Thus, IN 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, IN 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). IN leptin reversed upper airway obstruction during sleep in DIO mice by increasing V_{Imax}, whereas IP leptin had no effect. Taking together with pSTAT3 data showing ObRb signaling in the brain with IN but not IP 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 IN administration of the hormone.

IN leptin relieved upper airway obstruction during sleep by increasing maximal inspiratory flow without an effect on IFL prevalence. This apparent discrepancy is likely related to complexity of respiratory effects of leptin. As we discussed above, IN 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 V_{Imax}, indicating

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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 V_{Imax} and 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 (21,22,46). The melanocortin pathway has been identified as a putative downstream mechanism (47). 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 IN leptin will be less effective in humans due to differences in the anatomy of the cribriform plate and the olfactory bulb. *Second*, IN administration of leptin required anesthesia, which may have had an impact on respiration, food intake and weight gain. We used the IN vehicle control to mitigate this limitation. *Third*, IN

leptin decreased severity of upper airway obstruction during sleep and REM ODI, but IFL prevalence was not affected. This finding does not diminish significance of our data. By analogy in humans, it may mean converting of apneas to hypopneas, hypopneas to simple snoring or snoring to normal breathing. *Fourth*, although we provide evidence that IN 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 IN leptin's effect were not identified. *Fifth*, both IN and IP leptin increased energy expenditure, but only IN leptin induced weight loss. However, IN leptin, but not IP leptin decreased food intake. In DIO mice chronic IP leptin may have differential effects on food intake and energy expenditure (48). *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 IN 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 IN leptin should be tested as a potential therapy in patients with OSA and OHS.

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Figure legends

Figure 1. A representative sleep recording in the diet-induced obese mouse during non-rapid eye movement (NREM) sleep treated with intranasal bovine serum albumin alone (**IN VEHICLE, Left panel)** or intranasal leptin dissolved in BSA (**IN LEPTIN, Right panel)**. Electroencephalogram (EEG), nuchal electromyogram (EMG, arbitrary units, a.u.), pulse oximetry (SpO_2), respiratory flow and breathing effort (a.u., arbitrary units) were recorded simultaneously. *EXPIR* denotes expiration and *INSP* denotes inspiration.

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 minute ventilation (V_E/g of body weight) in individual mice during unobstructed breaths (ventilatory control, non-flow-limited breaths) in **(A)** non-rapid eye movement (NREM) and **(B)** REM sleep. The whiskers denote the second and third quartile 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 due 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.

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Figure 3. A representative sleep recording in the diet-induced obese mouse during rapid eye movement (REM) sleep treated with intranasal bovine serum albumin alone (**VEHICLE, Left panel)** or intranasal leptin dissolved in BSA (**LEPTIN, Right panel)**. Compressed recordings of electroencephalogram (EEG), nuchal electromyogram (EMG), pulse oximetry (SpO_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. *EXPIR* denotes expiration and *INSP* denotes 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-rapid eye movement (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 SpO_2 desaturations ≥ 4% from the baseline per hour of sleep (ODI4%) was measured; **(C)** The severity of upper airway obstruction was characterized as the maximal inspiratory flow (V_{Imax} IFL) ; **(D)** Minute ventilation (V_E/g of body weight) during flow-limited (obstructive) breathing was measured. Each dot represents data per one animal. The whiskers denote the second and third quartile divided by the median. *, $p < 0.05$ for the difference between IN leptin and IN vehicle (BSA) treatments. One mouse was excluded due 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.

Figure 5. Effect of a single dose of intranasal (IN) and intraperitoneal (IP) leptin on metabolism in diet-induced obese C57BL/6J mice. Determination of **(A)** $\dot{V}_{O2}$, **(B)** $\dot{V}_{CO2}$ and **(C)** Respiratory exchange ratio (RER) in DIO mice treated with IN or IP leptin vs vehicle controls over a 24-h period, which included the 12-h light and dark phases (mean±SEM., n=10 per group). The time course presented on the left, whereas mean values ± SEM during the light and dark phases are presented on the right. LP – light phase; DP – dark phase; *p < 0.05, between leptin and vehicle treatment.

Figure 6. Effect of IN and IP leptin on the leptin ObRb receptor signaling in the brain in diet-induced obese C57BL/6J mice. Representative photomicrographs of phosphorylated STAT3 (green) staining in the hypothalamus **(A)** and medulla **(B)** from mice 1 hour after IN or IP leptin injection (0.4 mg/kg) *vs* vehicle (BSA) control. DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus; ARC, arcuate nucleus; DMV, dorsal motor nucleus of vagus; XII, hypoglossal nucleus; CC, central canal. Cell nuclei were stained with DAPI (blue). Bar scales - 100μm. 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 to baseline, n = 4, for each treatment. *p<0.05.

Figure 7. Effect of chronic IN and IP leptin on food intake and body weight in diet-induced obese 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 *vs* 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 to mice treated with IP

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leptin or BSA controls. IP leptin had no effect on food intake and body weight as compared to BSA control. Mice treated with IN vehicle had lower food intake and body weight as compared to IP vehicle ($p<0.05$). In A, data are presented as cumulative pooled food intake per cage since each treatment group (n = 5 per group) was caged together. In B, data from individual mice are presented as mean ± SEM. N = 5 in each group.

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)** genioglossus muscles (n=3) or topically applied to the inferior surface of the **(B)** diaphragm (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 genioglossus muscles (n=3) or topically applied to the inferior surface of the diaphragm (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 dorsal medulla and RVLM, as expected. However, no Cre-dependent recombination of the viral genome and expression of EYFP/ mCerulean (cyan) reporter was observed. XII - Hypoglossal

nucleus; DMV, Dorsal Motor Nucleus of the Vagus; NA, Nucleus Ambiguous; CC, central canal. Bar scales - 100µm.

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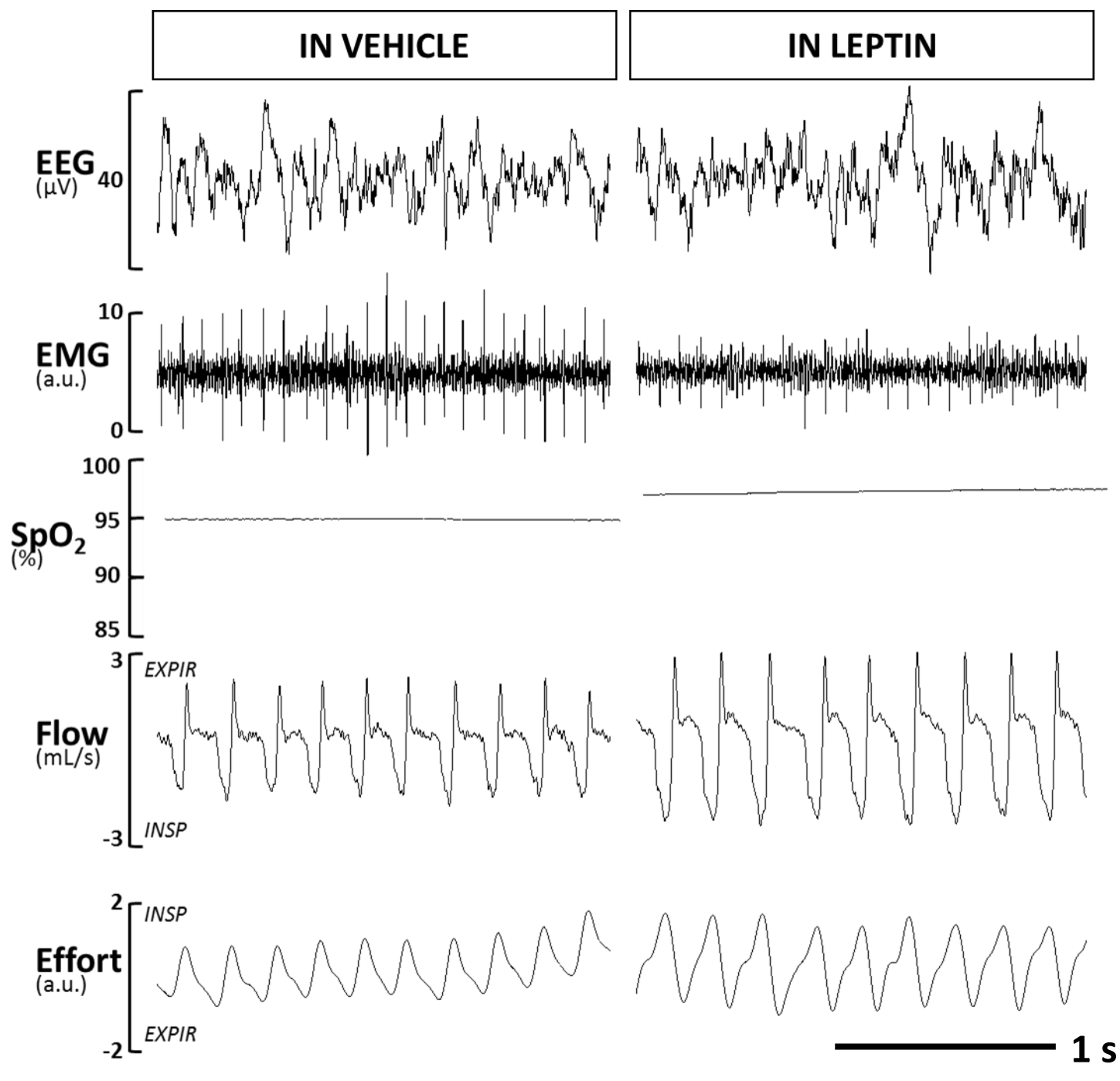

Figure 1.

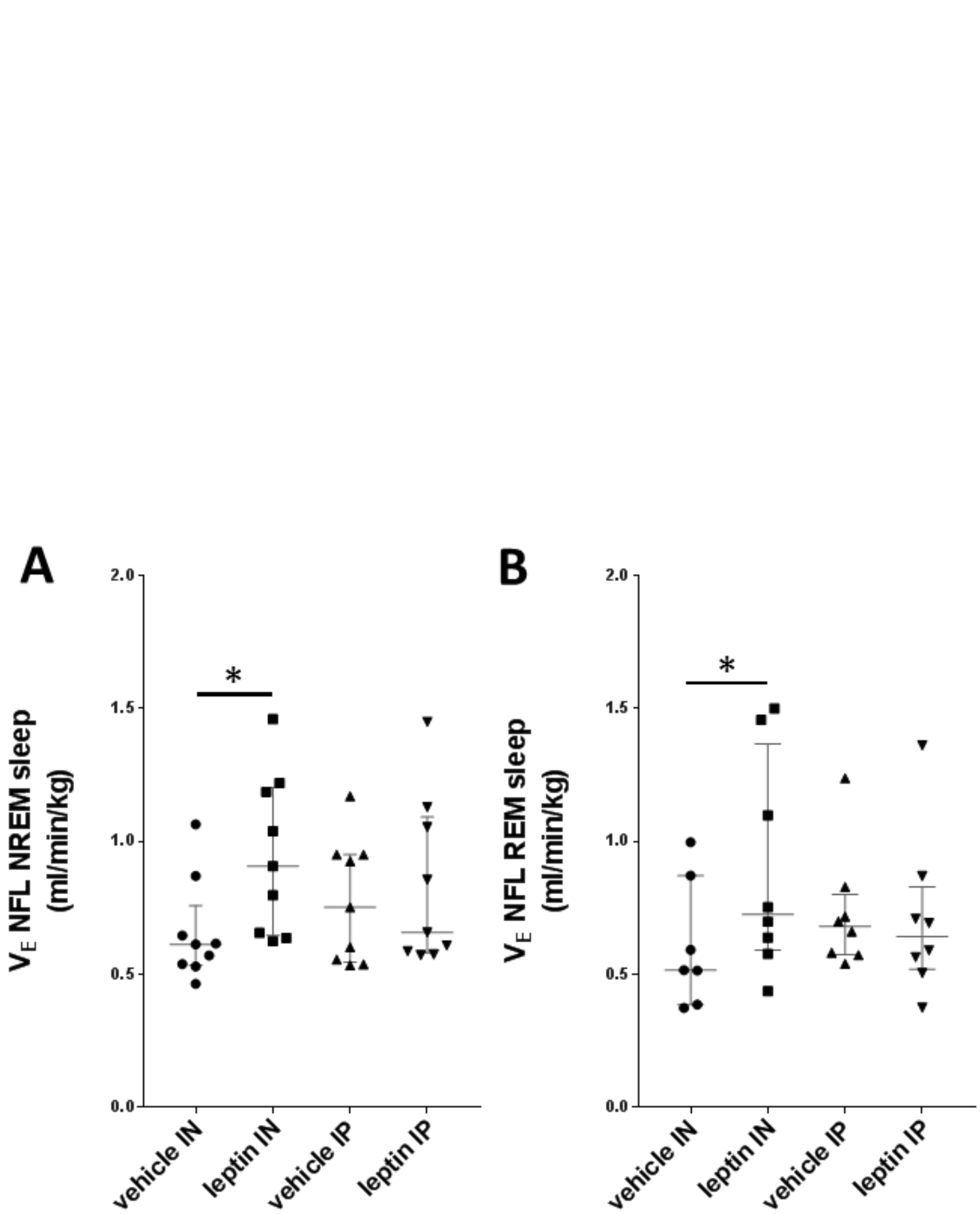

Figure 2

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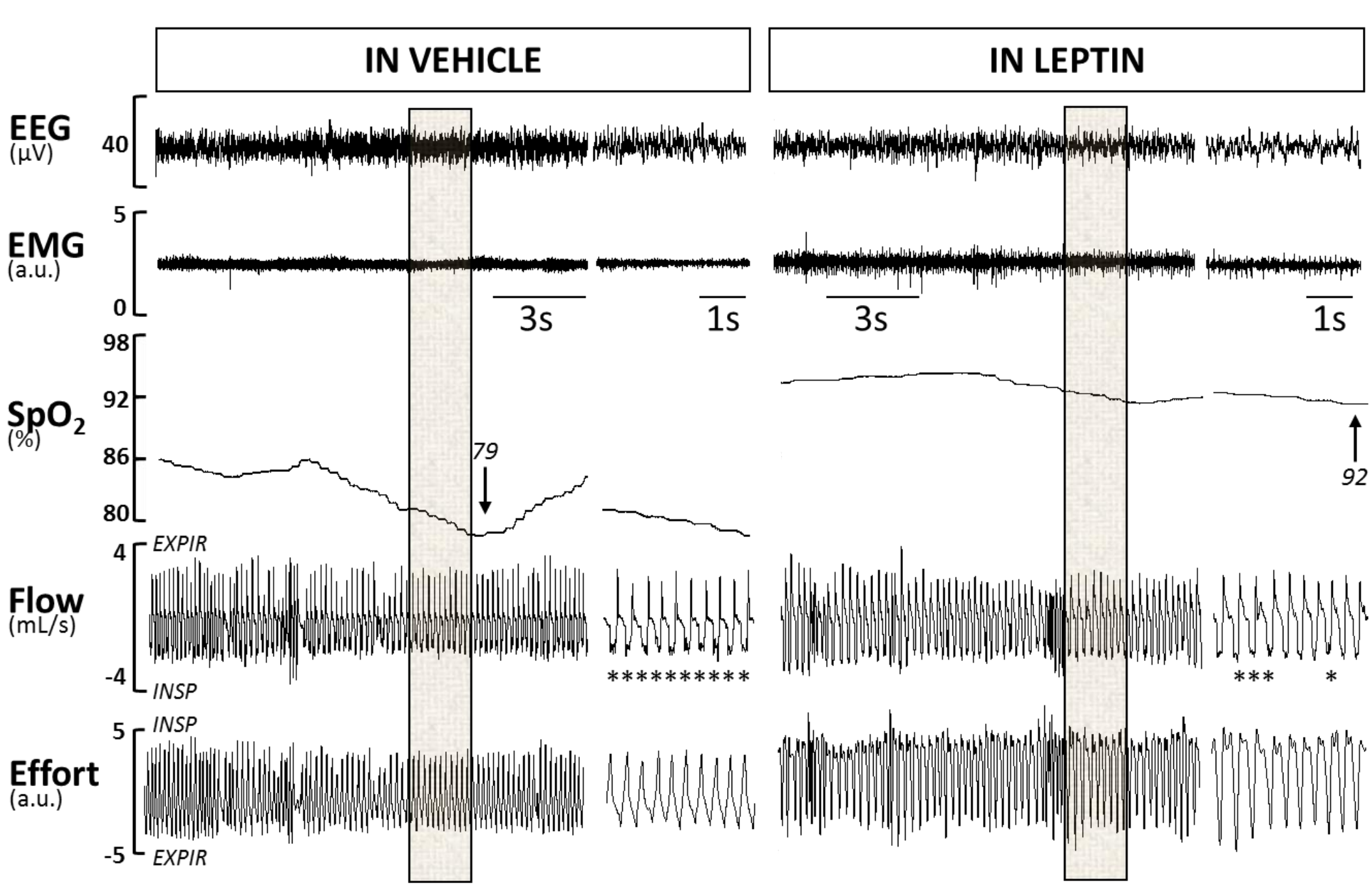

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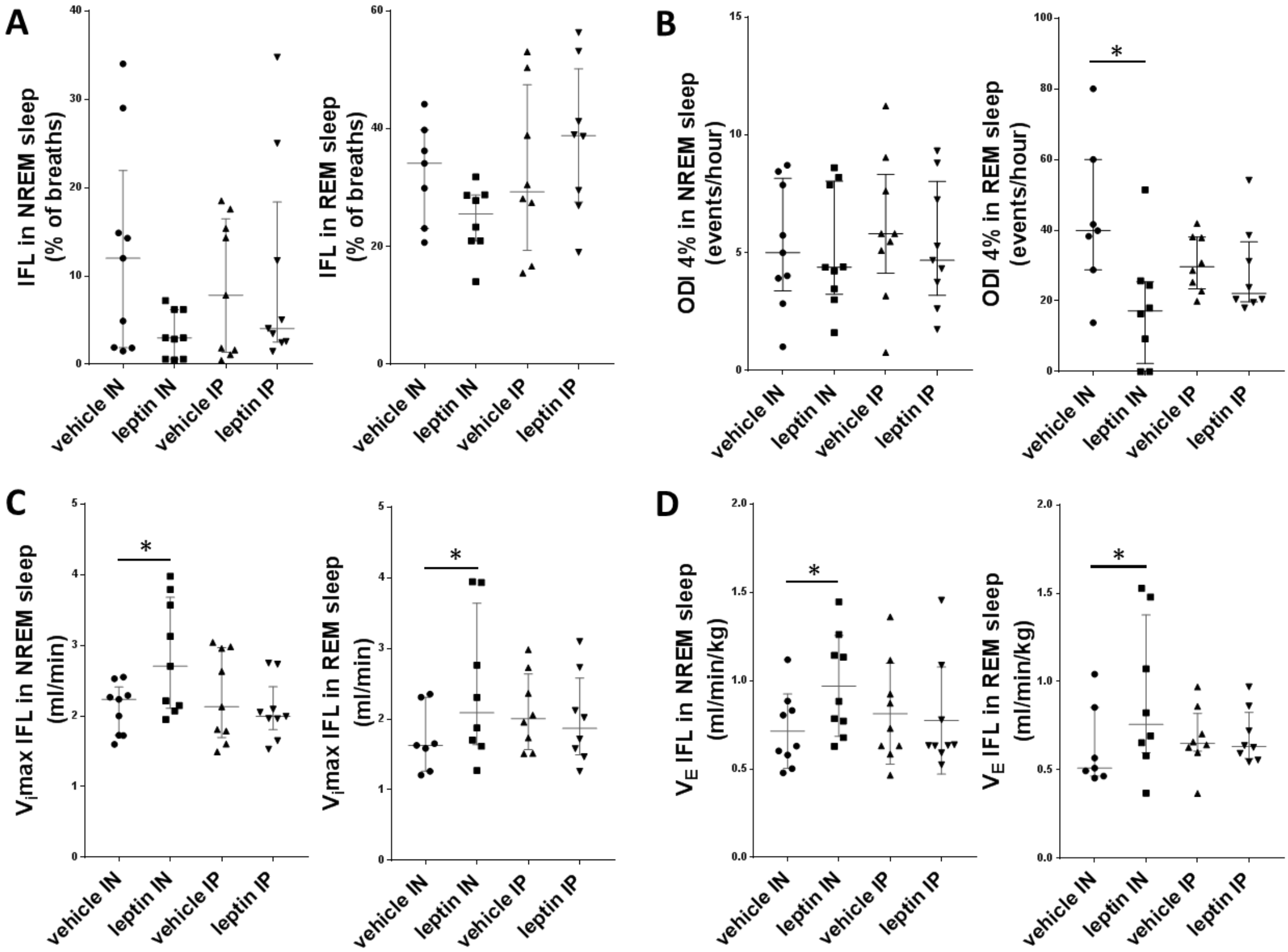

Figure 4.

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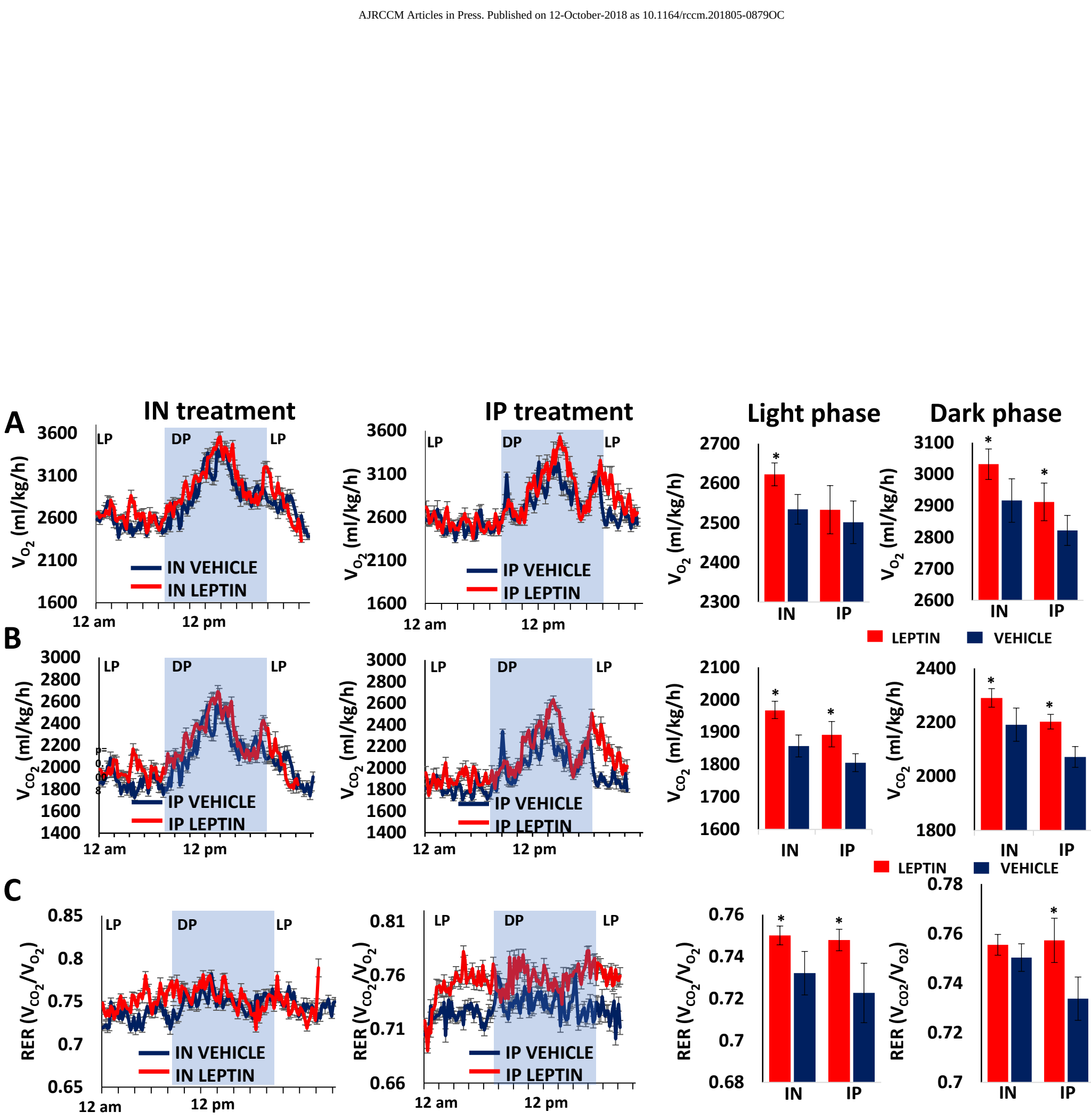

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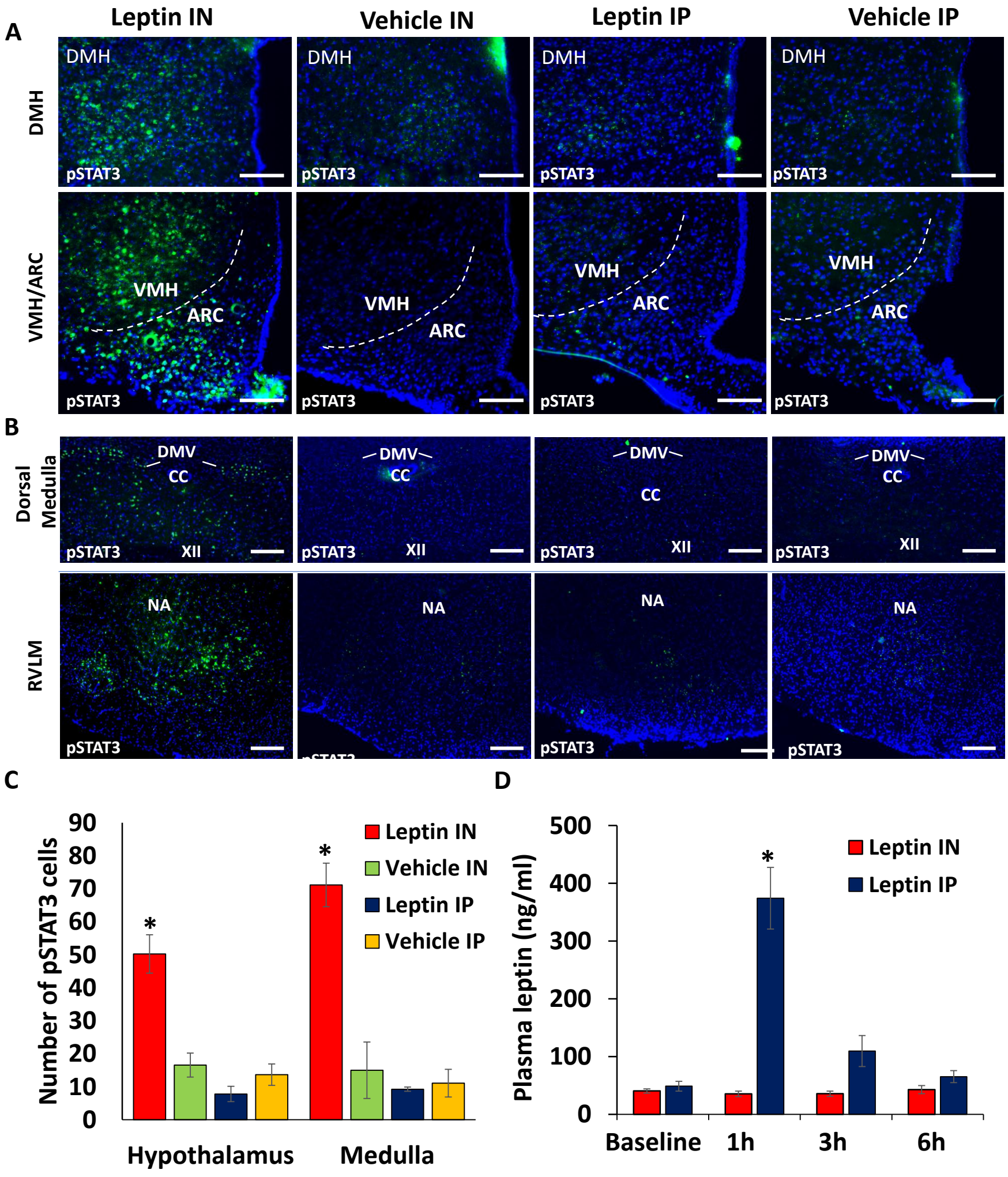

Figure 6.

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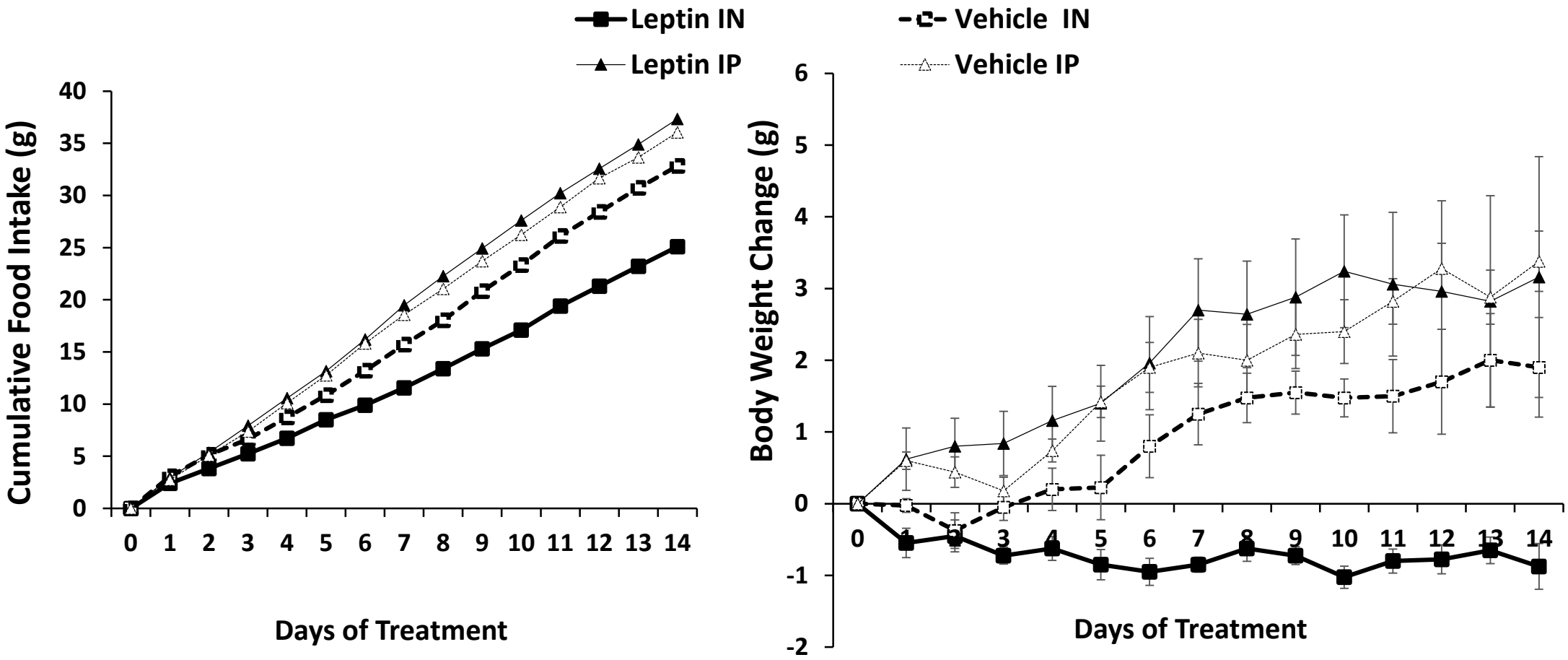

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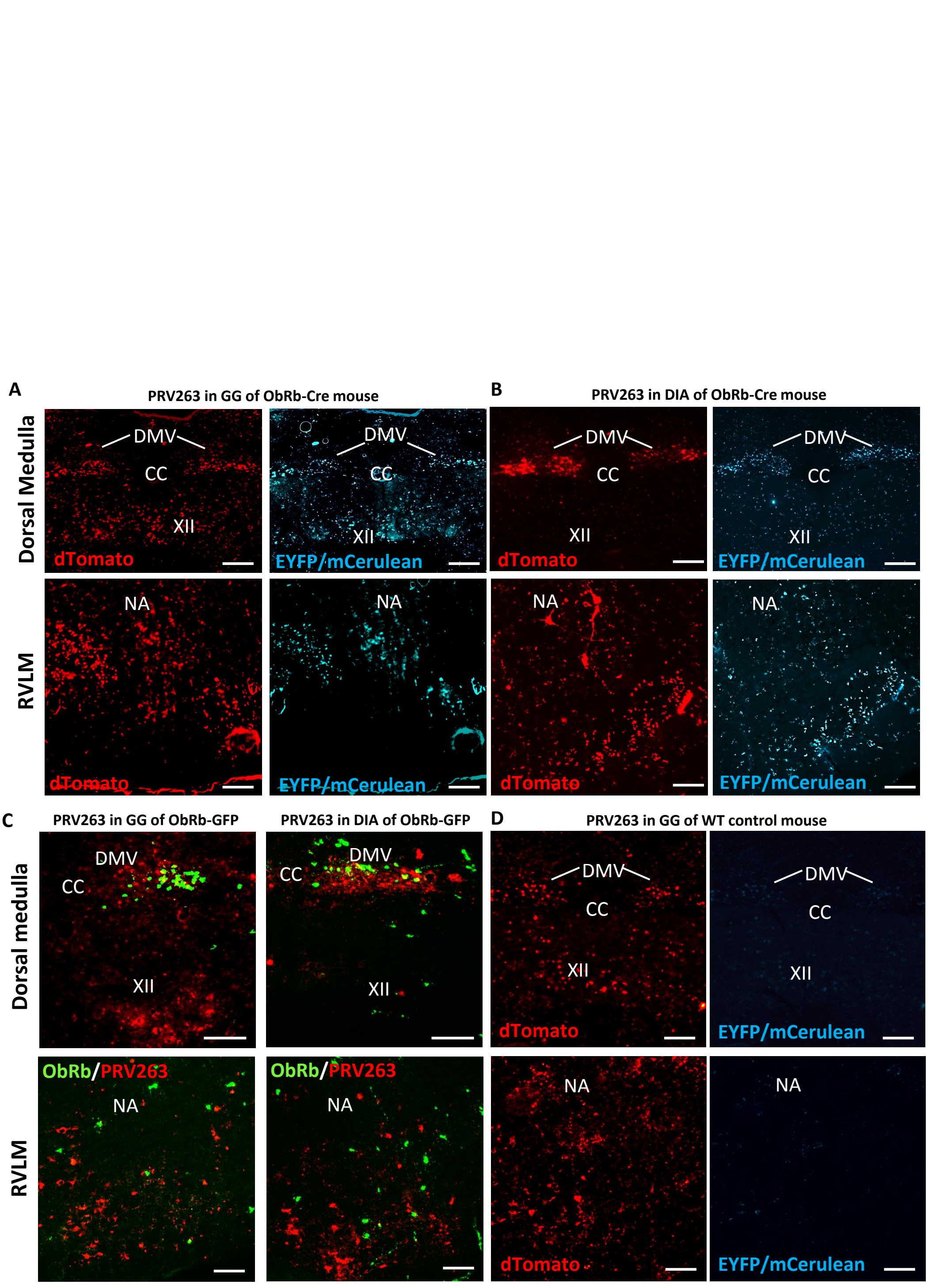

Figure 8.

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Intranasal leptin relieves sleep disordered breathing in mice with diet induced obesity

Slava Berger, Huy Pho, Thomaz Fleury-Curado, Shannon Bevans-Fonti, Haris Younas, Mi-Kyung Shin, Jonathan C. Jun, Frederick Anokye-Danso, Rexford S. Ahima, Lynn W Enquist, David Mendelowitz, Alan R Schwartz, and Vsevolod Y. Polotsky

Online Data Supplement

Supplemental Materials and Methods:

Animals

In total, forty eight adult male C57BL/6J mice (#000664), six adult male *ObRb-Cre* mice (B6.129(Cg)-Leprtm2(cre)Rck/J, #008320) and twelve *ObRb-GFP* mice were used in the experiments. *ObRb-GFP* mice were generated by crossing *ObRb-Cre* with GFP floxed mice (B6;129-Gt(ROSA)26Sortm2Sho/J, #004077). All mice were purchased from Jackson Laboratory (Bar Harbor, ME). Food and water were provided ad libitum. Mice were housed in a standard laboratory environment at 22° C in the 12 h light/dark cycle (9 am–9 pm lights on/9 pm–9 am lights off). Twenty-eight C57BL/6J male mice, 6–8 weeks of age (Jackson Laboratory, Bar Harbor, MA) were fed with a high fat diet (TD 03584, Teklad WI, 5.4kcal/g, 35.2% fat, 58.4% kcal from fat) for 16 weeks. Twenty C57BL/6J male mice, 6–8 weeks of age were fed with a high fat diet for 8 weeks prior to the chronic IN vs IP leptin experiment. *ObRb-Cre* and *ObRb-GFP* mice were fed a regular chow diet. All surgical procedures were performed under 1-2% isoflurane anesthesia. Burpenorphine (0.01 mg/kg) was administered subcutaneously at the end of surgery to minimize discomfort. 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.

Experimental Design:

1) Protocol #1: To study the effects of IN leptin treatment on SDB as a randomized crossover study was designed. Mice (n = 10) were treated with a single dose IN or IP leptin or vehicle, as described above and polysomnographic recordings were performed. On the injection day, previously acclimated animals were treated with leptin or vehicle at 11:00, and the recordings were performed for 6 h after until 17:00. After the sleep recordings, mice

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were given a week wash out resting period and then were treated with a single dose of IN/IP leptin or IN/IP vehicle and sacrificed one hour later for immunofluorescence. The acute experiment allowed avoiding a confounding effect of leptin-induced weight loss.

2) Protocol #2: A single dose of IN or IP leptin was administered to ten DIO mice as above and then VO_2 and VCO_2 were measured for 24 hs. Upon completion of all the experiments, mice in protocols #1 and #2 were sacrificed and leptin signaling in the hypothalamus and medulla was assessed by pSTAT3 immunofluorescence in 16 mice (from both protocols 1 and 2, combined).
3) Protocol #3: In eight mice, leptin was administered IN or IP as above (n = 4 per route) followed by serial retroorbital blood draws (under isoflurane anesthesia) for leptin levels, measured with an ELISA kit (Millipore, Burlington, MA).
4) Protocol #4: Chronic IN or IP leptin (0.4 mg/kg) *vs* vehicle (BSA) delivery (n = 5 per group or n = 20 in total) was performed to examine whether IN leptin affects food intake and body weight in leptin resistant DIO mice, which were injected daily for 14 days.
5) Protocol #5: six *ObRb-Cre* mice were infected in the diaphragm or genioglossus with a retrograde transneuronal tracer PRV263 carrying a cre-dependent Brainbow cassette to determine synaptic connections between respiratory motoneurons and ObRb expressing cells.
6) Protocol #6: As (5), except that six *ObRb-GFP* mice were used.
7) Protocol #7: Six *ObRb-GFP* mice were treated with IN leptin or vehicle (n = 3 per group) and sacrificed 1 hr later for pSTAT3 immunofluorescence and phenotyping ObRb positive cells.

Leptin Delivery

Intranasal leptin delivery was performed as described previously. Mice were anesthetized with 1-2% isoflurane. Leptin (0.4 mg/kg) was prepared in total volume of 24 ul of 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS). Leptin or BSA only (vehicle control) was delivered in 2 doses of 6 ul (3 ul drops) into each nostril. Same concentrations of leptin or BSA only were injected intraperitoneally (1, 2). In the chronic experiments, IN or IP leptin or vehicle were delivered daily for 14 days. Mice were kept in one cage per group of 5. Food intake was measured per cage, while body weight was measured individually in each mouse.

Mouse Polysomnography

Whole body plethysmography recordings (mouse whole body plethysmograph, WBP, Buxco, Wilmington, NC) were performed, as previously described (3). Briefly, the animals were implanted with an EEG/EMG Headmount (no. 8201, Pinnacle Technology, Lawrence, KS) as previously described to determine sleep/wake states in polysomnography recordings (4). After one week of recovery, mice were habituated to the WBP chamber from 11 am to 5 pm one day before the recording session. On the following day, sleep was recorded during ~6 hours, from 11 am to 5 pm. Positive and negative pressure sources were utilized in series with mass flow controllers and high-resistance elements to generate a continuous bias flow through the animal chamber while maintaining a sufficiently high time constant. Atmospheric pressure was equilibrated with slow leaks on both chambers. Mouse respiratory effort was measured using a sensor bladder that was placed under the mouse to transduce the mechanical pressure changes. Reference bladder signal was used for cancellation of the contaminating chamber pressure signal via the differential pressure transducer. The Drorbaugh and Fenn equation was used to calculate

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the WBP tidal volume signal from the WBP chamber pressure signal. Studies were scored by two independent scorers who were blinded to the study conditions (SBF or HY and HP).

Sleep-wake state was scored visually in 5 second epochs. Wakefulness was characterized by low-amplitude, high-frequency (~10 to 20 Hz) EEG waves and high levels of EMG activity compared with the sleep states. Non-rapid eye movement (NREM) sleep was characterized by high-amplitude, low frequency (~2 to 5 Hz) EEG waves with EMG activity considerably less than during wakefulness. Rapid eye movement (REM) sleep was characterized by low-amplitude, mixed frequency (~5 to 10 Hz) EEG waves with EMG amplitude either below or equal to that during NREM sleep.

Respiratory signals were analyzed from the entire REM sleep and from 20 s periods of NREM sleep sampled every 30 min throughout the sleep recording. REM sleep was not scored if it was shorter than 20 sec. Custom software was used to demarcate the start and end of inspiration and expiration for subsequent calculations of timing and amplitude parameters for each respiratory cycle. The instantaneous respiratory rate (RR, breaths/min) was calculated as the reciprocal of the respiratory period, and the instantaneous minute ventilation (V_E, mL/min) was product of the respiratory rate and tidal volume for each breath. Breaths were considered to be non-flow limited when a plateau in mid-inspiratory flow could not be discerned from flow timing indexes. We defined IFL based on detecting a plateau in mid-inspiratory flow, as follows: 1) breaths with an early peak of inspiratory flow followed by a plateau of sufficient duration, i.e. inspiratory time between early and late peaks in flow to be $\geq$ 20% of the total inspiratory time, 2) breaths with a late inspiratory airflow peak exceeding the early peak. Obstruction was characterized by the development of IFL in the presence of increased effort. Once breaths were deemed to be flow-limited, we defined the severity of airflow obstruction by the absolute peak

level of maximal inspiratory flow ($\dot{V}$imax) (30). The oxygen desaturation index (ODI) was defined as ≥4% oxyhemoglobin desaturation from the baseline (ODI) for at least two breaths.

The quality assessment was performed at multiple steps including plethysmography calibrations, signal quality and inadequate flow/effort tracing. One mouse was excluded from the analysis due to inadequate respiratory signal.

Indirect Calorimetry

Energy expenditure was assessed using indirect calorimetry (Oxymax/Comprehensive Laboratory Animal Monitoring System - CLAMS; Columbus Instruments), as previously described (5). DIO mice (age 26 weeks weight 45.1 ± 3.2g, n=10) were housed singly and acclimated for 2 days in the CLAMS cages in 12:12-h light-dark cycles and ambient temperature of 22°C and allowed free access to high-fat diet and water. Leptin or BSA was administered IN and oxygen consumption ($\dot{V}$O2) and carbon dioxide production ($\dot{V}$CO2) were measured for 1 day. A week later, the experiment of indirect calorimetry was performed in the same mice following IP leptin or BSA treatment. The respiratory exchange ratio (RER) was calculated as the ratio between carbon dioxide production and oxygen consumption (RER = $\dot{V}$CO2/$\dot{V}$O2).

Immunofluorescence

Immunofluorescent analysis of brain slices was performed as described previously (3) with modifications. Briefly, one hour after IN/IP leptin or vehicle injection, mice were anesthetized and rapidly perfused with ice-cold 4% paraformaldehyde in phosphate buffered saline (PBS). The brains were carefully removed, postfixed in 4% paraformaldehyde for 1 h at 4°C and cryoprotected in 20% sucrose in PBS overnight at 4°C. Then, brains were covered with

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O.C.T. Compound (Tissue Tek, cat# 4583) and frozen using 2-methylbutane on dry ice. Frozen brains were cut into 20-µm-thick coronal sections on a sliding microtome and stored at −20°C until further use. Sections were blocked for 2 h with 10% normal goat serum in PBS/0.5% TritonX-100 (PBST). Following primary antibodies were used in this study: anti-phosphorylated signal transducer and activator of transcription 3 (pSTAT3) (1:150, #9145, Cell Signaling Technology, Danvers, MA), Anti-NeuN as a neuronal marker (6) (1:100, ab104225, ABCAM, Cambridge, MA), anti- Glial Fibrillary Acidic Protein (GFAP) as an astrocyte marker (7) (1:100, N1506, DAKO, Carpinteria, CA), anti-ionized calcium binding adaptor molecule 1 (Iba1) as a microglia marker (8) (1:100. ab178846, ABCAM, Cambridge, MA) and incubated overnight at 4°C. On the next day, sections were washed at room temperature, incubated with Alexa Fluor 488 or Alexa 647 goat anti-rabbit antibodies (Invitrogen, Grand Island, NY) for 2 h (1:500). After that sections were washed with PBST and were mounted in Vectashield plus DAPI (Vector Labs) for nuclear staining and covered with coverslipps. The signals were then examined under fluorescent microscope (Olympus, Japan). Images were analyzed using ImageJ software (NIH). pSTAT3 positive cells were counted blindly in the hypothalamus including dorsal medial, ventromedial and arcuate nucleus (DMH, VMH and Arc); dorsal medulla, including nucleus of solitary tract (NTS), dorsal motor nucleus of the vagus (DMV) and hypoglossal nucleus (XII); and in rostral ventral lateral medulla (RVLM).

Pseudorabies Virus Infection (PRV)

To establish synaptic connections between ObRb positive cells and respiratory motoneurons, *ObRb-Cre* and *ObRb-GFP* mice were infected with the Bartha strain of PRV263 (a gift from Dr. Lynn W. Enquist) for retrograde labeling of respiratory neurons. PRV263 carries a

Brainbow 1.0L cassette, a Cre-dependent expression system of fluorescent reporters.(9) PRV-263 expresses a red dTomato cytoplasmic reporter but in the presence of Cre, dTomato gene is removed and either the cyan (mCerulean) or yellow (EYFP) reporter is expressed. Transsynaptically infected neurons will therefore express either cytoplasmic cyan or yellow or multiple cytoplasmic reporters of PRV-263 infection. The mice were infected with PRV263 either in the diaphragm (n = 3 per strain) or in the genioglossus muscle (n = 3 per strain), as described previously.(3) Successful application of the virus to the diaphragm was confirmed by positive PRV263 expression in the phrenic nucleus after 48 hours of infection (Figure E3). Ninety six hours after infection, mice were anesthetized and rapidly perfused, and brains were collected, as described in previous sections. Frozen brains were cut into 20-μm-thick coronal sections and analyzed under fluorescent microscope (Olympus). Images were analyzed using ImageJ software (NIH).

Statistical Analysis

Statistical analysis was structured to test a priori hypothesis that IN leptin will treat OSA and sleep-related hypoventilation in DIO mice. Our main predictors (independent variables) were leptin treatment (leptin *vs* BSA). The analyses of the effect of leptin were performed separately for IN and IP routes of administration. Our primary outcome was the number of oxygen desaturations of >4% (ODI4%). Our secondary outcomes were severity of disturbances in ventilation and prevalence of obstructive breaths. The statistical analysis was designed to examine effects of IN and IP leptin on measures of upper airway obstruction, ventilation and metabolic outcomes. Specifically, upper airway obstruction severity was characterized by the percentage of breaths that exhibited IFL and the level of V_{Imax} during flow limited breathing.

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Ventilation was assessed by V_E and its components, tidal volume and respiratory rate, during non-flow limited and flow limited breathing. The periodicity of gas exchange abnormalities (measures of sleep apnea) was assessed by ODI4%.

The sample size calculations were based on our primary outcome, ODI4%. Based on a previous study by Yao et al (3), intraventricular (ICV) leptin decreased the ODI4% in leptin deficient ob/ob mice from 48.1 ± 8.4 (SD) to 24.9 ± 4.7 events/hr, yielding a Cohen's *d* of 3.41. We anticipated a 50% reduction in effect size based on the differences in experimental design (DIO mice *vs ob/ob* mice, IN leptin *vs* ICV leptin). Therefore, we estimated a Cohen's d of 1.7. To detect a difference of this magnitude using in mice receiving IN vehicle *vs* IN leptin at 90% power with an alpha of 0.05 requires 9 mice/group. We used a sample size of 10/group to account for potential overestimation of effect size or data loss.

For time series data (calorimetry), we used mixed effects linear regressions to model the effects of leptin and its route of delivery on each metabolic outcome. Random intercepts were used to account for inter-subject differences in metabolism, and drug (leptin or vehicle) and route (IN or IP) as well as drug x route interactions were examined as fixed factors. Data were stratified by dark or light phase for this analysis. For respiratory outcomes, we compared effects of leptin vs. vehicle using the Kruskal-Wallis test (non-parametric) tests, stratifying the analysis by route of delivery. For the chronic IN and IP administration experiment we used repeated measures ANOVA. In the study by Yao et al (3), ICV leptin decreased food intake from 4 ± 0.1 (SD) to 0.9 ± 0.2 gr, resulting in a Cohen's *d* of 19.6. To detect a difference between IN and IP leptin on food intake at 90% power and alpha of 0.05 requires 2 mice per group. We used a sample size of 5 in each group to overcome potential overestimation of effect size and data loss. Because sleep stage can markedly affect sleep disordered breathing severity, we stratified

analyses by NREM vs REM sleep. The data derived from the parametric statistical analyses were presented as means ± SEM. The data derived from the non-parametric statistical analyses were presented as medians ± quartile. In all cases, a p value < 0.05 was considered significant. GraphPad Prism 7 software was used for statistical analysis.

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Supplemental Tables and Figures

Table E1. Basic characteristics of C57BL/6J mice with diet-induced obesity

Treatment	**Leptin IN**	**BSA IN**	**Leptin IP**	**BSA IP**
Age (weeks)	25 ± 1.6	27.4 ± 1.9	25.2 ± 1.1	24.7 ± 1.0
Body weight (g)	43.3 ± 5.8	42.7 ± 4.8	44.1 ± 7.1	43.9 ± 6.57
Body temp (C)	36.3 ± 0.24	36.2 ± 0.17	36.5 ± 0.7	36.3 ± 0.2

Table E2. Sleep architecture in diet-induced obese C57BL/6J mice

Treatment		**Leptin IN**	**BSA IN**	**Leptin IP**	**BSA IP**
Sleep efficiency	(% of total time)	55.3 ± 3.7	52 ± 7.4	50.1 ± 5.3	54.4 ± 4.9
NREM sleep	% of TST	91.3 ± 1.6	93.4 ± 2.3	94.1 ± 1.8	93.5 ± 2.1
	Number of bouts	68.5 ± 6.4	56.3 ± 11.8	56.2 ± 7.2	55.3 ± 5.8
	Length of the bout (min)	2.5 ± 0.3	2.9 ± 0.3	2.7 ± 0.3	2.8 ± 0.4
REM sleep	% of TST	8.7 ± 1.6	6.7 ± 2	5.8 ± 1.8	6.5 ± 2.1
	Number of bouts	11.6 ± 2.9	7.14 ± 3	8.0 ± 2.8	7.6 ± 2.7
	Length of the bout (min)	1.7 ± 0.16	1.4 ± 0.14	1.5 ± 0.13	1.5 ± 0.1

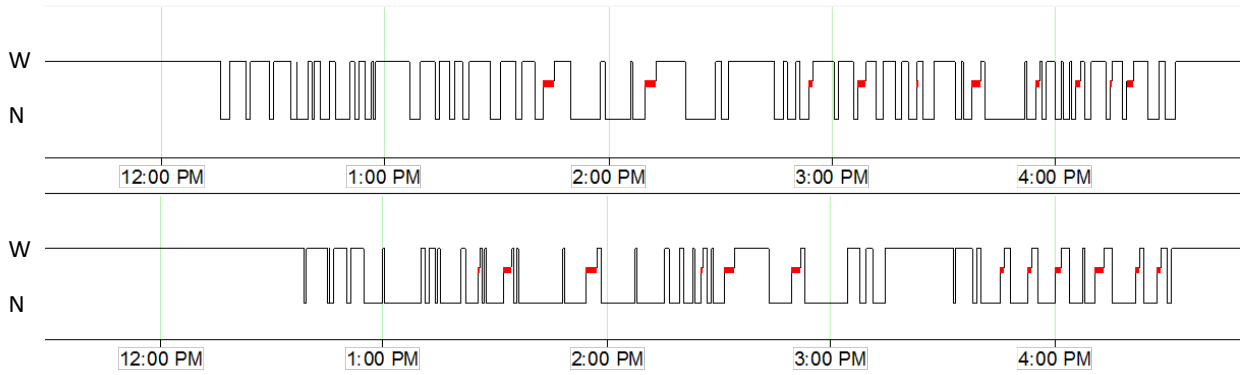

Figure E1. Representative hypnograms of two C57BL/6J mice with diet-induced obesity. The top thin lines represent wakefulness (W), the bottom thin lines represent NREM sleep (N). The red lines represent REM sleep.

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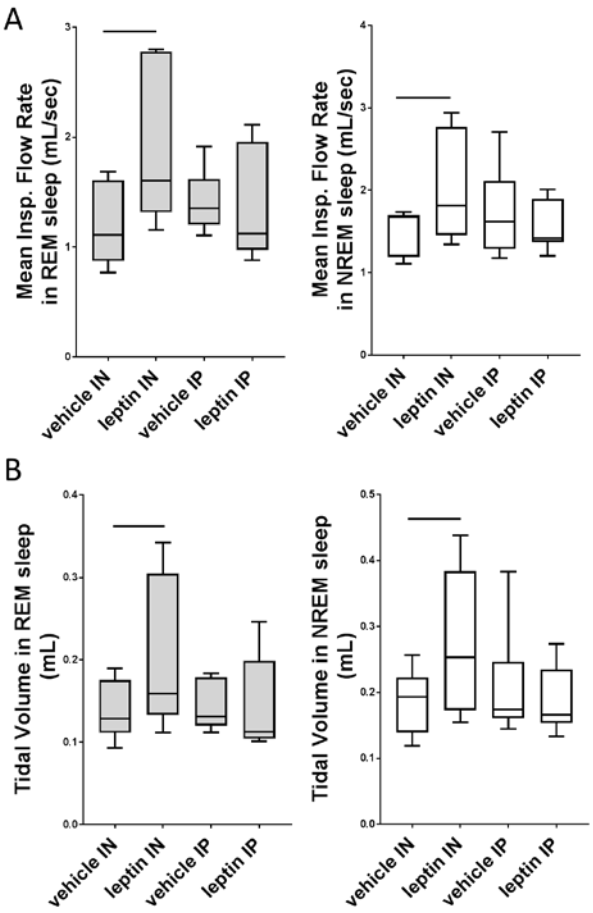

Figure E2. Effect of a single dose of intranasal (IN) and intraperitoneal (IP) leptin on (A) Mean Inspiratory Flow and (B) Tidal Volume during flow limited breathing in diet-induced obese C57BL/6J mice during non-rapid eye movement (NREM) and REM sleep. The boxes denote the second and third quartile divided by the median, whereas the whiskers show the maximum and the minimum values. *, $p < 0.05$ for the difference between IN leptin and IN vehicle (BSA) treatments (N = 10).

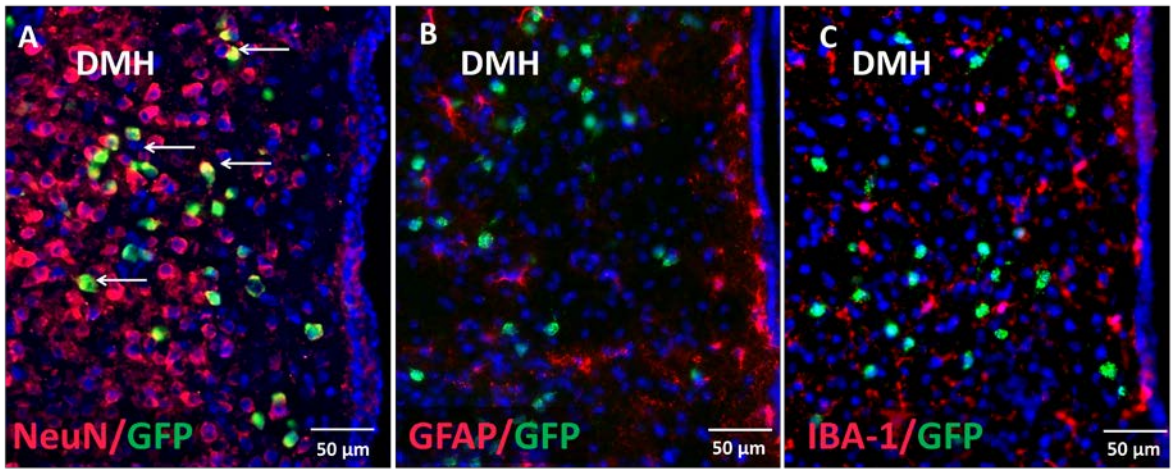

Figure E3. Phenotyping of ObRb positive cells (green) in the DMH of ObRb-GFP mouse. Sections were stained for **(A)** a neuronal marker NeuN, **(B)** an astrocyte marker, glial fibrillary acidic protein (GFAP) and **(C)** a microglia marker, ionized calcium binding adaptor molecule 1 (Iba1) antibodies (red), as indicated. Co-localization of the NeuN and GFP (yellow) is indicated by arrows. Nuclei were stained with DAPI.

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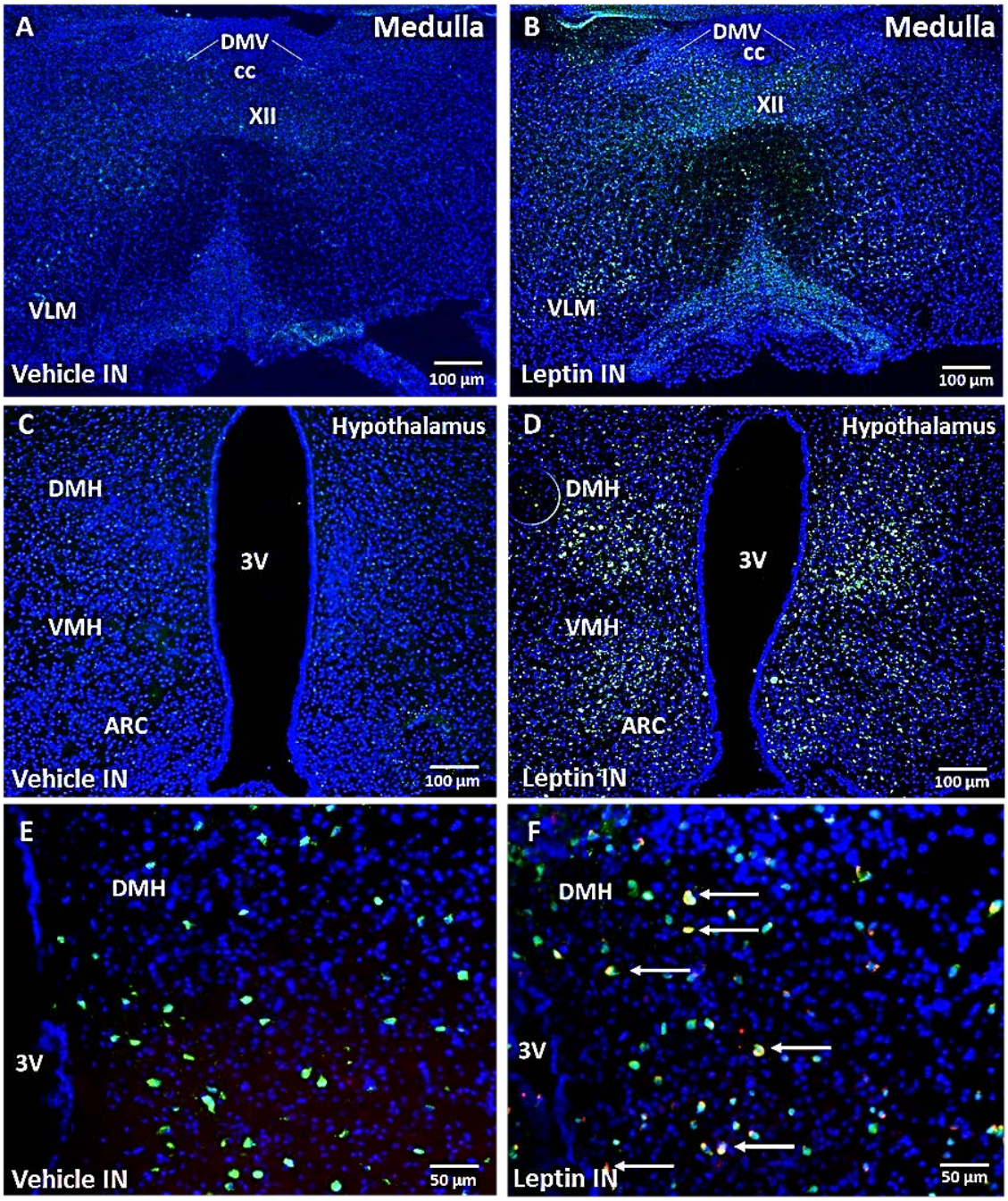

Figure E4. Low magnification representative photomicrographs of phosphorylated STAT3 (pSTAT3) (green) staining in the medulla **(A-B)** and the hypothalamus **(C-D)** from DIO mice one hour after IN vehicle or leptin (0.4 mg/kg). **E-F.** pSTAT3 staining in the hypothalamus of *ObRb-GFP* mouse. Co-localization of pSTAT3 and ObRb is indicated by arrows. DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus; ARC, arcuate nucleus; DMV, dorsal motor nucleus of vagus; XII, hypoglossal nucleus; CC, central canal; VLM, ventral lateral

medulla. Cell nuclei were stained with DAPI (blue). Bar scales as indicated. n = 3, for each treatment.

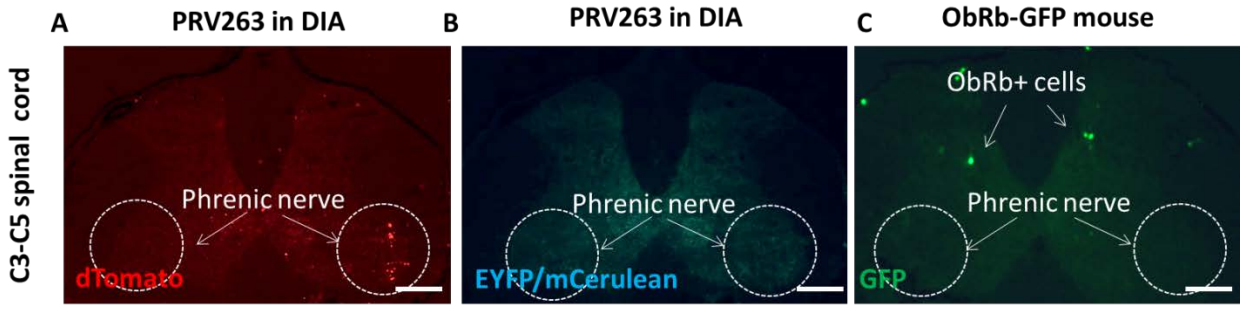

Figure E5. A. Diaphragm infection was confirmed by positive expression of PRV263 in the phrenic nucleus 48 hour after the infection. B. No Cre-dependent change of color was detected in the phrenic nerve. C. Analysis of the ObRb-GFP mice confirmed that phrenic nucleus does not contain ObRb positive cells.

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