Intranasal Leptin Prevents Opioid Induced Sleep Disordered Breathing in Obese Mice

Authors: Carla Freire, Huy Pho, Lenise J Kim, Xin Wang, Jhansi Dyavanapalli, Stone R Streeter,

Thomaz Fleury-Curado, Luiz U Sennes, David Mendelowitz, Vsevolod Y Polotsky

Online Data Supplement

Supplementary Methods

Animals

Sleep studies were conducted on 23 weeks old male diet-induced obese (DIO) C57BL/6J mice (n=9). Tail flick latencies were recorded in seven male DIO C57BL/6J mice (20 weeks old). Sixteen DIO C57BL/6J mice were purchased from the Jackson Laboratory (Stock No: 380050) at 12 weeks of age and maintained on a high fat diet (HFD, TD 03584, Teklad WI, 5.4kcal/g, 35.2% fat, 58.4% of kcal from fat) for 6 weeks until they reached at least 40g of weight. Mice were housed in a 12 h light/dark cycle (9 am–9 pm lights on/9 pm–9 am lights off) with food and water provided ad libitum. Excitatory post-synaptic currents (EPSCs) were recorded in vitro in medullary slices of seven C57BL/6J 5-10 day-old mice. Thus, in total we used 23 C57BL/6J mice for all experiments performed in the study. Animal care and experimental procedures were approved by the Johns Hopkins and the George Washington Universities Animal Use and Care Committees and complied with the American Physiological Society Guidelines for Animal Studies.

Intranasal Delivery of Leptin

After induction of anesthesia with Isoflurane 1-2% DIO mice were treated with a single dose of IN leptin or vehicle in a randomized crossover manner. A single dose of leptin (0.8 mg/kg) or bovine serum albumin 1% (vehicle) was administered intranasally using a micropipette with the mouse in supine position as previously described (1).

Opioid administration

Thirty minutes after intranasal administration of leptin, a 10mg/kg intraperitoneal (IP) bolus of morphine was injected. In sequence, morphine was infused via subcutaneous (SC) osmotic

pump delivering morphine at 2 mg/kg/h for the duration of the study (11am-5pm). An ALZET Osmotic Pump (2001D) was filled with morphine solution and primed 3 hours prior to implantation through mid-scapular incision. Baseline control mice were implanted with osmotic pump filled with saline. After the sleep study, the pump was explanted and the wound closed with sutures. Implant and explant were performed under 1-2% anesthesia.
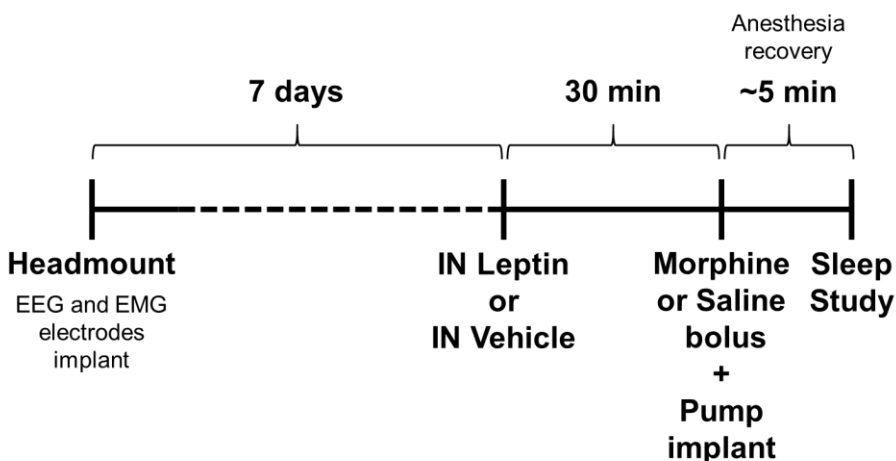
Anesthesia recovery

| 7 days | 30 min | ~5 min |

Headmount
EEG and EMG electrodes implant

IN Leptin or IN Vehicle

Morphine or Saline bolus + Pump implant

Sleep Study

Figure E1. Time course of the experiment.

Experimental Design

In sleep experiments, each mouse underwent three studies: baseline (B) receiving IN vehicle, IP saline and SC pump filled with saline; morphine+vehicle (M+V) receiving IN vehicle, IP morphine (10mg/kg) and SC pump filled with morphine solution; morphine+leptin (M+L) receiving IN leptin (0.8mg/kg), IP morphine (10mg/kg) and SC pump filled with morphine solution. Analgesia experiments had the same design except that the SC pump was not inserted. We performed studies in a randomized crossover manner and allowed a 1-week interval between studies.

Sleep Studies

Sleep studies were recorded in a modified plethysmography chamber as previously described (1,2). Briefly, under 1-2% Isoflurane anesthesia mice underwent headmount (no. 8201, Pinnacle Technology, Lawrence, KS) surgery with implantation of 4 (electroencephalogram) EEG electrode screws placed on frontal and parietal regions bilaterally and 2 (electromyography) EMG leads placed on nuchal muscles. After one-week recovery period, mice were placed in whole body plethysmography (WBP, Buxco, Wilmington, NC) chamber for acclimation. On the following day, tidal airflow, respiratory effort and sleep-wake state were continuously recorded (~6 hours) in the WBP chamber. The chamber was nearly airtight to allow high-fidelity tidal volume and airflow signals. A slow leak allowed atmospheric pressure equilibrium and the chamber was humidified to 90% relative humidity. Continuous airflow through the chamber was generated by positive and negative pressure sources in series with mass flow controllers. The Drorbaugh and Fenn equation was applied to convert the plethysmography chamber pressure signal to tidal volume.

All parameters were scored by two investigators, who were blinded to experimental conditions (CF and HP). Sleep-wake state was scored based on EEG and EMG in 5s epochs and respiratory signal was sampled from periods of stable NREM sleep throughout the recording. Given that morphine significantly decreased respiratory rate, apnea scoring was performed based on average respiratory rate at baseline with apneas scored when flow was absent for a period corresponding to two or more breaths or $\geq 0.7s$. Apnea was classified as obstructive, characterized by cessation of airflow in the presence of respiratory effort, or central, in which the effort was absent. In some instances, however, effort could not be quantified, and therefore these events were labeled as unidentified (Supplemental Tables 1 and 2). Obstructed breaths were defined by the occurrence of inspiratory flow limitation (IFL), characterized when a plateau in early inspiration was detected.

Tail flick test

The tail flick test was performed as previously described (5,6). For measurement of tail flick latencies after tail immersion in a hot water bath, mice were acclimated to restrainer tube for 3 days prior to test. All measures were recorded between 13:00-17:00. Mice were immobilized (25-30 sec) in an acrylic tube and the distal 1/3 of the tail was immersed in water at 50±1°C. Nociceptive latency was recorded the moment a tail flick was observed. A maximum of 15 seconds immersion time was used to avoid tissue damage. Baseline latencies were determined prior to any intervention and measured twice with 5 minutes interval between measures. Baseline values shown represent the mean of the two measurements acquired. After baseline measures, mice received IN vehicle or IN leptin (0.8 mg/kg) in a crossover randomized manner followed by IP morphine (10 mg/kg), 30 minutes after IN treatment. Tail flick latencies were measured 15, 30, 60, and 120 minutes after IP morphine/saline administration.

Brain slice preparation & Electrophysiological recordings

On the day of the experiment, 5-10 day old mice were anesthetized with isoflurane and sacrificed by cervical dislocation. The brain tissue was separated and placed in a physiological saline solution, PSS (in mM: 140 NaCl, 5 KCl, 2 CaCl2, 5 glucose and 10 Hepes) maintained at 4°C and continuously bubbled with 100% oxygen. The hindbrain was isolated from the brain tissue, glued on to a stage and placed in a slicing chamber of vibratome containing PSS. Brain slices of ~330µm thickness containing hypoglossal motor neurons (HMNs) were obtained(7). The slices were then mounted in a recording chamber that is constantly perfused with artificial cerebrospinal fluid (aCSF in mM: 125 NaCl, 3 KCl, 2 $CaCl_2$, 26 $NaHCO_3$, 5 glucose and 5 HEPES) oxygenated with carbogen (95% O_2-5% CO_2). Whole cell voltage clamp recordings from HMNs were obtained using Axopatch 200B and pClamp 8 software (Axon Instruments, Union City, NJ,

USA) at a holding voltage of -80mV at room temperature. The patch pipettes (2.5-5MΩ) were filled with a solution consisting (in mM) 150 K-gluconic acid, 10 Hepes, 10 EGTA, $MgCl_2$ and 1 $CaCl_2$ at a pH of 7.3. Gabazine (25 µM) and strychnine (1 µM) were applied focally (patch pipette positioned within 30µm of the patched MN; PV830 Pneumatic PicoPump pressure delivery system; WPI, Sarasota, FL) to block activation of GABA and glycine receptors respectively (all drugs from Sigma-Aldrich). DAMGO (200nM and 500nM, respectively) and leptin (200nM) were focally applied to the HMNs. The leptin and DAMGO solutions were ejected from a puffer pipette positioned within 30µm from the patched HMN localizing the site of action to HMN and their surrounding synaptic contacts and preventing any off-target or poly-synaptic effects (such as changes in bursting frequency due to inhibition of neurons in the preBőtzinger complex).

Statistical analysis

Statistical analysis was designed to test the hypothesis that IN leptin treats opioid-induced sleep disordered breathing for at least 2h after leptin administration. Our primary outcome was number of apneas and our secondary outcomes were changes in ventilation and prevalence of obstructed breaths. The effects of morphine and leptin treatments on ventilation, sleep architecture and pain sensitivity, were analyzed within the same mouse at 3 different conditions in a cross-over manner: at baseline (B), after morphine+vehicle (M+V) treatment, and after morphine+leptin (M+L). The variances of effects of treatments on each parameter in the 3 timepoints were assessed by repeated measures analysis, using univariate General Linear Model (GLM). The variances of the differences within-subjects in the repeated measures were evaluated by Mauchly's sphericity test, considering spherical data when p>0.05. When the data did not meet the sphericity requirement for Mauchly's test, F values, effect size (partial eta2), and observed power were corrected using the Greenhouse-Geisser correction. To ensure the validity of the analysis with a small sample size, all the data are represented as mean±SEM, including the F values, and effect size (partial

eta^2) for each parameter. Respiratory data obtained during REM sleep in baseline studies was not presented or utilized for comparisons because opioid administration abolished REM sleep. For IFL analysis, three of nine mice did not have any obstructed breaths during NREM sleep at baseline. One mouse was excluded from the percentage of flow limited breaths analysis because it exhibited artifacts on the flow tracing during baseline sleep recording interfering with the IFL detection. Data from one mouse was excluded from the maximal inspiratory flow (V_{Imax}) analysis during IFL because the pronounced negative effort dependence affected the measure of V_{Imax}. To verify whether these exclusions changed the effects on the main outcomes, we performed post hoc comparisons between M+V and M+L timepoints by independent samples t-test and similar significance was obtained. Statistical analyses were conducted in SPSS version 20.0 (IBM SPSS Inc, Chicago, IL).

Digitized (Clampex;10.2) electrophysiological data was analyzed (Clampfit;10.2) including for excitatory postsynaptic currents (EPSCs) frequency, amplitude, and baseline using MiniAnalysis version 6.0.7 (Synaptosoft, Decatur, GA). Statistical analysis was performed using Graphpad Prism 5.0 (Graphpad Software, San Diego, CA). Data are presented as mean ± SEM, normally distributed with equal variances using frequency distribution and Bartlett's correction, respectively. Comparisons between different DAMGO dosages (200nM and 500nM) and leptin (200nm) administrations within-subjects on each electrophysiological parameter were performed by repeated measures test using one-way ANOVA and Dunnett's post-hoc analysis. Statistical analysis for electrophysiological data was performed using Graphpad Prism 5.0 (Graphpad Software, San Diego, CA). Statistical significance was considered at a level of $p < 0.05$.

Supplementary tables

		B	M+V	M+L
Body weight	(g)	43.1±1.4	44.0±1.4	44.1±1.6
Age	(weeks)	24.1±1.4	24.3±1.6	24.5±1.4
Sleep efficiency	(% of total time)	63.7±3.5	84.3±3.2 *	92.3±1.0 *§
REM sleep	(% of TST)	2.3±0.1	0 *	0 *
	Number of bouts	3.1±0.7	0 *	0 *
	Duration of bout (min)	1.2±0.2	0 *	0 *
NREM sleep	(% of TST)	97.7±0.7	100 *	100 *
	Number of bouts	47.3±9.7	37.9±8.3	18±3.0 *§
	Duration of bout (min)	4.3±0.6	9.63±1.9 *	20.5±4.1 *§
Apnea index by type	Obstructive (/h)	1.3±0.6	9.2±3.9	6.1±1.6 *
	Central (/h)	7.9±2.0	12.8±3.4	11.8±1.9
	Unidentified (/h)	3.0±1.4	41.9±8.6 *	24.1±7.8 *

Table E1. General characteristics of mice studied, sleep architecture and apnea classification during the entire 6h recordings (n=9). Data are presented as means±SEM. B, Baseline; M+V, Morphine + IN Vehicle; M+L, Morphine + IN Leptin; TST, total sleep time.

* Significant difference ($p < 0.05$) from B; § Significant difference ($p < 0.05$) from M+V. All comparisons were analyzed with General Linear Model (GLM).

		B	**M+V**	**M+L**
Body weight	(g)	39.8±2.6	40.6±2.1	40.3±2.4
Age	(weeks)	20.3±1.8	20.7±1.7	20.3±1.5

Table E2. General characteristics of mice that underwent the tail flick test (n=7).). Data are presented as means±SEM. B, Baseline; M+V, Morphine + IN Vehicle; M+L, Morphine + IN Leptin
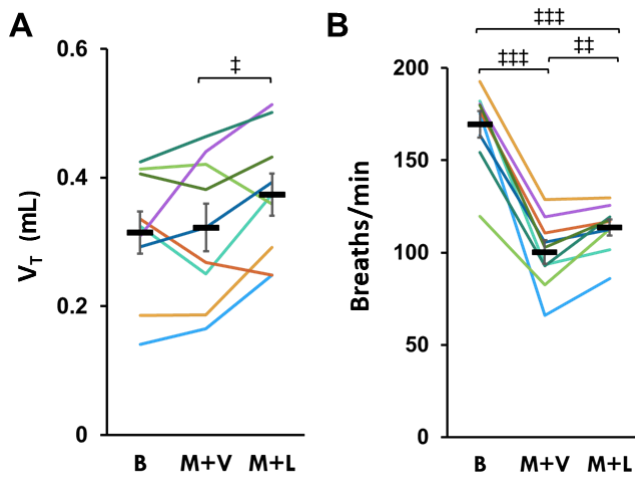
Supplementary figures

Figure E2. Leptin restored minute ventilation to baseline by increasing both tidal volume (V$_T$) (A) and respiratory rate (B) during first 2 hours of sleep recordings (n=9). Each line corresponds to individual data for one mouse. Bars show means±SEM. ‡, ‡‡ and ‡‡‡ denote p<0.05, p< 0.01 and p<0.001 respectively. All comparisons were analyzed with General Linear Model (GLM). B, Baseline; M+V, Morphine + IN Vehicle; M+L, Morphine + IN Leptin.
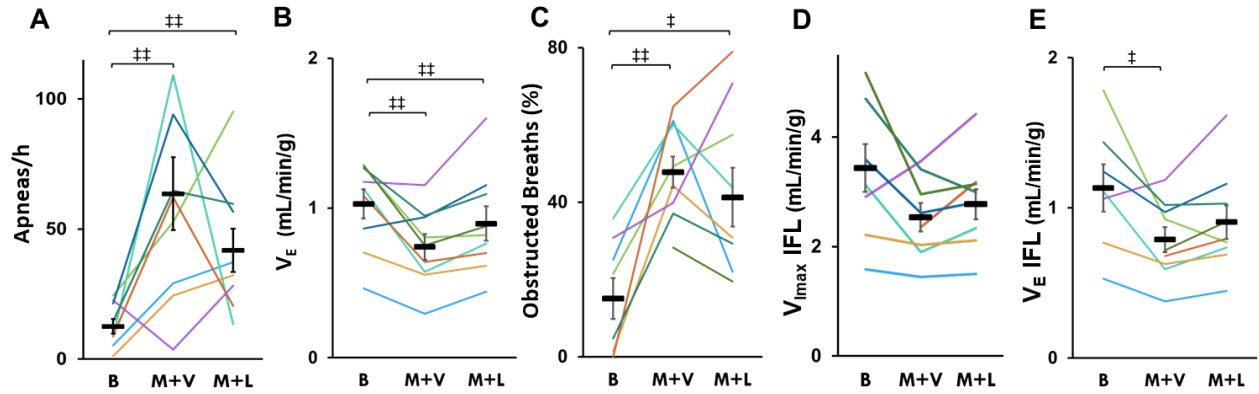
Figure E3: Leptin effects were no longer significant for the analysis of full 6h recordings (n=9). (A) Apneas, (B) minute ventilation (V_E), (C) frequency of obstructed (IFL) breaths and severity of upper airway obstruction evidenced by maximal inspiratory flow (V_{Imax}) (D), and V_E (E) during obstructed (IFL) breathing remained unchanged after leptin treatment compared to vehicle when the full 6h recordings were analyzed. Each line corresponds to individual data for one mouse. Bars show Means±SEM. ‡ and ‡‡ denote $p < 0.05$ and $p < 0.01$ respectively. All comparisons were analyzed with General Linear Model (GLM). B, Baseline; M+V, Morphine + IN Vehicle; M+L, Morphine + IN Leptin.

Supplementary References

E1   Berger S, Pho H, Fleury-Curado T, et al. Intranasal Leptin Relieves Sleep-disordered Breathing in Mice with Diet-induced Obesity. Am J Respir Crit Care Med 2018;199:773–83.

E2   Hernandez AB, Kirkness JP, Smith PL, et al. Novel whole body plethysmography system for the continuous characterization of sleep and breathing in a mouse. J Appl Physiol 1985 2012;112:671–80.

E3   Hübschle T, Thom E, Watson A, Roth J, Klaus S, Meyerhof W. Leptin-Induced Nuclear Translocation of STAT3 Immunoreactivity in Hypothalamic Nuclei Involved in Body Weight Regulation. J Neurosci 2001;21:2413–24.

E4   Yao Q, Pho H, Kirkness J, et al. Localizing Effects of Leptin on Upper Airway and Respiratory Control during Sleep. Sleep 2016;39:1097–106.

E5   Wang W, Baghdoyan HA, Lydic R. Leptin Replacement Restores Supraspinal Cholinergic Antinociception in Leptin-Deficient Obese Mice. J Pain 2009;10:836–43.

E6   Glovak Z, Mihalko S, Baghdoyan HA, Lydic R. Leptin status alters buprenorphine-induced antinociception in obese mice with dysfunctional leptin receptors. Neurosci Lett 2017;660:29–33.

E7   Wang X, Bryan C, LaMantia A-S, Mendelowitz D. Altered neurobiological function of brainstem hypoglossal neurons in DiGeorge/22q11.2 Deletion Syndrome. Neuroscience 2017;359:1–7.