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Leptin into the ventrolateral medulla facilitates chemorespiratory response in leptin-deficient (ob/ob) mice

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Abstract

Aim—Leptin, an adipocyte-derived hormone, is suggested to participate in the central control of breathing. We hypothesized that leptin may facilitate ventilatory responses to chemoreflex activation by acting on respiratory nuclei of the ventrolateral medulla. The baseline ventilation and the ventilatory responses to CO₂ were evaluated before and after daily injections of leptin into the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) for 3 days in obese leptin-deficient (ob/ob) mice.

Methods—Male ob/ob mice (40–45 g, $n = 7$ per group) received daily microinjections of vehicle or leptin (1 μ g per 100 nL) for 3 days into the RTN/pFRG. Respiratory responses to CO₂ were measured by whole-body plethysmography.

Results—Unilateral microinjection of leptin into the RTN/pFRG in ob/ob mice increased baseline ventilation (V_E) from 1447 ± 96 to 2405 ± 174 mL min⁻¹ kg⁻¹ by increasing tidal volume (V_T) from 6.4 ± 0.4 to 9.1 ± 0.8 mL kg⁻¹ ($P < 0.05$). Leptin also enhanced ventilatory responses to 7% CO₂ ($= 2172 \pm 218$ mL min⁻¹ kg⁻¹, vs. control: $= 1255 \pm 105$ mL min⁻¹ kg⁻¹), which was also due to increased V_T ($= 4.71 \pm 0.51$ mL kg⁻¹, vs. control: $= 2.27 \pm 0.20$ mL kg⁻¹), without changes in respiratory frequency. Leptin treatment into the RTN/pFRG or into the surrounding areas decreased food intake (83 and 70%, respectively), without significantly changing body weight.

Conflict of interest

We have no any actual or potential conflict of interest.

Conclusion—The present results suggest that leptin acting in the respiratory nuclei of the ventrolateral medulla improves baseline V_E and V_T and facilitates respiratory responses to hypercapnia in ob/ob mice.

Keywords

breathing; central chemoreception; leptin; obesity; ventrolateral medulla

Leptin is an adipocyte-derived hormone that acts in the central nervous system to regulate energy homeostasis (Grill 2006) and sympathetic activity (Rahmouni & Morgan 2007, Hall *et al.* 2010). In addition, previous studies demonstrated that leptin is also involved in the control of breathing (Polotsky *et al.* 2001, 2004, Inyushkin *et al.* 2009, Inyushkina *et al.* 2010, Malli *et al.* 2010, Bassi *et al.* 2012). Transgenic leptin-deficient (ob/ob) mice show attenuated breathing responses to CO₂, suggesting that leptin deficiency impairs ventilatory function. Moreover, systemic or intracerebroventricular leptin replacement reverses the deficit of ventilation in ob/ob mice by still unknown mechanisms (Tankersley *et al.* 1996, O'Donnell *et al.* 2000, Bassi *et al.* 2012).

Leptin receptors are present in several hindbrain regions including the nucleus of the solitary tract (NTS), locus coeruleus (LC), rostral ventrolateral medulla (RVLM) and Bötzing complex (BötC; Mercer *et al.* 1998, Elias *et al.* 2000, Hosoi *et al.* 2002, Grill & Hayes 2009, Bassi *et al.* 2012). The administration of leptin into the caudal portion of the NTS increases sympathetic nerve activity (Mark *et al.* 2009), and leptin-injected intracerebroventricularly activates pre-sympathetic neurones in the RVLM (Zhang & Felder 2004).

The activity of the respiratory system is controlled by a long column of cells extending laterally in the brainstem from the dorsolateral pons to the caudal ventrolateral medulla (Nattie 1999, Feldman *et al.* 2003, Guyenet *et al.* 2005, Moreira *et al.* 2006). The retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) in the medulla is considered a primary site for central chemoreception and also receives inputs from the carotid body providing important facilitatory signals for respiration (Guyenet *et al.* 2008). Acting in the RTN/pFRG or in other nuclei of the ventrolateral medulla involved in the control of breathing like Pre-Bötzing (pre-Böt) or Bötzing complex (BötC), leptin might influence breathing control and modulate chemoreception. However, to our knowledge, no previous studies tested the effects of leptin injected into the ventral areas of the medulla in controlling breathing and ventilatory responses to hypercapnia.

In this study, we investigated whether leptin microinjections chronically into the ventrolateral medulla, including the RTN/pFRG, would improve respiratory activity in ob/ob mice under resting condition and during central chemoreflex activation by hypercapnia.

Materials and methods

The experimental procedures and protocols used in this study were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical

Center and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals

Male C57BL/6J-Lep^{ob} leptin-deficient (ob/ob) mice from the Jackson Laboratory (Ann Arbor, MI, USA) between 7 and 9 weeks of age were used in these studies.

Surgery for brain cannula implantation

The ob/ob mice were anesthetized with ketamine (80 mg kg⁻¹ of body weight) and xylazine (7 mg kg⁻¹ of body weight) and immobilized in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). Under aseptic conditions, a unilateral stainless steel cannula (14 mm × 27 G) was implanted in the RTN/pFRG using the following stereotaxic coordinates: 1.3 mm caudal to lambda, 4.5 mm below the dura mater and 1.0 mm from the midline (Paxinos & Franklin 2001). The guide cannula was anchored to the skull with stainless steel screws and dental acrylic resin. A metal stylet was inserted into seal the guide cannulas when not in use. At the end of the surgery, the animals received a subcutaneous injection of the analgesic/anti-inflammatory Ketoflex (cetoprofeno 1%, 0.01 mL per animal) and were allowed to recover for 6 days before daily microinjections of leptin or vehicle, and ventilatory measurements were performed.

Leptin microinjections into the RTN/pFRG region

Leptin (1 µg per 100 nL) from National Hormone & Peptide Program (Torrance, CA, USA) or the vehicle (100 nL of phosphate buffer saline, PBS, pH = 7.4) was administered into the RTN/pFRG region of ob/ob mice once a day for three consecutive days. The injections were performed between 9:00 and 10:00 am under anaesthesia (isoflurane 1–3%) to avoid stress to the animals using a Hamilton syringe (1 µL) coupled with a PE-10 tube connected to a needle (15.7 mm × 33 G). This dose of leptin was chosen based on previous studies examining the acute effects of leptin on cardiovascular function (Harris *et al.* 1998, Rahmouni & Morgan 2007).

Ventilation measurements

Pulmonary ventilation (V_E) was measured using whole-body plethysmography as previously described (Malan 1973, Bassi *et al.* 2012). Briefly, mice were acclimatized to the plethysmography chamber (700 mL) at room temperature (25 °C) for 1 h. The ports for gas exit or entrance in the chamber were closed to produce an internal constant volume. The chamber temperature was <1 °C above room temperature (approx. 25 °C) with non-significant variations over the period of time (5 min) that all measurements were made. Rectal temperature was constant, and no significant changes were observed between groups (leptin treated: 37.0 ± 0.2 °C and vehicle treated: 37.3 ± 0.2 °C). Then, signals of breathing frequency (f_R) and tidal volume (V_T) were measured by changes in the pressure inside the chamber due to both temperature and humidification changes in the inspired/expired gases. A spirometer (model ML141; AD Instruments, Colorado Springs CO, USA) was used for measurements, and the signals were analysed using POWERLAB software (Ad Instruments, Colorado Springs, CO, USA). The system was calibrated with injections of 0.2 mL of room

air with the animal inside the plethysmography chamber. V_E was calculated as the product of f_R and V_T .

Experimental protocol

After recovery from surgery (6 days), each mouse was acclimatized inside the plethysmography chamber for V_E measurements. Baseline V_E was measured during room air conditions (21% O_2) followed by hypercapnia produced by switching the room air for a gas mixture containing 21% O_2 plus 4 or 7% CO_2 . The mice were exposed to hypercapnia for 5–10 min before measurements were obtained during one additional minute at the end of hypercapnia. The V_E measurements were made before treatment (control period), on the 3rd day of leptin or vehicle treatment and again 4 days after the treatment was stopped (recovery period). Food intake and body weight were measured daily. At the end of the recovery period, the brains were removed for histological analysis.

Histology

At the end of the tests, mice received injections of 2% Evans Blue solution into the RTN/pFRG region in the same volume used for drug injections. They were then deeply anesthetized with sodium thiopental (80 mg kg⁻¹ of body weight) and perfused transcardially with PBS pH 7.4 followed by 4% paraformaldehyde (Sigma-Aldrich, Saint Louis, MO, USA). Brains were removed, fixed in 4% paraformoldehyde (24 h) and submerged in 30% sucrose for cryoprotection. Frozen coronal section 50 μ m was cut and stained with Giemsa and analysed by light microscopy to confirm the injection sites into the RTN/pFRG region.

Statistical analysis

The results are presented as mean $\pm$ SEM. Two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test was used for comparisons, using GRAPHPAD PRISM 5 software (GraphPad Software, San Diego, CA, USA). Statistical significance was accepted at the level of $P < 0.05$.

Results

Histological analysis

Typical sites of leptin microinjections into the ventrolateral medulla are shown in the Figure 1a,b. The centre of the injections was typically located into the RTN/pFRG, medial and ventral to the facial nucleus, a region that contains the highest density of CO_2 -sensitive neurones that control cardiorespiratory responses (Takakura *et al.* 2006, 2011). The histological analysis showed that some mice received leptin injections outside the RTN/pFRG in sites more lateral and dorsal to the CO_2 -sensitive region (Fig. 1c). The results of these mice were also analysed and compared with the results of leptin injections specifically into the RTN/pFRG.

Baseline ventilation in leptin-deficient ob/ob mice treated with leptin injections into the RTN/pFRG region

At the end of 3 day treatment, microinjections of leptin ($1 \mu\text{g}$ per 100 nL day^{-1}) into the RTN/pFRG in leptin-deficient ob/ob mice increased baseline V_T ($9.1 \pm 0.8 \text{ mL kg}^{-1}$ vs. vehicle: $6.5 \pm 0.4 \text{ mL kg}^{-1}$) [$F_{1,29} = 4.23$; $P < 0.05$] and baseline V_E ($2406 \pm 174 \text{ mL min}^{-1} \text{ kg}^{-1}$, vs. vehicle: $1581 \pm 159 \text{ mL min}^{-1} \text{ kg}^{-1}$) [$F_{1,29} = 6.62$; $P < 0.02$], without changing f_R (Fig. 2, Table 1). The increased baseline V_T and V_E produced by leptin injections into the RTN/pFRG region returned to control values 4 days after stopping leptin treatment (Fig. 2, Table 1).

Ventilatory responses to 4 and 7% CO₂ in leptin-deficient ob/ob mice treated with leptin injections into the RTN/pFRG

Leptin ($1 \mu\text{g}$ per 100 nL day^{-1}) injected for 3 days into the RTN/pFRG region of leptin-deficient ob/ob mice markedly increased the ventilatory responses to 4 and 7% CO₂ (Fig. 3, Table 1). Leptin facilitated 4 and 7% CO₂-induced increase in V_T ($= 2.37 \pm 0.57$ and $4.71 \pm 0.51 \text{ mL kg}^{-1}$, respectively) and V_E ($= 1091 \pm 166$ and $2172 \pm 218 \text{ mL min}^{-1} \text{ kg}^{-1}$, respectively) compared with CO₂-induced increase in V_T ($= 0.91 \pm 0.29$ and $2.41 \pm 0.54 \text{ mL kg}^{-1}$, respectively) and V_E ($= 483 \pm 101$ and $1292 \pm 291 \text{ mL min}^{-1} \text{ kg}^{-1}$, respectively) in PBS-treated mice (Fig. 3). There was a significant difference in V_T comparing leptin and PBS-treated mice exposed to 4% [$F_{1,29} = 8.08$; $P < 0.01$] and 7% CO₂ [$F_{1,29} = 18.5$; $P < 0.0002$] and also in V_E to 4% [$F_{1,29} = 12.3$; $P < 0.002$] and 7% CO₂ [$F_{1,29} = 11.9$; $P < 0.002$; Fig. 3a,b). The slopes of the V_E/CO_2 curve responses for leptin- and PBS-treated mice were also significantly different (leptin: 325 ± 49 vs. vehicle: 157 ± 37 , $P < 0.01$; Fig. 3c).

The increases in V_T and V_E produced by 4 and 7% CO₂ after 3-day leptin treatment were also facilitated compared with the increases in V_T ($= 1.09 \pm 0.19$ and $2.27 \pm 0.20 \text{ mL kg}^{-1}$, respectively) and V_E ($= 488 \pm 53$ and $1255 \pm 105 \text{ mL min}^{-1} \text{ kg}^{-1}$, respectively) produced by 4 and 7% CO₂ in the control period in the same group of mice (Fig. 3, Table 1). The increased ventilatory response to CO₂ in leptin-treated mice returned to control values 4 days after stopping leptin treatment (Fig. 3, Table 1). Leptin injections into the RTN/pFRG region did not affect the increases in f_R produced by CO₂ (Fig. 3, Table 1).

Baseline ventilation and ventilatory responses to 4 and 7% CO₂ in leptin-deficient ob/ob mice treated with leptin injections in sites outside the RTN/pFRG region

Leptin ($1 \mu\text{g}$ per 100 nL day^{-1}) injected for 3 days in sites outside the RTN/pFRG did not significantly change baseline ventilation and the ventilatory responses to 4% CO₂ when compared to control period (before leptin) or vehicle PBS treatment (Table 1). However, leptin injected outside the RTN/pFRG increased V_T ($10.7 \pm 0.7 \text{ mL kg}^{-1}$, vs. control period: $8.1 \pm 0.5 \text{ mL kg}^{-1}$) and V_E ($3659 \pm 132 \text{ mL min}^{-1} \text{ kg}^{-1}$, vs. control period: $2407 \pm 212 \text{ mL min}^{-1} \text{ kg}^{-1}$) under 7% CO₂. Nevertheless, the increased V_E response to 7% CO₂ in mice that received leptin in the surrounding areas was less pronounced than the response observed in mice treated with leptin into the RTN/pFRG region ($3659 \pm 132 \text{ mL min}^{-1} \text{ kg}^{-1}$, vs. leptin inside: $4584 \pm 344 \text{ mL min}^{-1} \text{ kg}^{-1}$) [$F_{1,27} = 7.26$; $P < 0.01$].

Daily food intake and body weight in leptin-deficient ob/ob mice treated with leptin injections into the RTN/pFRG region or in the surrounding areas

Leptin microinjections into the RTN/pFRG or in the areas outside the RTN/pFRG similarly reduced daily food intake. Food intake was reduced from 5.2 ± 0.5 to 0.9 ± 0.2 g per 24 h (reduction of approx. 83%) in the mice that received injection of leptin into the RTN/pFRG and from 5.3 ± 0.4 to 1.7 ± 0.3 in those that received injections of leptin in sites outside the RTN/pFRG (reduction of approx. 70%). However, probably due to the short-term period of treatment, no significant changes in the body weight were observed comparing the weight of leptin-treated mice with that of vehicle-treated mice (37.6 ± 2.5 g and 35.1 ± 2.2 g vs. vehicle: 39.1 ± 2.9 g).

Discussion

The present study shows that leptin microinjected into the RTN/pFRG for three consecutive days increased baseline ventilation and the ventilatory responses to 4 and 7% CO₂ in obese leptin-deficient ob/ob mice. Although the injections of leptin in sites outside the RTN/pFRG also increased the ventilatory responses to CO₂, in this case, the effects were less intense. These results suggest that leptin acting in the respiratory nuclei of the ventrolateral medulla, including RTN/pFRG, improves baseline ventilation and facilitates the ventilatory responses to CO₂ in leptin-deficient ob/ob mice.

Leptin injected into the RTN/pFRG or in sites outside the RTN/pFRG similarly reduced food intake in obese leptin-deficient ob/ob mice (around 83 and 70%, respectively). Therefore, the reduction in food intake produced by the injections of leptin into the RTN/pFRG cannot be considered a consequence of the action of leptin specifically into the RTN/pFRG. Probably at the dose tested, leptin spread from the RTN/pFRG to other areas to affect the mechanisms involved in the control of food intake. Even the small amount of leptin that reached other brain areas was apparently enough to affect food intake. In spite of the reduction in food intake, the injections of leptin did not significantly change body weight, which supports previous suggestion that leptin acting centrally may improve ventilatory response to hypercapnia by mechanisms not dependent on changes in body weight (Bassi *et al.* 2012).

Although modest, the increase in the ventilatory response to 7% CO₂ when leptin was injected outside the RTN/pFRG suggests that leptin spreading to areas outside the RTN/pFRG may also produce respiratory effects. From the RTN/pFRG, leptin might spread to the surrounding areas that express leptin receptors like the Botzinger complex and the RVLM (Barnes *et al.* 2010, Bassi *et al.* 2012). Reaching these areas even in a small concentration, leptin might produce part of the ventilatory effects reported in the present study. Therefore, with the present results, it is not possible to exclude the action of leptin on other ventrolateral medullary areas outside RTN/pFRG to produce at least part of the respiratory responses in obese leptin-deficient ob/ob mice. In addition, although the present results suggest that leptin may facilitate respiratory responses also acting in the RTN/pFRG, it is necessary to consider that no study showed the presence of leptin receptors within the RTN/pFRG (Scott *et al.* 2009, Barnes *et al.* 2010), and more studies are necessary to solve these controversies. Previous studies have suggested that leptin acting centrally modulates

respiratory function (Inyushkin *et al.* 2009, Inyushkina *et al.* 2010, Bassi *et al.* 2012); however, to our knowledge, the present study is the first to demonstrate that leptin may act at the level of ventrolateral medulla to facilitate ventilatory responses to central chemoreflex activation.

A previous study showed that intracerebroventricular leptin administration also required few days to improve ventilatory responses to CO₂ in ob/ob mice (Bassi *et al.* 2012). Although the mechanisms by which leptin modulates respiratory function are not well understood, the current and previous data (Bassi *et al.* 2012) are consistent with the idea that these effects may involve changes in gene expression and protein synthesis, thus requiring chronic treatment of at least 3 days to have the effects. Also consistent with this possibility, preliminary slice-patch experiments showed that chemosensitive RTN/pFRG neurones and astrocytes elicited no change in membrane potential in response to acute exposure to leptin (data not shown). However, in this case, it is also necessary to consider that there is no study showing the presence of leptin receptors within the RTN/pFRG (Scott *et al.* 2009, Barnes *et al.* 2010).

The baseline V_E values reported in the present study for obese leptin-deficient ob/ob mice (1528 mL kg⁻¹ min⁻¹) are similar to baseline V_E values reported in the literature for obese ob/ob mice (1383 mL kg⁻¹ min⁻¹) or lean wild-type mice (1969 mL kg⁻¹ min⁻¹ when corrected to express the data using the same unit employed in the present study; O'Donnell *et al.* 1999) or to our previous data (1806 mL kg⁻¹ min⁻¹; Bassi *et al.* 2012) in obese ob/ob mice. However, it is important to note that although improved, the ventilation during 7% CO₂ in leptin-deficient ob/ob mice treated with leptin injected into the RTN/pFRG region (4584 ± 344 mL kg⁻¹ min⁻¹, present results) or i.c.v. (6156 ± 312 mL kg⁻¹ min⁻¹, Bassi *et al.* 2012) was still reduced compared with wild-type mice in the same condition (7747 ± 337 mL kg⁻¹ min⁻¹; Bassi *et al.* 2012).

It is important to consider that chronic leptin administration may also affect metabolic rate due to changes in O₂ consumption (VO₂) and/or CO₂ production (VCO₂), which might affect chemorespiratory responses. Previous studies have shown controversial results about leptin effects on respiratory quotient (RQ = CO₂/VO₂), such as a reduction in RQ due to increased VO₂ without changes in VCO₂ (O'Donnell *et al.* 1999) or due to decreased VCO₂ (Hogberg *et al.* 2006). According to these previous studies, leptin produces no increase in VCO₂. For this reason, it has been suggested that the stimulatory effects of leptin on ventilation may occur due to its action on central mechanisms that control respiratory system and not due to metabolic changes like increases in VCO₂. In the present study, we did not evaluate the metabolic rate during leptin administration into the RTN/pFRG, which limits any interpretations of the leptin effects on metabolic rates and possible metabolic modulation of ventilation. Further studies are necessary to better clarify this point.

Multiple mechanisms may contribute to chemoreception at the level of the ventral medulla. Among them, a population of glutamatergic neurones pH sensitive projects to respiratory centres (Nattie & Li 1995, Mulkey *et al.* 2004, Abbott *et al.* 2009). Leptin may facilitate the activity of these glutamatergic neurones in the ventral medulla as suggested for glutamatergic neurones located in the hippocampus and NTS (Williams *et al.* 2007, Guo *et*

al. 2012). In addition to the glutamatergic neurones, it has been suggested that glia cells in the ventral medulla, including the RTN, may also contribute to chemoreception (Gourine *et al.* 2005, Mulkey *et al.* 2006). Previous studies have shown that hypercapnia evokes discrete release of adenosine 5'-triphosphate (ATP) by astrocytes to stimulate respiratory output (Gourine *et al.* 2005, Wenker *et al.* 2010). Chronic central leptin administration activates astrocytes (García-Cáceres *et al.* 2011) by mechanisms involving calcium signalling and ATP release (Tu *et al.* 2008, Hsueh *et al.* 2009). Thus, it is possible to speculate that leptin may affect chemorespiratory response to hypercapnia by altering calcium and ATP signalling in glia cells located in the ventral medulla. Future studies should investigate the action of leptin on these mechanisms to produce respiratory effects.

In conclusion, the present results suggest that leptin improves baseline respiratory function and ventilatory responses to hypercapnia in ob/ob mice by acting in the respiratory nuclei of the ventrolateral medulla, including the RTN/pFRG. Additional studies are necessary to further examine the complex mechanisms and specific brain cells that mediate the actions of leptin on respiratory function.

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Figure 1.

(a) Photomicrograph showing a typical site of injection into the ventrolateral medulla (RTN/pFRG) in one of the mice tested. (b,c) Diagrams showing the sites of leptin microinjections inside (b) or outside (c) the RTN/pFRG region at different coronal levels (from top to bottom: Bregma -6.24, -6.36 and -6.48 mm, respectively). sp5, spinal trigeminal tract; 7N, facial nucleus; Rob, raphe obscurus nucleus; Rpy, parapyramidal region; RVL/C1, rostral ventrolateral and adrenergic C1 nuclei; Rpa, raphe pallidus nucleus; 4V, 4th ventricle. pFRG, parafacial respiratory group; RTN, retrotrapezoid nucleus.

Figure 2.

Baseline respiratory frequency (f_R), tidal volume (V_T) and ventilation (V_E) in control period (before leptin), after 3 days of leptin ($n = 7$) or PBS vehicle ($n = 5$) injections into the RTN/pFRG and after 4-day recovery period. $\alpha = P < 0.05$ compared with control and recovery period, and also compared with PBS injections. pFRG, parafacial respiratory group and RTN, retrotrapezoid nucleus.

Figure 3.

(a,b) Changes in respiratory frequency (f_R), tidal volume (V_T) and ventilation (V_E) in response to (a) 4% CO_2 or (b) 7% CO_2 during control period, after 3 days of leptin ($n = 7$) or PBS vehicle ($n = 5$) injections into the RTN/pFRG region and after 4-day recovery period. (c) V_E/CO_2 curve responses after 3 days of leptin or vehicle treatment into the RTN/pFRG in ob/ob mice. $\alpha = P < 0.05$ compared with control, recovery period or PBS injections. pFRG, parafacial respiratory group and RTN, retrotrapezoid nucleus.

Table 1

Respiratory frequency, tidal volume and ventilation in leptin-deficient ob/ob mice treated for 3 days with PBS or leptin injected into the RTN/pFRG or outside the RTN/pFRG in control condition and 4 or 7% CO₂

	PBS			Leptin inside RTN/pFRG			Leptin outside RTN/pFRG		
	Baseline	4% CO ₂	7% CO ₂	Baseline	4% CO ₂	7% CO ₂	Baseline	4% CO ₂	7% CO ₂
CTL									
f_R	248 ± 18	281 ± 8	310 ± 3	225 ± 9	281 ± 8	310 ± 3	240 ± 11	293 ± 11	300 ± 22
V_T	6.7 ± 0.4	7.7 ± 0.3	8.5 ± 0.4	6.5 ± 0.4	7.7 ± 0.3	8.5 ± 0.4	5.9 ± 0.6	6.6 ± 0.6	8.1 ± 0.5
V_E	1658 ± 107	2114 ± 100	2582 ± 111	1447 ± 96	2114 ± 100	2583 ± 112	1422 ± 178	1934 ± 229	2407 ± 212
3 days									
f_R	246 ± 15	276 ± 13	306 ± 8	273 ± 6	316 ± 11	340 ± 8	240 ± 31	308 ± 5	345 ± 10
V_T	6.5 ± 0.6	7.7 ± 0.3	8.5 ± 0.4	9.1 ± 0.8 ^a	10.9 ± 1.0 ^a	13.7 ± 0.9 ^a	6.9 ± 0.5 ^β	8.1 ± 0.58 ^β	10.7 ± 0.7 ^{aβ}
V_E	1581 ± 159	2109 ± 59	2592 ± 128	2406 ± 174 ^a	3261 ± 290 ^a	4584 ± 344 ^a	1780 ± 145 ^β	2574 ± 118 ^β	3659 ± 132 ^{aβ}
REC									
f_R	226 ± 24	284 ± 24	324 ± 17	242 ± 13	396 ± 13	326 ± 10	255 ± 16	305 ± 5	325 ± 3
V_T	6.1 ± 0.8	6.9 ± 1.0	7.9 ± 0.9	6.6 ± 0.4	8.1 ± 0.7	9.4 ± 0.5	6.2 ± 0.6	7.1 ± 0.9	7.6 ± 0.4
V_E	1362 ± 192	1967 ± 325	2554 ± 327	1552 ± 75	2202 ± 99	2949 ± 95	1578 ± 184	2174 ± 261	2712 ± 262

Values are mean ± SE. f_R , respiratory frequency (breaths min⁻¹), V_T , tidal volume (mL kg⁻¹) and V_E , pulmonary ventilation (mL min⁻¹ kg⁻¹) in control period (CTL), after 3 days of PBS ($n = 5$) or leptin treatment inside ($n = 7$) or outside ($n = 4$) the RTN/pFRG and in recovery period (4 days after stopping leptin or vehicle treatment).

^a $P < 0.05$ compared with control and recovery periods and also compared with PBS injections;

^β $P < 0.05$ compared with leptin treatment inside the RTN/pFRG region.