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High tidal volume ventilation affects neuronal activation in mechanically ventilated rats

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FA CONSTAR,

que el treball titulat **"High tidal volume ventilation affects neuronal activation in mechanically ventilated rats"** ha estat realitzat sota la meva direcció pel llicenciat **Mª Elisa Quílez Tierno**, trobant-se en condicions de poder ser presentat com a treball d'investigació de 12 crèdits, dins el programa de doctorat en Medicina Interna/Diagnòstic per la Imatge (curs 2010-2011), a la convocatòria de juny.

Sabadell, vint de març de dos mil onze.

Dr. Antoni Artigas Raventós

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FAN CONSTAR,

que el treball titulat **"High tidal volume ventilation affects neuronal activation in mechanically ventilated rats"** ha estat realitzat sota la seva direcció per la llicenciada **Mª Elisa Quílez Tierno**, trobant-se en condicions de poder ser presentat com a treball d'investigació de 12 crèdits, dins el programa de doctorat en Medicina Interna/Diagnòstic per la Imatge (curs 2010-2011), a la convocatòria de juny.

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Dra. Josefina López Aguilar

Dr. Lluís Blanch i Torra

INDEX

PRESENTACIÓ

El present treball de recerca constitueix la primera fase del projecte MEC BFU 2006 07124/BFI: "*Lesió pulmonar unilateral induïda per ventilació mecànica i/o endotoxina: mecanismes biofísics, cel·lulars i moleculars de lesió local, descompartimentalització multiorgànica remota i precondicionament"* i ha servit per fer un primer abordatge en l'estudi de l'afectació neuronal relacionada amb la lesió pulmonar aguda induïda per la ventilació mecànica

Els resultats obtinguts, han constituït la base pel desenvolupament de posteriors projectes dirigits a caracteritzar els mecanismes implicats en la comunicació pulmó-cervell (*crosstalk*) en la mateixa línia de recerca.

La doctoranda, Mª Elisa Quilez, és llicenciada en Biologia per la Universitat Autònoma de Barcelona (2006) i actualment, desenvolupa la seva carrera professional com a investigadora predoctoral, dins del grup 33 del CIBER de Malalties Respiratòries (IP: Lluís Blanch) al Laboratori de Recerca Translacional en Fisiopatologia Respiratòria de la Corporació Sanitària Parc Taulí.

La patologia crítica s'associa freqüentment amb el desenvolupament d' alteracions neurològiques a curt i/o llarg termini, que comporten un gran impacte tant social com econòmic. En els darrers anys, molts estudis han investigat si determinats procediments clínics aplicats de rutina sobre el pacient crític, com ara la ventilació mecànica, entre d'altres, podrien tenir efecte sobre aquestes alteracions. En aquest sentit, és conegut que la utilització d'un patró de ventilació mecànica inadequat pot contribuir a agreujar la lesió pulmonar aguda (LPA) preexistent o inclús induir-la en pulmons sans. La pèrdua de compartimentalització de la lesió local (al pulmó) pot

arribar a afectar altres òrgans i, en situacions extremes, conduir al desenvolupament de disfunció multiorgànica. Els mecanismes a través dels quals òrgans distals lesionats, com el pulmó, poden arribar a afectar al sistema nerviós central, són encara desconeguts i el seu estudi ha estat un dels objectius del present treball.

En un primer abordatge de l'anàlisi de la interacció pulmó - cervell, hem realitzat un estudi, basat en un model agut de ventilació mecànica en rates (3 hores), on s'ha caracteritzat el patró d'activació neuronal i la resposta inflamatòria local (pulmó) i sistèmica, així com d'altres paràmetres fisiològics en funció del volum corrent administrat.

ABSTRACT

Introduction: Survivors of critical illness often have significant long-term brain alteration and routine clinical procedures like mechanical ventilation (MV) may affect long-term brain outcome. We aimed to investigate the effect of the increase of tidal volume (Vt) on brain activation in a rat model.

Methods: Male Sprague Dawley rats were randomized to 3 groups: 1) control: anesthetized unventilated animals, 2) low Vt (LVt): MV for 3 hours with Vt 8 ml/kg and zero positive end-expiratory pressure (ZEEP), and 3) high Vt (HVt) MV for 3 hours with Vt 30 ml/kg and ZEEP. We measured lung mechanics, mean arterial pressure (MAP), arterial blood gases, and plasma and lung levels of cytokines. We used immunohistochemistry to examine c-fos as a marker of neuronal activation.

Results: After 3 hours on LVt, PaO_2 decreased and $PaCO_2$ increased significantly. MAP and compliance remained stable in MV groups. Systemic and pulmonary inflammation was higher in MV rats than in unventilated rats. Plasma TNFα was significantly higher in HVt than in LVt. Immunopositive cells to c-fos in the retrosplenial cortex and thalamus increased significantly in HVt rats but not in LVt or unventilated rats.

Conclusions: MV promoted brain activation. The intensity of the response was higher in HVt animals suggesting an iatrogenic effect of MV on brain. These findings suggest that this novel cross-talking mechanism between lung and brain should be explored in patients undergoing MV.

BACKGROUND

Acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) are associated with high morbidity and mortality [1], and ARDS survivors present significant long-term cognitive impairment [2]. These consequences may result from complex interactions between different clinical protocols and endogenous factors occurring simultaneously in critically ill patients [3]. In this context, mechanical ventilation (MV) is a lifesaving procedure but not without complications. Even in healthy lungs, MV may contribute to a positive feedback loop that starts with mechanotransduction at the epithelial and endothelial levels leading to a deleterious inflammatory cascade that might affect distant organs and systems [4-6]. Moreover, critical care patients who undergo long-term MV show distinctive neurological impairment, including memory and cognitive decline [7].

Many studies have examined the mechanisms involved in the neuroimmune crosstalk; most focus on the central nervous system (CNS) response to systemic inflammation. However, the mechanisms through which damage to remote organs can reach the brain are poorly understood [8, 9], including early neurological effects related to MV and the importance of settings used. The immediate early gene c-fos has been used as a marker of neuronal activity, and correlates with increase in electrical and metabolic activity in brain cells by pathological situations, also involved in phenomena of neuronal plasticity, amongst others. In a first approach, to study lung-brain crosstalk, we have used c-fos as a marker of areas sensible to injurious MV in the brain [10].

The main objective of the present study was to investigate the effect of the increase in tidal volume on activation in some areas of the brain in a rat model of MV, using c-fos. The second was to explore the effects of lung overstretching and the triggered inflammatory response. Therefore, we compared rats ventilated with two different levels of tidal volume, a high Vt group vs. a low Vt group, vs. non-ventilated control rats.

METHODS

Animal Preparation

Thirty male Sprague Dawley rats weighing 300-350gr where were housed in standard conditions of light-dark cycle regulated, air humid (60%) and temperature (22 ± 1ºC) Commercial chow pellets and tap water were available *ad libitum*.

Animals were treated following the Principles of Laboratory Animal Care (UE 609/86 CEE, Real Decreto 223/88 BOE-18/03) and all procedures were approved by the Animal Ethics Committee of Hospital de Sabadell (Spain).

Experimental protocol

Rats were anesthetized with intraperitoneal ketamine (75mg/kg, Parke-Davis, El Prat de Llobregat, Spain), and xylacine (10ml/kg, Rompun®, Bayer, Barcelona, Spain). One half of this dose of anesthesia was re-administered every 45 min. Right jugular vein and left carotid artery were cannulated for infusions, hemodynamic monitoring and to aspirate blood for blood gas analysis. An endotracheal tube (2 mm inner diameter) was inserted by a surgical tracheotomy and connected to a mechanical ventilator (300 Servo-Ventilator; Siemens, Solna, Sweden). Animals were paralized with an intravenous injection of 0.2 cc succinylcholine (1.5 mg/kg, Glaxo- Wellcome, Tres Cantos Madrid, Spain) immediately before measurements of lung mechanics.

Then animals were randomly assigned to one of three experimental groups (n=8 per group): 1) Control group (CRL), unventilated animals, were immediately exanguinated after the anesthesia, 2) Low Vt group (LVt), ventilated with 8 ml/kg and 0 cmH_2O of positive end-expiratory pressure (ZEEP) for 3 hours, and 3) High Vt group (HVt), ventilated with 30 ml/kg and ZEEP for 3 hours. To maintain normocapnia without decreasing respiratory rate, instrumental dead space was increased in the HVt group.

At baseline, animals in the MV groups underwent volume-controlled ventilation with 8 ml/kg Vt and 2 cmH_2O PEEP. Inspired oxygen fraction (FiO_2) was kept at 0.4

throughout the experiment, and the respiratory rate was adjusted for normocapnia. We measured values of MAP, arterial blood gases, and respiratory system parameters 15 minutes after initiating MV (baseline) and hourly thereafter after randomization.

Physiological parameters, including blood gas analysis, blood pressure, temperature, tidal volume, respiratory rate, and inspired gas composition, were measured recorded hourly. Inspiratory and expiratory pauses were applied to calculate static lung compliance (Crs). Fluid management was identical in all groups (Ringer-lactate, 10 $ml \cdot kg^{-1} \cdot h^{-1}$) to prevent differences that might favor edema formation, and vasoactive drugs were not used in any group. At the end of the 3-hour period, rats were euthanatized by exsanguination. We centrifuged 7 ml of blood from each animal and stored the plasma at -80º C for protein determinations. Hearts and lungs were removed en bloc, and the right lung was frozen for additional tissue analyses of proteins. Rats' brains were removed from the cranium by careful dissection and immediately frozen and stored at -80ºC.

Measurement of Cytokines/Chemokines

Commercially available enzyme linked immunosorbent assay (ELISA) kits (Biosource, Camarillo, CA, USA) were used to determine the following plasma protein levels: tumor necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-2 and interleukin (IL)-6.

Lung tissue was homogenized in a lysis buffer and the protein content of the lysate was quantified using the same commercially kits than we used in plasma samples.

Analyses of all samples, standards, and controls were run in duplicate following the manufacturer's recommendation.

Histological analysis

At the end of the experiment, the lungs were removed and fixed via intratracheal instillation of 4% buffered formaldehyde and immersed in the same fixative. After

fixation, the lungs were embedded in paraffin, and they were sectioned and processed for histological examination. Histological scores were calculated after hematoxylin-eosin (HE) staining as described elsewhere [12] and assessed intraalveolar neutrophil infiltration by counting the number of neutrophils in fifty fields per animal at a magnification of X400 using ImageJ v1.40g (Wayne Rasband, NIH, USA).

Lung damage was determined using a Lung Injury Score (LIS) based on the evaluation of alveolar edema, hemorrhage, neutrophil infiltration and alveolar septal thickening in each animal. Each parameter was scored from 0 to 4. Subsequently, the total LIS was calculated by adding the individual score for each parameter up to a maximum score of 16 [12].

Immunohistochemistry

At the end of the experiment, the brains were removed and immediately frozen and stored at -80ºC. Frozen brains were cut into 20-µm coronal sections (cryostat CM1900, Leica Microsystems, Spain) and stored at -80ºC until further processing. Sections were processed for c-fos immunohistochemistry to asses the neuronal activation in the thalamus, retrosplenial cortex (RS), central amygdala (CeA), hippocampus, paraventricular hypothalamic nuclei (PVN), and supraoptic nucleus (SON). Additional sections were stained with cresyl violet to identify the regions of interest.

Briefly, sections were first dried, post-fixed in 4% paraformaldehyde solution (PFA) for 10 minutes. Endogenous peroxidase was blocked with a solution at 1.5% H_2O_2. They were then rinsed with phosphate buffer saline (pH 7.4, 0.1M). Nonspecific binding was reduced by incubation with a blocking solution containing 5% normal goat serum, 3.5% egg whites and 0.35% triton X-100 for 60 min followed by incubation with 0.01% biotin. Sections were then incubated in a polyclonal rabbit antibody against c-fos (c-fos (4), Santa Cruz Biotechnology) at a dilution of 1:250 for 30 min at room temperature. Then sections were incubated with biotinylated goat anti-rabbit IgG for 30 min and then with avidin–biotin horseradish peroxidase solution (ABC kit, Vector laboratories). The c-fos

antibody-peroxidase complex was revealed using 0.06% diaminobenzidine (Sigma) and 0.01% H_2O_2. Finally, the slides were dehydrated with ethanol series and coverslipped with DPX.

Image analysis

After immunostaining, specific activated areas were identified by light microscopy (DM250, Leica, Wetzlar, Germany) with the aid of a stereotaxic atlas [14]. Images were digitized and a semi-quantitative analysis of c-fos positive cells was performed using the ImageJ software (ImageJ 1.40g, W.Rasband, NIH, USA). c-fos positive nuclei were evaluated according to the intensity of staining and an optimal threshold was set to avoid any background signal.

Statistical analysis

We used power analysis for ANOVA designs to estimate the sample size assuming an α error of 0.05 and β error of 0.2 (Granmo 5.2 software). U-Mann-Withney non-parametric tests were used to analyze differences between groups. All values are expressed as mean ± SEM. Significance was set at $p<0.05$.

RESULTS

Animal body weights were similar in all groups. At baseline, no differences in hemodynamics or gas exchange were observed between MV groups. Control rats were exanguinated at time zero and were used as the baseline group in comparison between groups.

Physiological variables

MAP remained stable within the normal range throughout the 3-hour period in all groups. Respiratory system compliance (Crs) and plateau pressure (Pplateau) increased with HVt MV, but both remained unchanged throughout the experimental period. Respiratory rates were not significantly different between LVt and HVt animals (mean 47.3 vs 47, respectively; p=0.7). Significant decreases in PaO_2/FiO_2 and pH and concurrent increases in $PaCO_2$ were found in LVt animals after 3 hours of MV **(figure 1)**

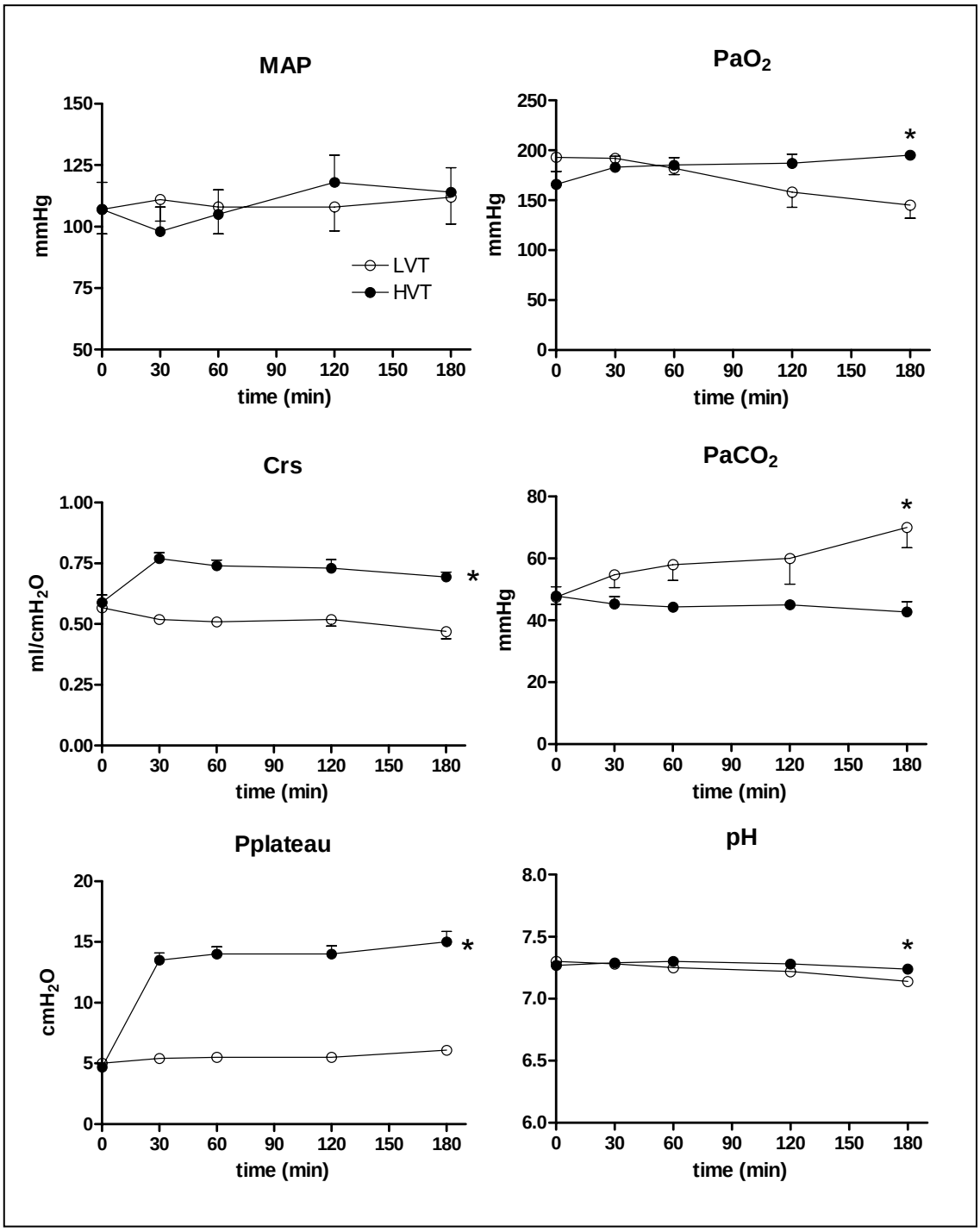

Figure 1. Hemodynamic and respiratory characteristics of rats during the 3-h period. No differences between groups were observed at baseline. MAP remained stable in both groups. Pplateau and Crs increased significantly during HVt ventilation but remained stable during the 3-h period. There were not differences between LVt and HVt in Pa/FiO_2. PCO2 increased only in LVt animals. Data are presented as mean± SE. *: $p < 0.05$ versus the HVt group. n= 8 animals per group. Abbreviations: MAP: mean arterial pressure; CRL: control; LVt: low tidal volume; HVt: high tidal volume; Pplateau: plateau pressure; Crs: static compliance of the respiratory system.

Histology

Figure 2 shows representative images of lungs in each experimental group. Lung neutrophilic infiltration and LIS were significantly higher in MV rats than in unventilated rats, but no differences between LVt and HVt were found **(figure 2)**

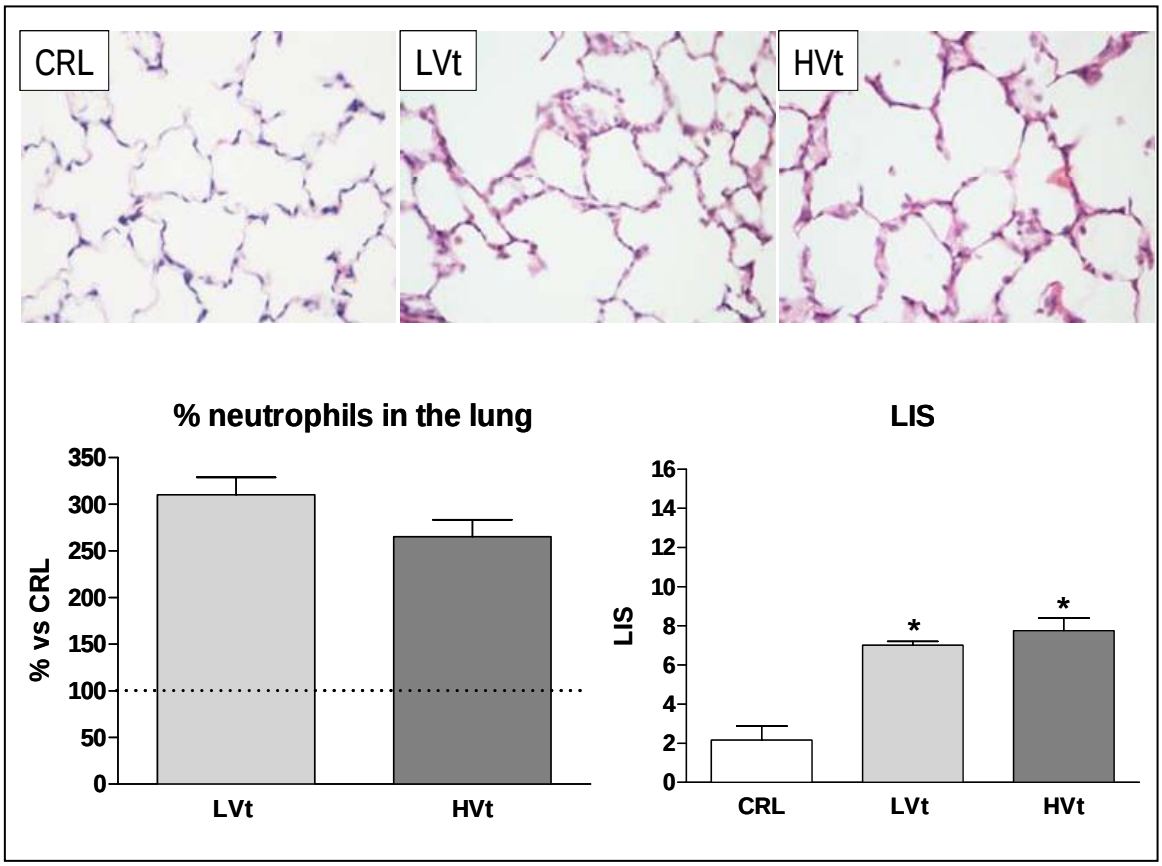

Figure 2. Representative images of lungs in each group after H-E staining and LIS.
% of lung neutrophil content and LIS increased with MV but was similar in animals receiving LVt and HVt. Results are represented as mean ± SE. *p<0.05 versus the unventilated control group. n= 8 animals per group. Abbreviations: CRL: control; LVt: low tidal volume; HVt: high tidal volume; LIS: Lung injury score.

c-fos immunopositive brain areas

Neuronal activation evidenced by an increased number of c-fos immunopositive cells was observed in the RS **(figure 3)** and thalamus **(figure 4)** of HVt rats, but not in LVt or control rats. Neuronal activation was also observed in the CeA **(figure 5)**, PVN **(figure 6)**, and SON (data not shown) of MV rats, although activation did not differ between HVt and LVt animals. Similarly, no differences in c-fos activation in other cortical areas or in the hippocampus were observed between the experimental groups (data not shown).

Coronal section diagrams encompassing areas of interest are represented at the top of **figures 3 to 6** (modified from reference 14).

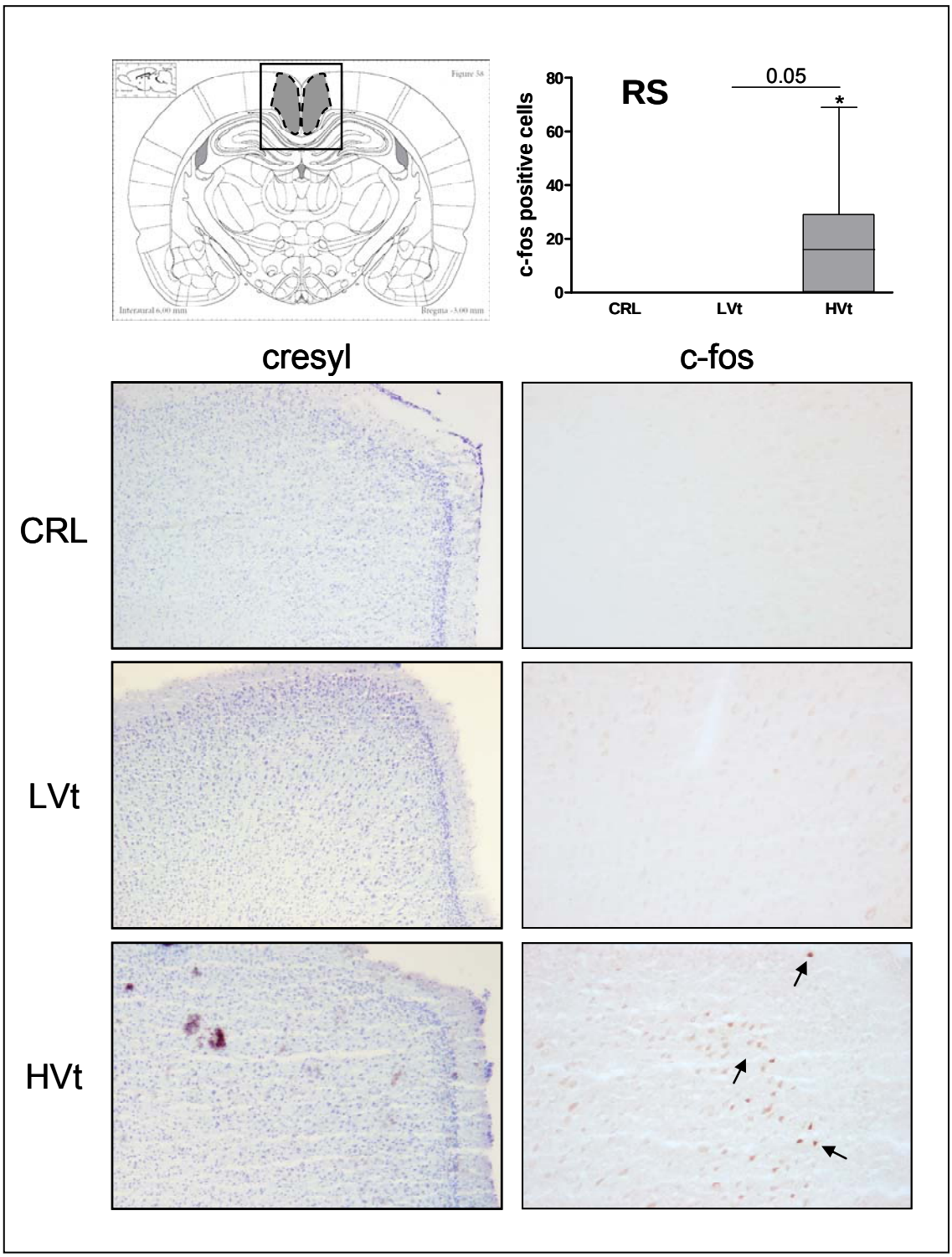

Figure 3. Brain activation evidenced by c-fos immunoreactivity in the retrosplenial cortex. Representative images of RS from each experimental group after immunohistochemical staining. Black arrows indicate c-fos positive cells: HVt increased the number of c-fos-positive neurons in the RS; lower levels of neuronal activation were found in unventilated and LVt animals. Data are presented as mean ± SE. *p<0.05 respect to unventilated control animals. Abbreviations: CRL: control; LVt: low tidal volume; HVt: high tidal volume; RS: retrosplenial cortex.

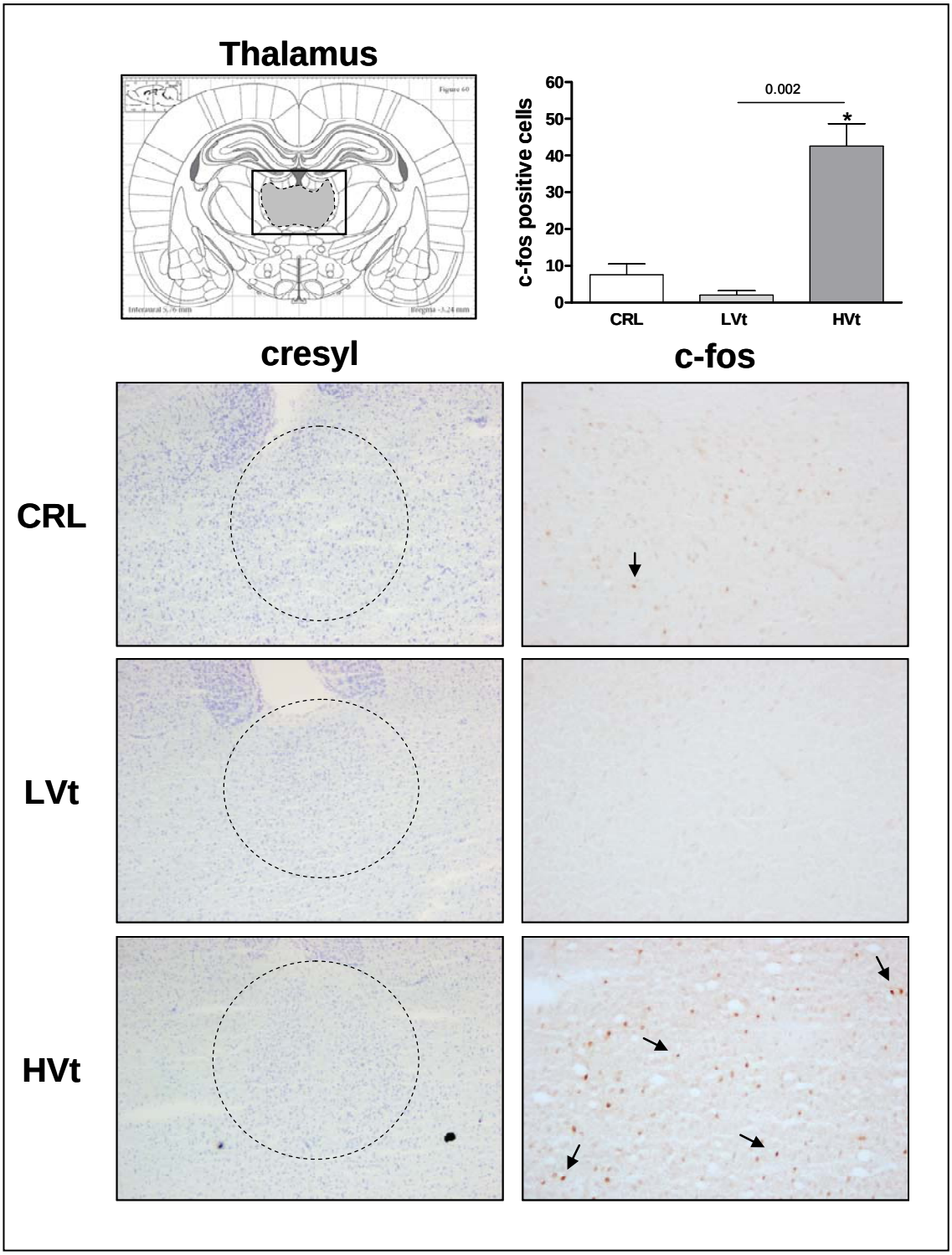

Figure 4. Brain activation evidenced by c-fos immunoreactivity in thalamus. Representative images of thalamus from each experimental group after immunohistochemical staining. Black arrows indicate c-fos positive cells: HVt increased the number of c-fos-positive neurons in the thalamus. Data are presented as mean ± SE. *p<0.05 respect to unventilated control animals. Abbreviations: CRL: control; LVt: low tidal volume; HVt: high tidal volume.

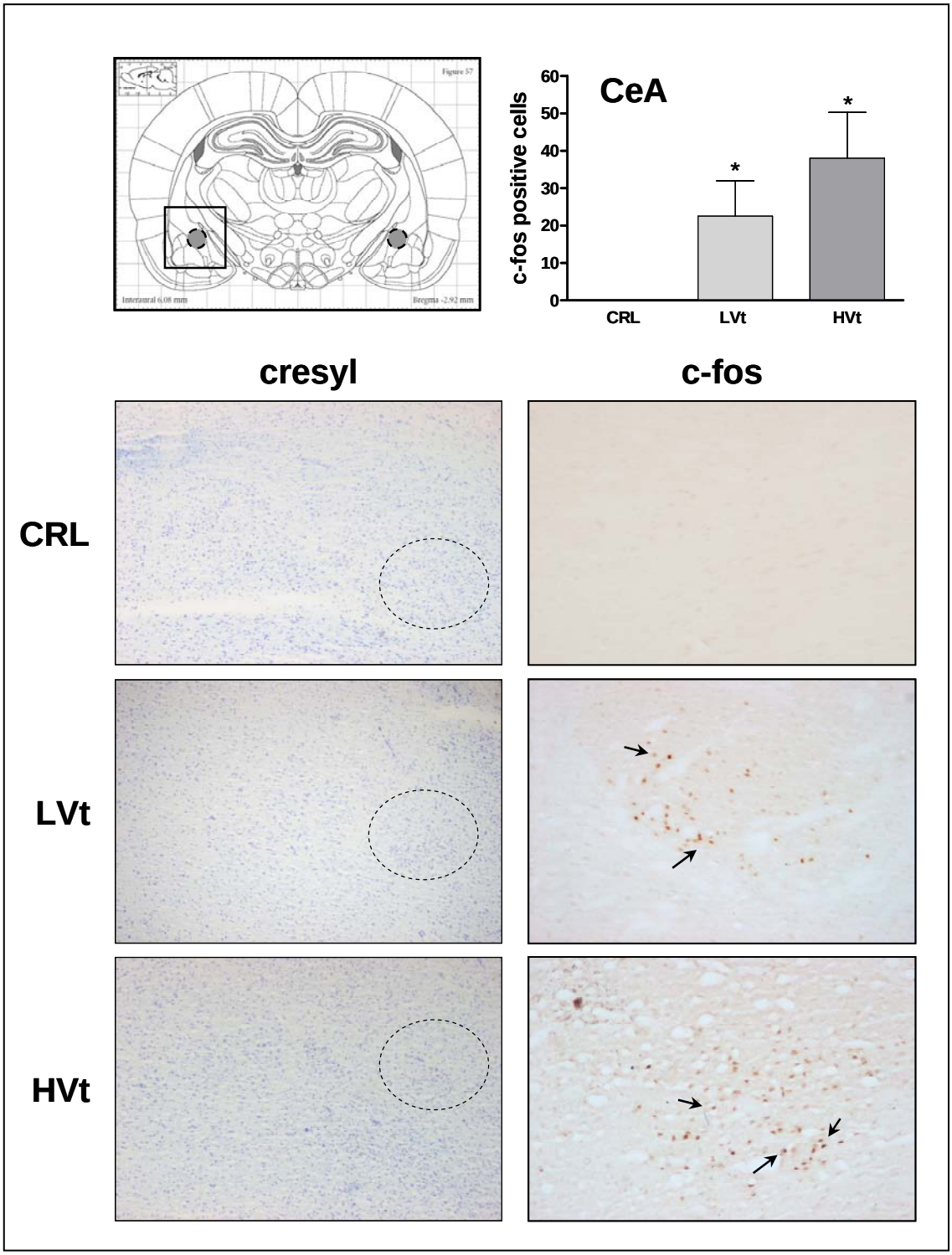

Figure 5. Brain activation evidenced by c-fos immunoreactivity in Central Amygdala. Representative images of CeA from each experimental group after immunohistochemical staining. Black arrows indicate c-fos positive cells: MV significantly increased neuronal activation in the CeA independently of the Vt level. Few c-fos positive cells were found in CeA of control animals. Data are presented as mean ± SE. *p<0.05 respect to unventilated control animals. Abbreviations: No MV: unventilated animals; LVt: low tidal volume; HVt: high tidal volume; CeA: Central amygdala.

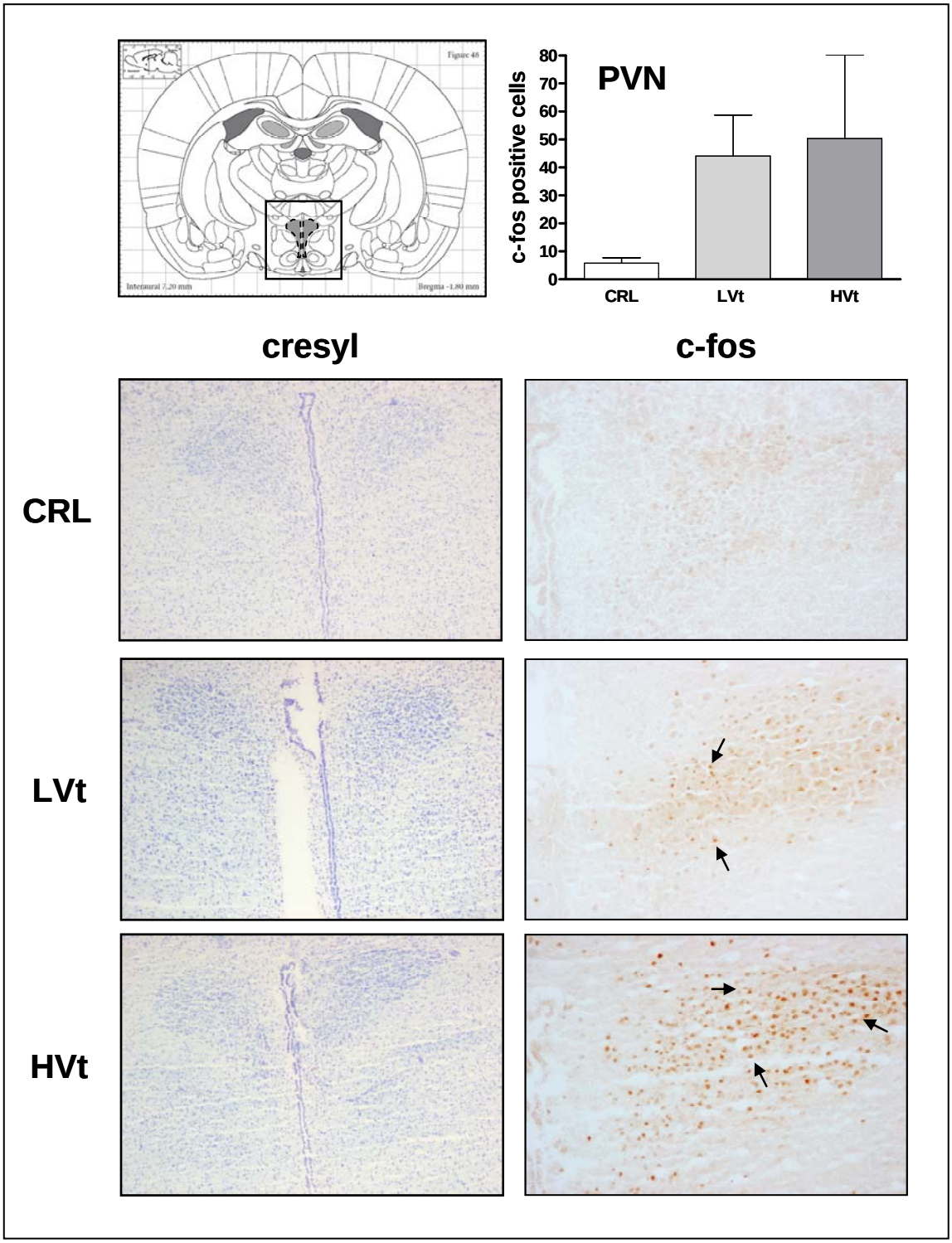

Figure 6. Brain activation evidenced by c-fos immunoreactivity in Paraventricular hypothalamic nuclei. Representative images of PVN from each experimental group after immunohistochemical staining. Black arrows indicate c-fos positive cells: Neuronal activation in the PVN tended to increase with MV, but this increase did not reach significance compared with controls. Data are presented as mean ± SE. *p<0.05 respect to unventilated control animals. Abbreviations: CRL: control; LVt: low tidal volume; HVt: high tidal volume; PVN: Paraventricular hypothalamic nuclei.

Inflammatory mediators

MV increased plasma levels of IL-6 and MIP-2 in LVt and HVt groups ($p< 0.05$). However, plasma TNFα levels increased significantly after 3 hours of HVt ventilation ($p=0.005$) but remained unaltered in the LVt group.

In the lungs, irrespective of the Vt level, MV increased IL-6 and MIP-2 levels. Lung TNFα levels were similar in MV and unventilated animals. Taken all together, the inflammatory response was higher (but also more variable) in the HVt group than in the LVt group **(figure 7)**

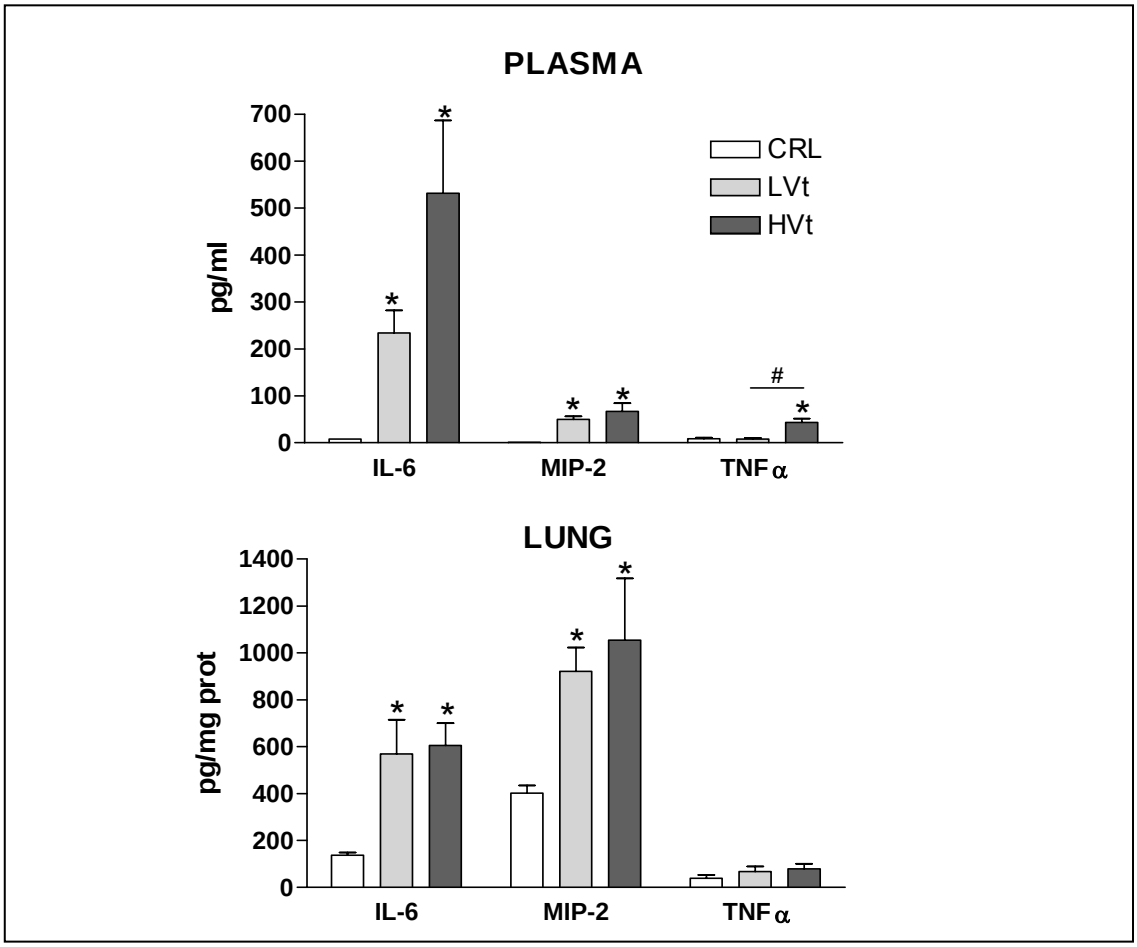

Figure 7. Plasma and lung levels of proteins involved in the inflammatory cascade. Mechanical ventilation triggered lung and systemic inflammatory responses. Compared to LVt, HVt promoted an increase in inflammatory markers mainly mediated by TNFα at the plasma level. Data are presented as mean ± SE. *$p<0.05$ respect to unventilated control animals, # $p<0.05$ vs LVt . n= 8 animals per group. Abbreviations: CRL: control; LVt: low tidal volume; HVt: high tidal volume; IL: interleukin, TNF: tumor necrosis factor; MIP: macrophage-inflammatory protein.

DISCUSSION

We found that MV induced c-fos expression in discrete areas of the brain in healthy and non-hypoxemic rats. Moreover, HVt ventilation caused more neuronal activation when compared to LVt ventilation, thus supporting the hypothesis that an iatrogeneous effect of MV may affect the brain. These results provide novel and important data that might have clinical relevance during the management of critically ill patients.

The immediate early gene *c-fos* [15] is rapidly induced and can be detected by immunochemistry; therefore it is a valuable tool for determining which brain areas are stimulated [16, 17]. The basal expression of c-fos is low, but can be dramatically induced by a variety of stimuli and conditions such as metabolic stress, ischemia, and inflammation, among others [18, 19]. Various mechanisms are probably involved in the response to MV. Lungs can "sense" mechanical stimuli by lung mechanoreceptors that can communicate to the brain. The autonomic nervous system is also involved in this crosstalk [20-22].

The ventilatory strategy may also affect the CNS by altering the inflammatory cascade promoted by mechanotransduction. In the present study, as reported elsewhere, we have used two different MV strategies that triggered proinflammatory responses even in subjects receiving LVt [4, 5, and 23]. The proinflammatory response to HVt was found mainly at the systemic level and was mediated by TNFα [6, 23, and 24]. Only minimal differences in other cytokines, lung function parameters or LIS were found between MV groups. The release of inflammatory mediators [23, 24] or certain metabolites to the bloodstream can be sensed by the brain, altering the permeability of the blood brain barrier [22, 25] or modifying cerebral blood flow. No data is available about the contribution of these two mechanisms in the activation observed in the brain areas studied in our model.

HVt consistently increased c-fos in the RS and thalamus, neither of which were activated in LVt or CRL animals. Moreover, in the literature RS and thalamus have

been linked to neurological disorders after stress [26, 27], fatigue-loading in rats [28, 29]; emotional or psychological stress might also induce neuronal activation in cortical and limbic regions [16, 30].

In the present study we cannot determine whether the regional brain activation observed in LVt group was caused by moderate hypercapnia. This impaired gas exchange in the LVt group is compatible with progressive alveolar de-recruitment in the absence of PEEP. The higher level of brain activation observed in the HVt group occurred in the context of normocapnia, thus suggesting that the mechanisms inducing cell activation in these brain areas are different in HVT, which deserves being explored in further investigations.

Our results were obtained in the context of preserved lung function and hemodynamic stability. The magnitude of the response to HVt observed by different authors varies [23, 24, 31-33], and some authors have reported detrimental effects of HVt on MAP [34]. However, we found that adequate fluid management ensured MAP stability throughout the experimental procedure (3h), corroborating previous findings in our laboratory [11, 23]. Therefore, the differences in the results could not be attributed to differential organ perfusion.

Clinical relevance

Due to the novelty of this issue (brain activation and MV) and the limitations of the study, we can only speculate about the translation of these results to the clinical setting. The etiology of cognitive impairment in critically ill patients is undoubtedly multifactorial and is the subject of ongoing discussion [2, 3]. Nevertheless, crosstalk between the lung and brain is poorly understood [22], and although many randomized controlled clinical trials have evaluated the efficacy and safety of various methods of MV in ARDS and ALI patients, few studies have explored the influence of MV patterns at the neuronal level. Our findings about regional brain activation during MV could help define particular areas susceptible to be activated by mechanoreceptors in the lung. Those areas might play a crucial role in regulating early events occurring during the application of non-adequate MV patterns. Our findings might have implications for understanding how the brain senses incoming signals or insults from the lungs in anesthetized and paralyzed subjects.

In summary, our data further support the concept of brain-lung interaction during MV and indicate the importance of the ventilatory settings used. These findings may therefore have clinical relevance and emphasize the importance of further research in this field.

REFERENCES

1. Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD: **Incidence and outcomes of acute lung injury.** *N Engl J Med* 2005, **353**:1685-93.
2. Hopkins RO, Jackson JC: **Long-term neurocognitive function after critical illness**. *Chest* 2006, 130:869-78.
3. Jackson JC, Girard TD, Gordon SM, Thompson JL, Shintani AK, Thomason JW, Pun BT, Canonico AE, Dunn JG, Bernard GR, Dittus RS, Ely EW: **Long-term cognitive and psychological outcomes in the Awakening and Breathing Controlled trial**. *Am J Respir Crit Care Med* 2010, 182:183-91.
4. Wolthuis EK, Vlaar AP, Choi G, Roelofs JJ, Juffermans NP, Schultz MJ. **Mechanical ventilation using non-injurious ventilation settings causes lung injury in the absence of pre-existing lung injury in healthy mice.** *Crit Care* 2009, 13: R1.
5. Vaneker M, Joosten LA, Heunks LM, Snijdelaar DG, Halbertsma FJ, van Egmond J, Netea MG, van der Hoeven JG, Scheffer GJ: **Low-tidal-volume mechanical ventilation induces a toll-like receptor 4-dependent inflammatory response in healthy mice**. *Anesthesiology* 2008, 109:465-72.
6. Ranieri VM, Suter PM, Tortorella C, De Tullio R, Dayer JM, Brienza A, Bruno F, Slutsky AS: **Effect of mechanical ventilation on inflammatory mediators in patients with acute respiratory distress syndrome: a randomized controlled trial**. *JAMA* 1999, 282:54-61.
7. Pustavoitau A, Stevens RD: **Mechanisms of neurologic failure in critical illness**. *Crit Care Clin* 2008, 24: 1-24.
8. Akrout N, Sharshar T, Annane D: **Mechanisms of brain signaling during sepsis**. *Curr Neuropharmacol* 2009, 7: 296-301.

9. Quan N: **Brain's firewall: blood-brain barrier actively regulates neuroimmune information flow**. *Brain Behav Immun* 2006, 20: 447-8.
10. Sanz O, Estrada A, Ferrer I, Planas AM: **Differential cellular distribution and dynamics of HSP70, cyclooxygenase-2, and c-Fos in the rat brain after transient focal ischemia or kainic acid**. *Neuroscience* 1997, 80: 221-32.
11. López-Aguilar J, Villagrá A, Bernabé F, Murias G, Piacentini E, Real J, Fernández-Segoviano P, Romero PV, Hotchkiss JR, Blanch L: **Massive brain injury enhances lung damage in an isolated lung model of ventilator-induced lung injury.** *Crit Care Med* 2005, 33:1077-83.
12. Murakami K, Bjertnaes LJ, Schmalstieg FC, et al: **A novel animal model of sepsis after acute lung injury in sheep.** *Crit Care Med* 200, 30: 2083-2090.
13. Morgan JI, Curran T: **Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes fos and jun.** *Annu Rev Neurosci* 1991. 14:421-51.
14. Paxinos G and Watson C: **The rat brain in stereotaxic coordinates** 6th eds Academic Press; 2007.
15. Akazawa KH, Cui Y, Tanaka M, Kataoka Y, Yoneda Y, Watanabe Y: **Mapping of regional brain activation in response to fatigue-load and recovery in rats with c-Fos immunohistochemistry**. *Neurosci Res* 2010, 66:372-379.
16. Jankord R, Herman JP: **Limbic regulation of hypothalamo-pituitary-adrenocortical function during acute and chronic stress**. *Ann N Y Acad Sci* 2008, 1148:64-73.
17. Zhang J, Zhang D, McQuade JS, Behbehani M, Tsien JZ, Xu M: **c-fos regulates neuronal excitability and survival**. *Nat Genet* 2002, 30:416-20.
18. Chaudhuri A, Zangenehpour S, Rahbar-Dehgan F, Ye F: **Molecular maps of neural activity and quiescence**. *Acta Neurobiol Exp* 2000, 60: 403-10.
19. Tracey KJ: **The inflammatory reflex**. *Nature* 2002, 420:853-859.

20. Dos Santos CC, Shan Y, Akram A, Slutsky AS, Haitsma JJ: **Neuroimmune Regulation of Ventilator-Induced Lung Injury**. *Am J Respir Crit Care Med* 2010, Sep 24. [Epub ahead of print].

21. Kubin L, Alheid GF, Zuperku EJ, McCrimmon DR: **Central pathways of pulmonary and lower airway vagal afferents.** *J Appl Physiol* 2006, 101:618-27.

22. Gonzalvo R, Martí-Sistac O, Blanch L, López-Aguilar J: **Bench-to-bedside review: brain-lung interaction in the critically ill--a pending issue revisited**. *Crit Care* 2007, 11: 216.

23. López-Aguilar J, Quilez ME, Martí-Sistac O, García-Martín C, Fuster G, Puig F, Flores C, Villar J, Artigas A, Blanch L: **Early physiological and biological features in three animal models of induced acute lung injury**. *Intensive Care Med* 2010, 36:347-55.

24. Imai Y, Parodo J, Kajikawa O, de Perrot M, Fischer S, Edwards V, Cutz E, Liu M, Keshavjee S, Martin TR, Marshall JC, Ranieri VM, Slutsky AS: **Injurious mechanical ventilation and end-organ epithelial cell apoptosis and organ dysfunction in an experimental model of acute respiratory distress syndrome.** *JAMA* 2003, 289:2104-12.

25. Niimi M, Wada Y, Sato M, Takahara J, Kawanishi: **Effect of continuous intravenous injection of interleukin-6 and pretreatment with cyclooxigenase inhibitor on brain c-fos expression in the rat.** *Neuroendocrinol* 1997, 66:47-53.

26. Senba E, Ueyama T: **Stress-induced expression of immediate early genes in the brain and peripheral organs of the rat**. *Neurosci Res* 1997, 29:183-207.

27. Senba E, Matsunaga K, Tohyama M, Noguchi K: **Stress-induced c-fos expression in the rat brain: activation mechanism of sympathetic pathway**. *Brain Res Bull* 1993, 31: 329-44.

28. Pothuizen HH, Davies M, Aggleton JP, Vann SD: **Effects of selective granular retrosplenial cortex lesions on spatial working memory in rats.** *Behav Brain Res* 2010, 208:566-75.

29. Dumont JR, Petrides M, Sziklas V: **Fornix and retrosplenial contribution to a hippocampo-thalamic circuit underlying conditional learning**. *Behav Brain Res* 2010, 209:3-20.
30. Herman JP, Cullinan WE: **Neurocircuitry of stress: central control of the hypothalamo-pituitary- adren ocortical axis**. *Trends Neurosci* 1997, 20:78-84.
31. Ricard JD, Dreyfuss D, Saumon G: **Ventilator-induced lung injury**. *Eur Respir J* 2003, 42: 2-9.
32. Matute-Bello G, Frevert CW, Martin TR: **Animal models of acute lung injury**. *Am J Physiol Lung Cell Mol Physiol* 2008, 295:379-99.
33. Dos Santos CC, Slutsky AS: **Mechanisms of ventilator-induced lung injury: A perspective**. *J Appl Physiol* 2000, 89:1645–1655.
34. Martínez-Caro L, Lorente JA, Marín-Corral J, Sánchez-Rodríguez C, Sánchez-Ferrer A, Nin N, Ferruelo A, de Paula M, Fernández-Segoviano P, Barreiro E, Esteban A: **Role of free radicals in vascular dysfunction induced by high tidal volume ventilation**. *Intensive Care Med* 2009, 35:1110-1119.