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Research report

# Pilocarpine-induced *status epilepticus* reduces chemosensory control of breathing

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ABSTRACT

One of the possible causes of death in epilepsy is breathing disorders, especially apneas, which lead to an increase in $CO_2$ levels (hypercapnia) and/or a decrease in $O_2$ levels in arterial blood (hypoxemia). The respiratory neurons located in the ventral brainstem respiratory column are the main groups responsible for controlling breathing. Recent data from our group demonstrated respiratory changes in two experimental models of epilepsy, i.e. audiogenic epilepsy, and amygdala rapid kindling. Here, we aimed to evaluate respiratory changes in the classic model of temporal lobe epilepsy induced by intra-hippocampal injection of pilocarpine. Adult Wistar rats with stainless-steel cannulas implanted in the hippocampus region were used. The animals were submitted to pilocarpine injection (2.4 mg/μL, $N$ = 12–15) or saline ($N$ = 9) into the hippocampus. The respiratory parameters analyzed by whole-body plethysmography were respiratory rate ($f_R$), tidal volume ($V_T$) and ventilation ($V_E$). Respiratory mechanics such as Newtonian airway resistance ($R_n$), viscance of the pulmonary parenchyma ($G$) and the elastance of the pulmonary parenchyma ($H$) were also investigated. No changes in baseline breathing were detected 15 or 30 days after pilocarpine-induced *status epilepticus* (SE). However, 30 days after pilocarpine-induced SE, a significant reduction in $V_E$ was observed during hypercapnic (7% $CO_2$) stimulation, without affecting the hypoxia (8% $O_2$) ventilatory response. We also did not observe changes in respiratory mechanics. The present results suggest that the impairment of the hypercapnia ventilatory response in pilocarpine-induced SE could be related to a presumable degeneration of brainstem respiratory neurons but not to peripheral mechanisms.

## 1. Introduction

Epilepsy is considered a common neurological disease and approximately 70 million people worldwide have been diagnosed (Ngugi et al., 2010). Epilepsies are characterized by spontaneous recurrent seizures and loss of neuronal cells by abnormal rhythmic firing neurons in the brain (Duncan et al., 2006). In both humans and experimental models of epilepsy, a decline in the cognitive abilities (Bell et al., 2011), as well as cellular modifications characterized by neuronal loss in the hippocampal region, inflammation, gliosis, neurogenesis and autonomic imbalance have been observed (Lopim et al., 2016; Totola et al., 2019; Vitorino et al., 2016).

Pilocapine-induced *status epilepticus* (SE) is one of the most widely used model of the seizure (Turski et al., 1989; Cavalheiro, 1995). The hallmark mortality in epilepsy highly account for a cascade of cardiorespiratory events preceding seizures and death (Ryvlin et al., 2013; Totola et al., 2019; Marincovich et al., 2019).

Experimental evidence recapitulates a seizure-induced respiratory arrest phenotype. Perhaps the most well-known models are the DBA/1 and DBA/2 mice audiogenic seizure models (Marincovich et al., 2019). These mice commonly exhibit respiratory arrest following audiogenic seizure. In addition, we have also reported that Wistar Audiogenic Rat (WAR) and the rapid amygdala kindling models of epilepsy also have respiratory impairments (Totola et al., 2017, 2019). Collectively, these findings, together with a recent literature on breathing disorders and epilepsy, demonstrated that breathing abnormalities could represent a potential cause of death in epilepsy (Totola et al., 2017, 2019; Kim et al., 2018; Kuo et al., 2019).

Several studies have recognized that seizures commonly cause apnea and oxygen desaturation (Bateman et al., 2008), and given the

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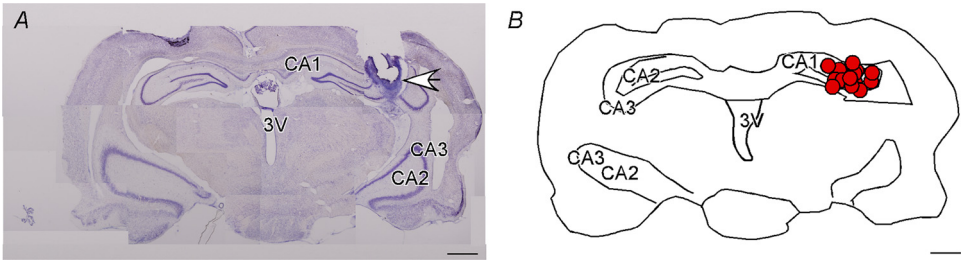


**Fig. 1.** Location of pilocarpine injections in hippocampus.
(A) Photomicrograph of a coronal section showing the site of a unilateral injection in the dorsal hippocampus. (B) Computer-generated plots of injections that were confined to the dorsal hippocampus region (Bregma level -4.4 mm according to the Paxinos and Watson atlas, 2007). Abbreviations: CA1, CA1 region of the hippocampus; CA2, CA2 region of the hippocampus; CA3, CA3 region of the hippocampus; 3 V, third ventricle. Scale bar = 1 mm for (A)–(B).

fact that we observed a significant breathing impairment in both WAR and amygdala kindling models of epilepsy (Totola et al., 2017, 2019), in the current study, we aimed to evaluate the breathing activity and cellular lung profile using the experimental model of SE induced by intra-hippocampal injection of pilocarpine.

## 2. Results

### 2.1. Histological analysis

Injections of pilocarpine (2.4 mg/μL – 1 μL) were placed unilaterally in the right dorsal hippocampus ($N$ = 15) in unrestrained conscious rats (Figs. 1A and B). The injections centers were located 3.0 mm below the dura mater, 4.4 mm caudal to the Bregma level and 4.5 mm lateral to the midline. The majority of the cannulas implanted outside the hippocampus region reached the ventro postero lateral or medial aspect of the thalamic nuclei (VPL or VPM) (data not shown).

### 2.2. Breathing responses in pilocarpine-induced status epilepticus

The first series of experiments were performed to characterize baseline breathing patterns in the control and pilocarpine-induced SE (15 or 30 days) in unrestrained conscious rats.

Fifteen days after pilocarpine-induced SE, we did not observe significant changes in baseline $V_T$ (5.9 ± 0.6 vs. control: 5.5 ± 1.5 mL/kg, $p > 0.05$), $f_R$ (87 ± 4.1 vs. control: 86 ± 5 breaths/min, $p > 0.05$), $V_E$ (503 ± 43 vs. control: 454 ± 30 mL/kg/min, $p > 0.05$) (Fig. 2A–C). Challenging the animals to 8% $O_2$ (hypoxia ventilatory response – HVR), the increase in $V_T$ (9.5 ± 0.4 vs. control: 8.6 ± 0.5 mL/kg, $p > 0.05$), $f_R$ (139 ± 4.2 vs. control: 141 ± 4.4 breaths/min, $p > 0.05$) and $V_E$ (1323 ± 173 vs. control: 1240 ± 114 mL/kg/min, $p > 0.05$) were not affected in pilocarpine-treated rats (Fig. 2A–C). On the other hand, challenging the animals to 7% $CO_2$ (hypercapnia ventilatory response – HCVR), we found a significant reduction in the increase in $V_T$ (9.9 ± 0.4 vs. control: 11.2 ± 0.5 mL/kg, $p < 0.05$) and $V_E$ (1321 ± 57 vs. control: 1617 ± 65 mL/kg/min, $p < 0.001$), without changing the tachypneic response to hypercapnia (133 ± 3.4 vs. control: 144 ± 3 breaths/min, $p > 0.05$) (Figs. 2A–C).

We also analyzed breathing parameters 30 days after pilocarpine-induced SE. The group data showed that 30 days after SE no changes in baseline breathing parameters were noticed. For example, baseline $V_T$ (4.3 ± 0.2 vs. control: 4.2 ± 0.13 mL/kg/min, p > 0.05), $f_R$ (88 ± 5 vs. control: 92 ± 3 breaths/min, p > 0.05) and $V_E$ (371 ± 20 vs. control: 384 ± 10 mL/kg/min, $p > 0.05$) were not affected 30 days after pilocarpine-induced SE (Fig. 3A–C). As expected, hypoxia increased breathing parameters, but pilocarpine-induced SE did not change the increase in $V_T$, $f_R$ and $V_E$ (Fig. 3A–C). Hypercapnia increase $V_T$, $f_R$ and $V_E$ in both control and pilocarpine-induced SE groups; however, 30 days after SE, the increase in $V_T$ (6.2 ± 0.4 vs. control: 8.3 ± 0.6 mL/kg, $p < 0.001$), $f_R$ (132 ± 1.6 vs. control: 152 ± 2 breaths/min, $p < 0.001$) and $V_E$ (817 ± 39 vs. control: 1266 ± 28 mL/kg/min, $p < 0.001$) were reduced during $CO_2$ challenge (Fi$CO_2$ = 7%) (Fig. 3A–C).

We did not find significant changes in body temperature during

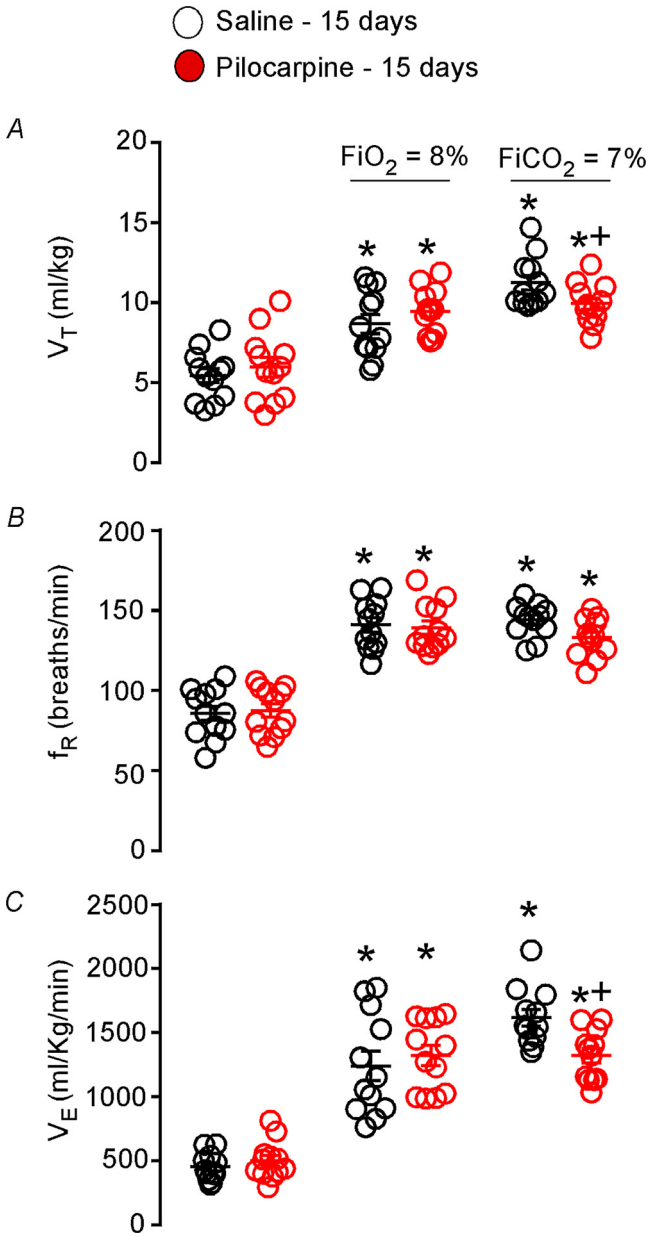


**Fig. 2.** Respiratory changes 15 days after pilocarpine-induced *status epilepticus*. Changes in (A) tidal volume ($V_T$, mL/kg), (B) respiratory rate ($f_R$, breaths/min), and (C) ventilation ($V_E$, mL/kg/min) under normoxia (baseline), lower levels of $O_2$ (hypoxia: 8%, bal $N_2$) and increased levels of $CO_2$ (hypercapnia: 7% $CO_2$, 21 % $O_2$, bal $N_2$) in saline (control) and pilocarpine-treated rats. Results represented as mean ± SEM. *Different from normoxia; +different from saline-treated rats. The significance level was set at $p < 0.05$, $N$ = 12 animals per group.

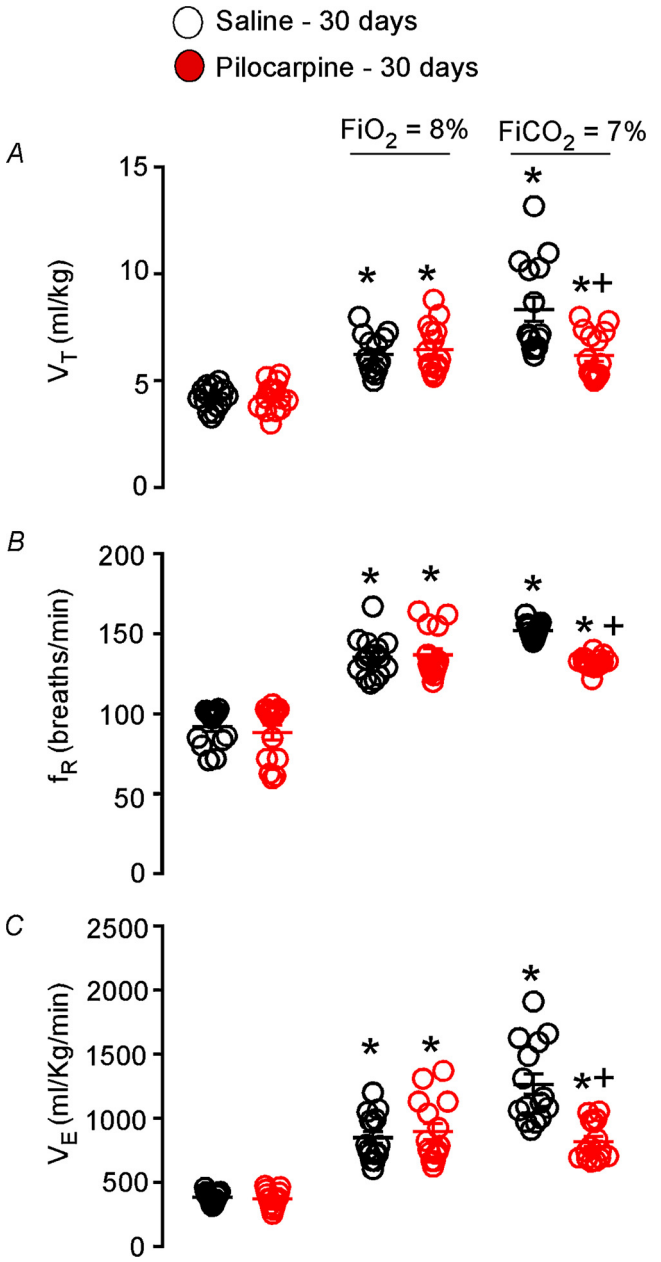


**Fig. 3.** Respiratory changes 30 days after pilocarpine-induced *status epilepticus*. Changes in (A) tidal volume ($V_T$, mL/kg), (B) respiratory rate ($f_R$, breaths/min), and (C) ventilation ($V_E$, mL/kg/min) under normoxia (baseline), lower levels of $O_2$ (hypoxia: 8%, bal $N_2$) and increased levels of $CO_2$ (hypercapnia: 7% $CO_2$, 21 % $O_2$, bal $N_2$) in saline (control) and pilocarpine-treated rats. Results represented as mean ± SEM. *Different from normoxia; +different from saline-treated rats. The significance level was set at $p < 0.05$, $N = 15$ animals per group.

normoxic/normocapnic conditions 15 or 30 days after pilocarpine-induced SE. For example, 15 and 30 days after pilocarpine-induced SE did not change body temperature (15 days after SE: 37.1 ± 0.15; 30 days: 36.9 ± 0.14 vs. saline: 37.2 ± 0.12 °C; one-way RM; $p > 0.05$). In addition, during the experiments, the mean chamber temperature was 26.5 ± 0.2 °C, and the mean room temperature was 25.1 ± 0.2 °C.

### 2.3. Peripheral breathing parameters in pilocarpine-induced status epilepticus

In order to evaluate the resistance of upper airways and lung properties, we used a subset of the rats that underwent the plethysmography experiment [$N = 6$ out of 12 (15 days after pilocarpine-induced SE) and $N = 9$ out of 15 (30 days after pilocarpine-induced SE)]. The analysis of respiratory mechanics was performed 15 and 30 days after pilocarpine-induced SE, both before and after opening of the thorax. No significant changes on airway resistance ($R_n$), tissue viscance ($G$) and tissue elastance ($H$) were observed between saline and pilocarpine-treated group (Fig. 4A–F). Important to point out that once the thorax had been opened, the tissue viscance ($G$) and tissue elastance (H) were reduced in both the saline and the pilocarpine-treated animals (Fig. 4B–C and E–F).

We also used the methacholine (MCh, muscarinic cholinergic receptor agonist: 100 μg/kg, i.v.) challenge and found no significant differences in saline and pilocarpine-treated animals (15 and 30 days) (Fig. 5).

In addition, we also quantified lung volume and the presence of mono- or polymorphonuclear cells in the bronchoalveolar lavage (BAL) in both saline and pilocarpine-treated rats. We did not find significant changes in lung volume or BAL when comparing saline and pilocarpine-treated rats (15 and 30 days) (Table 1).

## 3. Discussion

In the present study, we demonstrated a reduced HCVR in an experimental model of SE induced by pilocarpine into the hippocampus. We believe that our time-dependent analysis may have triggered a degeneration mechanism culminating in breathing impairment. Interestingly, we did not observe changes in respiratory mechanics, not even in the number of cells in the bronchoalveolar after pilocarpine-induced SE, suggesting a central mechanism that could compromise breathing.

## 4. The respiratory components of the central chemoreflex are affected in pilocarpine-induced *status epilepticus*

Recently, we demonstrated a considerable reduction of baseline breathing and the HCVR in two experimental models of epilepsy, i.e. the audiogenic epilepsy (Wistar audiogenic rat – WAR model) and the rapid amygdala kindling (ARK) model (Totola et al., 2017, 2019). Therefore, we suggested that WAR and the ARK animal models are not able to compensate for metabolic alterations, thus impairing the maintenance of basal oxygen consumption. In addition, we also observed a significant reduction in the number of serotonergic neurons located in the medullary raphe region, which could also explain the reduction of ventilation and oxygen consumption, since the importance of the neurons in this region to control metabolism has already been demonstrated (Ootsuka and Blessing, 2005). Differently from the former models, the classic experimental model of SE with intra-hippocampal injection of pilocarpine did not show a reduction in baseline breathing (present results). The lack of effect could be due to the fact that in general the pilocarpine model became chronic after an average of 44 days (Cavalheiro et al., 1991, 1994). We recorded breathing at 15 and 30 days after pilocarpine-induced SE. Thirty days after pilocarpine injection into the hippocampus, animals experienced the following steps: (i) an acute period which lasted 24 h which corresponds to the pattern of repetitive seizures and *status epilepticus*, and (ii) a silent period (4–44 days) characterized by a progressive return to normal EEG and behavior. Therefore, it is conceivable that pilocarpine-treated rats did not reach the generation, spread and motor expression of spontaneous recurrent seizures. Further experiments will be necessary to support the hypothesis that recurrent seizures are necessary to produce baseline respiratory changes, as demonstrated before in a different model of chronic temporal lobe epilepsy model, the WAR model (Garcia-Cairasco et al., 2017; Totola et al., 2019).

The grouped data observed in the present study are similar to the results observed in the ARK models (Totola et al., 2019). We noticed a

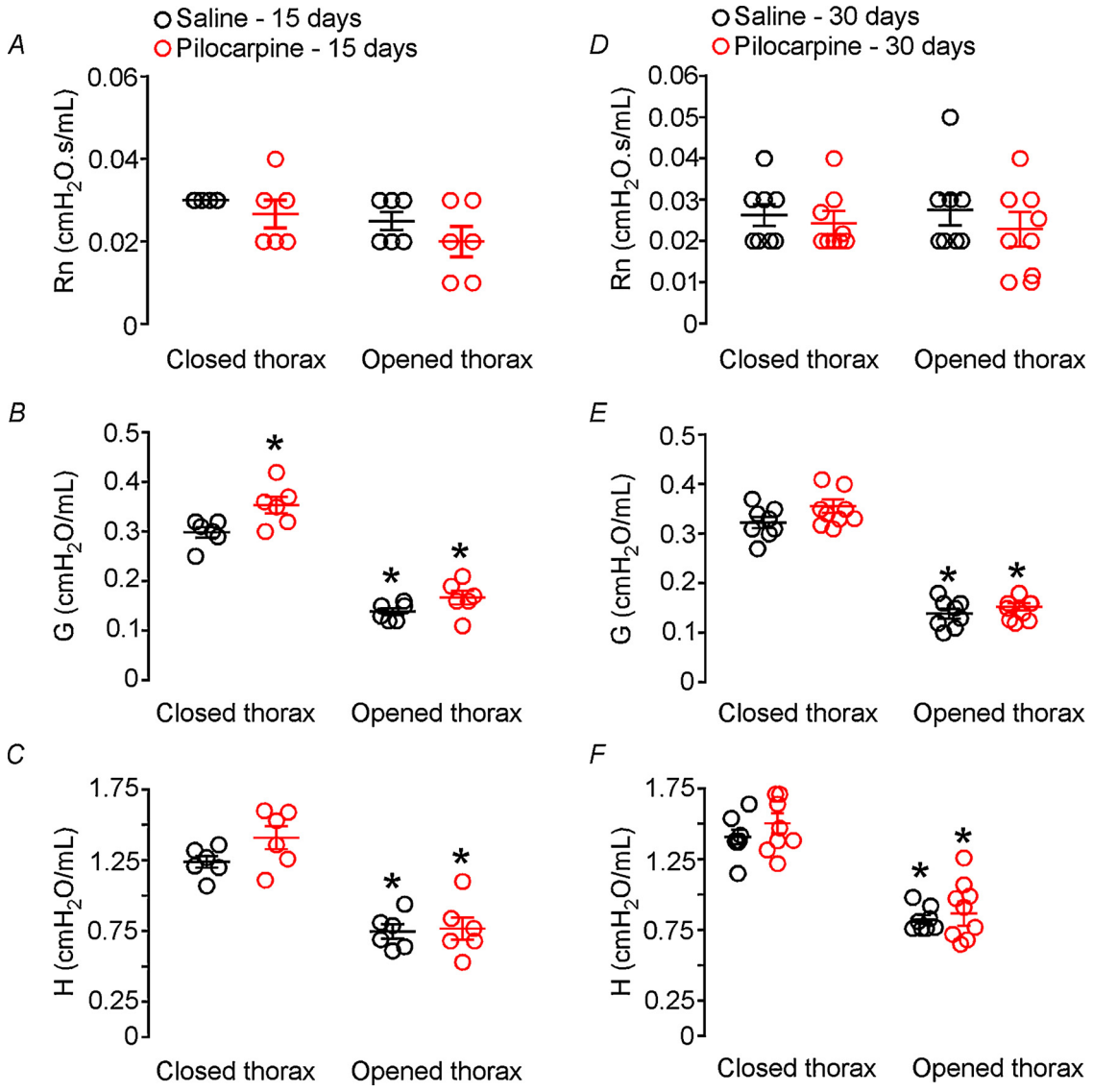


**Fig. 4.** Analysis of lung properties in pilocarpine-treated rats.
Changes in (A) and (D) airway resistance ($R_n$, $cmH_2O$ s/mL), (B and (E) tissue viscance (G, $cmH_2O$/mL) and (C and F) tissue elastance (H, $cmH_2O$/mL) while the thorax had been preserved or after thoracotomy in saline (control) and pilocarpine-treated rats. *Different from closed thorax. $N$ = 6–9 animals per group.

significant reduction in the HCVR 15 and 30 days after pilocapine-induced SE. Our time dependent analysis of pilocarpine-induced SE is considered an acute model of epileptogenesis, not yet reaching the chronicity of the pathology. It is noteworthy that in the ARK model we observed changes in the number of fos-expressing neurons in the RTN, medullary raphe and NTS regions after a hypercapnic stimulus. The RTN is, in fact, an important brainstem region enrolled in the chemosensory control and breathing automaticity (Onimaru et al., 2012; Wang et al., 2013; Takakura et al., 2006, 2014; Kumar et al., 2015). The mechanisms described seem to involve inhibition of TASK-2 subtype potassium channels and activation of G protein-associated receptors (GPR4) (Kumar et al., 2015). Indirectly, RTN neurons could also be activated by astrocytes releasing ATP (Gourine et al., 2010; Huckstepp et al., 2010; Wenker et al., 2012; Sobrinho et al., 2014). We speculate that some of this machinery is compromised during the pilocarpine-induced SE.

In addition to the presumable impairment of RTN neurons, it is possible that we have a significant reduction in the number of serotonergic raphe and NTS neurons. Evidence suggest that serotonergic neurons in the medullary raphe may be enrolled in breathing control, including those associated with chemoreception (Ray et al., 2011; Hodges et al., 2008). Serotonergic neurons in the medullary raphe seems to be activated under hypercapnia and their selective lesions reduce the HCVR (Teppema et al., 1997; Dias et al., 2007; Iceman et al., 2013). NTS is considered the main site of viscerosensory afferents to the brain, contributing to the homeostatic regulation of the cardiovascular, gastrointestinal and respiratory systems. The caudal aspect of the NTS contribute to central chemoreception since increased levels of $CO_2$ were able to promote increased excitability of a subset of neuronal profile and thereby increase respiratory activity (Dean et al., 1990; Nichols et al., 2009; Fu et al., 2017). Thus, we believe that a reduction in the number of neurons in the RTN and NTS region, during exposure to high levels of $CO_2$, could be a potential mechanism to justify the reduction of the HCVR in pilocarpine-treated rats.

Interestingly, the HVR was intact in pilocarpine-treated rats, suggesting that the CNS was able to reorganize in order to maintain ventilatory response during low $O_2$ stimulation. In a condition of hypoxia, peripheral chemosensors are able to detect changes in arterial blood $O_2$ and send information to the CNS, more precisely to the NTS. From the NTS, a series of neural communications are triggered with the purpose of regulating cardiovascular, endocrine, behavioral and respiratory homeostasis. Even believing that NTS neurons may be compromised,

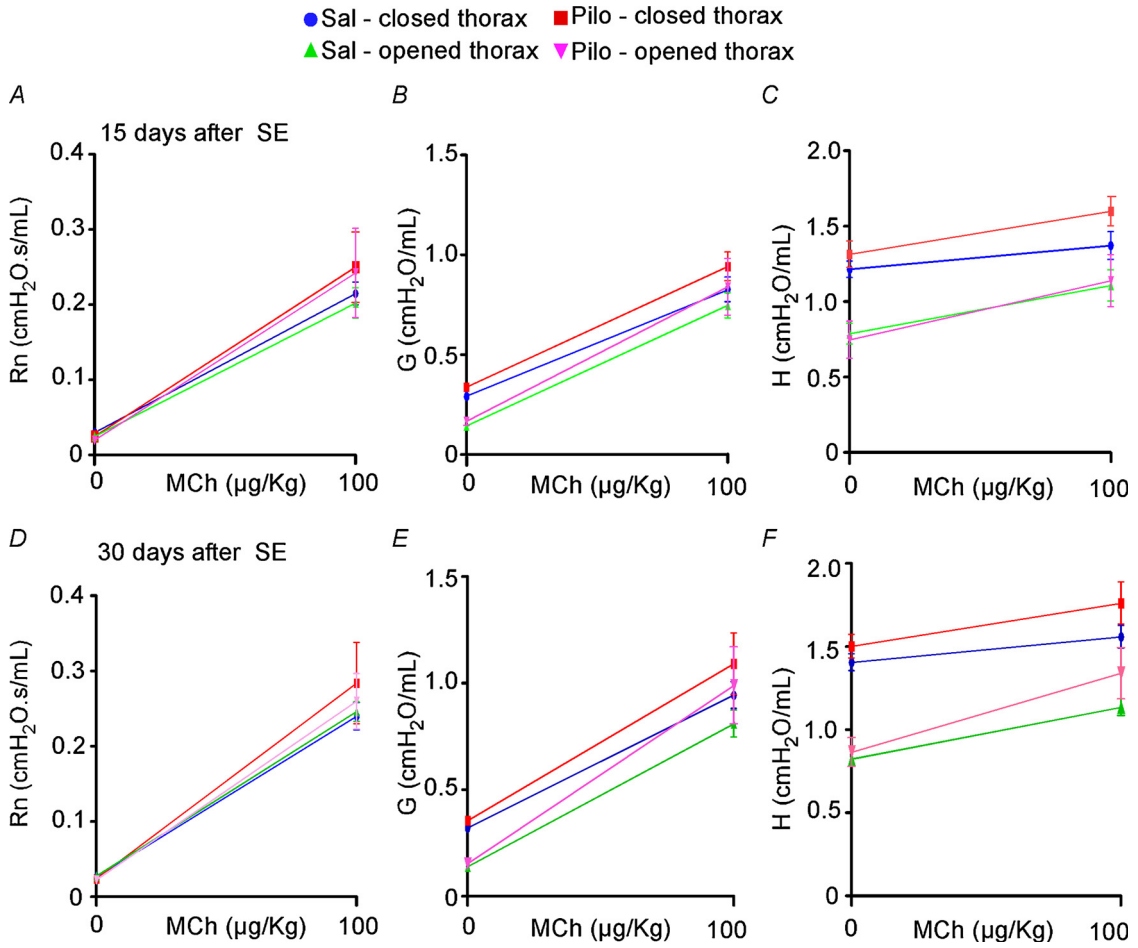


**Fig. 5.** Effect of methacholine on lung properties in pilocarpine-treated rats.
Effects produced by intravenous injection of methacholine (MCh – 100 µg/kg) in (A and D) airway resistance ($R_n$, $cmH_2O$ s/mL), (B and E) tissue viscance (G, $cmH_2O$/mL) and C and F) tissue elastance (H, $cmH_2O$/mL) while the thorax had been preserved or after thoracotomy in saline (control) and pilocarpine-treated rats (15 or 30 days). $N$ = 6–9 animals per group.

**Table 1**
Number of cells recovered in bronchoalveolar lavage and lung volume of rats submitted to *Status epilepticus* induced by pilocarpine.

| Groups | Number of cells (104 /mL) | | | |
|---|---|---|---|---|
| | Total | PMN | Mn | Lung volume |
| 15 days | | | | |
| Saline | 51.8 ± 15.5 | 2.2 ± 0.7 | 49.6 ± 15.8 | 17.3 ± 1 |
| Pilocarpine | 27.2 ± 12.8 | 0.7 ± 0.2 | 26.5 ± 12.5 | 18.5 ± 1.3 |
| 30 days | | | | |
| Saline | 32.2 ± 9.1 | 1.3 ± 0.4 | 30.9 ± 3.4 | 16.7 ± 0.7 |
| Pilocarpine | 30.1 ± 11.1 | 0.3 ± 0.05 | 29.8 ± 4.1 | 16.9 ± 1.1 |

Values are expressed as mean ± SEM of 6 animals/group. PMN: polimorphonuclear cells; MN: mononuclear cells.

the emergency response to changes in systemic $O_2$ levels would be intense enough to recruit a constellation of brainstem neurons in an attempt to maintain respiratory responses. In addition, astrocytes at the level of pre-Bötzinger Complex would be candidates for $O_2$ sensors and therefore maintain the ventilatory response to hypoxia in pilocarpine-treated rats (Funk and Gourine, 2018).

### *4.1. The peripheral components of the respiratory system are preserved in pilocarpine-induced status epilepticus*

Methacholine, a muscarinic cholinergic agonist, is routinely used in medicine for the diagnosis of asthma and it is well described that its use is not capable of promoting epileptic seizures in humans and experimental animals (Buels and Fryer, 2012). However, methacholine is not indicated in patients diagnosed with epilepsy and may cause involuntary seizures of greater intensity due to the fact that epileptic seizures produce blood-brain barrier disruption and thus allow access to methacholine in the CNS (Roundtree et al., 2016). Here, we did not notice epileptogenic changes after the methacholine challenge of the respiratory system by three possible mechanisms: i) the dose used is too small to induce epileptic seizures such as pilocarpine (another muscarinic cholinergic agonist) (100 µg methacholine × 2.4 mg pilocarpine); (ii) the chemical structure of methacholine differs from the pilocarpine. Methacholine has a chemical structure with a quaternary amine, hindering access to the CNS and (iii) in our epilepsy model, the blood-brain barrier was not compromised, at least during 15 and 30 days (Mendes et al., 2019).

Methacholine is capable of promoting increased airway resistance and bronchoconstriction. Methacholine responses, as well as changes in the static parameters of respiratory mechanics were not different between experimental groups, suggesting that in our epilepsy model with intra-hippocampal injection of pilocarpine the airway resistance responses, parenchymal viscance and elastance were not compromised.

Our data differ from the experimental model of another major neurodegenerative disease with impaired respiratory function, i.e. Parkinson's Disease (PD) (Tuppy et al., 2015; Fernandes-Junior et al., 2018; Oliveira et al., 2019). In the experimental model of PD, in addition to impaired respiratory activity and loss of neurons located in the brainstem respiratory column, significant changes in respiratory mechanics were observed with changes in airway resistance parameters and pulmonary parenchyma viscance (Oliveira et al., 2019). Thus, it is conceivable that upon different neurological diseases present different respiratory pathways and disturbance patterns. Future experiments may demonstrate the real mechanism in which the pilocarpine-epilepsy model promotes respiratory system impairment.

## 5. Conclusion

Our study showed an impairment in the HCVR in animals that received pilocarpine injection into the hippocampus. Thereby, we speculate that ventilation reduction to $CO_2$ challenge was due to a degeneration of brainstem respiratory neurons elicited by the process of epileptogenesis, especially in the brainstem respiratory column.

## 6. Materials and methods

### 6.1. Animals

All experiments were conducted using male Wistar rats (290 − 350 g at the time of experimentation) in accordance with NIH Guide for the Care and Use of Laboratory Animals and approved by the Animal Experimentation Ethics Committee of the Institute of Biomedical Sciences at the University of São Paulo (CEUA – ICB/USP; protocol number: 5009060818/2018). All experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. The animals had free access to tap water and food (Standard rat chow: BioBase Rat Chow, Bioquímica Produtos Químicos LTDA, Águas Frias, Santa Catarina, Brazil), and were housed in a temperature and humidity-controlled chamber maintained at 24 − 26 °C and 55 ± 10%, respectively with a 12:12 h light/dark cycle (lights on at 6:00 a.m.). The experimental protocols were performed between 9:00 a.m. and 5:00 p.m.

### 6.2. Surgical procedure

Rats were anesthetized with intraperitoneal (i.p.) injection of ketamine (100 mg/kg) combined with xylazine (10 mg/kg) and placed in a stereotaxic frame (model 900; David Kopf Instruments). The skull was leveled between bregma and lambda. Stainless steel 23-gauge cannulas (15 × 0.6 mm) were implanted in direction to the right dorsal hippocampus using the following coordinates: AP: 4.4 mm caudal to bregma, 4.5 mm lateral to the midline and 3.0 mm below the dura mater (Paxinos and Watson, 2007).

The cannulas were fixed to the cranium using dental acrylic resin and jeweler screws. Prophylactic dose penicillin (benzylpenicillin – 30,000 IUs plus streptomycin – 16 mg; Pentabiotico Veterinário, Fort Dodge Saude Animal Ltda, Campinas, Brazil) was given intra-muscularly and the anti-inflammatory Ketoflex (ketoprofen 1%, 0.1 mL/rat, Mundo Animal, Sao Paulo, Brazil) was given subcutaneously post surgically. After the surgery, rats were allowed to recover for one week before starting the experimental protocols.

### 6.3. Status epilepticus induced by pilocarpine

A Hamilton syringe (5 μL) connected by polyethylene tubing (PE-10) to an injection needle (1.5 mm longer than the guide cannulas) was used to manual deliver pilocarpine (muscarinic agonist: 2.4 mg/μL – 1 μL, pH 7.4; from Sigma Chemical Co.) or saline into the hippocampus of awake freely moving rats. The injector needle was gently inserted into the cannula direct to the hippocampus region. All injections were made with a volume of 1 μL and were performed over a period of 30 s, with one additional minute allowed to elapse before the injection needle was removed from the guide cannula to avoid reflux. The pilocaprine drug concentration was selected based on previous studies (Furtado et al., 2002, 2011; Melo et al., 2016) and was dissolved in sterile saline (pH 7.4). Sterile saline was used as a control. After reaching the SE phase, the animals remained for ninety minutes in this condition. Then, the animals received an injection of diazepam (5 mg/kg, i.p., União Química, Minas Gerais, Brazil) in order to stop the SE (Furtado et al., 2011).

As expected, pilocarpine (2.4 μg/μL) injected into the hippocampus developed a tonic-clonic limbic seizures with maximum score (5), assessed by the Racine scale (Racine et al., 1972). In general, the behavior response is characterized by piloerection, followed by episodes of whole body tremor and/or restricted head and face myoclonic spasms, accompanied by sialorrhea. *Status epilepticus* (SE) was characterized by repeated cycles of myoclonus of the head, forelimbs and hindquarters, elevation and fall, and eventually hyperextension of the forelimbs and hindquarters. As expected, none of these events were observed in animals receiving hippocapmpus injection of saline (control). Signs of inappetence were observed in the 4–5 days after pilocarpine-induced SE, as well as elevated levels of environmental exploitation and reactivity compared to control rats during the first week after SE (data not shown).

### 6.4. Whole body plethysmography

The ventilatory response was assessed using barometric, unrestrained whole body plethysmography (EMKA Technologies, Falls Church, VA, USA) 15 or 30 days after the induction of SE protocol. Briefly, freely moving rats were kept in a 5-L plethysmography chamber with room air for 45 − 60 min before starting to record the ventilatory parameters. The plethysmography chamber was continuously flushed with 1.5 L/min, regulated by computer-driven mass flow controllers for $O_2$, $N_2$, and $CO_2$ (Alicat Scientific, Inc., Tucson, AZ, USA). The flow controllers were adjusted as follow: (a) normoxia condition ($FiO_2$ = 21%, balanced with $N_2$); (b) hypercapnia ($FiCO_2$ = 7%; $FiO_2$ = 21% and balanced $N_2$) and c) hypoxia ($FiO_2$ = 8%, balanced with $N_2$). Room temperature (23 − 26 °C) and humidity (50–60 %) were continuously recorded inside the plethysmography chamber and used to calculate the tidal volume ($V_T$). Rectal temperature was used as a core body temperature index. The ventilatory parameters measured by the plethysmography system were: respiratory frequency ($f_R$, breaths/min), $V_T$ (mL/kg), and minute volume ($V_E$, mL/min/kg) (Malheiros-Lima et al., 2017).

### 6.5. Respiratory mechanics and bronchoalveolar lavage

The same rats used for whole body plethysmograph experiments were anesthetized with intraperitoneal injection of ketamine (110 mg/kg) combined with xylazine (10 mg/kg) and underwent a tracheostomy surgical procedure to insert a 14 G metal cannula (Höppner, 40 × 20, with smoothed bevel). In parallel, the right jugular vein was cannulated with a polyethylene catheter. At the end of surgical procedures, the animals received the muscle relaxant pancuronium at the initial dose of 1 mg/kg intravenously (i.v.) and were placed in a small rodent mechanical ventilation device (model: flexiVent, SCIREQ® Montreal, Quebec, Canada) and adjusted with the following parameters: $V_{T:}$ 10 mL/kg; $f_R$: 120 rpm and positive pressure at the end in expiration (PEEP): 3 $cmH_2O$. After preparation stabilization, the animals received either phosphate buffered saline (PBS) injection (control) or methacholine stimulation (muscarinic cholinergic agonist - 100 μg/kg, i.v.) to assess pulmonary responsiveness, airway resistance, viscance, and elastance of the pulmonary parenchyma with closed and open thorax in 5-

minute intervals. After euthanasia, the animals were submitted to bronchoalveolar lavage (BAL) by injecting in the metal cannula previously introduced into the trachea, a total of 10 mL of PBS (twice of 5 mL) and the population of collected cells were identified and quantified. In addition, the lungs were used for volume measurement.

### 6.6. Histology

At the end of the respiratory mechanics and bronchoalveolar lavage experiments, rats were deeply anesthetized with sodium thiopental (60 mg/kg of body weight i.p.) and a 2% solution of Evans blue was injected into the hippocampus (1 μL). Saline (150 – 200 mL) followed by 4% buffered formalin (pH 7.4; 500 mL) was perfused through the heart. The brains were removed and processed as described previously (Damasceno et al., 2014; Lima et al., 2019). Injections sites in the hippocampus were confirmed by visual inspection using an Axioskop 2 microscope (Carl Zeiss Microscopy, Germany) and according to the atlas of Paxinos and Watson (Paxinos and Watson, 2007). Only animals with injections into the hippocampus were considered for statistical analysis.

### 6.7. Statistical analysis

Microsoft Excel 2010 and GraphPadPrism 6 were used to collect and analyze data. The distribution of the data was tested for normality (Kolmogorov–Smirnov test with Dallal–Wilkinson–Lillie for $p$ value), and significant differences between samples were determined with one-way ANOVA (Holm–Sidak multiple comparisons test) with a significance threshold of $p < 0.05$. Results are presented as mean ± standard error of the mean.

## Authors contributions

Octávio A.C. Maia: designed research, performed research, analyzed data, approved the final version of the manuscript

Milene R. Malheiros-Lima: designed research, performed research, analyzed data, approved the final version of the manuscript

Maria A. Oliveira: performed research, analyzed data, approved the final version of the manuscript

Claudio L. Castro: performed research, approved the final version of the manuscript

Henrique T. Moriya: analyzed data, approved the final version of the manuscript

Wothan Tavares-de-Lima: analyzed data, approved the final version of the manuscript

Ana C. Takakura: designed research, wrote the paper, approved the final version of the manuscript

Thiago S. Moreira: designed research, analyzed data, wrote the paper, approved the final version of the manuscript

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## Declaration of Competing Interest

None.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.brainresbull.2020.05.002.

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