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M4-muscarinic acetylcholine receptor into the pedunculopontine tegmental nucleus mediates respiratory modulation of conscious rats

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ABSTRACT

The pedunculopontine tegmental nucleus (PPTg) has been shown to have important functions relevant to the regulation of behavioral states and various motor control systems, including breathing control. The PPTg is considered an important nucleus in the mesopontine region with considerably cholinergic input to the ventral respiratory column. In addition, recent studies indicate that cholinergic innervation of the ventral respiratory column may play an important role in modulation of breathing. Here, we investigated the cholinergic stimulation of the PPTg and the changes in breathing output in conscious rats. Male Wistar rats (280–350 g, N = 5–12/group) with unilateral stainless steel cannula implanted into the PPTg were used. Respiratory parameters (tidal volume (V_T), respiratory frequency (f_R) and ventilation (V_E)) were analyzed by whole body plethysmography. In unrestrained awake rats, unilateral injection of the cholinergic muscarinic agonist carbachol (10 mM–100 nL) in the PPTg decreased f_R, and increase V_T, without changing V_E. The changes in f_R and V_T elicited by carbachol into the PPTg are abolished by previous blockade of the M4 muscarinic cholinergic receptors tropicamide into the PPTg. No significant changes in f_R and V_T elicited by carbachol were observed after blockade of the M1 and/or M3 muscarinic cholinergic receptors pirenzepine or 4-DAMP into the PPTg. Our data suggest that the changes in f_R and V_T produced by muscarinic cholinergic stimulation of PPTg is presumably mediated through a Gi-coupled M4 muscarinic receptors.

1. Introduction

The pedunculopontine tegmental nucleus (PPTg) and the laterodorsal tegmental nucleus (LDT) are heterogeneous neighbors brainstem structures that contains cholinergic (ChAT), glutamatergic, and GABAergic neurons (Kroeger et al., 2017; Mena-Segovia and Bolam, 2017; Luquin et al., 2018). Several evidence suggests that PPTg neurons play key roles in both motor and non-motor behaviors (Morita et al., 2014; Mena-Segovia and Bolam, 2017). The PPTg projects to multiple targets in the brain, including the reticular formation (Jenkinson et al., 2009; Benarroch, 2013), and is involved in a wide range of physiological functions such as state dependent and behavioral functions. Stimulation of PPTg increases rapid eye movement (REM) sleep and wakefulness in rats and cats (Calvo et al., 1992; Datta et al., 2001a, 2001b). The PPTg also participates in regulation of motor control (Garcia-Rill, 1991; Winn, 2006), modulation of sensation (Reese et al., 1995), and attention (Rostron et al., 2008), reaction time, learning and memory (Datta, 1997; Datta and Hobson, 1995; Garcia-Rill, 1991) and autonomic and respiratory regulation (Saponjic et al., 2005, 2006; Topchiy et al., 2010; Lima et al., 2019). Electrical stimulation of the PPTg produced reduction in respiratory activity (Lydic and Baghdoyan, 1993), whereas pharmacological manipulation of the PPTg increased respiratory instability during sleep in conscious rats (Radulovacki et al., 2004).

It has long been hypothesized that cholinergic transmission is a requisite component of breathing activity (Dev and Loeschcke, 1979; Fukuda and Loeschcke, 1979; Nattie et al., 1989; Sobrinho et al., 2016; Lima et al., 2019). Defects in the cholinergic muscarinic system within the brainstem may play a role in disorders of respiratory control such as sudden infant death syndrome (SIDS) (Kinney et al., 1995).

Of the five muscarinic cholinergic receptor subtypes described (Brann et al., 1993), at least four of them (M1-M4) can be detected in the rat brain (Messer et al., 1989; Tice et al., 1996; Zubieta and Frey, 1993). Carbachol has a different affinity for each muscarinic receptors

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subtypes; however, depending on the dose used, it can activate all of them (Mayorga et al., 1999). The M3 receptors are thought to control salivary gland secretion, vasodilation and breathing activity at the level of the brainstem (Borella et al., 2008; Sobrinho et al., 2016; Lima et al., 2019). M1 and M3 receptors are involved in acetylcholine-induced water intake (Massi et al., 1989; Polidori et al., 1990; Rowland et al., 2003; Borella et al., 2008), and M1 receptors are also thought to be involved in pressor responses (Borella et al., 2008). Although central cholinergic system may be involved in breathing activity, it is still not known which muscarinic receptors subtypes are involved in these responses at the level of PPTg, a classic region that harbors cholinergic neurons. In this manuscript, we address the following questions: Does cholinergic stimulation of PPTg affect breathing output? What type of cholinergic receptors mediates the cholinergic activation of PPTg neurons? What is the relationship between the cholinergic stimulation within the PPTg and its effects on the respiratory-related motor pattern?

2. Methods

2.1. 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: 81/2015). All animal 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 6:30 a.m.). The experimental protocols were performed between 9:00 a.m. and 5:00 p.m

2.2. Surgical procedures

Rats were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) combined with xylazine (7 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 PPTg using the following coordinates: 7.9 mm caudal to bregma, 1.7 mm lateral to the midline and 5.2 mm below the dura mater (Paxinos and Watson, 2007). The cannulas were fixed to the cranium using dental acrylic resin and jeweler screws. A prophylactic dose penicillin (benzylpenicillin - 30,000 IUs plus streptomycin - 16 mg; Pentabiotico Veterinario, 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.

2.3. In vivo recordings of physiological variables

One week after the stereotaxic surgery, when the rats were recovered from the surgeries and adapted to the environment of the recording room, whole-body plethysmography was used to measure respiratory activity in awake rats, based on the technique described by Drorbaugh and Fenn (Drorbaugh and Fenn, 1955) and used previously by our group (Damasceno et al., 2014; Falquetto et al., 2018; Lima et al., 2019). Adult rats were placed individually into a plexiglass recording chamber (5 L) that was flushed continuously with a mixture of 79% nitrogen and 21% oxygen at a rate of 1.3 L/min. During the measurements, the flow was interrupted, and the chamber was sealed for approximately 2 min; the pressure oscillations due to ventilation were monitored by a differential pressure transducer (MLT844; ADInstruments, Sydney, NSW, Australia). The signals were fed into a preamplifier (Bridge Amp, ML221; ADInstruments)), passed through an analog-to-digital converter, and digitized on a microcomputer equipped with data acquisition software (LabChart Software, version 7.3; ADInstruments, Sydney, Australia). The sampling frequency was 200 Hz. The results were analyzed using data analysis software (LabChart Software, version 7.3; ADInstruments, Sydney, Australia). Tidal volume (V_T, measured in ml, normalized to body weight and corrected to account for chamber and animal temperature, humidity, and atmospheric pressure) and respiratory frequency (f_R, breaths/min) were calculated to estimate ventilation per breath. V_T was calculated using an appropriate formula (Drorbaugh and Fenn, 1955). Rectal temperature was used as a core body temperature index and was measured twice: before and at the end of the experiments. The calibration for volume was obtained during each experiment by injecting 1 mL of air into the animal chamber.

2.4. Drugs

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 the following drugs into the PPTg of awake freely moving rats: a) carbachol (muscarinic agonist: 10 mM–100 nL, pH 7.4; from Sigma Chemical Co.), pirenzepine (muscarinic M1 antagonist: 10 mM–100 nL, pH 7.4; from Sigma Chemical Co.), 4-DAMP (muscarinic antagonist M1/M3: 10 mM–100 nL, pH 7.4; from RBI), and tropicamide (muscarinic M4 antagonist: 10 mM–100 nL, pH 7.4; from RBI). The injector needle was gently inserted into the cannula direct to the PPTg region. All microinjections were made with a volume of 100 nL and were performed over a period of 30–45 s, with one additional minute allowed to elapse before the injection needle was removed from the guide cannula to avoid reflux. All drugs concentrations were selected based on previous studies (Borella et al., 2008; Gouveia et al., 2016; Anesio et al., 2019; Sá et al., 2019) and were dissolved in sterile saline (pH 7.4). Sterile saline was used as a control.

2.5. Histology

At the end of the experiments, rats were deeply anesthetized with pentobarbital (60 mg/kg of body weight i.p) and a 2% solution of Evans blue was injected into the PPTg (100 nL). 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 PPTg 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 PPTg were considered for statistical analysis.

2.6. 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 (Tukey's multiple comparisons test) with a significance threshold of $p < 0.05$. Results are presented as mean ± standard error of the mean unless noted otherwise.

2.7. Experimental protocols

All studies were performed in unanesthetized rats and the chamber temperature was maintained between 24 and 26 °C. Seven days after

surgery, the rats were gently handled and the injector needle was inserted into the guide cannula. The animals were then placed into a 5 L plethysmograph chamber and allowed 45–60 min to acclimate. During this period, the chamber was flushed with room air without any measurement. After this acclimation period, the experimental recording began. In room-air conditions, tidal volume (V_T, mL/kg), respiratory frequency (f_R, breaths/min) and minute ventilation (V_E, mL/kg/min) were recorded before the PPTg injections. Subsequently, muscarinic antagonists (pirenzepine, 4-DAMP or tropicamide, respectively) or saline was microinjected into the rat PPTg. Ten minutes later, carbachol (muscarinic cholinergic agonist) or saline was microinjected into the PPTg. Respiratory measurements were performed 1, 5, 15, and 30 min after the microinjection under normocapnic conditions. The experiments were performed in different groups of animals as described in the next section.

2.7.1. Effects of the combination of pirenzepine and carbachol injected into the PPTg on breathing measurements

Carbachol (10 mM–100 nL) or saline was injected into the PPTg 10 min after the injection of pirenzepine (10 mM–100 nL) or saline in the same place. Four groups of rats were used in order to investigate the respiratory effects produced by the combination of PPTg injections of pirenzepine and carbachol:

1) Saline into the PPTg followed by saline into the PPTg (control group);
2) Saline into the PPTg followed by carbachol into the PPTg;
3) Pirenzepine into the PPTg followed by saline into the PPTg;
4) Pirenzepine into the PPTg followed by carbachol into the PPTg.

2.7.2. Effects of the combination of 4-DAMP and carbachol injected into the PPTg on breathing measurements

Carbachol (10 mM–100 nL) or saline was injected into the PPTg 10 min after the injection of 4-DAMP (10 mM–100 nL) or saline in the same place. Four groups of rats were used in order to investigate the respiratory effects produced by the combination of PPTg injections of 4-DAMP and carbachol:

1) Saline into the PPTg followed by saline into the PPTg (control group);
2) Saline into the PPTg followed by carbachol into the PPTg;
3) 4-DAMP into the PPTg followed by saline into the PPTg;
4) 4-DAMP into the PPTg followed by carbachol into the PPTg.

2.7.3. Effects of the combination of tropicamide and carbachol injected into the PPTg on breathing measurements

Carbachol (10 mM–100 nL) or saline was injected into the PPTg 10 min after the injection of tropicamide (10 mM–100 nL) or saline in the same place. Four groups of rats were used in order to investigate the respiratory effects produced by the combination of PPTg injections of tropicamide and carbachol:

1) Saline into the PPTg followed by saline into the PPTg (control group);
2) Saline into the PPTg followed by carbachol into the PPTg;
3) Tropicamide into the PPTg followed by saline into the PPTg;
4) Tropicamide into the PPTg followed by carbachol into the PPTg.

3. Results

3.1. Histological analysis

Typical unilateral injections sites in the PPTg are shown in Fig. 1A. A total of 84 rats were used, most of the injections (N = 46, representing 55% of all the histological analysis) are located in the PPTg (Fig. 1B–F). The majority of the cannulas implanted outside the PPTg region reached the oral aspect of the pontine reticular nucleus (PnO) or the paralemniscal nucleus (PL) (data not shown).

3.2. Carbachol injection into the pedunculopontine tegmental nucleus changes respiratory parameters

The first series of experiment was designed to evaluate the effect of muscarinic cholinergic stimulation of the PPTg on V_T, f_R and V_E in conscious rats. Cholinergic stimulation of PPTg reduced respiratory rate and increased V_T, without affecting V_E. For example, carbachol (10 mM–100 nL) injection into the PPTg decreased f_R (66 ± 4 *vs.* saline: 93 ± 2.4, breaths/min; *t*-test t = 4.827; p = 0.0009) and increased V_T (11 ± 0.5, *vs.* saline: 7.9 ± 0.3 mL/kg; *t*-test t = 5.023; p = 0.0007). Considering the opposite effects on f_R and V_T after carbachol injection into the PPTg, V_E was not changed (723 ± 40, *vs.* saline: 736 ± 40 mL/kg/min; *t*-test t = 3.129; p = 0.001) (Fig. 2A–C).

3.3. Effects of central muscarinic blockade on carbachol-induced changes in respiratory parameters

To determine whether cholinergic transmission at the level of the PPTg contributes to breathing modulation, we injected muscarinic receptor antagonists and analyzed the effects on baseline f_R, V_T and V_E. The data showed that pirenzepine, 4-DAMP and tropicamide did not change the resting breathing (Table 1).

We also wanted to investigate which cholinergic receptors could be involved in the carbachol-induced changes in f_R and V_T and we tested the effects of different muscarinic receptor antagonists followed by the muscarinic cholinergic agonist carbachol into the PPTg while measuring respiratory activity under normoxic condition.

In awake rats, injections of the M1 muscarinic cholinergic antagonist pirenzepine (10 mM - 100 nL) did not affect the reduction in fR (72 ± 3 *vs.* saline + carbachol: 66 ± 4, breaths/min; One-way ANOVA; p > 0.05), and the increase in V_T (10.6 ± 0.6 *vs.* saline + carbachol: 11 ± 0.5 ml/kg; One-Way RM; p > 005) elicited by carbachol (Fig. 2A–C). In the same magnitude, injections of the M1/M3 muscarinic cholinergic antagonist 4-DAMP (10 mM - 100 nL) did not affect the reduction in fR (68 ± 5 *vs.* saline + carbachol: 66 ± 4, breaths/min; One-way ANOVA; p > 0.05), and the increase in V_T (10.8 ± 0.7 *vs.* saline + carbachol: 11 ± 0.5 ml/kg; One-Way RM; p > 005) elicited by carbachol (Fig. 2A–C). However, injection of the M4 muscarinic cholinergic antagonist tropicamide (10 mM - 100 nl) blocked the reduction in fR (86 ± 7 *vs.* saline + carbachol: 66 ± 4, resp/min; One-Way RM; p < 0.01), and the increase in V_T (7.7 ± 0.3 *vs.* saline + carbachol: 11 ± 0.5 ml/kg; One-Way RM; p < 0.05) elicited by carbachol into the PPTg (Fig. 2A–C).

Injections of the muscarinic cholinergic antagonists (pirenzepine, 4-DAMP or tropicamide) and/or carbachol outside the PPTg, *i.e* in the pontine reticular nucleus (PnO) or the paralemniscal nucleus (PL) did not change f_R ($F_(1165)$ = 1.57; p > 0.05), V_T ($F_(1165)$ = 0.84; p > 0.05) and V_E ($F_(1165)$ = 1.13; p > 0.05) (data not shown).

Treatment with muscarinic cholinergic antagonists and/or carbachol in the PPTg did not affect body temperature during normoxic/normocapnic conditions. For example, injections of the muscarinic cholinergic antagonists (pirenzepine, 4-DAMP or tropicamide) and/or carbachol into the PPTg did not change body temperature (pirenzepine + carbacol: 37.2 ± 0.17; 4-DAMP + carbacol: 37 ± 0.11 or tropicamide + carbacol: 37.1 ± 0.18 *vs.* saline + carbachol: 37.2 ± 0.11 °C; One-Way RM; p > 005). In addition, during the experiments, the mean chamber temperature was 26.4 ± 0.2 °C, and the mean room temperature was 24.9 ± 0.2 °C.

3.4. Discussion

The literature has shown that the cholinergic system within the PPTg is an important mediator of cardiorespiratory function (Padley

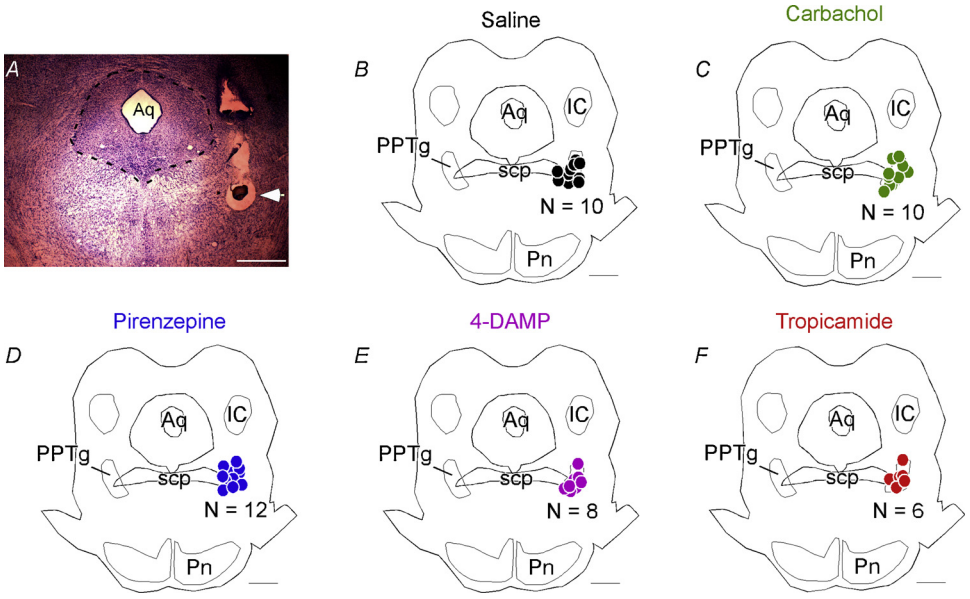

Fig. 1. Location of PPTg injections.
A) Photomicrograph of a coronal section showing the site of a unilateral injection in the pedunculopontine tegmental nucleus (PPTg). B–F) Computer-generated plots of injections that were confined to the PPTg region (Bregma level -7.92 mm according to the Paxinos and Watson atlas, 2007). Abbreviations: Aq, aqueduct mesencephalic; IC, inferior colliculus; Pn, pontine nuclei; scp, superior cerebelar peduncle. Scale bar = 1 mm for A, B.

et al., 2007; Topchiy et al., 2010; Fink et al., 2017; Lima et al., 2019). In the present study, we investigated the role of a muscarinic cholinergic agonist in respiratory modulation through M4 receptor-mediated mechanisms in the PPTg of awake unrestrained rats. We thereby provided the first functional evidence of a cholinergic signaling within the PPTg that is important for breathing modulation in conscious animals.

3.5. Cholinergic signaling and breathing control at the level of mesopontine region

Microinjection of the muscarinic cholinergic agonist carbachol into the PPTg elicited a significant decrease in respiratory frequency compared to the control group. The data surprised us since our main hypothesis was that the stimulation of PPTg would produce an increase in respiratory activity. This information is based on the fact that PPTg is considered the main source of cholinergic excitation to the brainstem, including the ventral respiratory group (Padley et al., 2007; Lima et al., 2019).

Carbachol injections in the PPTg has been demonstrated to elicit a decrease in the locomotor activity in spontaneous freely moving rats (Brudzynski et al., 1988). In accordance with the data described above, we demonstrated that injections of carbachol in the PPTg produce a decrease in the respiratory rate. In view of our experimental findings, we have two possible new hypotheses: i) the PPTg region does not send direct excitatory projections to the respiratory column within the brainstem and ii) the cholinergic stimulation of PPTg is mediated by the activation of inhibitory M2 and M4 subtypes of cholinergic receptors.

Our first hypothesis could be justified by a mechanism in which cholinergic stimulation in the PPTg region leads to an activation of cholinergic neurons producing release of acetylcholine as a neurotransmitter, which in turn activate glial cells in the PPTg region. Activation of the glial cells induces glutamate release, as gliotransmitter, activating GABAergic cells present in the PPTg complex. Inhibitory interneurons project to the ventral respiratory column inducing inhibition of respiratory output. Although PPTg is one of the main sources of cholinergic innervation in the brainstem (Kubin and Fenik, 2004; Lima et al., 2019), it also has distinct populations of neurons (Wang and Morales, 2009). The literature showed different effects induced by chemogenetic activation of the cholinergic, glutamatergic or GABAergic PPTg neurons in wakefulness/sleep. The glutamatergic PPTg neurons increase wakefulness, whereas cholinergic PPTg neurons decrease EEG slow waves during NREM sleep (Kroeger et al., 2017). Possibly, in wakefulness states or during REM sleep, the stimulation of PPTg produces the activation of a circuit involving inhibitory neurons, producing a reduction of respiratory activity. PPTg neurons have ipsilateral and contralateral projections to the parabrachial nucleus and this network can certainly modulate and increase REM sleep (Quattrochi et al., 1998). Our results are in agreement with the literature, since PPTg neurons seem to activate the pontine respiratory centers such as the Kölliker-Fuse and Parabrachial Complex that are involved in expiratory activity (Saponjic et al., 2006). In addition, other studies have shown that the electrical stimulation of PPTg was able to increase the release of acetylcholine in the gigantocellular tegmental region and cause respiratory rate depression (Lydic and Baghdoyan, 1993).

Our second hypothesis would be that the M2/M4 muscarinic cholinergic receptors of the PPTg are inhibitory receptors, thus involving the activation of an inhibitory G-couple protein cascade, triggering neuronal hyperpolarization. We believe that carbachol could act on M2 and/or M4 type receptors, activating Gi/Go inhibitory proteins that leads to activation of GTP. This process produce the inhibition of the adenylate cyclase enzyme, decreasing cAMP levels and K^+ influx, as well as an inhibition of the voltage-regulated Ca^{2+} channels. The triggering of this cellular response would be able to elicit a hyperpolarizing response and inhibition of excitable membranes (Kruse et al., 2014). Thus, cholinergic stimulation of PPTg, *via* M2/M4 receptors, produce a reduction of PPTg neuronal activity, eliciting a reduction of excitatory signals to the ventral respiratory column and a reduction of breathing rate. Our results showed that injection of the M1 receptor antagonist (pirenzepine) or the M1/M3 receptor antagonist (4-DAMP) into PPTg did not change the respiratory inhibitory response of carbachol on breathing rate, whereas injection of the M4 receptor antagonist (tropicamide) was able to attenuate the reduction in breathing rate elicited by carbachol in the PPTg.

Our data showed that the cholinergic stimulation of PPTg elicited a reduction of breathing frequency. In a more general perspective, stimulation of the PPTg elicits respiratory behavior reminiscent of REM sleep, *i.e.*, suppressed respiratory output (Lydic and Baghdoyan, 1993) and/or irregular breathing patterns (Saponjic et al., 2003). This is not unexpected since stimulation of the PPTg is known to promote wakefulness and REM sleep-like behavior. Considering that ACh is excitatory at most level of the respiratory circuit and since PPTg neurons innervate many brainstem structures including pontine respiratory centers associated with expiration (*e.g.*, parabrachial complex and Kölliker-Fuse) (Saponjic et al., 2006), PPTg-mediated respiratory depression and variability likely results from cholinergic activation of expiratory drive.

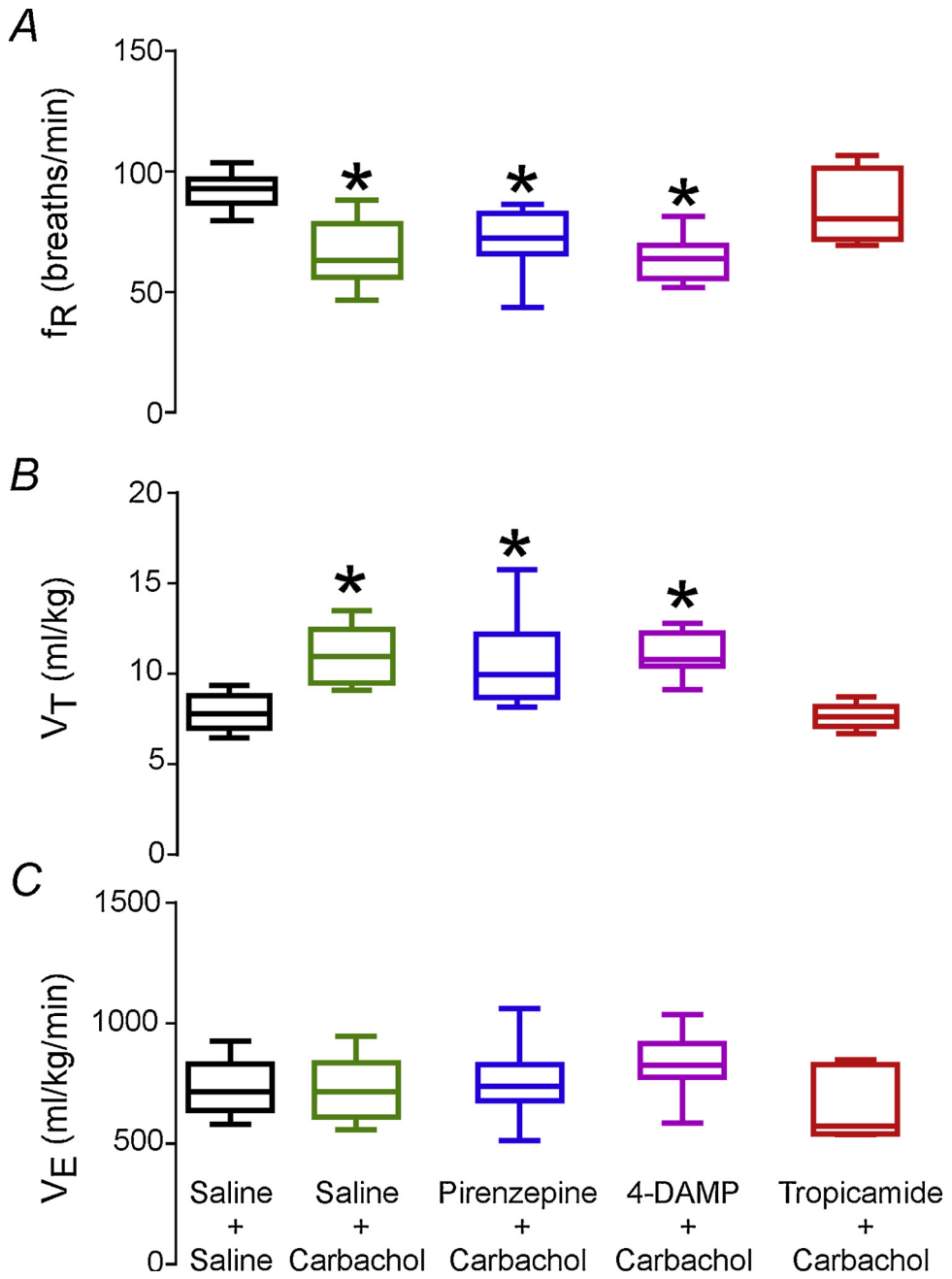

Fig. 2. Effect of blockade of M1, M1/M3 or M4 muscarinic cholinergic receptors in the PPTg on carbachol-induced respiratory depression.
Changes in A) respiratory frequency (f_R, bpm), B) tidal volume (V_T, mL/kg), and C) Minute ventilation (V_E, mL/kg/min) elicited by unilateral injection of saline or pirenzepine or 4-DAMP or tropicamide followed by saline or carbachol in the PPTg region under normoxic/normocapnic condition. *different from saline + saline; One-Way ANOVA; $p < 005$; N = 6–12/group of rats.

Table 1
Resting respiratory changes produced by muscarinic cholinergic receptors blockade.

	Groups				
Breathing Parameters	Saline + Saline	Pirenzepine + Saline	4-DAMP + Saline	Tropicamide + Saline	p
Tidal Volume (V_T) (mL/kg)	7.9 ± 0.3	8.3 ± 0.4	7.9 ± 0.5	7.7 ± 0.3	$p > 0.05$
Respiratory Frequency (f_R) (bpm)	93 ± 2.4	96 ± 12	95 ± 4	99 ± 2.6	$p > 0.05$
Ventilation (VE)(mL/kg/min)	736 ± 40	792 ± 47	756 ± 44	765 ± 57	$p > 0.05$

Values are means ± SEM. N = 6–12/group. One-way ANOVA.

Consistent with this possibility, we previously showed that ACh injections into another expiratory region, the Bötzinger region, suppressed respiratory activity in awake rats, presumably by activation of expiratory activity (Lima et al., 2019). To fully understand the contribution of cholinergic drive to state-dependent control of breathing, it will be important for future work to identify and selectively manipulate subsets of cholinergic PPTg neurons with discrete projections to various levels of the respiratory system across natural sleep-wake states.

4. Conclusion

Neurons within the PPTg region exhibit wakefulness- and REM-dependent firing behavior (Kubin and Fenik, 2004) and are known to participate in a wide range of state-regulating functions including breathing control (Boutin et al., 2017; Lydic and Baghdoyan, 1993; Saponjic et al., 2003). Therefore, connections between cholinergic PPTg neurons and ventral respiratory column within the brainstem may serve as the anatomical basis for state-dependent control of chemoreceptor activity. More generally, stimulation of the PPTg elicits respiratory behavior reminiscent of REM sleep (Saponjic et al., 2003). This is not unexpected since stimulation of the PPTg is known to promote wakefulness and REM sleep-like behavior. The evidence is consistent with our data, *i.e.*, PPTg cholinergic stimulation, specifically through M4 receptors, plays an inhibitory role in the breathing rate.

In addition, we should consider that acetylcholine is excitatory at most level of the respiratory circuit and since PPTg neurons innervate many brainstem structures including pontine respiratory centers associated with expiration (*e.g.*, parabrachial complex and Kölliker-Fuse), PPTg-mediated respiratory depression and variability could also results from cholinergic activation of expiratory drive (Boutin et al., 2017; Saponjic et al., 2006). To fully understand the contribution of cholinergic drive to state-dependent control of breathing, it will be important for future work to identify and selectively manipulate subsets of cholinergic PPTg neurons with discrete projections to various levels of the respiratory system across natural sleep-wake states.

Author contributions

JDL, CRS, ACT, and TSM designed research; JDL, and LKS performed research; JDL, CRS, and TSM analyzed data; JDL, CRS, ACT, and TSM wrote the paper. JDL, CRS, LKS, ACT, and TSM preformed critical review of the manuscript. All authors approved the final version.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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