α1- and α2-adrenergic receptors in the retrotrapezoid nucleus
differentially regulate breathing in anesthetized adult rats

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Norepinephrine (NE) is a potent modulator of breathing that can increase/decrease respiratory activity by α₁-/α₂-adrenergic receptors (AR) activation, respectively. The retrotrapezoid nucleus (RTN) is known to contribute to central chemoreception, inspiration and active expiration. Here we investigate the sources of catecholaminergic inputs to the RTN and identify respiratory effects produced by activation of ARs in this region. By injecting the retrograde tracer FluorGold into the RTN we identified back-labeled catecholaminergic neurons in the A7 region. In urethane-anesthetized, vagotomized and artificial ventilated male Wistar rats unilateral injection of NE or moxonidine (α₂-AR agonist) blunted DiaEMG frequency and amplitude, without changing AbdEMG. Those inhibitory effects were reduced by pre-application of yohimbine (α₂-AR antagonist) into the RTN. Conversely, unilateral RTN injection of phenylephrine (α₁-AR agonist) increased DiaEMG amplitude, frequency and facilitated active expiration. This response was blocked by prior RTN injection of prazosin (α₁-AR antagonist). Interestingly, RTN injection of propranolol (β-AR antagonist) had no effect on respiratory inhibition elicited by applications of NE into the RTN, however, the combined blockade of α₂- and β-ARs (co-application of propranolol and yohimbine) revealed an α₁-AR-dependent excitatory response to NE that resulted in increase in DiaEMG frequency and facilitation of active expiration. However, blockade of α₁-, α₂-, or β-ARs in the RTN had minimal effect on baseline respiratory activity, on central or peripheral chemoreflexes. These results suggest that NE signaling can modulate RTN chemoreceptor function; however, endogenous NE signaling does not contribute to baseline breathing or the ventilatory response to central or peripheral chemoreceptor activity in urethane-anesthetized rats.
NEW & NOTEWORTHY:

Disruption of norepinephrine signaling contributes to respiratory problems associated with disease states as Rett syndrome. The presence of catecholaminergic varicosities is observed in the retrotrapezoid nucleus (RTN), a ventrolateral medullary region that contributes to central chemoreception and breathing. This study demonstrates that activation of RTN $\alpha_1$- and $\alpha_2$- adrenergic receptors leads to activation and inhibition of breathing, respectively, and the main source of catecholaminergic inputs to RTN is from A7 region.
INTRODUCTION

Breathing is essential for life. Central respiratory chemoreception is a mechanism by which very small changes in tissue CO$_2$/H$^+$ produce very large changes in breathing. One region that is involved in central respiratory chemoreception is the retrotrapezoid nucleus (RTN) (Guyenet et al. 2010; Kumar et al. 2015; Nattie and Li 2012). This region is located in the ventrolateral surface of the medulla with neurons that have a genetic lineage (derived from neurons that express Phox2b, Atoh-1, and Egr-2) and express unique phenotype (Phox2b, NK1 receptors, VGlut2, TASK-2, GPR4, and galanin, but not GABA, glycine, acetylcholine, or catecholamines) (Kumar et al. 2015; Lazarenko et al. 2009; Ruffault et al. 2015; Takakura et al. 2014; Takakura et al. 2008; Wang et al. 2013). In addition to being intrinsically active and CO$_2$/H$^+$-sensitive, the activity of chemosensitive RTN neurons is also controlled by a variety of inputs such as glutamatergic, GABAergic, serotoninergic, cholinergic and adrenergic (Rosin et al. 2006). It is also well known that catecholamines, norepinephrine (NE) in particular, modulate respiratory rhythm (Tryba et al. 2008; Viemari et al. 2011; Zanella et al. 2014) and respiratory motor output (Funk and Feldman 1995; Funk et al. 2011; Parkis et al. 1995). For example, activation of $\alpha_1$-adrenoceptors ($\alpha_1$-AR) in the pre-Bötzinger complex and hypoglossal motor nucleus has been shown to enhance respiratory output, while activation of $\alpha_2$-AR produced an inhibition in breathing activity (Viemari and Hilaire 2002; Viemari et al. 2004; Viemari and Ramirez 2006; Zanella et al. 2006). However, potential contributions of these receptors upstream of the pre-Bötzinger complex in chemoreceptor regions are not clear.

Several brainstem nuclei could be the catecholaminergic source for respiratory modulation. For example, the A6 noradrenergic neurons modulate hypercapnic ventilatory response, project to the pre-Bötzinger complex and regulate the respiratory frequency (Biancardi et al. 2008; Hilaire et al. 2004; Viemari et al. 2011). Also, A5 neurons have been shown to regulate cardiovascular tone, sympathetic outflow and contribute to increased respiratory activity elicited by central chemoreflex activation (Koshiya and Guyenet 1994; Taxini et al. 2011). The A7, A1/C1, A2/C2 may also be involved in control of breathing (Abbott et al. 2012; Bruinstroop et al. 2012; Li et al. 2008). Together, these studies support the hypothesis that NE is an important modulator of cardiorespiratory function (St-John and Leiter 2008; Viemari et al. 2011).
The aims of this study are to further examine sources of catecholaminergic inputs to the RTN, as well as identify cardiorespiratory effects produced by activation of ARs in this region. We show that A7 region of the dorsolateral pons is the main source of NE to the RTN. We also demonstrate that injections of NE into the RTN decrease respiratory activity by an $\alpha_2$-AR-dependent mechanism. Conversely, selective activation of $\alpha_1$-AR caused an increase in respiratory output. However, despite the presence of functional ARs in the RTN, our results show that those receptors do not contribute to the respiratory response evoked by activation of central or peripheral chemoreflexes. Together, these results identify NE as an important modulator of RTN chemoreceptor function, and provide insight into how neuromodulators differentially affect distinct aspects of breathing.

METHODS

Animals

Animal use was in accordance with guidelines approved by the University of São Paulo Institutional Animal Care and Use Committee. Experiments were done on male Wistar rats weighing 250-350 g (8-10 months old; $N = 89$). All efforts were made to minimize animal discomfort and the number of animals used.

Physiological experiments

Surgery and anesthesia

The surgical procedures and experimental protocols were similar to those described previously (Takakura et al. 2011; Takakura and Moreira 2011; Takakura et al. 2006). Briefly, general anesthesia was induced with 5% isoflurane in 100% oxygen inhaled. The rats were submitted to the following procedures: i) tracheostomy cannulation, ii) femoral artery and vein cannulation for arterial pressure (AP) measurement and administration of fluids and drugs, respectively, iii) removal of the occipital plate to insert a recording electrode into the medulla oblongata via a dorsal transcerebellar approach. Artificial ventilation with 1.5% isoflurane in 100% oxygen was maintained throughout surgery. A bilateral vagotomy was performed distal to the carotid bifurcation as described previously (Guyenet et al. 2005). Bipolar electrodes were coupled to record the activity of diaphragm ($\text{Dia}_{\text{EMG}}$) and abdominal ($\text{Abd}_{\text{EMG}}$) muscles and the amplification used for the signal was
5,000-10,000 times. The rats were ventilated with 100% oxygen throughout the experiment. Rectal temperature (maintained at 37°C) and end-tidal CO₂ were monitored throughout the experiment with a capnometer (Columbus Instruments, Ohio, USA) that was calibrated twice per experiment.

On completion of surgical procedures, isoflurane was gradually replaced by urethane (1.2 g/kg i.v. over 30 min). After injection of the intravenous anesthetic, the anesthesia level was monitored by testing for absence of withdrawal response and lack of AP changes due to firm paw pinch. Activation of peripheral chemoreflex was done by intravenous bolus injections of potassium cyanide (KCN; 40 µg/0.1 ml iv.) and activation of central chemoreflex was done by hypercapnia (10% of CO₂).

All analog data (end-expiratory CO₂, EMG activities and AP) were stored on a computer via a micro1401 digitizer from Cambridge Electronics Design (CED, Cambridge, UK) and were processed using version 5 of the Spike 2 software (CED). Integrated electromyography activity (∫EMG) was obtained after rectification and smoothing (τ = 0.003 s) (Mulkey et al. 2007). Neural minute x volume (mvDiaEMG and mvAbdEMG, a measure of the total diaphragm and abdominal muscles activities per unit of time) was determined by averaging ∫EMG over 30s and normalizing the result by assigning a value of 0 to the dependent variable recorded at 3-4% of end-expiratory CO₂ and a value of 1 at the 9-10% of end-expiratory CO₂.

**Drugs and intraparenchymal injections**

All drugs were purchased from Sigma Aldrich (Sigma Chemicals Co.) unless otherwise stated. NE (0.03, 0.075, 0.15, 0.75, 1.5, 7.5, 15 and 30 pmol/30 nl in sterile saline pH 7.4), moxonidine, an α₂-AR agonist (0.6 pmol/30 nl in sterile saline pH 5.6, gift from Solvay Pharma, Germany), phenylephrine, an α₁-AR agonist (0.3 pmol/30 nl in sterile saline pH 7.4), prazosin hydrochloride, an α₁-AR antagonist (6 pmol/30 nl in propilenoglycol pH 7.4), yohimbine hydrochloride, an α₂-AR antagonist (3 pmol/30 nl in propilenoglycol pH 7.4), or propranolol hydrochloride, a β-AR antagonist (3 pmol/30 nl in sterile saline pH 7.4) were pressure injected (Picospritzer III, Parker Hannifin Corp, USA) (30 nl in 3 s) through single-barrel glass pipettes (20 µm tip diameter). Injections into RTN
were placed 8.8 mm below the dorsal surface of the cerebellum, 1.7-1.8 mm lateral to the midline and 2.6 to 2.8 mm caudal to the lambda.

All drugs contained a 5% dilution of fluorescent latex microbeads (Lumafluor, New City, NY, USA) for later histological identification of the injection sites. Complete recovery of respiratory responses to RTN injection of AR antagonists occurred within 45 minutes.

**Anatomical experiments**

Retrograde tracer injection: Retrograde tracer injections were made while the rats were anaesthetized with a mixture of ketamine (80 mg/kg) and xylazine (7 mg/kg) administered intraperitoneally (i.p.). Surgery used standard aseptic methods. After surgery, the rats were treated with the antibiotic ampicillin [100 mg/kg, intramuscular (i.m.)] and analgesic ketorolac [0.6 mg/kg, subcutaneous (s.c.)]. A group of four rats received injections of 2% FluorGold (FG, Fluorochrome, Inc., Englewood, USA) in sterile saline in the left RTN to retrogradely label catecholaminergic neurons that innervate RTN. These injections were made by iontophoresis through a glass micropipette (tip diameter 20 µm, 7 μA positive-current pulses of 5 seconds duration every 10 seconds for 10 minutes). The coordinates to reach RTN were: 8.8 mm below the dorsal surface of the cerebellum, 1.7-1.8 mm lateral to the midline and 2.6 to 2.8 mm caudal to the lambda (Paxinos and Watson 1998). Seven to ten days following the FG application, the rats were anesthetized with pentobarbital (60 mg/kg, i.p.) and immediately perfused transcardially with fixative.

**Histology, cell mapping, and imaging**

The rats were deeply anesthetized with 60 mg/kg sodium pentobarbital (i.p.), injected with heparin (500 U, intracardially), and perfused through the ascending aorta with 250 ml phosphate-buffered saline (pH 7.4) followed by 4% phosphate-buffered paraformaldehyde (0.1 M, pH 7.4; Electron Microscopy Sciences, Fort Washington, PA, USA). The brain was removed and stored in the perfusion fixative for 24-48 h at 4°C. A series of coronal sections (40 µm) from the brain was cut using a microtome and stored in cryoprotectant solution at -20°C for up to 2 weeks (20% glycerol plus 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) until histological processing. All of the histochemical
procedures were performed using free-floating sections according to previously described protocols (Barna et al. 2014; Rosin et al. 2006). Tyrosine hydroxylase (TH; a limiting enzyme in the synthesis of catecholamines) was detected using a mouse monoclonal antibody (1:1000; Chemicon, Temecula, CA) followed by Alexa 488-tagged goat anti-mouse IgG (1:200; Invitrogen, Carlsbad, CA). The sections were mounted onto slides in rostrocaudal sequential order, dried and covered with DPX (Aldrich, Milwaukee, WI, USA). Coverslips were affixed with nail polish.

A conventional multifunction microscope (fluorescence; Nikon, Japan) was used for all observations. ImageJ software (http://rsb.info.nih.gov/ij/) was used to count the various types of neuronal profiles within a defined area and merge the colour channels in photographs in the dual-labelling experiments. Section alignment between brains was performed relative to a reference section. To identify the bregma level of drugs injections that targeted the RTN or catecholaminergic neurons of A1/C1 and A5 regions, the most caudal section that contained an identifiable cluster of facial motor neurons was identified in each brain and assigned a level of 11.6 mm caudal to Bregma. To align sections around A2/C2 level, the section containing mid-area postrema was identified in each brain and assigned the level 13.8 mm caudal to Bregma. The same method was also used to identify the Bregma level of A6, the section containing the most rostral section of facial motor neurons assigned the level 10.3 mm caudal to Bregma and the A7, the section containing the most rostral section of superior cerebellar peduncle assigned the level 8.3 mm caudal to Bregma. Levels rostral or caudal to this reference section were determined by adding a distance that corresponded to the interval between sections multiplied by the number of intervening sections. It was analyzed 11 sections to A2/C2, 11 sections to A1/C1, 9 sections to A5, 4 sections to A6 and 4 sections to A7.

All files were exported to the Canvas 9 software drawing program for final modifications. The neuroanatomical nomenclature and Bregma levels are according to the atlas of Paxinos & Watson (Paxinos and Watson 1998).

Statistics
Data were analyzed using one-way repeated measures ANOVA followed by Newman Keul’s or Dunn’s post hoc tests. All results are presented as means ± SEM. Differences were considered significant when p < 0.05.

RESULTS

1) Injection of NE into the RTN inhibits breathing

To determine whether noradrenergic transmission at the level of the RTN is able to produce changes on breathing, we injected NE into the RTN while measuring respiratory activity. We recorded AP, Dia_{EMG} and Abd_{EMG} in a total of 54 animals that received increasing doses of NE injected unilaterally into the RTN. The same animal received 2 different doses of NE one/side. We found that RTN injections of NE caused a dose-dependent decrease in respiratory activity. For example, RTN injections of NE (1.5, 7.5, 15 and 30 pmol/30 nl) reduced Dia_{EMG} amplitude (1.5 pmol: 34 ± 4.9%; 7.5 pmol: 41 ± 4.1%; 15 pmol: 48 ± 2.9%; 30 pmol: 65 ± 2% of inhibition; H = 54.30, ID_{50} = 1.31 pmol/30 nl, p < 0.001) (Figs. 1A and B). These injections also decrease Dia_{EMG} frequency, albeit with a 10-fold lower sensitivity (7.5 pmol: 10 ± 4.3%; 15 pmol: 14 ± 6.8% and 30 pmol: 27 ± 10.7% of inhibition; H = 19.31, ID_{50} = 15.10 pmol/30 nl, p < 0.001) (Figs. 1A-C). The reduction in inspiratory activity produced by unilateral injection of NE (1.5 - 30 pmol/30 nl) had duration of 2 ± 1 seconds (range: 2 to 5 s). All doses of NE-tested were unable to produce changes in AP (p = 0.726) and Abd_{EMG} activity (p = 0.959) (Fig. 1A). As shown in Figures 1D-E, all injections were centered 250 μm below of the facial motor nucleus and 200 μm rostral regarding the caudal portion of the facial nucleus, lateral to pyramidal tract and medial to the spinal trigeminal tract (Takakura et al. 2006). Note that microbeads used to identify injection sites showed a dispersion of ~250 μm in the rostro-caudal direction from the center of the injection.

2) α2-ARs contribute to NE-mediated inhibition of inspiratory activity

Next, we made RTN injections of selective ARs blockers to determine which class of receptor contributes to NE-mediated inhibition inspiratory activity. We found that prior RTN injection of the α2-AR antagonist yohimbine (3 pmol/30 nl) reduced subsequent inhibitory effects of NE on inspiratory amplitude (22 ± 15, vs. saline + NE: 61 ± 5%, p =
0.048, n = 6/group) (Figs. 2A-B) but not frequency (8 ± 8%, vs. saline + NE: 16 ± 4% of inhibition, p = 0.46, Figs. 2A and 2C). Consistent with this preferential effect on amplitude over frequency, we found that when α₂-ARs were blocked with yohimbine, NE increased inspiratory time (2.2 ± 0.9 s vs. saline: 0.6 ± 0.0 s, p = 0.022) (Fig. 2D) but did not significantly affect expiratory time (Fig. 2E). As shown in Figures 2F-G, these injections were centered in the RTN.

We also tested for potential involvement of α₁- and β-ARs in the respiratory response produced by NE injections into the RTN. We found that RTN injections of prazosin (specific α₁-AR blocker) did not significantly affect NE-mediated inhibition of respiratory frequency (18.3 ± 3.0% vs. saline: 15.2 ± 5.1% of inhibition, p = 0.827) (Figs. 3A-B and 3D). However, prazosin did enhance NE-mediated inhibition of inspiratory amplitude (83 ± 5% vs. saline: 63 ± 4% of inhibition, H = 10.29 and p = 0.006) (Fig. 3E), suggesting α₁-ARs in the RTN provide an excitatory drive that partially offsets the inhibitory effects of α₂-AR activation. Conversely, RTN injections of propranolol (β-AR blocker) did not blunt the inhibitory effect of NE on DiaEMG amplitude (58.3 ± 5.2% vs. saline: 60.1 ± 2.0% of inhibition, p = 0.923) or frequency (15.1 ± 2.1% vs. saline: 14.2 ± 3.7% of inhibition, p = 0.505 (Figs. 3C-E). In addition, we also found that RTN injections of α₁-, α₂- and β-AR blockers had negligible effects on MAP (130 ± 5 mmHg, 129 ± 4 mmHg and 135 ± 6 mmHg, respectively vs. saline: 132 ± 3 mmHg, p = 0.381).

To further explore the role of RTN ARs in control of breathing, we tested effects of unilateral RTN injections of moxonidine (α₂-AR agonist) or phenylephrine (α₁-AR agonist) on breathing. Consistent with the possibility that activation of α₂-AR in the RTN inhibits breathing, we found that unilateral RTN injection of moxonidine (0.6 pmol/30 nl) reduced DiaEMG amplitude by 64 ± 3.8% (p < 0.001) and frequency by 18 ± 4.6% (p = 0.014) (Figs. 4A-C). The inhibitory effect of moxonidine on breathing lasted 2 ± 2 seconds (range 1 - 4 s) and was blocked by prior injection of the α₂-AR antagonist yohimbine in the RTN (5 ± 3% vs. Moxo + saline: 64 ± 3.8% of inhibition, p < 0.001, Figs. 4A-C). We also found that yohimbine into the RTN was able to increase inspiratory time (1.7 ± 0.2 vs. saline: 0.6 ± 0.1 s, p < 0.001) (Fig. 4D) without changing expiratory time (Fig. 4E).

Despite the consistent inhibitory effect of NE on inspiratory activity, we found that selective activation of α₁-AR by RTN injection of phenylephrine (Phe, 0.3 pmol/30 nl)
increased DiaEMG amplitude by 62 ± 8.1% (p < 0.001) and frequency by 75 ± 18.6% (p = 0.005) as well as facilitated AbdEMG activity (Figs. 5A1 and 5B-E). These effects were completely blocked by prior injection of the α1-AR antagonist prazosin in the RTN (DiaEMG amplitude: 7.5 ± 4.6% and DiaEMG frequency: 6 ± 12% vs. Phe + saline, p < 0.001, Figs. 5A2 and 5B-E). These results suggest that α1-ARs are expressed in the RTN and when activated can enhance both inspiratory and expiratory activity. Consistent with our evidence that disruption of NE signaling minimally affected MAP, we found that RTN injection of moxonidine or phenylephrine had no measurable effect on arterial pressure (132 ± 3 mmHg and 148 ± 9 mmHg vs. saline: 137 ± 9 mmHg, p = 0.18 and p = 0.19, respectively).

Although RTN injection of phenylephrine clearly enhanced respiratory activity (Fig. 5), we did not observe an α1-AR-mediated excitatory response to RTN injection of NE when α2-ARs were blocked with yohimbine (Fig. 2). Considering that RTN neurons may express α1-, α2- and β-ARs, and in other tissues downstream signaling pathways activated by these receptors can reciprocally interact (Bawa-Khalfe et al. 2007; Yue et al. 2004), we decided to test effects of RTN injections of NE when both α2- and β-ARs were blocked. Despite our evidence presented above that shows blockade of β-AR did not affect the α2-AR-mediated inhibitory response (Fig. 3C-E), when both α2- and β-AR are blocked by co-application of yohimbine and propranolol (i.e., blocker cocktail) into the RTN, subsequent injection of NE increased in DiaEMG amplitude and frequency and stimulated AbdEMG activity (Fig. 6). These results suggest β-ARs are functionally expressed in the RTN where they serve to fine tune NE regulation of respiratory drive by influencing activity of α1-ARs.

3) Endogenous NE signaling in the RTN does not contribute to ventilatory responses to central or peripheral chemoreceptor activation in anesthetized rats

In the next series of experiments, we tested effects of RTN injections of α- (prazosin, yohimbine), β- (propranolol) AR blockers alone and in combination (cocktail: yohimbine + propranolol) on baseline breathing and the ventilatory response to CO2 (9-10% CO2 for 1 min) or intravenous injection of KCN (40 μg/0.1 ml) in anesthetized rats. We found that bilateral RTN injections of prazosin (6 pmol/30 nl; n = 8), yohimbine (3 pmol/30 nl; n = 6), propranolol (3 pmol/30 nl; n = 6) or the cocktail (yohimbine; 3 pmol/30
nl + propranolol; 3 pmol/30 nl; n = 5) had minimal effect on resting inspiratory activity (Dia\textsubscript{EMG} frequency [p = 0.524] and Dia\textsubscript{EMG} amplitude [p = 0.27]) or expiratory activity (Abd\textsubscript{EMG} frequency [p = 0.639] and Abd\textsubscript{EMG} amplitude [p = 0.699]) (data not shown). Furthermore, blockade of these receptors at the level of the RTN also did not affect the inspiratory (mvDia\textsubscript{EMG} [CO\textsubscript{2}: p = 0.634, KCN: p = 0.192]) or expiratory (mvAbd\textsubscript{EMG} [CO\textsubscript{2}: p = 0.858, KCN: p = 0.185]) response to CO\textsubscript{2} or KCN (Figs. 7A-B). These results indicate that NE signaling in the RTN does not contribute to central or peripheral chemoreceptor regulation of breathing in anesthetized rats.

4) Catecholaminergic innervations of the retrotrapezoid nucleus

The RTN receives dense catecholaminergic innervations as denoted by the presence of terminals contained the dopamine β hydroxylase (DβH) immunoreactive (Rosin et al. 2006). To identify the source of catecholaminergic inputs, the retrograde tracer FluorGold 2% (FG) was injected by iontophoresis unilaterally under the caudal end of the facial motor nucleus (Figs. 8A-B). Consistent with previous work (Rosin et al. 2006), FG-labeled neurons were present in many brainstem respiratory centers (e.g., nucleus of the solitary tract, pre-Bötzinger complex, medullary raphe and Kolliker-Fuse region (data not shown). A significant proportion of FG-labeled A7 neurons were also immunoreactive for TH (38 ± 2%), thus confirming these cells are catecholaminergic (Figs. 8C-E). A small number of double labeled (TH\textsuperscript{+}/FG\textsuperscript{+}) neurons were also detected in the A2/C2 region (13 ± 2%) (Figs. 9A-B) and A1/C1 (9 ± 2%) (Figs. 9C-D). We did not find double-labeled neurons within the A5 or A6 regions (data not shown).

DISCUSSION

NE provides a powerful stimulus for breathing and respiratory chemoreception and catecholaminergic terminals are diffuse throughout the brain (Li et al. 2008; Sun et al. 1994; Viemari and Ramirez 2006) including at the level of the RTN. However, despite the potentially important role of NE in control of breathing, virtually nothing is known regarding mechanisms underlying adrenergic modulation of chemoreceptor function. In the present study, we identify adrenergic pontine A7 neurons as the main source of NE to the RTN, and we show that the primary effect of NE injection into the RTN is to inhibit
respiratory output in anesthetized adult rats by an α2-AR dependent mechanism. These results are mechanistically similar to our in vitro data described in the companion paper (Kuo et al. 2016); activation of α1- and α2-AR increases and decreased RTN chemoreceptor activity, respectively. However, contrary to our in vivo evidence shown here, at the cellular level NE primarily activates RTN chemoreceptors in neonatal slices (Kuo et al. 2016). The reasons for these divergent results are not clear, however, based on previous evidence suggesting brainstem levels of α1-ARs peaks during the neonatal period followed by decreased expression to low levels in adulthood, it is reasonable to speculate that they reflect developmental differences in brainstem expression of α1- and α2-ARs (Viemari and Hilaire 2002). Evidence also suggests that α2-ARs have a higher affinity for NE compared to α1- or β-ARs (Ramos and Arnsten 2007), thus giving preference to α2-mediated inhibition. Furthermore, our evidence suggests that α1-dependent excitation might be limited by the activity of β-ARs. For example, RTN injections of NE increase breathing when α2- and β-ARs are blocked (Fig. 6) but not when just α2-ARs are blocked (Fig. 2). Together, these factors favor a net inhibitory response to exogenous NE. Nevertheless, we show that selective activation of α1-AR in the RTN increases inspiratory and expiratory activity in adult rats, suggesting these receptors are present and can enhance RTN chemoreceptor function. We also show here that blockade of α1-, α2- and β-ARs in the RTN had no effect on basal breathing or the ventilatory response to central or peripheral chemoreceptor activation, thus suggesting that endogenous adrenergic signaling in the RTN does not regulate breathing in urethane-anesthetized rats. However, considering that noradrenergic neurons are most active during wakefulness, it remains possible that NE signaling in the RTN contributes to breathing in the awake state.

**Catecholaminergic inputs to RTN**

Previous evidence (Rosin et al. 2006) showed that there is a dense catecholaminergic number of varicosities in the ventral medullary surface, including the RTN region. Previous studies identified the source of noradrenergic innervation of the RTN as coming primarily from the A5 and subceruleous regions (Rosin et al. 2006). However, our evidence suggests the A7 region provides a major source of adrenergic input to the RTN. The A7 region has been shown to enhance activity of respiratory motor neurons and
withdrawal of this excitatory drive during sleep is thought to increase airway resistance and promote obstructive sleep apnea (Funk et al. 2011). Based on our evidence that NE signaling in the RTN inhibits breathing in adults, it is conceivable that concurrent withdrawal of NE drive from A7 to the RTN would help maintain respiratory activity during sleep. Therefore, it will be important for future work to determine the role of endogenous NE on RTN function across sleep-wake states. We also observed diffuse projections from A1/C1 and A2/C2 to the RTN region. Previous studies have shown that A1/C1 and A2/C2 inputs to pre-Bötzinger complex act by modulating the respiratory frequency and are involved in stabilization of the respiratory rhythm (Tryba et al. 2008; Viemari 2008; Viemari and Ramirez 2006). Therefore, it is possible that A1/C1 and A2/C2 input to the RTN provide a similar function.

**Activation of α2-adrenoceptors in the RTN inhibits breathing**

Neurons and astrocytes in this region sense changes in CO₂/H⁺ to produce an integrated CO₂/H⁺-dependent drive to other components of the respiratory circuit to regulate both inspiratory and expiratory activity (Abbott et al. 2011; Abdala et al. 2009; Gourine et al. 2010; Kumar et al. 2015). Chemosensitive RTN neurons also contribute to respiratory rhythm generation, thus the RTN has a powerful influence on all aspects of breathing (Janczewski and Feldman 2006; Onimaru et al. 2008; Thoby-Brisson et al. 2009). Based on evidence that i) the RTN receives catecholaminergic innervation (Cream et al. 2002; Rosin et al. 2006); ii) at the cellular level NE primarily activates RTN chemoreceptors in neonatal brain slices (Kuo et al. 2016); and iii) since disruption of catecholaminergic neurons disrupts breathing and respiratory chemoreception (Li et al. 2008), we hypothesized that NE signaling in the RTN would enhance breathing and chemoreceptor function. However, contrary to our expectation, we found that RTN injection of NE decreased respiratory activity in anesthetized rats by a mechanism involving α₂-AR. Furthermore, RTN applications of moxonidine (α₂-AR agonist) confirm that activation of α₂-AR in the RTN inhibits breathing. We also found that RTN injections of phenylephrine (an α₁-AR agonist) enhanced respiratory activity by increasing DiaEMG activity and facilitating active expiration. These results are consistent with the well-established effects of α₁-AR and α₂-AR activation on breathing in rats and mice (Tryba et
al. 2008; Viemari et al. 2011; Zanella et al. 2014). However, the net effect of NE on respiratory activity is dependent on the relative balance between α₁- and α₂-adrenergic receptor activation; which can vary depending on several factors including species, brain region, and age (Hilaire et al. 2004). For example, previous studies using the rhythmically active brain slice or brainstem spinal-cord preparations isolated from neonatal rats reported that bath application of NE (Al-Zubaidy et al. 1996; Errchidi et al. 1991) or focal application of NE to the pre-Bötzinger complex (Al-Zubaidy et al. 1996) suppressed respiratory activity. Conversely, in mice exogenous application of NE into the pre-Bötzinger complex typically stimulates respiratory activity in neonatal preparations (Viemari et al. 2005) and in anesthetized adult animals (Viemari and Ramirez 2006). At the level of respiratory motor neurons, NE has been shown to activate hypoglossal motor neurons in rats (Parkis et al. 1995) and mice (Funk et al. 1995) but this response decreases during the transition from juvenile to adulthood (Funk et al. 2011). In the RTN, we show in neonatal rat slices that NE activates the majority of RTN chemoreceptors by an α₁-AR dependent mechanism (Kuo et al., 2016). This study also showed that NE inhibits a small subset of RTN chemoreceptors by an α₂-AR dependent mechanism. Based on previous evidence that brainstem levels of α₁-ARs peaks during the neonatal period followed by decreased expression to low levels in adulthood, it is reasonable to speculate that the observed differences in the effects of NE on RTN activity reflects developmental differences in the expression of α₁- and α₂-ARs (Viemari and Hilaire 2002). However, we also show in vitro that α₁-ARs influence inhibitory input to RTN chemoreceptors (Kuo et al., 2016). Although injections of prazosin did not affect basal respiratory activity, it remains possible that injection of NE into the RTN could indirectly inhibit breathing by activation of α₁-mediated synaptic inhibition of RTN chemoreceptors.

It is also possible that RTN astrocytes contribute to the effects of NE on RTN function. For example, RTN astrocytes are known to function as chemoreceptors by providing a CO₂/H⁺-dependent ATP-purinergic drive to enhance activity of chemosensitive neurons (Gourine et al. 2010; Wenker et al. 2010). In addition, astrocytes in other brain regions have been shown to express all three classes of adrenergic receptors, and when activated by the diffuse release of NE, can influence neural activity directly by releasing transmitters or indirectly by regulating glutamate uptake and glycogen synthesis/glycogenolysis.
(O'Donnell et al. 2012; Paukert et al. 2014). The possibility that astrocytes respond to NE and influence breathing is supported by recent evidence that astrocytes in the pre-Bötzinger complex show a $\text{Ca}^{2+}$ response to NE (Schnell et al. 2015). Although our evidence presented in the companion paper suggests that chemosensitive RTN astrocytes do not response to NE with a change in voltage or by releasing ATP to activate chemosensitive neurons (Kuo et al. 2016), it remains possible that RTN astrocytes respond to NE with an increase in intracellular $\text{Ca}^{2+}$ and communicate with chemosensitive neurons by alternative signaling mechanisms.

It is well described that the RTN neurons are located in close proximity to C1 and non-C1 neurons which regulate blood pressure (Guyenet 2014; 2006) and evidence suggests that NE inhibits these cells by an $\alpha_2$-AR-depenent mechanism (Hayar and Guyenet 1999) leading to decreased MAP (Schreihofer and Guyenet 2000). We found that NE in the RTN did not change blood pressure, suggesting that our injections did not reach the core of the cells that control sympathetic nerve activity.

**Physiological Significance**

NE is a potent modulator of the respiratory network and it is considered one of the most prominent neuromodulators in the mammalian central nervous system. Noradrenergic neurons project from discrete brainstem nuclei (Bolme and Fuxe 1973) to a variety of brain areas where NE can switch networks into different activity states such as the regulation of the sleep-wake cycle, feeding, and cardiorespiratory activity (Aston-Jones et al. 2001; Krantz et al. 2004; Ouyang et al. 2004). In addition, NE dysregulation has been reported in several pathologies affecting the control of breathing, such as Rett Syndrome, Sudden Infant Death Syndrome, Congenital Central Hypoventilation Syndrome and Obstructive Sleep Apnea (Hilaire 2006; Nobuta et al. 2015; Rukhadze et al. 2010; Santos et al. 2010). Furthermore, in some of these pathologies treatment with a NE re-uptake blocker or NE agonists have been shown to improve the respiratory outcome (Kubin 2014; Pattyn et al. 2000; Viemari et al. 2004), thus strongly suggesting loss of NE signaling contributes to disordered breathing.

Previous evidence showed that the NE system provides a wakefulness-related excitatory drive to the respiratory motor neurons to help maintain activity airway patency
(Kubin 2014). A major NE input to XII motoneurons originates in the A7 pontine neurons that have state-dependent activity, i.e. maximal during active wakefulness and minimal or absent during REM sleep (Rukhadze and Kubin 2007). We also found that the majority of our NE projections to the RTN are from A7 region. Therefore it is likely that NE input from A7 contributes to state-dependent control of upstream chemoreceptors, and depending on the relative involvement of α1- or α2-AR’s, this contribution could be excitatory or inhibitory.

Conclusions

In conclusion, we show that NE modulates respiratory output by different mechanisms involving α1-, α2- and β-AR. These results identify key components of the role of catecholaminergic control of RTN chemoreceptors function, and in doing so provide potential avenues for therapeutic treatment of respiratory disorders.

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Conflict of interest

The authors state no conflict of interest.
FIGURE LEGENDS

Figure 1: Activation of ARs in the RTN inhibits breathing in vago-sino-aortic denervated rats.

(A) recording from one rat showing the effect of unilateral injections of vehicle or NE (30 pmol/30 nl) on arterial pressure (AP), diaphragm (Dia\textsubscript{EMG}) and abdominal (Abd\textsubscript{EMG}) electromyography activities. Changes in (B) Dia\textsubscript{EMG} amplitude and (C) Dia\textsubscript{EMG} frequency elicited by vehicle or NE injections into the RTN. (D) Photomicrograph showing typical sites of unilateral injections in the RTN region. The latex microspheres are located ventral to the caudal portion of the facial nucleus in the RTN region (arrows). (E) Computer-generated plot of 67 vehicle or NE injections that were confined to the RTN/pFRG region (Bregma level -11.12 to -11.6 mm according to the Paxinos and Watson atlas). *, different from vehicle (Newman-Keul’s test, p < 0.05). Abbreviations: py, pyramid; Sp5, spinal trigeminal tract; VII, facial motor nucleus; RPa, raphe pallidus. Scale bars in D and E are 1 mm.

Figure 2: Blockade of α\textsubscript{2}-AR in the RTN attenuates the respiratory inhibition caused by RTN injections of NE in vago-sino-aortic denervated rats.

(A) recording from one rat showing the effect of unilateral injections of NE (30 pmol/30 nl) on diaphragm (Dia\textsubscript{EMG}) electromyography activity after bilateral injection of vehicle or yohimbine (3 pmol/30 nl) into the RTN. Changes in (B) Dia\textsubscript{EMG} amplitude, (C) Dia\textsubscript{EMG} frequency, (D) inspiratory time, (E) expiratory time elicited by vehicle, NE and yohimbine + NE injections in the RTN region. (F) Photomicrograph showing typical sites of bilateral injections in the RTN region. The latex microspheres are located ventral to the caudal portion of the facial nucleus in the RTN region. (G) Computer-generated plot of 6 injections that were confined to the RTN/pFRG region (Bregma level -11.12 to -11.6 mm according to the Paxinos and Watson atlas). * indicates different from vehicle and # indicates different from NE (Newman-Keul’s test, p < 0.05). Abbreviations: py, pyramid; Sp5, spinal trigeminal tract; VII, facial motor nucleus; RPa, raphe pallidus. Scale bars in F and G are 1 mm.
Figure 3: RTN injections of NE inhibit respiratory activity in vago-sino-aortic denervated rats by a mechanism that is independent of $\alpha_1$- and $\beta$-ARs. (A-C) recording from two rats showing the effect of unilateral injections of NE (30 pmol/30 nl) on diaphragm (DiaEMG) electromyography activity following the injections of vehicle (A), prazosin (6 pmol/30 nl) (B) or propranolol (3 pmol/30 nl) (C) into the RTN. Changes in (D) DiaEMG amplitude and (E) DiaEMG frequency elicited by vehicle, NE, prazosin + NE and propranolol + NE injections in the RTN region. *, different from vehicle. #, different from NE (Newman-Keul’s test, $p < 0.05$).

Figure 4: Activation of $\alpha_2$-AR in RTN inhibits breathing in vago-sino-aortic denervated rats. (A) recordings from one rat showing the effect of unilateral injections of moxonidine (0.6 pmol/30 nl) on diaphragm (DiaEMG) electromyography activity in response to injections of vehicle (A1) or yohimbine (3 pmol/30 nl) (A2) into the RTN. Changes in (B) DiaEMG amplitude, (C) DiaEMG frequency, (D) inspiratory time and (E) expiratory time elicited by vehicle, moxonidine and yohimbine + moxonidine injections in the RTN region. * indicates different from vehicle and # indicates different from Moxo (Newman-Keul’s test, $p < 0.05$).

Figure 5: RTN injection of a selective $\alpha_1$-AR agonist (phenylephrine) increased breathing in vago-sino-aortic denervated rats. (A), recordings of diaphragm (Dia) and abdominal (Abd) show that application of Phe (0.3 pmol/30 nl) into the RTN increased breathing when preceded by injection of saline into this same region (i.e., control condition), but not after injection of prazosin (6 pmol/30 nl) into this region. Respiratory responses to Phe partly recovered after washing prazosin for ~1 hr. B-E, summary data (n = 8) show changes in DiaEMG amplitude (%DiaEMG ampl) (B), AbdEMG amplitude (%AbdEMG ampl) (C), DiaEMG frequency (%DiaEMG freq) (D) and AbdEMG frequency (%AbdGEMG freq) (E) elicited by injection of saline or prazosin + Phe into the RTN. * indicates different from saline and # indicates different from Phe (Newman-Keul’s test, $p < 0.05$).
Figure 6: RTN injection of NE increases breathing after blockade of both α2- and β-ARs in vago-sino-aortic denervated rats.

(A) recordings from one rat showing the effect of unilateral injections of NE (30 pmol/30 nl) on diaphragm (DiaEMG) and abdominal (ABD_EMG) electromyography activities in response to injections of vehicle (A1) or cocktail of α2- and β-ARs blockers (yohimbine: 3 pmol/30 nl and propranolol: 3 pmol/30 nl) (A2) into the RTN. Changes in (B) DiaEMG amplitude, (C) DiaEMG frequency, (D) AbdEMG amplitude and (E) AbdEMG frequency elicited by vehicle + NE and cocktail + NE injections in the RTN region. *indicates different from vehicle and #indicates different from NE (Newman-Keul’s test, p < 0.05).

Figure 7: Endogenous NE signaling in the RTN does not contribute to the ventilatory response to central or peripheral chemoreceptor activation in anesthetized rats. (A) summary data plotted as mvDiaEMG (the sum of diaphragm frequency and amplitude) shows that RTN injections of prazosin, yohimbine, propranolol or yohimbine and propranolol (cocktail) minimally affect the inspiratory response to central (stepping end-expiratory CO2 from 3-4 to 9-10%) or peripheral (intravenous injections of KCN: 40 μg/0.1 ml) chemoreflexes (one-way ANOVA on ranks, n = 5). (B) summary data plotted as mvAbdEMG show that RTN injections of prazosin, yohimbine, propranolol or yohimbine and propranolol (cocktail) minimally affect the expiratory response to central or peripheral chemoreceptor activation.

Figure 8: A7 neurons project to RTN.

(A) Photomicrograph shows the typical injection site of the retrograde tracer FluorGold (FG) in the retrotrapezoid nucleus (RTN) region. (B) Computer-generated plot of four injections of FG that were confined to the RTN (Bregma level -11.6 to -11.1 mm according to the Paxinos and Watson atlas). (C) FG-labelled neurons in the A7 region (white arrows). (D) Mean number of A7 neurons (means ± SEM of four rats) detected in 4 sections per brain (TH, all TH-ir neurons; FG, all FG neurons). (E) Representative coronal section at bregma -9.0 mm. Each dot represents a neuron. Abbreviations: Aq, aqueduct; scp, superior cerebellar peduncle; PnO, nucleus pontis oralis; py, pyramid; Sp5, spinal trigeminal tract;
VII, facial motor nucleus; RPa, raphe pallidus. Scale bars in A = 1 mm, B = 1 mm, C = 100 μm, C3 = 20 μm (applies to C1-C3) and E = 200 μm.

Figure 9: A2/C2 and A1/C1 neurons project to RTN

(A) FluorGold (FG)-labelled neurons in the A2/C2 region (white arrows) (Bregma –13.8 mm). (B) Mean number of A2/C2 neurons (means ± SEM of four rats) detected in 11 sections per brain (TH, all TH-ir neurons; FG, all FG neurons). (C) FG-labelled neurons in the A1/C1 region (white arrows) (Bregma –13.8 mm). (D) Mean number of A1/C1 neurons (means ± SEM of four rats) detected in 11 sections per brain (TH, all TH-ir neurons; FG, all FG neurons). Abbreviations: AP: area postrema; cc: central canal. Scale bars in A = 250 μm, A3 = 20 μm (applies to A1-3) and C = 100 μm, C3 = 20 μm (applies to C1-3).


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