Submitted to the Journal of Neurophysiology

In vitro characterization of noradrenergic modulation of chemosensitive neurons in the retrotrapezoid nucleus

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Abbreviated Title: α₁- and α₂-adrenoceptors differential regulate RTN chemoreceptors

Keywords: neonatal brain slice, norepinephrine, adrenergic receptors, chemoreception, KCNQ channels

Number of figures: 8
Number of words in Abstract: 236
Number of words main text: 6,913

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ABSTRACT

Chemosensitive neurons in the retrotrapezoid nucleus (RTN) regulate breathing in response to CO₂/H⁺ changes, and serve as an integration center for other autonomic centers including brainstem noradrenergic neurons. Norepinephrine (NE) contributes to respiratory control and chemoreception, and since disruption of NE signaling may contribute to several breathing disorders; we sought to characterize effects of NE on RTN chemoreception. All neurons included in this study responded similarly to CO₂/H⁺ but showed differential sensitivity to NE; we found that NE activated (79%), inhibited (7%), or had no effect on activity (14%) of RTN chemoreceptors. The excitatory effect of NE on RTN chemoreceptors was dose dependent, retained in the presence of neurotransmitter receptor blockers, and could be mimicked and blocked by pharmacological manipulation of α₁-adrenergic receptors (ARs). In addition, NE-activation was blunted by XE991 (KCNQ channel blocker), and partially occluded the firing response to serotonin, suggesting involvement of KCNQ channels. However, in whole-cell voltage-clamp, activation of α₁-ARs decreased outward current and conductance by what appears to be a mixed effect on multiple channels. The inhibitory effect of NE on RTN chemoreceptors was blunted by an α₂-AR antagonist. A third group of RTN chemoreceptors was insensitive to NE. We also found that chemosensitive RTN astrocytes do not respond to NE with a change in voltage or by releasing ATP to enhance activity of chemosensitive neurons. These results indicate NE modulates subsets of RTN chemoreceptors by mechanisms involving α₁- and α₂-ARs.
News and Noteworthy Section

Chemosensitive neurons in the retrotrapezoid nucleus (RTN) provide a CO₂/H⁺-dependent drive to breathe. Here, we show that norepinephrine (NE) differentially modulates activity of subsets of chemosensitive RTN neurons by α1- and α2-adrenergic receptor-dependent mechanisms. These results identify key components of the mechanism by which NE modulates activity of RTN chemoreceptors, and suggest there is a degree of neuromodulatory specialization among RTN chemoreceptors that may correlate with function.
INTRODUCTION
Carbon dioxide (CO₂) provides the primary stimulus to breathe; it is sensed by respiratory chemoreceptors which regulate depth and frequency of breathing in order to maintain CO₂/H⁺ homeostasis (Nattie & Li, 2012). A region of the brainstem called the retrotrapezoid nucleus (RTN) is an important site of chemoreception (Guyenet & Mulkey, 2010). Neurons (Kumar et al., 2015; Wang et al., 2013) and astrocytes (Gourine et al., 2010) in this region sense changes in CO₂/H⁺ to produce an integrated CO₂/H⁺-dependent drive to breathe. The RTN also receives input from several sleep-state-dependent centers including cholinergic, serotonergic and noradrenergic neurons (Rosin et al., 2006). In particular, serotonergic and noradrenergic neurons are most active during wakefulness (Brown et al., 2012), and so are a likely source of wakeful drive to the respiratory system. Withdrawal of this wake-on drive likely contributes to suppression of breathing and the chemoreflex during sleep (Guyenet et al., 2010). Consistent with this possibility, disruption of the serotonergic (Hodges et al., 2009; Ray et al., 2011) or catecholaminergic (Li & Nattie, 2006) systems have been shown to decrease basal respiratory activity and central respiratory chemoreception. Catecholaminergic neurons produce norepinephrine (NE), adrenaline or dopamine. Of these, we focus on NE for this study because there is compelling evidence suggesting that reduced NE levels contribute to respiratory problems associated with Rett syndrome (Katz et al., 2009; Viemari et al., 2005), which typically presents as irregular breathing during wakefulness (Ramirez et al., 2013), a state during which NE levels are normally high and serve to help stabilize breathing (Zanella et al., 2006; Zanella et al., 2014). Despite this important physiological role, little is known regarding the effects of NE on chemosensitive neurons or astrocytes in the RTN.
Norepinephrine modulates neuronal excitability through three classes of adrenergic receptors (ARs), the α1-, α2- and β- ARs, each of which consist of three subtypes (Marzo et al., 2009). The α1- ARs are Gq-coupled and primarily responsible for excitatory effects of NE on respiratory activity (Hilaire et al., 2004; Viemari & Ramirez, 2006; Viemari, 2008). Considering that Gq-signaling by other wake-on neurotransmitters including serotonin (Hawkins et al., 2015; Hawryluk et al., 2012) and acetylcholine (Sobrinho et al., 2015) increase RTN chemoreceptor activity, in part, by inhibition of KCNQ channels, we wondered whether NE activates RTN chemoreceptors by a common mechanism that targets KCNQ channels. α2- ARs are Gi-coupled and typically associated with NE-mediated inhibition of respiratory activity by either pre- or post-synaptic mechanisms (Errchidi et al., 1991; Hilaire et al., 2004; Nasse & Travers, 2014; Rekling et al., 2000). β-ARs are Gs-coupled and although relatively little is known regarding roles of these receptors in control of breathing (Arata et al., 1998), early evidence suggests that application of a β-AR agonist either systemically (Folgering, 1980) or centrally (Burton et al., 1990) increased respiratory output in anesthetized animals, whereas systemic blockade of these receptors decreased the ventilatory response to CO2 in awake humans (Patrick & Pearson, 1980). More recently, it has been shown that activation of β-ARs in the pre-bötzingher complex, a brainstem region critical for respiratory rhythm generation, selectively increased the frequency of sighs (augmented breaths) but not eupneic breathing, suggesting β-ARs selectively control a subset of inspiratory pacemaker neurons (Viemari et al., 2013). Furthermore, in other brain regions, astrocytes have been shown to express all three classes of ARs (O'Donnell et al., 2012), and when activated by the diffuse release of NE at non-junctional varicosities, can influence neural network activity directly by releasing transmitters or indirectly by regulating glutamate uptake and glycogenesis/glycogenolysis (O'Donnell et al., 2012; Paukert et al., 2014).
Therefore, the main objectives of this study are to determine the effects of NE on electrical activity of chemosensitive neurons and astrocytes in the RTN, and to gain insight into mechanisms by which NE modulates RTN chemoreceptor function.

Here, we use the neonatal brain slice preparation and electrophysiological techniques to demonstrate that NE differentially modulates activity of discrete subsets of chemosensitive RTN neurons by α1- and α2-AR-dependent mechanisms. We also present evidence suggesting chemosensitive RTN astrocytes do not respond to NE with a change in voltage or by releasing ATP to activate chemosensitive RTN neurons. These results identify key components of the mechanism by which NE modulates the activity of RTN chemoreceptors. In the companion paper, we test the functional significance of NE signaling in the RTN on cardiorespiratory function in adult anesthetized rats.

METHODS

Animals
Animal use was in accordance with guidelines approved by the University of Connecticut Institutional Animal Care and Use Committee. Brain slices were isolated from neonatal rat pups (7-11 days old; n = 86). All efforts were made to minimize animal discomfort and the number of animals used.

Brain slice preparation and slice-patch electrophysiology
Slices containing the RTN were prepared as previously described (Mulkey et al., 2004; Wenker et al., 2012). Briefly, neonatal rats were decapitated under ketamine/xylazine anesthesia, and
transverse brainstem slices (300 μm) were cut using a microslicer (DSK 1500E; Dosaka) in ice-
cold substituted Ringer solution containing (in mm): 260 sucrose, 3 KCl, 5 MgCl2, 1 CaCl2, 1.25
NaH2PO4, 26 NaHCO3, 10 glucose, and 1 kynurenic acid. Slices were incubated for ~30 min at
37°C and subsequently at room temperature in normal Ringer solution (in mm): 130 NaCl, 3
KCl, 2 MgCl2, 2 CaCl2, 1.25 NaH2PO4, 26 NaHCO3, and 10 glucose. Both substituted and
normal Ringer solutions were bubbled with 95% O2-5% CO2, extracellular pH 7.30.

Individual slices containing the RTN were transferred to a recording chamber mounted on a
fixed-stage microscope (Zeiss Axioskop FS) and perfused continuously (~2 ml min⁻¹) with
normal Ringer solution bubbled with 95% O2-5% CO2. Slices were exposed to hypercapnia by
equilibrating bath solution with 10% (pH = 7.0) or 15% CO2 (pH = 6.9). All recordings were
made with an Axopatch 200B patch-clamp amplifier, digitized with a Digidata 1322A A/D
converter, and recorded using pCLAMP 10.0 software (Molecular Devices, Sunnyvale, CA).
Recordings were obtained at room temperature (~22°C) with patch electrodes pulled from
borosilicate glass capillaries (Harvard Apparatus, Molliston, MA) on a two-stage puller (P89;
Sutter Instrument, Novato, CA) to a DC resistance of 5–7 MΩ when filled with an internal
solution containing the following (in mm): 120 KCH3SO3, 4 NaCl, 1 MgCl2, 0.5 CaCl2, 10
HEPES, 10 EGTA, 3 Mg-ATP, and 0.3 GTP-Tris (pH 7.2); electrode tips were coated with
Sylgard 184 (Dow Corning, Midland, MI). Spontaneous neuronal activity was measured in the
cell-attached voltage-clamp configuration with holding potential matched to resting membrane
potential (Vhold = -60 mV) and zero current is generated by the amplifier (Iamp = 0 pA)
(Perkins KL, 2006). To validate this approach, we compared basal neuronal activity measured in
cell-attached current- or voltage clamp using the above internal solution as well as to recordings
made in the loose-patch mode (i.e., no tight seal) with electrodes filled with Ringer’s solution. We found no difference in baseline firing between these three recording configurations (F_{2,33}=0.0107, \ p = 0.989), thus confirming that our recording configuration allows for accurate assessment of cell excitability while minimizing alterations of the intracellular milieu as previously described (Perkins, 2006). Firing rate histograms were generated by integrating the number of action potential currents generated in 10-s bins and plotted using Spike 5.0 software.

Whole-cell voltage-clamp recordings were made from chemosensitive RTN neurons (V_{hold} = -60 mV) and astrocytes (V_{hold} = -80 mV) in the presence of tetrodotoxin (TTX, 0.1 µM) to block neuronal action potentials. Holding current, conductance, and current-voltage (I-V) relationships were determined using voltage steps between -30 and -150 mV (increments of Δ 10 mV). The CO_{2}/H^{+}- and NE-sensitive I-V relationships were determined by subtracting I-V relationships obtained during exposure to 10% CO_{2} or NE (1 NE) from those recorded under control conditions.

Drugs

Tetrodotoxin was purchased from Alomone Labs (Bethlehem, Israel); other chemicals were obtained from Sigma-Aldrich Co (St. Louis, MO) unless otherwise stated. All drugs were bath applied at the following concentrations: alprenolol (25 nM; Tocris), idazoxan (10 µM; Tocris), isoproterenol (10 µM; Tocris), NE (0.005 - 5 µM), nicergoline (0.1 µM), phenylephrine (10 µM; Tocris), prazosin (1 µM), XE991 (10 µM; Tocris), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 µM), DL-2-Amino-5-phosphonopentanoic acid (APV; 50 µM), strychnine (2 µM) and gabazine (10 µM).
**Statistical analysis**

Data are reported as mean ± standard error of the mean. In most cases, data was normally distributed (Shapiro-Wilk test) and statistical significance was determined using paired t-test, one- or two-way ANOVA with Newman-Keuls multiple comparisons as appropriate. Some data sets with low sample sizes were not normally distributed; therefore, these data were compared using the Wilcoxon test (paired comparison), Mann-Whitney test (independent comparison) or one-way ANOVA on ranks. When non-parametric analysis was performed, we identified the specific test in the results and figure legends. All relevant values used for statistical analysis are provided in the results and figure legends.

**RESULTS**

As part of this study, we characterized the effects of NE on a total of 87 chemosensitive RTN neurons isolated from the same number of animals (we typically isolate 2 RTN slices per animal and obtain a successful recording with ~50% efficiency). These cells were functionally identified based on their firing rate response to CO₂/H⁺. As previously defined (Sobrinho et al., 2015), neurons were considered chemosensitive if they show some level of spontaneous active under control conditions and a robust firing rate response to 10% CO₂ (1.3 ± 0.05 Hz). RTN neurons that showed < 0.8 Hz firing response to 10% CO₂ were considered non-chemosensitive and excluded from this study. We also characterized the effects of NE on chemosensitive RTN astrocytes. These cells were identified by their location near the ventral medullary surface, passive electrical signature and expression of a CO₂/H⁺-sensitive inward rectifying K⁺ current as described previously (Mulkey & Wenker, 2011; Wenker et al., 2010).
Differential effects of NE on excitability of chemosensitive RTN neurons

We found that NE differentially modulates activity of RTN chemoreceptors (Figs. 1A-D). For example, bath application of NE (1 µM) increased activity in 69 of 87 (79%) of RTN chemoreceptors by 1.3 ± 0.05 Hz ($T_{68} = 19.573, p = < 0.0001$), decreased activity of 7% RTN chemoreceptors by 1.0 ± 0.3 Hz (Wilcoxon test; $Z = -2.201, p = 0.028$), or had no measurable effect on the activity of 14% RTN chemoreceptors ($T_{11} = 0.13, p = 0.323$). Although all three groups showed a similar degree of CO$_2$/H$^+$-sensitivity (one-way ANOVA on ranks; $X^2_{2,87} = 0.958, p = 0.619$), they did exhibit different levels of baseline activity; under control conditions NE-inhibited cells were most active (1.1 ± 0.1 Hz), NE-insensitive cells were least active (0.1 ± 0.01 Hz) and NE-activated cells showed an intermediate level of basal activity (0.5 ± 0.1 Hz) (one-way ANOVA on ranks; $X^2_{2,87} = 11.446, p = 0.003$). However, despite these differences in average basal activity, the distribution of NE-activated cells is highly variable (Fig. 1E) and linear regression analysis of these results failed to detect a significant regression equation ($F_{1,84} = 0.17, p = 0.68, R^2 = 0.02$), suggesting there is not a significant relationship between basal activity and the firing response to NE. Nevertheless, these results are consistent with evidence from mice that showed chemosensitive RTN neurons can be sub-classified based on basal activity (Lazarenko et al., 2009), and suggest that NE differentially modulates discrete subsets of RTN neurons that may have divergent roles in control of breathing. Considering that the majority of RTN neurons are activated by NE, we next sought to characterize the excitatory effects of NE on RTN chemoreceptor function, as well as to identify receptors contributing to the differential effects of NE on RTN chemoreceptor excitability.

RTN chemoreceptors are intrinsically activated by NE through an $\alpha_1$-AR mechanism.
NE increased activity of RTN chemoreceptors in a dose dependent manner with an EC$_{50}$ of 235 nM (Figs. 2A-B). For subsequent blocker experiments, we use 1 µM NE to ensure that we elicit a near maximal response of RTN chemoreceptors to NE. We also assessed CO$_2$/H$^+$-sensitivity of RTN chemoreceptors during sustained exposure to NE (Figs. 2C-D). As before, NE (1 µM) increased the baseline level of RTN discharge, but the magnitude of firing rate responses to changes in CO$_2$/H$^+$ was similar in the presence and absence of NE (F$_{2,21}$= 0.449, p = 0.645). For example, 10% CO$_2$ increased RTN chemoreceptor activity by 1.3 ± 0.1 Hz under control conditions and by 1.36 ± 0.1 Hz in the presence of NE (1 µM). Likewise, exposure to 15% CO$_2$ increased RTN chemoreceptor activity by 1.71 ± 0.3 Hz under control conditions and by 1.67 ± 0.1 Hz in the presence of NE. Thus, as was the case for other excitatory monoamines (i.e., serotonin) (Hawryluk et al., 2012), NE caused an upward shift in the CO$_2$/H$^+$ sensitivity curve of chemosensitive RTN neurons in a manner that is independent of transmitter modulation (F$_{2,21}$ = 0.449, p = 0.645).

To determine which class of AR mediates the excitatory effects of NE on RTN chemoreceptors, we characterized the excitatory effects of NE in the presence of α- and β-AR blockers. We found that the firing rate response to NE was retained when β-ARs were blocked with alprenolol (25 nM) (1.4 ± 0.3 Hz vs. 1.6 ± 0.3 Hz in alprenolol; (Wilcoxon test; Z = -0.9448, p = 0.345) (Fig. 3A). Likewise, bath application of isoproterenol (10 µM), a non-selective β-AR agonist, had minimal effect on the activity of RTN chemoreceptors (-0.02 ± 0.1 Hz; T$_{6}$=0.15; p = 0.886). Conversely, bath application of a non-selective α-AR blocker (nicergoline; 0.1 µM) did not significantly affect chemoreceptor activity (Wilcoxon test; Z = -0.948, p = 0.68); however, in the continued presence of nicergoline, exposure to NE increased chemoreceptor activity by only 0.38
± 0.1 Hz (one-way ANOVA on rank; $X^2_{2,17} = 10.1$, $p = 0.006$) (Figs. 3B and D), i.e., 42% of the increase seen in control conditions. However, CO\textsubscript{2}/H\textsuperscript{+}-sensitivity of RTN chemoreceptors was retained in nicergoline (Wilcoxon test; $Z = -0.948$, $p = 0.34$) (Fig. 3C), thus further reinforcing the possibility that RTN chemoreceptors are intrinsically CO\textsubscript{2}/H\textsuperscript{+}-sensitive (Wang et al., 2013). In addition, the excitatory response of RTN chemoreceptors to NE was mimicked by phenylephrine (a selective α1-AR agonist) (Fig. 4A-B); bath application of phenylephrine (10 µM) increased chemoreceptor activity by $1.8 \pm 0.2$ Hz ($T_{12} = 10.05$; $p < 0.0001$). Together, these results suggest that α1-ARs contribute to the excitatory effects of NE on RTN chemoreceptors. Consistent with this possibility, we found that blockade of α1-ARs with prazosin (1 µM; a selective α1-AR blocker) decreased NE-mediated activation from $1.12 \pm 0.2$ Hz to $0.16 \pm 0.1$ ($T_{6} = 3.842$, $p = 0.009$) (Fig. 4C), i.e., 15% of the increase seen in control condition. We also found that NE-mediated activation of chemosensitive RTN neurons was retained in the presence of mixture of ionotropic receptor blockers that included CNQX (10 µM) to block AMPA/kainite receptors, gabazine (10 µM) to block GABA\textsubscript{A} receptors, and strychnine (2 µM) to block glycine receptors. For example, under control conditions bath application of NE (1 µM) increased firing rate by $1.3 \pm 0.1$ Hz. After returning to control conditions, exposure to the blocker cocktail increased activity from $0.3 \pm 0.1$ Hz to $0.8 \pm 0.1$ Hz ($T_{7} = 5.04$, $p = 0.001$) (Fig. 4D). In the continued presence of these blockers, exposure to NE significantly increased firing rate by $1.05 \pm 0.2$ Hz ($T_{6} = 3.52$, $p = 0.013$) in a manner similar to control (Figs. 4D-E). Furthermore, based on recent evidence that activation of NMDA receptors in the RTN stimulated breathing (Silva et al., 2016), we repeated these experiments with an NMDA receptor blocker (APV; 50 µM) included in the blocker cocktail. Again, NE significantly increased firing rate by $1.04 \pm 0.2$ Hz ($T_{7} = 3.845$, $p = 0.006$) which is similar to the control response. However, as
before the NE response was blocked by the addition of prazosin to the cocktail ($F_{2,17} = 7.864$, $p < 0.01$) (Figs. 4D-E). These results show that NE stimulates RTN chemoreceptors directly by activation of $\alpha_1$-ARs.

In addition to this direct excitatory effect of NE on RTN chemoreceptors, $\alpha_1$-ARs may also influence synaptic input to RTN. For example, we found that exposure to a prazosin increased chemoreceptor by $0.62 \pm 0.2$ Hz ($T_{12} = 3.761$, $p = 0.003$) under control conditions but not when inhibitory receptors were blocked ($-0.31 \pm 0.1$ Hz; $T_6 = -2.776$, $p = 0.03$) (Figs. 4D, F). Note that the firing response to $CO_2/H^+$ was fully retained in the combined presence of the blocker cocktail and prazosin (Fig. 4D), thus confirming the recording is healthy and $CO_2/H^+$ sensitivity of RTN chemoreceptors is retained under these conditions. These observations suggest that, in addition to controlling RTN chemoreceptor activity directly, $\alpha_1$-ARs also regulate a local tonic inhibitory drive to RTN chemoreceptors.

We also found that in addition to blocking the excitatory effect of NE on RTN chemoreceptors, prazosin also revealed an inhibitory effect of NE in 6 of 13 cells. For example, in the continued presence of prazosin, exposure to NE decreased chemoreceptor activity by $0.73 \pm 0.1$ Hz (Figs. 4A,C) (one-way ANOVA on rank; $X^2_{2,16} = 12.813$, $p = 0.002$). The residual inhibitory effect of NE in the presence of prazosin could be eliminated by the addition of idazoxan (10 $\mu$M; selective $\alpha_2$-AR blocker) to the bathing medium (Fig. 4C) (one-way ANOVA on rank; $X^2_{2,16} = 12.813$, $p = 0.002$). These results suggest that NE can increase or decrease activity of RTN chemoreceptors depending on involvement of $\alpha_1$- and $\alpha_2$-ARs.
NE activates RTN chemoreceptors by multiple mechanisms including KCNQ channels.

KCNQ channels regulate activity of RTN chemoreceptors by controlling resting membrane potential and serving as downstream targets for Gq-coupled neuromodulators including serotonin (Hawkins et al., 2015; Hawryluk et al., 2012). Considering that α1-ARs are also Gq-coupled, we wondered whether KCNQ channels also contribute to NE-mediated activation of RTN chemoreceptors. We found that pharmacological blockade of KCNQ with XE991 (10 µM) increased baseline firing by 0.7 ± 0.2 Hz, as expected for inhibition of a sub-threshold $K^+$ conductance. In the continued presence of XE991 (with baseline activity adjusted to near control levels by application of a negative current) exposure to NE increased activity of chemosensitive RTN neurons by only 1.36 ± 0.1 Hz, this response represents a 26% decrease compared to the control NE response (1.77 ± 0.1 Hz; $T_7 = 4.869$, $p = 0.002$) (Figs. 5A-B). Together with previous evidence that XE991 also blunted the firing response of RTN chemoreceptors to serotonin, suggests that KCNQ channels serve as a common target for both NE and serotonergic modulation of RTN chemoreceptors. Considering that NE and serotonin are both wake-on neurotransmitters that likely converge upon RTN chemoreceptors simultaneously, we next wanted to determine whether NE-mediated activation of chemosensitive neurons occludes serotonin sensitivity. To test this possibility, we characterized the firing response to serotonin under control conditions and during sustained activation by NE. Consistent with previous studies (Hawkins et al., 2015; Hawryluk et al., 2012), we found that bath applications of serotonin (5 µM) increased activity of RTN chemoreceptors by 1.51 ± 0.2 Hz ($T_{12} = 9.127$, $p < 0.0001$, Figs. 5 C-D), and it did so in a reversible and repeatable manner (ratio of the third serotonin response divided by the second response was 0.9 ± 0.1; Hawryluk et al., 2012). As described above, bath application of NE (1 µM) also increases chemoreceptor activity by 2.03 ±
0.2 Hz ($T_6 = -10.782$, $p < 0.001$) (Fig. 5C). During sustained NE-mediated activation of RTN chemoreceptors, subsequent exposure to serotonin increased chemoreceptor activity by only 0.5 ± 0.1 Hz (Figs. 5C-D) ($T_5 = 4.18$, $p = 0.009$). Although adrenergic receptors can desensitize during several minutes of agonist exposure (Leeb-Lundberg et al., 1987; Wakabayashi et al., 1989), we found that NE-mediated activation of RTN chemoreceptors was maintained for ~30 minutes, suggesting that α1-ARs in chemosensitive RTN neurons are not subject to functional desensitization during this time period. For this reason, it is also unlikely that prolonged activation of α1-ARs triggers negative feedback regulation of serotonergic receptors. Therefore, these results indicate that NE-mediated activation of RTN chemoreceptors partially occludes the excitatory effects of serotonin on these cells. Conversely, the firing response to 10% CO$_2$ was retained under these same conditions (Figs. 5C-D) ($p = 0.360$), suggesting that the reduced serotonin response is not caused by a ceiling effect that limits firing rate. These results are consistent with the possibility that NE and serotonin modulate RTN chemoreceptor activity by a common mechanism may involve KCNQ channels. However, since the majority of the firing response to NE was retained when KCNQ channels are blocked indicates involvement of other downstream targets. To explore this possibility further, we made whole-cell voltage-clamp recordings ($V_{hold} = -60$ mV; TTX) of holding current and conductance during exposure to phenylephrine. We found that RTN chemoreceptors respond to phenylephrine (10 µM) with a decrease in holding current and conductance of 10.9 ± 1.7 pA and 0.17 ± 0.04 nS, respectively ($T_5 = 6.256$, $p = 0.001$; $T_5 = 3.8$, $p = 0.01$). (Fig 5 E-F). Current responses to depolarizing and hyperpolarizing voltage steps obtained under these conditions show the phenylephrine-sensitive current (obtained by subtracting control currents from those obtained in the presence of
phenylephrine) did not reverse over the voltage range tested (Fig. 5G). Together, these observations suggest that activation of α1-ARs has off-setting effects on multiple conductances.

**NE inhibits a subset of RTN chemoreceptors by an α2-adrenergic receptor-dependent mechanism**

We found that 6 of 87 (7%) of RTN chemoreceptors showed a strong inhibitory response to NE under control conditions (Figs. 6A-B); NE decreased activity by $1.0 \pm 0.3$ Hz (Wilcoxon test; $Z = -2.2$, $p = 0.02$). This NE-inhibitory response was eliminated by incubation (10 min) in idazoxan (a selective α2-AR blocker) (Mann-Whitney test; $U = 0.0$, $Z = -2.34$, $p = 0.02$) (Fig. 6B). Likewise, as noted above, an additional number of NE-activated neurons also showed an inhibitory response to NE during α1-AR blockade that was also mediated by α2-ARs (Figs. 4A,C). These results suggest RTN chemoreceptors can express both α1- and α2-ARs that have opposing effects on excitability. However, we were unable to explore the role of α2-ARs in more detail due to the scarcity of encountering NE-inhibited neurons.

**NE-insensitive chemosensitive neurons and astrocytes**

We found that 12 of 87 (14%) chemosensitive RTN neurons showed no observable firing response to NE. This group of neurons exhibits low baseline activity ($0.1 \pm 0.01$ Hz) but an otherwise robust firing response to CO$_2$ ($1.2 \pm 0.1$ Hz, $T_{11} = 13.5$, $p <0.0001$) that was similar in magnitude to NE-activated and –inhibited RTN neurons. However, bath application of NE had no effect on resting or CO$_2$/H$^+$-stimulated activity (Figs. 7A-B). In addition, this group of neurons also did not respond to bath application of phenylephrine ($F_{2,29} = 0.44$, $p = 0.64$) (Figs.
Because we find NE-insensitive RTN chemoreceptors infrequently, we did not attempt to further characterize the noradrenergic or transmitter pharmacological profile of this subtype.

Astrocytes in the RTN also function as respiratory chemoreceptors (Gourine et al., 2010), and since astrocytes in other brain regions (e.g. cortex, pre-bötzinger complex) express ARs and respond to NE with an increase in intracellular Ca^{2+} (Schnell et al., 2015), we considered the possibility that NE may also modulate RTN astrocyte function. In previous work we identified chemosensitive astrocytes based on their electrical response to CO_{2}/H^{+} (Wenker et al., 2010), and since the function of RTN astrocytes as chemoreceptors does not appear dependent on intracellular Ca^{2+} (Huckstepp et al., 2010; Wenker et al., 2012), we chose to characterize the effects of NE on RTN astrocytes using the slice-patch electrophysiological approach. Chemosensitive RTN astrocytes were identified in whole-cell voltage clamp (V_{hold} = -80 mV) by their location near the ventral medullary surface, passive electrical signature and expression of a CO_{2}/H^{+}-sensitive current that corresponds with a > 40 pA change in holding current, or 30% change in conductance during exposure to 10% CO_{2}. On average, CO_{2}/H^{+}-sensitive astrocytes respond to 10% CO_{2} (which corresponds with a ΔpH of ~0.3 pH unit acidification) with a decrease in holding current of -46 ± 6 pA (Wilcoxon test; Z = 2.02, p = 0.04) (Figs. 8A, C). The CO_{2}/H^{+}-sensitive current exhibits modest inward rectification and reverses slightly negative to EK^{+} (Fig. 8B), consistent with previous evidence that Kir4.1-Kir5.1 channels and the electrogenic sodium/bicarbonate cotransporter contribute to CO_{2}/H^{+}-sensitivity of RTN astrocytes (Wenker et al., 2010). Once a CO_{2}/H^{+}-sensitive astrocyte has been identified, we then tested effects of NE (1 µM) on holding current and conductance. These experiments were performed in the presence of TTX (0.1 µM) to block neuronal actitation potentials. We found
that bath application of NE did not significantly affect holding current (Δ = 6.12 ± 2.0 pA) or conductance (Δ = 3.7 ± 2.9 nS) (Wilcoxon test; Z = -1.753, p = 0.08) (Figs. 8A-D). Furthermore, considering that RTN astrocytes have been shown to contribute to RTN chemoreception by releasing ATP to activate local chemosensitive neurons by a P2 receptor-dependent mechanism (Gourine et al., 2010; Huckstepp et al., 2010; Wenker et al., 2012), we also tested the firing response of chemosensitive RTN neurons to NE when P2 receptors were blocked with suramin (100 µM). We found that the firing response to NE was unaffected by 10 minute incubation in suramin ($T_5 = 2.07, p = 0.1$) (Figs. 8E-F). These results suggest that chemosensitive RTN astrocytes do not respond to NE with a change in voltage or by releasing ATP to activate local chemosensitive neurons.

**DISCUSSION**

The RTN receives sleep-wake state dependent modulatory input, such as that from serotonergic, cholinergic and noradrenergic systems, which likely contribute to RTN chemoreceptor control of basal respiratory drive and the ventilatory response to CO$_2$. Here, we show at the cellular level that NE can directly and bi-directionally modulate the excitability of chemosensitive RTN neurons via activation of α1- and α2-ARs. The majority of chemosensitive neurons respond to NE with a firing rate increase that is mediated by α1-ARs and involves modulation of multiple downstream ion channels that may include KCNQ. Based on previous evidence that other wake-on transmitters like serotonin (Hawkins et al., 2015; Hawryluk et al., 2012) and acetylcholine (Sobrinho et al., 2015) also stimulate chemoreceptor activity by targeting KCNQ channels, these results suggest KCNQ channels serve as a common substrate for arousal-dependent control of respiratory drive. A second group of chemosensitive RTN neurons were inhibited by NE by a
mechanism involving post-synaptic α2-ARs. A third group of chemosensitive RTN neurons showed no measurable response to NE. We also found that chemosensitive RTN astrocytes do not respond to NE with a change in voltage or by releasing ATP to gain-up activity of chemosensitive neurons. Intermingled within NE-activated and –inhibited populations were neurons that could switch the polarity of their NE response under control conditions as compared to during α1- or α2-AR blockade, thus suggesting some RTN chemoreceptors express both α1- and α2-ARs. Our evidence that NE differentially modulates RTN chemoreceptors, suggests there is a degree of neuromodulatory specialization among RTN chemoreceptors that may correlate with function.

**Experimental Limitations**

Important limitations of the brain slice preparation include the use of tissue that has been traumatized, subjected to oxidative stress (i.e., high oxygen incubation conditions; Mulkey *et al.*, 2001) and neural network connections have been disrupted. Therefore, cellular activity and modulation by neurotransmitters may not mirror *in vivo* conditions. Our experiments were also limited to the use of animals less than ~2 weeks of age. This is a potential issue because chemosensitive RTN neurons show a much larger firing response to CO₂ in anesthetized adult rats *in vivo* as compared to in the neonatal slice preparation (Guyenet *et al.*, 2005). Furthermore, the noradrenergic system takes several weeks to reach maturity (Murrin *et al.*, 2007). Therefore, the response of RTN chemoreceptors to CO₂/H⁺ or neurotransmitters in neonatal slices may not fully recapitulate their response in adult animals *in vivo*. In addition, our experiments are limited by the use of exogenous drug application. This is an issue because bath application of NE may not mimic the discrete and rapid transient nature of endogenous

neurotransmitter release. It should also be recognized that NE may activate astrocytes by mechanisms that are independent of voltage. For example, NE has been shown to trigger intracellular Ca\(^{2+}\) release from astrocytes in the pre-bötzing complex (Schnell et al., 2015), and this response does not necessarily correspond with a change in voltage. However, the transmitter basis for communication between RTN astrocytes and local chemosensitive neurons involves purinergic signaling, and our evidence that neuronal sensitivity to NE is retained during P2 receptor blockade, does suggest that RTN astrocytes do not contribute to the excitatory effect of NE on RTN chemoreceptors. Nevertheless, it remains possible that RTN astrocytes respond to NE with an increase in intracellular Ca\(^{2+}\) and communicate with chemosensitive neurons by alternative signaling mechanisms.

**NE modulation of RTN chemoreceptors**

Noradrenergic neurons (A1-7) contribute to several autonomic functions including modulation of respiratory rhythm (Hilaire et al., 2004; Viemari & Ramirez, 2006; Viemari, 2008; Viemari & Tryba, 2009), respiratory motor output (Funk et al., 2011; Horner, 2009; Parkis et al., 1995), and central respiratory chemoreception (Li & Nattie, 2006; Nattie & Li, 2009; Pineda & Aghajanian, 1997). There are seven brainstem noradrenergic centers (A1-7); of these, previous evidence showed that the RTN receives input from A5 and the subceruleus regions (Cream et al., 2002; Rosin et al., 2006). Furthermore, in the companion paper we report that the RTN also receives input from the A7 region (Oliveira et al., 2016). At the cellular level, we found that NE can increase or decrease activity of RTN chemoreceptors by activation of \(\alpha_1\)- and \(\alpha_2\)-ARs, respectively. A third group of chemosensitive RTN neurons did not respond to NE under control conditions or when activity was stimulated by high CO\(_2\). Although neurons in each group
showed a similar firing response to $\text{CO}_2/\text{H}^+$, each group exhibited different levels of baseline activity. We did not systematically characterize firing behavior of each group of chemoreceptor under synaptic block conditions; therefore, we do not know whether these differences in baseline activity can be attributed to intrinsic or synaptic properties. In any case, as described below the effects of NE on RTN chemoreceptor activity was retained during synaptic blockade, suggesting direct involvement of $\alpha_1$- and $\alpha_2$-ARs. Contrary to previous evidence that Type I and Type II RTN chemoreceptors show similar responses to other neurotransmitters like substance P (Lazarenko et al., 2009), our evidence suggests subsets these subsets of RTN chemoreceptors are differentially modulated by NE mediated activation of $\alpha_1$- and $\alpha_2$-ARs. Therefore, it is tempting to speculate that differential expression of $\alpha_1$- and $\alpha_2$-ARs by subsets of RTN chemoreceptors may confer specialized roles of NE in regulation of discrete aspects of breathing. However, this possibility has yet to be determined.

The molecular basis for NE-activation of RTN chemoreceptors involves activation of $\alpha_1$-ARs and downstream inhibition of KCNQ channels. For example, we show that NE primarily stimulates activity of RTN chemoreceptors in a dose dependent manner, and this response could be mimicked by phenylephrine (specific $\alpha_1$-AR agonist) and blocked by prazosin (specific $\alpha_1$-AR antagonist), thus indicating $\alpha_1$-ARs mediate NE activation of RTN chemoreceptors. Furthermore, this excitatory effect was retained in the presence of a cocktail of ionotropic receptor blockers that included APV (to block NMDA receptors), CNQX (to block AMPA/kainite receptors), gabazine (to block $\text{GABA}_A$ receptors), and strychnine (to block glycine receptors), suggesting NE directly activates RTN chemoreceptors. These results are consistent with previous evidence that showed NE activates respiratory neurons in the pre-
bötzheimer complex (Hilaire et al., 2004; Viemari & Ramirez, 2006; Viemari, 2008) and hypoglossal motor nucleus (Funk et al., 1994; Funk et al., 2011; Parkis et al., 1995) primarily by activation of α1-ARs. Previous studies suggest that β-ARs also contribute to the excitatory effects of NE by activating a subset of inspiratory pacemaker neurons in the pre-bötzheimer complex (Viemari et al., 2013) and potentiating inspiratory output of the hypoglossal motor nucleus (Selvaratnam et al., 1998), however, we found that pharmacological manipulations of β-ARs had negligible effect on activity of RTN chemoreceptors under our experimental conditions and so were not considered further.

Interestingly, we also found that bath application prazosin increased activity of RTN chemoreceptors. Furthermore, the excitatory effect of prazosin was eliminated when inhibitory synaptic transmission was disrupted by gabazine andstrychnine. These results suggest that α1-ARs also regulate an unidentified inhibitory input to RTN chemoreceptors. This is interesting because NE, when paired with bouts of hypoxia, has been shown to facilitate synaptic inhibition of the pre-bötzheimer complex and destabilize breathing (Zanella et al., 2014). Therefore, it is possible that NE-dependent synaptic inhibition of the RTN may also contribute to respiratory problems under certain pathological states. We did not attempt to identify the source of α1-mediated inhibitory drive to RTN chemoreceptors; however, we believe it to be local since α1-ARs are exclusively post-synaptic.

Several ion channels are thought to contribute to α1-AR-mediated activation of respiratory neurons including a Ca^{2+}-activated non-selective cation conductance (Viemari & Ramirez, 2006; Viemari & Tryba, 2009), persistent Na^{+} channels (Viemari & Ramirez, 2006; Viemari & Tryba,
2009), TASK channels (Parkis et al., 1995; Talley et al., 2000), and medium afterhyperpolarization current (Parkis et al., 1995) which is likely conferred in part by KCNQ channels (Bond et al., 2004; Tzingounis & Nicoll, 2008). Of these, we consider KCNQ a likely candidate since these channels have been shown to regulate basal activity of RTN chemoreceptors and serve as downstream targets for other Gq-coupled neuromodulators like serotonin (Hawkins et al., 2015; Hawryluk et al., 2012) and acetylcholine (Sobrinho et al., 2015). Consistent with this possibility, we found that the excitatory effects of NE on RTN chemoreceptor activity was blunted by bath application of a selective KCNQ channel blocker (XE991) (Figs. 5A-B) and partially occluded the firing response to serotonin (Figs. 5C-D).

These results are consistent with evidence from other brain regions that showed serotonin, NE and other Gq-coupled neurotransmitters converge on common targets (Parkis et al., 1995; Talley et al., 2000). Clearly, there is a residual excitatory response to NE when KCNQ channels are blocked, suggesting involvement of multiple effectors. This was also evident in whole-cell voltage-clamp where phenylephrine decreased outward current but with only a small change in conductance, and the phenylephrine-sensitive current did not reverse over the voltage range tested.

A subset of chemosensitive RTN neurons were inhibited by NE under control conditions and ~30% of NE-activated neurons showed an inhibitory response to NE during α1-AR blockade. The inhibitory effect of NE on RTN chemoreceptors was blocked by idazoxan (a selective α2-AR blocker) and in some cases converted to an excitatory response, thus further suggesting RTN chemoreceptors can express both α1- and α2-ARs and the net effect of NE reflects the sum contribution of both receptors. Furthermore, the inhibitory effect of NE was blocked by
idazoxan, thus indicating involvement of α2-ARs. Considering that the noradrenergic system takes several weeks to reach maturity (Murrin et al., 2007) and since levels of α2-ARs tend to increase during development in certain brainstem regions (Happe et al., 2004), whereas brainstem expression of α1-ARs peaks at around 2 weeks of age followed by a decline to low levels in adulthood (Funk et al., 2011), perhaps the differential effects of NE on RTN chemoreceptors from neonatal rats reflects a developmental transition that favors excitation in neonates and inhibition in adult. Consistent with this possibility, we report in the companion paper that NE injection into the RTN of anesthetized adult rats caused a dose-dependent inhibition of breathing by an α2-AR-dependent mechanism (Oliveira et al., 2016). However, at this point we cannot exclude the possibility that the differential effects of NE on RTN chemoreceptors may reflect specialized roles for α1- and α2-ARs in the regulation of RTN function.

Astrocytes in other brain regions have been shown to express all three classes of ARs, and when activated can influence neural network activity directly by releasing transmitters or indirectly by regulating glutamate uptake and glycogenesis/glycogenolysis ((O'Donnell et al., 2012; Paukert et al., 2014)). In addition, a recent study showed that bath application of NE elicited Ca\(^{2+}\) responses in astrocytes located in the nearby pre-böttinger complex (Schnell et al., 2015), suggesting that astrocytes may contribute to NE modulation of breathing. In the RTN, astrocytes function as chemoreceptors by sensing CO\(_2\)/H\(^+\) in part by inhibition of an inward rectifying K\(^+\) channel (i.e., Kir4.1-Kir5.1) (Wenker et al., 2010) and releasing ATP in a CO\(_2\)/H\(^+\)-dependent manner to enhance the activity of chemosensitive neurons (Gourine et al., 2010; Huckstepp et al., 2010; Wenker et al., 2012). However, we found that RTN astrocytes do not respond to NE
with a change in voltage or by releasing ATP to activate chemosensitive neurons (Fig. 8). These results suggest that RTN astrocytes do not contribute to NE modulation of RTN chemoreception. However, it remains possible that RTN astrocytes respond to NE with an increase in intracellular Ca$^{2+}$ and communicate with chemosensitive neurons by alternative signaling mechanisms.

In sum, we show that NE directly and bi-directionally modulates activity of chemosensitive RTN neurons by mechanisms involving α1 and α2-ARs. The differential effects of NE on RTN chemoreceptors is likely due to differences in the expression of α1- and α2-ARs by subsets of RTN chemoreceptors. We speculate that these differences in NE-sensitivity reflect a level of functional specialization of noradrenergic control of respiratory activity. Furthermore, our finding that the majority of RTN chemoreceptors in slices from neonatal rats were activated by NE by a mechanism that involves multiple channels including inhibition of KCNQ channels is consistent with the possibility that NE facilitates breathing and respiratory central chemoreception (Li & Nattie, 2006), and suggests that KCNQ channels serve as a common target for wake-on neurotransmitters including serotonin and acetylcholine (Hawryluk et al., 2012; Sobrinho et al., 2015). However, in the companion paper, we show that injection of NE into the RTN of adult rats has a net inhibitory effect on breathing by a mechanism involving α2-ARs (Oliveira et al., 2016). The reasons for these divergent results are not clear, but may involve developmental changes in the level of α1-and α2-AR expression by discrete subsets of RTN chemoreceptors.
FIGURE LEGEND:

**Figure 1.** Differential effects of NE on activity of RTN chemoreceptors. A-C, traces of firing rate and segments of membrane potential show the characteristic firing responses of NE-activated (A), -inhibited (B) and –insensitive (C) neurons to 10% CO₂ and NE (1 µM). D, summary data (N=87) shows the average firing rate response to CO₂/H⁺ and NE (1 µM) for each group of chemoreceptors sub-classified based on their firing response to NE. Note that all neurons included in this analysis showed a similar firing response to 10% CO₂. However, subsets of cells within this population showed differential responses to NE; the majority were of cells tested were activated by NE (79%), while 7% were inhibited by NE and the remaining 14% showed no obvious response to NE. *, different from control (paired t-test, p < 0.001). †, different from NE activation (one-way ANOVA on rank, p < 0.0001). E, summary data (N=87) shows that NE-activated, -inhibited and –insensitive cells exhibited different levels of baseline activity. *, one-way ANOVA on ranks, p = 0.003.

**Figure 2.** NE stimulates activity of RTN chemoreceptor in a dose-dependent manner and results in an upward-shift in the CO₂/H⁺ response profile. A, trace of firing rate shows the response of a chemosensitive RTN neuron to 10% CO₂ and to graded increases in NE. B, average firing rate at each concentration plotted (black dots) were fitted (continuous red line) to a logistic equation of the form: \( y = \frac{a - c}{1 + \left(\frac{[NE]}{EC_{50}}\right)^b} + c \), where \( a \) and \( c \) are the theoretical minimum and maximum, respectively, and \( b \) is a slope function (Li et al., 1998). Only neurons tested with at least two concentrations of NE are included; each point represents data from 3-8 neurons. The calculated NE EC_{50} was 235 nM. C, trace of firing rate shows that chemosensitive
RTN neurons respond similarly to 10 and 15% CO₂ under control conditions and in the continued presence of NE (1 µM). D, average data (N=5) show that exposure to NE caused a parallel upward shift in the CO₂/H⁺ response curve of RTN chemoreceptors. *, different from control (two-way ANOVA; p < 0.0001).

Figure 3. α- but not β-ARs mediate the effects of NE on RTN chemoreceptors.

A, firing rate trace shows the response of a chemosensitive RTN neuron to NE (1 µM) under control conditions and after ~15 minute incubation in 25 nM alprenolol (non-specific β-AR blocker). B, trace of firing rate from a chemosensitive RTN neuron shows the firing response to 10% CO₂ and NE (1 µM) under control conditions and in the presence of nicergoline (0.1 µM), an non-specific α-AR blocker. C, summary data (N = 5) shows that CO₂/H⁺-sensitivity of RTN chemoreceptors was retained in the presence of nicergoline (Wilcoxon test, p = 0.34). D, summary bar graph shows the firing response to NE under control conditions and in the presence of nicergoline and alprenolol. *, different from control (one-way ANOVA on rank, p = 0.006).

Figure 4. α₁-ARs mediate the excitatory effects of NE on chemosensitive RTN neurons.

A, firing rate trace shows an example where the excitatory effect of NE (1 µM) on chemoreceptor activity was mimicked by phelylephrine (10 µM, Phe). However, when α₁-ARs are blocked with prazosin, approximately half of NE-activated neurons showed an inhibitory response to NE, suggesting some RTN chemoreceptors express both α₁ and α₂-ARs. Note that application of prazosin alone under control conditions typically increased chemoreceptor activity. B, summary data (N=13) shows that phelylephrine (10 µM, Phe) mimicked the excitatory effects of NE on chemoreceptor activity. C, summary data of those cells inhibited by
NE in the presence of prazosin (N=6) show the NE-induced change in firing rate under control conditions, in the presence of prazosin alone, and in the combined presence of prazosin and idazoxan (10 µM). The residual inhibitory response to NE in the presence of prazosin was eliminated by blockade of α2-ARs. * different from control (one-way ANOVA on ranks, p = 0.002). D, trace of firing rate shows the response of an RTN chemoreceptor to NE (1 µM) under control conditions, in the presence of a neurotransmitter receptor blocker cocktail containing CNQX (10 µM), gabazine (gab; 10 µM) and strychnine (strych; 2 µM), and the cocktail plus prazosin. Note that application of the blocker cocktail typically increases chemoreceptor activity by ~1 Hz, suggesting these neurons receive a tonic inhibitory drive. After ~10 minutes incubation in the blocker cocktail exposure to NE increased chemoreceptor activity by an amount similar to control conditions. However, the combined effects of the blocker cocktail plus prazosin eliminated the excitatory effects of NE on RTN chemoreceptor activity. E, summary data (N=8) shows that the excitatory effects of NE are retained in the presence of the synaptic blocker cocktail this time also including APV (50 µM) but eliminated by prazosin. *, different from control (one-way ANOVA on ranks, p < 0.01). F, summary data (N=7) shows that the excitatory effects of prazosin was eliminated by gabazine (gab; 10 µM) and strychnine (strych; 2 µM). *, different from control (paired t-test, p < 0.01).

Figure 5. NE activates RTN chemoreceptors by multiple mechanisms including inhibition of KCNQ channels. A, firing rate trace shows that bath application of the KCNQ channel blocker XE991 (10 µM) increased basal activity by 1.0 Hz. In the continued presence of XE991 with baseline activity was adjusted to near control levels by DC current injection (arrow), the firing response to NE was reduced by 26% compared to control. B, summary data (N=8) show
that XE991 blunted the NE response of RTN chemoreceptors. // designate 30 minute time break.

*, different from control (paired t-test, p = 0.002). C, firing rate trace from a chemosensitive RTN neuron shows typical excitatory responses to (5-HT) and NE. During sustained NE-activation, a second exposure to serotonin increased activity by only 0.5 Hz. However, under these same experimental conditions exposure to 10% CO₂ increased activity by an amount similar to control conditions. D, summary data (N=6) shows the firing response to serotonin and 10% CO₂ under control conditions and in the continued presence of NE. Note that activation of RTN chemoreceptors by NE blunted subsequent activation by serotonin. *, (RM ANOVA, p < 0.01). E-F, in whole-cell voltage-clamp mode, bath application of phenylephrine (10 µM, Phe) caused a pronounced decrease holding current (E) in conjunction with a modest decrease in conductance (F). G, I-V plot of the phenylephrine-sensitive current (obtained by subtracting current responses to voltage steps ranging from -130 to -30 mV under control conditions from those recorded in the presence of phenylephrine) does not reverse over the voltage range tested, suggesting activation of α1-AR has mixed effects on multiple conductances. Together, these results suggest that NE activates RTN chemoreceptors by a mechanism involving α1-ARs and modulation of multiple downstream ion channels including KCNQ.

**Figure 6. NE inhibits a subset of RTN chemoreceptors by an α2-AR-dependent mechanism.** A, representative traces of firing rate from NE-inhibited chemosensitive RTN neurons that the inhibition is blunted by idazoxan. Note that RTN chemoreceptors of this subtype exhibit high basal activity under control conditions but otherwise show a typical 1 Hz firing rate increase in response to 10% CO₂. (paired t-test, p=0.001) Also note that repeated applications of NE consistently inhibited activity under control conditions. The inhibitory effect
of NE was totally eliminated by ~10 minute incubation in idazoxan (10 µM). B, summary data 
(N = 3) shows that incubation in idazoxan (10 µM) converted the inhibitory effect of NE into an 
excitation. *, different from control (Mann-Whitney test, p = 0.02).

Figure 7. A subset of RTN chemoreceptors do not respond to NE under control conditions 
or during CO2/H+-stimulation. A, trace of firing rate shows that this chemosensitive RTN 
neuron has low basal activity under control conditions and exhibits a robust firing response to 
10% CO2. This cell did not show excitatory responses to bath applications of either NE (1 µM) 
or phenylephrine (10 µM; Phe) under control conditions nor did it respond to bath applied NE 
when neural activity was stimulated by 10% CO2. B, summary bar graph (N=12) shows the 
pharmacological profile of NE-insensitive RTN chemoreceptors. *, different from control (RM ANOVA, p < 0.0001)

Figure 8. chemosensitive RTN astrocytes do not respond to NE with a change in voltage or 
by releasing ATP to activate chemosensitive neurons. A, traces of holding current (top) and 
conductance bottom) show that exposure to 10% CO2 decreases outward current and 
conductance. After returning to control conditions bath application of NE (1 µM) had no notable 
effect on holding current or conductance. (Wilcoxon test, p = 0.08) B, under each experimental 
condition we delivered voltage steps from -80 mV to between -40 and -150 mV and the 
corresponding current-voltage (I-V) relationship for the CO2/H+-sensitive and NE sensitive 
currents are shown here. Note that the CO2/H+-current (black) has properties reminiscent of 
Kir4.1-Kir5.1 channels; i.e., it reverses near the equilibrium potential for K+ and shows a modest 
degree of inward rectification at the more depolarized voltages. However, the NE-sensitive
current (red) is negligible. **C-D**, summary data (N=5) shows the effects of 10% CO₂ and NE on holding current (C) and conductance (D). *, different from control. (Wilcoxon test, p = 0.04) **E**, trace of firing rate from a NE-activated chemosensitive RTN neuron shows that ~10 minute incubation in suramin (100 µM), a non-specific P2 receptor blocker, had no discernable effect on the excitatory response to NE (1 µM). **F**, summary data (N=6) shows NE-mediated activation of RTN chemoreceptors was unaffected by blockade of P2 receptors by suramin (paired t-test, p = 0.1). These results indicate that chemosensitive RTN astrocytes do not respond to NE with a change in voltage or by releasing ATP to activate local chemosensitive neurons.
ACKNOWLEDGEMENTS

This work was supported by funds from the National Institutes of Health Grants HL104101 (DKM), Connecticut Department of Public Health Grant 150263, and public funding from the São Paulo Research Foundation (FAPESP) grants 2014/22406 (ACT) and FAPESP fellowship 2013/26540-1 (LMO); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); grant: 471263/2013-3 (ACT). CNPq fellowship 301651/2013-2 (ACT).


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