Adrenoreceptor Modulation of Oromotor Pathways in the Rat Medulla

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Abstract:

Regulation of feeding behavior involves the integration of multiple physiologic and neurologic pathways that control both nutrient seeking and consummatory behaviors. The consummatory phase of ingestion includes stereotyped oromotor movements of the tongue and jaw that are controlled through brainstem pathways. These pathways not only encompass cranial nerve sensory and motor nuclei for processing feeding-related afferent signals and supplying the oromotor musculature, but also reticular neurons for orchestrating ingestion and coordinating it with other behaviors that utilize the same musculature. Based on decerebrate studies, this circuit should be sensitive to satiety mechanisms mediated centrally by A2 noradrenergic neurons in the caudal nucleus of the solitary tract (cNST) that are potently activated during satiety. Because the first observable phase of satiety is inhibition of oromotor movements, we hypothesized that norepinephrine (NE) would act to inhibit pre-hypoglossal neurons in the medullary reticular formation. Using patch clamp electrophysiology of retrogradely labeled pre-hypoglossal neurons and calcium imaging to test this hypothesis, we demonstrate that norepinephrine can influence both pre- and postsynaptic properties of reticular neurons through both $\alpha_1$- and $\alpha_2$-adrenoreceptors. The $\alpha_1$-adrenoreceptor agonist phenylephrine (PE) activated an inward current in the presence of TTX, and increased the frequency of both inhibitory and excitatory miniature postsynaptic currents. The $\alpha_2$-adrenoreceptor agonist dexmedetomidine (DMT) inhibited caudal NST-evoked excitatory currents as well as spontaneous and miniature excitatory currents through presynaptic mechanisms. The diversity of adrenoreceptor modulation of these pre-hypoglossal neurons may reflect their
role in a multifunctional circuit coordinating both ingestive and respiratory lingual function.

Key Words: Norepinephrine, Adrenoreceptor, Feeding, NST
Introduction

Regulation of feeding behavior involves a complex network of brain regions and pathways that include both forebrain and hindbrain structures. Forebrain structures such as the hypothalamus regulate the appetitive aspect of the feeding while, as demonstrated in decerebrate rat studies, the hindbrain controls the consummatory phase (Grill and Smith, 1988; Seeley et al., 1994; Grill, 2010; Schneider et al., 2013). Neurons in the caudal nucleus of the solitary tract (cNST) play a critical role in the control of the consummatory response. During the course of a meal, satiety signals, like those arising from gastric stretch and the release of gut peptides, activate neurons located in the cNST including the A2 noradrenergic group (Smith et al., 1981; Willing and Berthoud, 1997). These neurons receive direct input from the gastrointestinal tract via vagal afferents and transection of the vagus nerve attenuates the effects of satiety factors such as CCK (Smith et al., 1985; Rinaman et al., 1998). While much attention has focused on the influence of norepinephrine in regulating the dorsal vagal complex (Fukuda et al., 1987; Rogers et al., 2003; Martinez-Peña y Valenzuela et al., 2004; Appleyard et al., 2007) and gastric reflexes (Hikasa et al., 1992; Hermann et al., 2005), little research has explored the neural pathways through which visceral cNST neurons influence the first observable consequence of satiety, i.e. the inhibition of ingestive oromotor activity that defines meal termination.

In the decerebrate rat, normal ingestive mechanisms such as suppression of feeding by CCK (Grill and Smith, 1988) persist, indicating that the neuronal substrate controlling this behavior resides in the brainstem. However, because ingestion and respiration share a common lingual musculature (Miller, 2002), ingestive oromotor behaviors including
swallowing must also be coordinated with respiratory activity to protect and maintain the
patency of the airway. The neuronal architecture controlling this orolingual coordination
may derive from a multifunctional substrate that is housed in the brainstem, as respiratory
inhibition during a swallow is also evident in the decerebrate rat (Saito et al., 2003). The
reticular formation lateral to the hypoglossal nucleus may be involved in this coordination.
Neurons in this region of the reticular formation project to the hypoglossal nucleus and are
active during licking, swallowing, and respiration (Ono et al., 1994; 1998; Travers et al.,
2000; Travers et al., 2005). Furthermore, pharmacological manipulation of this area
modulates hypoglossal inspiratory-phase activity (Okabe et al., 1994; Chamberlin et al.,
2007).

Norepinephrine is also implicated in coordinating these disparate oromotor
dbehaviors. Norepinephrine microinjection or stimulation of noradrenergic centers,
including the A2 noradrenergic group, inhibit the swallow reflex induced by superior
laryngeal nerve stimulation and prolong the time between inspiratory activity following a
swallow (Kessler and Jean, 1986a; 1986b; Yamanishi et al., 2010). Since noradrenergic
neurons are potently activated during normal satiety (Rinaman, 2011), we predicted that
norepinephrine, acting through one or more of its known receptors, would inhibit either
pre-hypoglossal neurons in the medullary reticular formation directly, or inhibit the
release of excitatory neurotransmitter from the presynaptic fibers that drive them.

The mechanisms by which norepinephrine operates within this multifunctional
network are unclear. Norepinephrine binds to multiple adrenoreceptor subtypes and leads
to multiple complex intracellular signaling pathways that can influence both postsynaptic
membrane properties and presynaptic signaling mechanisms (Moore and Guyenet, 1983;
The current study tests the hypothesis that multiple adrenoreceptors modulate pre-hypoglossal neurons in the medullary reticular formation.

Methods

Animals

Sprague-Dawley rat pups with dam (Harlan Industries, Indianapolis, IN) were maintained on a 12:12 light/dark cycle with constant temperature and humidity control. The dam was given *ad libitum* access to both food and water. Pups remained with the dam until the time of each experimental procedure. All experimental procedures were conducted in accordance with National Institutes of Health guidelines, and were approved by The Ohio State University Institutional Animal Care and Use Committee.

Retrograde Tracer Injections

Neonatal pups (P6-9) were initially anesthetized with 5% Isoflurane and then placed into a stereotaxic device. Once in the stereotaxic holder, a surgical level of anesthesia was maintained with 1-3% Isoflurane. A midline incision was made over the back of the skull, and neck musculature was retracted to expose the dura covering the foramen magnum. A small incision was made through the dura using a 26 Ga subcutaneous needle and subsequently expanded in the caudal direction with vannascissors to expose
the area postrema and obex. Retrograde tracer injections (30-50 nL, carboxylated-
polysytrene beads, 100 nm diameter, Life Technologies, Grand Island, NY) into the
hypoglossal nucleus were made at the level of obex, 0.2 mm off the midline and 1.0 mm
from the brain surface using a pulled glass pipette beveled to a nominal tip diameter of 22-
50 µm. These injection coordinates were chosen to be just caudal of the area postrema
where patch clamp recordings were conducted to minimize the potential for the bright
fluorescent signal from the injection site to bleed over into the recording area. Injections
were made at a rate of 10 nL/min and the injection pipette was left in place for 5 min
before being slowly removed. The wound was closed with 6.0 silk sutures, and topical
antibiotic was applied to the wound site. The pups were given 0.15 mL of lactated ringers
and returned to the dam following recovery from anesthesia.

Brain Slices

24-72 hours after retrograde tracer injection, pups were anesthetized with ethyl
carbamate (Urethane, Sigmal-Aldrich, St. Louis, MO, 2gm/kg, dissolved in sterile distilled
H₂O) by intra-peritoneal injection and euthanized by rapid decapitation. The brain was
rapidly removed from the skull and cooled to 4°C in a modified artificial cerebral spinal
fluid containing (in mM) 110 choline chloride, 25 NaHCO₃, 2.5 KCl, 7 MgSO₄·7 H₂O, 1.5
NaH₂PO₄, 10 D-glucose, and 0.5 CaCl₂·2 H₂O. The cerebellum was removed and the
brainstem blocked rostrally at the ponto-medullary junction and caudally at the spino-
medullary junction. The brain was sectioned in the coronal plane using a sapphire blade
(Delaware Diamond Knives, Wilmington, DE) on a vibrating tissue slicer (Vibratome
classic-1000) at a nominal thickness of 350 µm. Following sectioning, slices were transferred to warmed (32°C) carboxygenated normal artificial cerebral spinal fluid (ACSF) containing (in mM) 124 NaCl, 25 NaHCO₃, 3 KCl, 1 MgSO₄·7·H₂O, 1.5 NaH₂PO₄, 10 D-glucose, and 1.5 CaCl₂·2·H₂O and allowed to equilibrate for 30-60 minutes. Slices were then held at room temperature until used for individual experiments.

Drugs

All drugs were administered through the perfusion system at a rate of 2 mL/min. The concentrations of antagonists for the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, and N-methyl-D-aspartate (NMDA) receptor were: 6-Cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX: 10 µM), 6,7-Dinitroquinoxaline-2,3-dione (DNQX: 10 µM) and 5S,10R-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801: 10 µM) (AMPA and NMDA respectively) and were chosen to be consistent with our previous study in the rostral reticular formation, and with a previously published concentrations shown to be effective (Nasse et al., 2008). Norepinephrine (Sigma-Aldrich, St. Louis, MO) was delivered at a concentration of 20µM. Dexmedetomidine (DMT) was chosen as the α₂-adrenoreceptor agonist for its greater selectivity over more common agonists such as clonidine or UK14-304 (Virtanen et al., 1988; Scheinin et al., 1989), and delivered at a concentration of 100 nM. This concentration is approximately 100 times greater than the binding affinity for the α₂-adrenoreceptor and 10 times lower than the binding affinity for the α₁-adrenoreceptor (Virtanen et al., 1988) and was selected in order to achieve potent, yet selective activation.
of only the $\alpha_2$-adrenoreceptor. DMT was perfused for 5 min prior to taking any measurements to provide adequate time to reach an equilibration state. Phenylephrine was delivered at a concentration of 20 µM which is 2-fold greater than the EC$_{50}$ for rat $\alpha_1$-adrenoreceptor activation (Minneman et al., 1994). Phenylephrine has been shown to increase intracellular calcium concentrations and depolarize neuronal membrane potentials (Martinez-Peña y Valenzuela et al., 2004; Hermann et al., 2005); as such we limited the exposure time in each experiment to prevent potential excitotoxic effects on the recorded neurons. In calcium imaging experiments a 30 second exposure was chosen to provide an adequate signal to noise ratio while minimizing exposure times to the excitation laser. This protocol allowed us to repeatedly stimulate the slices with minimal photo-bleaching of the fluorescent indicator dye and without causing photo-toxicity in the tissue. For patch clamp experiments, we increased the exposure time to 90 seconds in order to characterize the maximum increase of induced currents and allow adequate time to characterize miniature and spontaneous synaptic currents. TTX concentration (1µM) was based on previous experiments performed in our lab (Boxwell et al., 2013) and shown to be effective at blocking voltage gated Na$^+$-currents. TTX was perfused for 5 minutes prior to any measurements being taken in order to assure complete blocking of the channels and equilibration throughout the tissue. All drugs were purchased from Tocris Biosciences (Bristol, UK) unless otherwise specified in the text.
For patch clamp experiments, slices were transferred to a custom-made polycarbonate recording chamber attached to a fixed-stage upright microscope (Nikon EF-600), and superfused with warm (32°C) ACSF at a rate of 2ml/min. Slices were held in place with a custom-made gold-wire “harp” strung with elastic nylon strings. The strings of the harp were oriented so that they did not overlay the NST or underlying reticular formation. A bipolar twisted-wire stimulating electrode (formvar-coated, silver wire, 75µm O.D., A-M systems, Sequim, WA) was placed in the cNST and used to deliver short (0.1ms) current pulses to the nucleus. Patch electrodes were pulled from 1.5mm O.D. borosilicate glass (A-M Systems, Carlsborg, WA) on a Narashige P-83 pipette puller (Narashige International LTD. Tokyo, JP) to a nominal resistance of 3-5 MΩ.

Electrophysiologic recordings were performed with an A-M systems 2400 patch amplifier and recorded using a Digidata 1300 and pClamp 9 software (Molecular Devices, Sunnyvale, CA). Signals were sampled at 20kHz and low-pass filtered at 3kHz. Patch pipettes were directed towards retrogradely labeled neurons in the medullary reticular formation at the level of the area postrema. Following the formation of a giga-seal, a brief pulse of suction was applied with a 5ml syringe to rupture the cell membrane and gain electrical access to the cell. The pipette solution consisted of (in mM) 130 K-gluconate, 10 EGTA, 10 HEPES, 1 CaCl₂, 1 MgCl₂, and 2 ATP, at pH 7.2–7.3 and osmolality 290–295 mosmol/kg H₂O. Lucifer yellow (0.1%) was included in the internal pipette solution to mark recorded neurons. Series resistance and cellular capacitance were compensated and monitored throughout the experiment. Cells were included in the study if the input resistance was greater than
100 MΩ and compensated series resistance was less than 40 MΩ. Short paired-pulse (inter-stimulus interval = 50 ms) stimulation was delivered to the cNST with a Grass model S88 stimulator equipped with a stimulus isolation unit. Stimulus current amplitude ranged from 30-150 µA and was determined empirically for each cell by increasing the stimulating current until consistent postsynaptic currents were elicited. Excitatory postsynaptic currents (EPSCs) were recorded in voltage-clamp at a holding potential of -60 to -70 mV. Holding potential was increased to -40 mV in a subset of cells in order to potentiate and record inhibitory postsynaptic currents (IPSCs). Membrane resistance was monitored throughout recordings using norepinephrine and phenylephrine with a short (50 ms) hyperpolarizing (-70mV) pulse. For DMT experiments the membrane resistance was determined in current clamp using injected current steps from -200 nA + 200 nA and plotting an I-V curve from the linear portion of the response. The chloride reversal potential was -91.2 mV. Voltages reported in the text are not corrected for the liquid junction potential, calculated to be -13.9 mV.

Calcium Imaging

Neonatal pups were injected in the hypoglossal nucleus and coronal sections were made as described above. Following a 30-minute recovery at 32°C, slices were incubated in 16 µM Fluo-8AM (AAT Bioquest, Sunnyvale, CA) in a 22 mm petri dish containing approximately 3 mL ACSF continuously bubbled with 95% O₂/5% CO₂ for 15-20 min. Fluo-8 (50 µg) was first dissolved in 40 µL of 10% pluronic F-120 in DMSO, and then further diluted with 200 µL ACSF. The diluted solution was pipetted over the slices with gentle
agitation to ensure complete mixing of the dye and media. Following an incubation time of 15-20 minutes, the slices were rinsed 2x with carboxygenated ACSF at 32°C for 30 minutes each, and then held at room temperature until used for individual experiments. Images were collected on an Olympus fixed-stage upright microscope equipped with an Infinity-3 live-cell confocal imaging system (Visitech, Sunderland, UK). The calcium indicator dye was excited with a 491nm LED laser. Typical exposure time was 110 ms and images were acquired at the maximum frame rate for approximately 2.5 minutes to allow adequate time for fluorescent signals to return to baseline after drug application. Phenylephrine (20 µM) was applied through the perfusion system for 30 seconds and a 5 minute washout period was given between drug applications. Data from an individual cell was excluded if it did not respond with at least a 5% increase in fluorescent signal from baseline values following the first exposure to phenylephrine.

Data and Analysis

For evoked currents, a minimum of 10 sweeps were averaged for each treatment to determine amplitude, latency, and jitter values. Spontaneous- and miniature postsynaptic currents were quantified using MiniAnalysis software (Synaptosoft, Inc., Decatur, GA). Detection thresholds were set at 3x the root mean square of the noise and then manually verified. For cases without TTX in the media, we quantitated currents for 10 sec just prior to the drug entering the chamber and for either 10 sec after the equilibration period (5 min, DMT) or when the drug had reached peak concentration in the bath (phenylephrine) at approximately 2 minutes. TTX substantially reduced the frequency of spontaneous
postsynaptic currents. We therefore quantitated miniature postsynaptic currents over a
period of 60 sec to adequately measure changes in response to each drug condition. Action
potential-independent activity was converted to frequency by dividing the total number of
currents measured per given time, and reported as the average number of currents per
second in the text. In most cases, a one-way ANOVA with Dunnett’s post-hoc tests were
used to determine statistical significance for drug effects that included washout data. We
report the overall ANOVA value. All significant ANOVAs were accompanied by significant
post-hoc tests for drug effects. However, since drug effects were not always reversible, we
specifically indicate instances where washout occurred. Note that because a Dunnett test
compares a treatment to the control value (ACSF baseline), a non-significant effect for the
washout period indicates a return to baseline and is denoted as such in the figures. When
sufficient washout data were not available, we conducted t-tests between baseline and
drug conditions as indicated in the text. For the analysis of spontaneous and miniature
postsynaptic currents, we report both group statistics (ANOVA) and the results of a
Kolmogorov-Smirnov 2 sample test (KS) on individual cells to determine statistical
significance between control and drug conditions. A significance level was set at P<0.05.

For calcium imaging experiments, regions of interest were drawn over individual
cells using Metamorph Imaging software (Molecular Devices, Sunnyvale, CA) and changes
in fluorescent intensity were measured. We calculated the changes in intensity (ΔF/F) by
subtracting the baseline values from the peak change during the drug application and then
dividing by the baseline value. Statistical analysis was performed using a one-way ANOVA
with a Dunnett’s multiple comparison post-hoc test, significance was set at P≤0.05.
Oscillations in fluorescent intensity were manually counted for each condition and compared with a Student's t-test.

**Results**

_Synaptic Connectivity_

Anterograde tracing studies have demonstrated that cNST fibers traverse the medullary reticular formation in which pre-hypoglossal neurons are located (Cunningham and Sawchenko, 1989; 2000) but physiological connectivity has not been well established. Thus, our first experiment focused on demonstrating synaptic connections between the cNST and pre-hypoglossal neurons (Fig. 1). cNST time-locked evoked postsynaptic currents were recorded in 29 neurons, 21 of which were labeled by retrograde tracer injection into the hypoglossal nucleus. No differences in synaptic connectivity or membrane properties were observed between retrogradely traced and untraced neurons. Hence, all data were pooled for statistical analysis. EPSCs recorded in 27 neurons had an average latency of 4.6±0.30 ms, with a range of 2.3 to 9.1 ms (Fig. 1H). This range is similar to those obtained from a previous study where stimulation of the rostral NST (rNST) evoked excitatory currents in pre-hypoglossal neurons further rostral in the medullary reticular formation (Nasse et al., 2008). The jitter of the elicited currents (standard deviation of the latencies) ranged from 0.12 to 2.41 ms (Fig. 1I) and was used to classify mono- vs. polysynaptic responses (Doyle and Andresen, 2001). Previous recordings in the rostral reticular formation study showed a bimodal distribution of jitter with a clear separation at 0.5 ms. When we used this criteria to classify mono- and polysynaptic
responses in the current data set, our results demonstrated that 55% of neurons received polysynaptic input (15 of 27 neurons), and 45% (12 of 27) received monosynaptic input from the cNST. Unlike the rNST, there was no correlation between latency and jitter. In 12 neurons, we blocked ionotropic glutamate AMPA and NMDA receptors with CNQX (10 µM), and/or MK-801 (10 µM) respectively (Fig. 1G&J). No differences in the level of suppression were observed in response to either CNQX alone (n=5) or in combination with MK-801 (n=7); therefore, the data were pooled for statistical analysis. Blocking AMPA and/or NMDA receptors suppressed the evoked excitatory current amplitude by an average of 74% with 8 of the 12 cases showing complete suppression of the evoked current (Fig. 1J: ACSF: 67.2±19.2 pA, CNQX/MK-801: 6.2±3.3 pA, paired t-test, P≤0.01). For six neurons for which washout data was available (four of which showed complete suppression), there was a significant drug effect (ANOVA: P≤0.002) followed by washout. Evoked inhibitory currents were observed in a small number of recorded neurons (5 out of 29). The mean latency of inhibitory currents was 4.9±0.19 ms with a mean jitter of 0.37±0.06 ms (data not shown). Jitter analysis of evoked inhibitory currents showed that 3 of the 5 were monosynaptic and the remaining 2 were polysynaptic using the same criteria as for excitatory currents. Because evoked inhibitory currents were often observed in conjunction with excitatory currents (3/5), obtaining reliable amplitude values was not possible.
The endogenous adrenoreceptor agonist, norepinephrine (20 µM,) was bath-applied while recording from 12 reticular formation neurons. In 3 cases where cNST stimulation evoked time-locked excitatory currents, norepinephrine completely suppressed these responses, and in 2 instances these responses recovered following a 10-minute washout (Fig. 2A-B). In 9 neurons that were not driven, we measured holding current, membrane resistance, and changes in the frequency and amplitude of spontaneous EPSCs (sEPSC) and spontaneous IPSCs (sIPSC). Norepinephrine significantly decreased the holding current (classified as Group 1, Fig. 3A&B) by approximately 35% in 4 cells (ACSF: -42.2±12.9 pA, NE: -27.4±13.5 pA, P≤0.015) followed by washout, and there was a concomitant decrease in membrane resistance (ACSF: 307.3±28.5 MΩ, NE: 268.8±26.4 MΩ, P≤0.003) (Fig. 3B) that also recovered with washout. Although these data are suggestive of a postsynaptic inhibitory effect, this was subsequently not born out using selective adrenoreceptor agonists in the presence of TTX (below).

There were also significant excitatory effects with norepinephrine application. Five cells showed a non-significant increase in holding current (classified as Group 2, Fig. 3C&D) by approximately 26% (ACSF: -40.8±4.48, NE: -51.3±6.0, P=0.11) suggestive of depolarization, and there was a significant increase in the frequency of sEPSCs from 3.9±1.4 EPSC/s to 8.0±1.9 EPSC/s (P≤0.002) (Fig. 3E-F). Although only the neurons with an increase in holding current had a significant increase in the frequency of sEPSCs, both Group 1 and Group 2 neurons had significant increases in sIPSC frequency following norepinephrine (Fig. 3H: P’s≤0.03). When analyzed separately, Group 1 and Group 2
neurons did not show significant changes in sIPSC amplitude (Fig. 3I), however when
neurons from both groups were combined, there was a significant (47%) increase in IPSC
amplitude (ACSF: 13.0±1.1 pA, NE: 19.1±2.8 pA, P≤0.02). Additional statistical testing
showed significant differences in the distribution of IPSC amplitudes after drug infusion in
all cells (KS: P's <0.03). These data suggest that multiple, or different, adrenoreceptors are
involved in the oromotor pathway. Thus, we conducted experiments with selective α-
adrenoreceptor agonists to determine which receptors may contribute to the contrasting
effects observed with norepinephrine and where they may be expressed.

α₂-Adrenoreceptor

Evoked Synaptic Currents

The selective α₂-adrenoreceptor agonist DMT (100 nM) was applied through the
perfusion system while recording from 11 pre-hypoglossal neurons. cNST stimulation-
evoked EPSCs were seen in 9 of the 11 neurons (Fig. 2C-D). When slices were exposed to
DMT, on average, the amplitude of evoked EPSCs was diminished by 53.5±11.15% of
control values (Fig 2E: ACSF: 52.2±17.0 pA, DMT: 19.8±6.2pA), which trended toward, but
did not reach significance (P<0.09, paired t-test). However, individual paired t-tests
showed that DMT induced suppression of the excitatory current in 7 of the 9 cases (P's
≤0.05). In conjunction with a decrease in amplitude, DMT also caused a significant increase
in the failure rate of evoked currents (Fig. 2D&F: DMT: 67.5%, ACSF: 29.9%, P≤0.05, paired
t-test). Even with up to 35 minutes of continuous perfusion with ACSF, we observed
washout in only 2/5 neurons. DMT binds with high affinity to the $\alpha_2$-adrenoreceptor and others have reported only partial washout after even longer (1-2 hrs) time frames (Chiu et al., 1995; Shirasaka et al., 2007).

**Spontaneous Synaptic Currents**

In this same group of neurons, the application of DMT significantly reduced the frequency of sEPSCs by 72%, compared to control values (Fig. 4B: ACSF: 13.4±2.4 EPSCs/s, DMT: 4.1±0.79 EPSCs/s, P=0.0002, n=11) and there was a trend towards a decrease in amplitude (47% decrease, ACSF: 32.0±2.0 pA, DMT: 16.9±0.6 pA, P=0.18). Consistent with this trend, were significant reductions in the cumulative distributions of the amplitudes of the sEPSCs in 8 of the 11 neurons (KS: P’s<0.03). In contrast to sEPSCs, sIPSCs showed no significant changes in either frequency (ACSF: 9.9±4.8 sIPSCs/s, DMT: 8.5±4.4 sIPSCs/sec) or amplitude (ACSF: 21.5±3.5 pA, DMT: 18.6±3.6 pA, n=5, data not shown). This suggests that $\alpha_2$-adrenoreceptors may be expressed selectively on excitatory neurons or terminals, but not on inhibitory neurons.

While the reductions in sEPSC frequency observed with DMT suggest a presynaptic site of action, the reduction in sEPSC amplitude could be due to either pre- or postsynaptic sites. To further disambiguate the site of action, we recorded miniature EPSCs (mEPSC) in the presence of the sodium-channel blocker TTX (1 $\mu$M). With TTX in the media, DMT did not significantly reduce mEPSC amplitude (Fig. 4G: ACSF: 13.6±0.7 pA, DMT: 13.6±0.55 pA, P≥0.52). In addition, when current amplitudes were plotted with a cumulative histogram, no significant changes in mEPSC amplitude distribution were detected (KS: P’s>0.05). If
the site of action was on the postsynaptic cell, than we would expect to observe decreases in the amplitude of mEPSCs when slices are exposed to DMT in the presence of TTX. Unlike sEPSCs, there was no group effect for DMT to reduce the frequency of mEPSCs (Fig. 4F, P>0.05), although 2/5 neurons did show significantly reduced frequencies (KS: P’s<0.001). Membrane resistance and holding current did not vary with DMT in either experimental paradigm (TTX or no TTX). These results strongly support the assertion that the site of action for DMT induced changes in sEPSCs is presynaptic. Hence, the tendency for a reduction in sEPSC amplitudes caused by DMT in ACSF is likely due to the inhibition of spontaneous action potentials in presynaptic neurons, and not to postsynaptic activation of the receptor.

α₁-Adrenoreceptor

Prolonged application of the α₁-adrenoreceptor agonist phenylephrine increased the holding current to levels where we were unable to maintain adequate space clamp without damaging the postsynaptic neuron even at concentrations as low as 5 µM. This is perhaps not surprising given that pre-hypoglossal neurons can have very long (>1000µm) dendrites with multiple branches (Nasse et al., 2008). As such, we were unable to reach an adequate equilibration state that would allow us characterize the effects of α₁-adrenoreceptor agonists on evoked synaptic currents. However, at the beginning of the equilibration period before the holding current became unstable, we observed a dramatic increase in sEPSC frequency coincident with increases in holding current. This led us to hypothesize that presynaptic release of glutamate was acting in conjunction with
postsynaptic activation of the $\alpha_1$-adrenoreceptor leading to a dual excitatory effect. To test this hypothesis, we initially performed calcium imaging studies with ionotropic glutamate receptor antagonists in the media. Subsequently, we conducted a series of patch clamp experiments in which we used shorter applications of phenylephrine both with and without TTX to evaluate the pre- and postsynaptic effects.

**Calcium Imaging**

To screen the interaction of PE and glutamate across a large population of reticular formation neurons, we used live-cell confocal calcium-imaging to record changes in intracellular calcium dynamics in response to short (30s) exposures of phenylephrine (20 $\mu$M) with and without ionotropic glutamate antagonists. A total of 11 pups were used to image 245 cells, from 26 individual slices. Although we attempted to label pre-hypoglossal neurons with a retrograde marker, this was not very successful and there were few such neurons that took up the calcium indicator.

The application of phenylephrine in ACSF resulted in a dramatic increase in fluorescent intensity values ([Fig. 5A]: ACSF PE1: 20.1±1.3, $\Delta F/F$, n=193). To assess the contribution of presynaptic glutamate release to this increase in Ca$^{2+}$, we blocked ionotropic glutamate receptors with DNQX, (AMPA) (10$\mu$M) and MK-801 (NMDA) (10$\mu$M) following the first exposure to phenylephrine and observed a significant 34% decrease in fluorescent intensity levels compared to the first phenylephrine exposure ([Fig. 5C]: PE2 GluR Block: 13.4±1.2, $\Delta F/F$, 126 cells, paired t-test: $P$.≤0.05). The attenuation in the response to phenylephrine in the presence of glutamate antagonists was greater than the
non-significant 20% attenuation to a second application of phenylephrine alone (ACSF PE2; paired t-test: P=0.17). To verify that glutamate contributes to changes in intracellular calcium and that the decrease in the phenylephrine response was not merely due to receptor desensitization or run-down of the Ca\(^{2+}\) response, DNQX/MK-801 was added to the bath prior to the first exposure of phenylephrine and again, the increase in fluorescent intensity evoked by the \(\alpha_1\) agonist was significantly smaller (31%) than that observed in ACSF (Fig. 5C: GluR Block PE1: 14.2±1.2, P≤0.002, 52 cells). Thus, phenylephrine applied in the presence of ionotropic glutamatergic blockade still elicits an increase in intracellular calcium, but less so than when phenylephrine is applied alone. The blockade of ionotropic glutamate receptors revealed an additional interesting phenomena in reticular formation neurons. When AMPA and NMDA receptors were blocked prior to the first phenylephrine exposure, we observed significantly more oscillations in fluorescent intensity during drug exposure compared to when phenylephrine was delivered in normal ACSF (Fig. 5D: number of peaks ACSF: 3.5±0.21, PE: 7.9±0.72, two-sample t-test: P≤0.001). These data suggest that glutamate released from presynaptic sources contributes to the excitatory response elicited by \(\alpha_1\)-adreoreceptor agonists, and that there are multiple sites of action within the oromotor circuit where phenylephrine can cause changes in neuronal physiology.

**Patch Clamp**

Because the Ca\(^{2+}\) imaging experiments included few pre-hypoglossal neurons, similar experiments were conducted in patch-clamp to further differentiate pre- and
postsynaptic effects elicited by phenylephrine, and to explore the PE-glutamate interaction in this defined population of cells. First, we blocked action potential-dependent synaptic activity by including TTX in the media and limited the time of phenylephrine application. With TTX in the media, a 90-sec application of phenylephrine (20 µM) produced a significant increase in holding current across all cells tested (n=6, [Fig. 6A&B]). The average increase in holding current was 41.2±3.7 pA (P≤0.0001). We also quantified the frequency and amplitude of mEPSCs in response to phenylephrine in the presence of TTX. Compared to ACSF, there was a significant increase in the frequency of mEPSCs with phenylephrine ([Fig. 6D&F]: ACSF: 5.9±1.2, PE: 9.9±1.4, mEPSCs/s, P≤0.003), an effect reversed during washout. In addition, there was a small but significant increase in the mean amplitude of mEPSCs ([Fig. 6E&G]: ACSF: 12.6±0.78 pA, PE: 13.5±0.92 pA, P≤0.02), and a significant rightward shift in the cumulative histogram in 5 of 6 neurons (KS: P's≤0.019). This increase in mEPSC amplitude, along with the increase in holding current, suggests that α1-adrenoreceptors are housed on the postsynaptic neuron. Furthermore, since we also observed an increase in the mEPSC frequency it is likely that α1-adrenoreceptors are also located on presynaptic neurons.

In a small number of cells (n=3) we measured the response to phenylephrine in the absence of TTX and observed a significant increase in either or both frequency and amplitude of sEPSCs (KS: P's<0.02), similar to that observed under TTX. In addition, phenylephrine increased sIPSCs frequency in all 3 neurons (KS: P's<0.005) and a change in amplitude in 1 (KS:P<0.0001), an effect not observed with TTX in the media. This increase in both the frequency and amplitude of spontaneous postsynaptic currents is again consistent with phenylephrine acting at both pre- and postsynaptic sites. However, in
contrast to the TTX data where we observed an increase in holding current in every instance, a short exposure of phenylephrine produced no significant change in holding current without TTX (data not shown) and one cell actually presented with a reduction in holding current of -34.1 pA similar to what we observed in Group 1 cells with norepinephrine application. This suggests that action potential driven IPSCs can mitigate the depolarizing current induced by postsynaptic $\alpha_1$-adrenoreceptor activation and lead to an overall inhibitory effect in some cells. To provide further evidence that the $\alpha_1$-adrenoreceptor is expressed directly on presynaptic inhibitory neurons, we blocked ionotropic glutamate receptors with DNQX and MK-801 (10µM) prior to phenylephrine. After blocking ionotropic glutamate receptors, phenylephrine exposure increased the frequency of spontaneous IPSCs by 314% (Fig. 6J: DNQX/MK-801: 3.4±0.87 IPSCs/s, PE DNQX/MK-801: 12.4±3.3 IPSCs/s, P≤0.006), an effect reversed during washout. Although there was no group effect on sIPSC amplitude (Fig. 6K: DNQX/MK-801: 14.7±2.4 pA, PE DNQX/MK-801: 24.5±6.5 pA), individual tests were highly significant in all cells (KS: P's<0.0001). These data indicate that increases in sIPSCs induced by phenylephrine are not due to a network effect involving glutamate, and suggest that the $\alpha_1$-adrenoreceptor is expressed directly on presynaptic inhibitory neurons.

With TTX in the media the mean change in holding current with phenylephrine application was 41.2 pA. When MK-801/DNQX was added to the media, the mean change in holding current induced by PE (-16.2 pA) was significantly less than the change induced in TTX alone (unpaired t-test, P=0.037, n=6 TTX, n=5 non-TTX). This suggests that network inhibitory currents counteract the inward current in response to phenylephrine.
This study demonstrates that the cNST, the main sensory nucleus receiving signals from the viscera, sends both inhibitory and excitatory connections to pre-oromotor neurons in the caudal reticular formation. The evoked currents demonstrated properties consistent with both mono- and polysynaptic connectivity, similar to the results obtained by stimulating the rostral (gustatory) NST (Nasse et al., 2008; Chen et al., 2012). This suggests a common blueprint through which oral and visceral signals impact oromotor neurons associated with brainstem-mediated consummatory responses.

Both pre- and postsynaptic properties of pre-hypoglossal neurons were modulated by adrenoreceptor agonists. Norepinephrine suppressed cNST driven EPSCs in pre-hypoglossal neurons, an effect duplicated with the \( \alpha_2 \)-adrenoreceptor agonist DMT. Changes in the holding current in response to norepinephrine could be accounted for by a combination of both pre- and postsynaptic effects, further delineated by the use of selective adrenoreceptor agonists. The inward current (depolarization) produced by norepinephrine was likely caused by \( \alpha_1 \)-adrenoreceptor activation via postsynaptic mechanisms and through increases in EPSCs via presynaptic (network) pathways. The reduction in holding current (inhibition) seen with norepinephrine was not likely induced by a postsynaptic \( \alpha_2 \)-adrenoreceptor mechanism, but more likely by \( \alpha_1 \)-adrenoreceptor activation of inhibitory presynaptic neurons from an as yet unknown location.
Pre-hypoglossal neurons, especially those receiving input from the cNST provide a pathway for the visceral modulation of consummatory oromotor function. These neurons, however, are likely involved in other functions of the oral cavity, e.g. respiratory entrainment of the tongue. Pre- and postsynaptic modulation of these neurons by multiple classes of adrenoreceptors provides a substrate for the multifunctional role these neurons play in homeostatic regulation.

Technical Considerations

The medullary reticular formation becomes heavily myelinated after postnatal day 12 and renders it impossible to visualize individual neurons with infrared differential interference optics used for slice preparations. As such, P7-12 pups were used for the experiments in this study. Significant developmental changes do occur over this time period. Nevertheless, neonates in this age range respond in a similar manner as adults with regard to both satiety behavior (Smith et al., 1991) and oromotor pattern generation (Ganchrow et al., 1986; Nasse and Travers, 2006). While it is true that neonatal animals can suckle and breath simultaneously, experimental data from an in vitro brainstem block preparation in young (P0-P3) animals, demonstrated that swallowing-like activity briefly inhibits respiratory activity in C3 nerve rootlets (Kogo et al., 2002), i.e. respiration and the consummatory response of suckling interact at the brainstem level. Therefore, while the use of young animals entails limitations, this preparation has the important advantage of allowing identified single-cell physiologic experiments in a key brain region that are not possible using adult animals.
\( \alpha_2 \)-Adrenoreceptors

\( \alpha_2 \)-adrenoreceptor stimulation provided direct evidence for noradrenergic modulation of cNST input onto pre-hypoglossal neurons by decreasing the amplitude of cNST evoked EPSCs. Because cNST evoked synaptic currents were blocked with ionotropic glutamate receptor antagonists, and there were no changes in membrane resistance, holding current, or mEPSC amplitude in response to DMT with TTX pre-treatment, it is likely that \( \alpha_2 \)-adrenoreceptors are expressed on the presynaptic terminals or cell bodies of glutamatergic neurons, but not on the pre-oromotor neurons themselves. Consistent with this, \( \alpha_2 \)-adrenoreceptor mRNA expression in the (presynaptic) cNST is denser than in the subjacent reticular formation (Rosin et al., 1993). In contrast to the effects on sEPSCs, \( \alpha_2 \)-adrenoreceptor agonists affected neither the frequency nor amplitude of sIPSCs suggesting that \( \alpha_2 \)-adrenoreceptors are not expressed on presynaptic inhibitory neurons projecting to pre-hypoglossal neurons (at least those within the confines of the slice).

The present results are similar to previous studies focused on other components of the medullary circuits of the gastrointestinal and feeding systems, where \( \alpha_2 \)-adrenoreceptor activation was observed to suppress the activity of both cNST and dorsal motor nucleus of the vagus neurons \textit{in vitro} and \textit{in vivo} (Moore and Guyenet, 1983; Martinez-Peña y Valenzuela et al., 2004). Furthermore, studies in many different brain regions show that \( \alpha_2 \)-adrenoreceptor activation attenuates EPSC amplitude (Bertolino et al., 1997; Hayar and Guyenet, 1999; Jiménez-Rivera et al., 2012). The \( \alpha_2 \)-adrenoreceptor is coupled to \( G_{i/o} \) intracellular signaling pathways and its activation can inhibit both neuronal
activity and neurotransmitter release from synaptic terminals. For example, when the G-protein subunits are dissociated upon receptor binding, the \( \beta\gamma \)-subunit activates G-protein activated inwardly-rectifying potassium currents (GIRKs) and increases outward potassium conductance leading to hyperpolarization of the neuronal membrane potential (Hein, 2006). In fact, based on the concordant decrease in holding current and membrane resistance seen in Group 1 neurons following norepinephrine application, we predicted such an \( \alpha_2 \)-adrenoreceptor-mediated effect would be observed following stimulation with DMT in the presence of TTX. This was not the case, however, suggesting that the decrease in holding current observed with norepinephrine had a different origin, e.g. increases of inhibitory (likely GABA or glycine) input to pre-hypoglossal neurons mediated by \( \alpha_1 \). Adrenoreceptor activation (read below). In addition to membrane potential hyperpolarization, inhibition of transmitter release can also be attributed to \( \alpha_2 \)-adrenoreceptor activation through dissociation of the \( \beta\gamma \)-subunit. In this case the \( \beta\gamma \)-subunit binds directly to N- and P/Q-type calcium channels in a voltage dependent manner and reduces channel conductance. This results in lower intra-terminal calcium concentration and can inhibit neurotransmitter release (Bean, 1989; Hille, 1994; Zamponi and Snutch, 1998; Tedford and Zamponi, 2006). Indeed, this may be the mechanism for the reduction in evoked-response amplitude and increase in failure rate we observed with DMT application in patch clamp experiments.
Prolonged stimulation with the $\alpha_1$-adrenoreceptor agonist phenylephrine caused a large increase in holding current prior to reaching an equilibration point that precluded determining if this agonist modulated cNST evoked currents in pre-hypoglossal neurons. Nevertheless, we did observe postsynaptic changes in the presence of TTX with short (90s) applications of the drug. Furthermore, we also observed presynaptic modulation of spontaneous and miniature postsynaptic currents, evidenced by increases in the frequency of these currents. In the presence of TTX, phenylephrine consistently produced an inward current similar to what was observed in a subset of neurons stimulated with norepinephrine (in the absence of TTX). Although this indicates depolarization in response to $\alpha_1$-adrenoreceptor activation, we did not see a simultaneous increase in membrane resistance as would be expected if this inward current was mediated by inhibition of two-pore potassium channels via the PLC/DAG intracellular signaling pathway (Talley et al., 2000). A lack of change in the membrane resistance might be explained by the simultaneous activation and inactivation of two different channels following $\alpha_1$-adrenoreceptor stimulation. Yamanaka et al. (Yamanaka et al., 2006) demonstrated that $\alpha_1$-adrenoreceptor activation induced an inward current in hypothalamic orexin neurons through the opening of a non-selective cation channel, potentially a member of the canonical transient receptor potential (TRPC) family, which should decrease membrane resistance. On the other hand, the same PLC/DAG signaling pathway that opens TRPC channels also inhibits GIRK and two-pore potassium channels (reviewed in (Albert, 2011)) which would increase the
membrane resistance. As such, it is possible that the inward current seen with
phenylephrine is the result of the simultaneous activation of TRPC channels and the
inhibition of GIRK channels with no net change in membrane resistance.

In addition to the increased holding current observed with phenylephrine in TTX,
we also observed a significant increase in both the frequency and amplitude of excitatory
and inhibitory spontaneous postsynaptic currents in ACSF. This suggests that $\alpha_1$-
adrenoreceptors are expressed directly on pre-hypoglossal neurons and on the cell bodies
or terminals of neurons that synapse on to them. Since our patch clamp studies
demonstrated that electrical stimulation of the cNST produced excitatory glutamatergic
currents in pre-hypoglossal neurons, it is presumed that increased cNST activity in
response to phenylephrine would also increase glutamatergic excitation in pre-hypoglossal
neurons. Indeed, (Hermann et al., 2005), demonstrated that phenylephrine causes
oscillations in intracellular calcium and increased activity in cNST neurons that respond to
gastric stretch. In our studies, the $\alpha_1$-adrenoreceptor agonist phenylephrine produced a
robust increase in intracellular calcium signal which was attenuated by blocking ionotropic
 glutamate receptors. Although we could not identify these reticular formation neurons as
pre-hypoglossal, they were located in the same region as the identified pre-hypoglossal
cells, and thus suggest that presynaptic glutamate release contributes to the increased
intracellular calcium signal produced by $\alpha_1$-adrenoreceptor activation.

Very few inhibitory currents were observed when slices were pretreated with TTX
in any paradigm tested in this study. However, both norepinephrine and phenylephrine
significantly increased the frequency and amplitude of spontaneous inhibitory currents
when TTX was not included. This may indicate that spontaneous inhibitory currents
require action potentials in spontaneously active inhibitory neurons. This result is consistent with respiratory and cardiovascular circuits where presynaptic GABA and glycine currents can influence autonomic output (O'Brien and Berger, 1999; O'Brien et al., 2004; Boychuk et al., 2011). Inhibitory currents recorded from cardiac vagal neurons located in the nucleus ambiguous were potentiated in a similar fashion as our pre-hypoglossal neurons when exposed to norepinephrine or phenylephrine (Boychuk et al., 2011); that is, the increase in spontaneous inhibitory currents was dependent on action potentials since the potentiation was not observed in the presence of TTX.

β-adrenoreceptors are also expressed in low to moderate levels in the region where we recorded (Wanaka et al., 1989). Therefore, it is possible that some of the effects observed with norepinephrine and attributed to α₁-adrenoreceptors could also be mediated by the β-adrenoreceptor, which we did not test. For example, β-adrenoreceptor activation can inhibit K⁺ channels leading to postsynaptic depolarization (Madison and Nicoll, 1986a; 1986b), and thus could also lead to an increase in the frequency of excitatory and inhibitory postsynaptic currents via presynaptic network activity (Bateman et al., 2012).

Functional considerations

The main findings of this study indicate that norepinephrine activates multiple adrenoreceptor subtypes both pre- and postsynaptic to pre-hypoglossal neurons and exerts a host of physiologic changes in reticular neurons. These complex effects are consistent with the complex and multifunctional nature of this region of the reticular...
Adrenoreceptor Modulation

formation as well as the known involvement of norepinephrine with a wide variety of functions. We propose that the excitatory and inhibitory effects of norepinephrine on these reticular neurons may represent two arms of autonomic regulation (Fig. 7). Suppression of excitatory currents from the cNST through $\alpha_2$-adrenoreceptor mechanisms may support a role for inhibiting sensory stimuli from propagating through the system during the satiety phase of ingestion, or in times of high sympathetic activation. Here, we posit that an excitatory viscero-oromotor pathway from the cNST to (excitatory) pre-hypoglossal neurons is suppressed by norepinephrine acting presynaptically via $\alpha_2$-adrenoreceptors.

For instance suppression of afferent signals from the oral cavity that activate oromotor and swallowing central pattern generators could be suppressed following a meal. Indeed it has been shown that both locally infused norepinephrine and leptin, which are released during satiety, can inhibit reflexive swallowing induced by stimulation of the superior laryngeal nerve (Kessler and Jean, 1986b; Félix et al., 2006). Furthermore, the swallow central pattern generator is located in the cNST and acts to both recruit oropharyngeal muscles to perform a swallow and inhibit respiration (reviewed in (Jean, 2001; Lang, 2009). In addition, the suppression of excitatory currents in pre-hypoglossal neurons elicited from the rostral (taste) NST are also attenuated by norepinephrine (Chen et al., 2012), suggesting that taste and visceral NST afferents modulate pre-hypoglossal neurons through similar pathways. The origin of the norepinephrine could potentially be A2 neurons located in the cNST that show enhanced activity during satiety (Rinaman et al., 1998; Rinaman, 2003; Wellman, 2005; Appleyard et al., 2007).

While it may be advantageous to suppress chemosensory inputs from the tongue during satiety to inhibit consummatory behavior, respiratory-related signals need access to
the tongue regardless of metabolic state. Here, we posit a role for $\alpha_1$-adrenoreceptors influencing GABAergic pre-hypoglossal neurons via a second GABAergic interneuron (Fig. 7). GABAergic neurons are well represented in the reticular formation lateral to the hypoglossal nucleus in the region we recorded (Li et al., 1997; Travers et al., 2005), and electrical stimulation of this area can elicit inhibitory currents in hypoglossal motor neurons (O’Brien and Berger, 1999; O’Brien et al., 2004). While the infusion of GABA receptor agonists into the hypoglossal nucleus reduces inspiratory-phase related hypoglossal nerve activity (Okabe et al., 1994), the infusion of GABA$_A$ receptor agonists into the reticular area subjacent to the cNST in the region from which we recorded, results in increased activity of the hypoglossal motor nucleus. This suggests a mechanism of disinhibition for potentiating hypoglossal inspiratory-phase activity (Chamberlin et al., 2007). Thus, simultaneous with norepinephrine suppressing a viscero-oromotor circuit related to metabolic state via $\alpha_2$-adrenoreceptors on excitatory projections to pre-hypoglossal neurons, norepinephrine could also be disinhibiting lingual motorneurons via $\alpha_1$-adrenoreceptor excitation of GABAergic interneurons projecting to GABAergic pre-hypoglossal neurons.

While we did not test the phenotypes of neurons recorded in this study, it is possible that a proportion of the cells are GABAergic, and the increased inhibitory drive we observed is evidence for an $\alpha_1$-adreneroreceptor mediated disinhibition of hypoglossal activity. Future studies could test this hypothesis using a transgenic mouse line currently available that expresses a variant of yellow-fluorescent protein (Venus) in inhibitory neurons (Wang et al., 2009). While the exact details of mechanisms and the overall neurophysiologic circuit remain to be determined, this study provides evidence for
noradrenergic modulation of a multifunctional substrate that could support both respiratory and oral consummatory behavior.
Acknowledgements

We thank Alexandra Toole for excellent technical assistance, and Dr. Susan Travers for invaluable insights and critical reading of the manuscript. Calcium imaging experiments were performed using the instruments and services at the Campus Microscopy and Imaging Facility at The Ohio State University.

Grants

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

J.S.N. and J.B.T. design of research; J.S.N. performed experiments; J.S.N and J.B.T interpreted results, analyzed data, and prepared the manuscript. J.S.N and J.B.T approved the final version of the manuscript.
**Figure Captions**

Fig. 1. A. Low magnification photomicrograph of brainstem slice indicating location of the stimulating electrode and recording pipette. Reticular formation neurons were recorded from the area subjacent to the cNST. B. IR-DIC image of recorded cell. C. Fluorescent photomicrograph of recorded cell (green) showing label from the retrograde tracer (red). D. Summary diagram of recorded neurons showing the location of a subset of neurons recorded in this study. On the left are neurons not driven by cNST stimulation, on the right are neurons driven by cNST stimulation. Stimulation sites are shown in the cNST (yellow). Representative examples of monosynaptic (E) and polysynaptic (F) excitatory currents evoked by electrical stimulation of the cNST. G. Electrical stimulation of a cNST-evoked short latency EPSCs (black trace) in a pre-hypoglossal neuron that was blocked with ionotropic glutamate receptor antagonists (CNQX/MK-801, 10 µM) (red trace) showed some recovery with washout (blue trace). H. Evoked responses demonstrated short latencies ranging from 2-9ms. I. The standard deviation of evoked current onset (jitter) indicates the majority (15 of 27) of the EPSCs were polysynaptic. Jitter values >0.5ms were classified as polysynaptic based on criteria from a previous study in the rostral reticular formation (Nasse, et. al., 2008). J. Excitatory currents were significantly suppressed by a mean of 74% (P≤0.01, paired t-test, n=12) following CNQX/MK-801 with 8/12 neurons completely suppressed. Abbreviations: AP; area postrema, cNST: caudal nucleus of the solitary tract, RF: medullary reticular formation, mXII: hypoglossal motor nucleus.
Fig. 2. A. Example of a neuron in which excitatory currents elicited by electrical stimulation of the cNST (black trace) were inhibited by 20 µM norepinephrine (red trace). B. In the same neuron following washout (black trace) application of the ionotropic glutamate receptor antagonist CNQX blocked the excitatory current (green trace). C. cNST-evoked EPSCs (black trace) were attenuated by bath application of DMT (red trace). D. Representative traces showing failures to evoke an excitatory postsynaptic current (red arrowheads). E. With each neuron serving as its own control, there was a significant decrease following DMT in 7/9 neurons ($P'\leq0.05$) for an overall reduction of 53.5%. F. DMT significantly increased the failure rate of evoked currents by 37.6% ($P\leq0.05$, paired t-test).

Fig. 3. A. Representative decrease in holding current in response to norepinephrine. The apparent high noise level is actually short hyperpolarizing currents injected to measure membrane resistance and is not noise per se, a moving average of the holding current is indicted in white. B. These Group 1 neurons ($n=4$) showed a significant 35% decrease ($P\leq0.015$) in mean holding current compared to baseline (ACSF) and a significant decrease ($P\leq0.003$) in membrane resistance. During the washout period, neither of these measures were significantly different from the pre-drug ACSF period indicating a reversal of the drug effect. C. Other neurons showed an increase in holding current in response to norepinephrine. D. These Group 2 neurons ($n=5$) had a non-significant ($P=0.11$) 26% increase in holding current compared to baseline and no change in the membrane resistance. E. Representative traces exhibiting an increase in both sEPSCs (arrows) and sIPSCs (arrowheads) in response to norepinephrine (lower trace) compared to ACSF.
(upper trace). F. Norepinephrine significantly increased the frequency of spontaneous EPSCs in Group 2 cells (those showing an increase holding current, $P \leq 0.002$), but not in Group 1 cells (those showing a decrease in holding current). G. The amplitude of EPSCs was not changed in either group. H. The frequency of IPCSs was significantly increased by norepinephrine in both groups (Group 1: $P \leq 0.03$; Group 2: $P < 0.03$) but significant changes in amplitude (I.) were only observed when the data from both groups were combined ($P \leq 0.02$).

Fig. 4. A. Representative traces in normal ACSF (upper trace) and after 5 min of DMT (lower trace) showing suppression of spontaneous excitatory postsynaptic currents (sEPSCs). B. DMT (100 nM) significantly decreased the frequency of sEPSCs by 72% of control values ($P = 0.0002$, $n = 11$) and there was no washout effect, i.e. the frequency was significantly different compared to baseline (ACSF). C. DMT also reduced the amplitude of sEPSCs however the reduction did not reach significance ($P = 0.18$). D. Representative trace of mEPSCs after TTX (1 µM) pretreatment (top) and in the presence of DMT (bottom). E. Cumulative histogram of mEPSCs from the same case showing no change in the slope, indicating that the distribution of mEPSC amplitudes is the same in all treatment groups. F. In the presence of TTX the 26% reduction in mEPSC frequency to DMT was not significant ($n = 5$) and the mEPSC amplitude (G) remained constant.

Fig. 5. A. Representative traces of responses to bath-applied phenylephrine (20 µM) showing increase in the fluorescent intensity (green trace) that is attenuated following a second application of phenylephrine combined with NMDA and AMPA receptor antagonists.
(DNQX & MK-801: red trace). B. When glutamate receptors are blocked prior to the first exposure of phenylephrine (blue trace) there was a reduced response to phenylephrine compared to the first exposure of phenylephrine in ASCF (green trace). C. Mean Ca\textsuperscript{2+} responses to phenylephrine applied in the presence or absence of ionotropic glutamate receptor blockade. An initial application of phenylephrine alone (ACSF PE1) evoked a large response. A subsequent application of PE evoked only a nominally smaller response (ACSF PE2: P=0.17, n=67 cells) but when the 2\textsuperscript{nd} application of phenylephrine occurred in the presence of glutamate blockade, the reduction was larger and significant (PE2 GluR block: P≤0.05, n=126 cells). In a second set of experiments, an initial application of phenylephrine in the presence of glutamate blockade elicited a significantly smaller response than the initial phenylephrine response observed in the first experiment (GluR block PE1, P≤0.002, n=52), and a 2\textsuperscript{nd} application of phenylephrine under the same conditions elicited only a nominally smaller response. D. Blocking ionotropic prior to the first phenylephrine application significantly increases the oscillations of intracellular calcium (P≤0.001).

Abbreviations: U = arbitrary fluorescent intensity units.

Fig. 6. A. Representative trace showing an increase in holding current in response to phenylephrine in the presence of TTX. B. When the cells are held at -60 mV, phenylephrine significantly increased the holding current by approximately 84\% (P≤0.0001, n=6). A second exposure of phenylephrine after a 5-minute recovery (PE2) reached a similar value (P≤0.001). C. No statistically significant changes in the membrane resistance were observed. D. Representative trace of mEPSCs with TTX in the media. When phenylephrine is perfused through the chamber, both the frequency and amplitude of mEPSCs is increased
(lower trace) compared to TTX alone (upper trace). E. Cumulative histogram from the
same cell in A. Phenylephrine produced a significant rightward shift indicating an increase
in the frequency of higher amplitude mEPSCs (KS: P≤0.02). F. There was a significant
increase in mEPSC frequency (P≤0.003) and amplitude (G) (P≤0.02) in the presence of
phenylephrine, which returned to baseline following washout. H. Representative trace
showing an increase in spontaneous inhibitory postsynaptic currents (sIPSCs) in the
presence of glutamate antagonists (DNQX and MK-801) following phenylephrine exposure
(lower trace) compared to control (upper trace). I. Cumulative histogram of sIPSC
amplitudes from the same cell in H (KS: P≤ 0.0001). J. Phenylephrine significantly
increased the frequency of sIPSCs by 314% (P≤0.006, n=5). K. The mean amplitude of
sIPSCs was not significantly increased following phenylephrine exposure (P≤0.112), but
individual tests for each cell were highly significant (KS: P’s≤.0001).

Fig. 7. Schematic representation hypothesized integrative circuits that inhibit orosensory
pathways and allow respiratory input to entrain the hypoglossal motor nucleus. On the
left, glutamatergic sensory input from the NST is inhibited by pre-synaptic activation of α2-
adrenoreceptors and prevents information from propagating through the circuit by
inhibiting glutamate release onto glutamatergic pre-oromotor neurons. Additionally,
GABAergic interneurons are activated through an α1-adrenoreceptor mechanism to further
inhibit pre-oromotor neurons that govern ingestive oromotor activity. Respiratory related
neurons from other medullary regions, such as the pre-Bötzinger complex, could still either
entrain the pre-oromotor neuron, or the hypoglossal motor neurons themselves, to
maintain airway patency. On the right, a disinhibitory circuit that withdraws tonic
inhibition from the hypoglossal neuron is diagrammed. Here, activation of $\alpha_2$-
adrenoreceptors inhibit excitatory currents from the NST that project to a GABAergic pre-
oromotor neuron. However, this GABAergic pre-oromotor neuron is inhibited through $\alpha_1$-
adrenoreceptor mediated activation of a GABAergic interneuron leading to the
disinhibition of the hypoglossal neuron. This would then allow direct projections from the
pre-Bötzinger complex to entrain the hypoglossal motor neuron directly, and entrain it to
inspiratory activity.


O’Brien JA, Sebe JY, Berger AJ. GABA(B) modulation of GABA(A) and glycine receptor-


Shirasaka T, Kannan H, Takasaki M. Activation of a G protein-coupled inwardly rectifying K+ current and suppression of Ih contribute to dexmedetomidine-induced inhibition of rat


Figure 2

A. ACSF Norepinephrine

B. NE Washout CNQX

C. Dimensions: 612.0x792.0

D. Evoked EPSC Amplitude

E. Evoked EPSC Failure Rate

F. ACSF DMT Wash
Figure 3

A. 

![Graph showing IPSC Amplitude over time with 50 pA and 1 min. NE 20μM](image)

B. 

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<td>ACSF NE Wash</td>
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<td>Group 1</td>
<td>Group 2</td>
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C. 

![Graph showing IPSC Amplitude over time with 50 pA and 1 min. NE 20μM](image)

D. 

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E. 

![Graph showing EPSC Amplitude over time with 60 pA and 100 ms](image)

F. 

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<td>ACSF NE Wash</td>
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Figure 4.

A. ACSF

B. sEPSC Frequency

C. sEPSC Amplitude

D. ACSF

E. Cumulative Fraction

F. mEPSC Frequency

G. mEPSC Amplitude
Figure 5

A.

B.

C.

D.
Figure 6

A.  

B.  

C.  

D.  

E.  

F.  

G.  

H.  

I.  

J.  

K.
Figure 7. Sensory Inhibitory Circuit

Disinhibitory Circuit

α₂-AR
Activation NE/DMT

α₁-AR
Excitatory Pre-oromotor Neuron

GABA Neuron

α₂-AR
Activation NE/DMT

α₁-AR
Excitatory Pre-botzinger Neuron

GABA Neuron

AMAP/NMDA Receptor
Action Potentials

GABA-A Receptor
Excitatory Pre-botzinger Neuron

Sensory Inhibitory Circuit:

Disinhibitory Circuit: