Physiological and anatomical properties of intramedullary projection neurons in rat rostral nucleus of the solitary tract

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Corson JA, Bradley RM. Physiological and anatomical properties of intramedullary projection neurons in rat rostral nucleus of the solitary tract. J Neurophysiol 110: 1130–1143, 2013.—The rostral nucleus of the solitary tract (rNTS), the first-order relay of gustatory information, not only transmits sensory information to more rostral brain areas but also connects to various brain stem sites responsible for orofacial reflex activities. While much is known regarding ascending projections to the parabrachial nucleus, intramedullary projections to the reticular formation (which regulate oromotor reflexive behaviors) remain relatively unstudied. The present study examined the intrinsic firing properties of these neurons as well as their morphological properties and synaptic connectivity with primary sensory afferents. Using in vitro whole cell patch-clamp recording, we found that intramedullary projection neurons respond to depolarizing current injection with either tonic or bursting action potential trains and subsets of these projection neurons tested received monosynaptic innervation from primary afferents, while the rest received polysynaptic innervation, indicating that at least a subpopulation of these neurons can be directly activated by incoming sensory information. Neuron morphological reconstructions revealed that many of these neurons possessed numerous dendritic spines and that neurons receiving monosynaptic primary afferent input have a greater spine density than those receiving polysynaptic primary afferent input. These results reveal that intramedullary projection neurons represent a heterogeneous class of rNTS neurons and, through both intrinsic voltage-gated ion channels and local circuit interactions, transform incoming gustatory information into signals governing oromotor reflexive behaviors.

taste; patch clamp; primary afferent

GUSTATORY INFORMATION is transmitted from taste receptors in the oral cavity to the rostral nucleus of the solitary tract (rNTS) via the seventh and ninth cranial nerves (King 2007). This information is then either relayed via an ascending pathway to the parabrachial nucleus or via a descending, intramedullary pathway to brain stem oromotor nuclei controlling salivary secretion and orofacial reflex motor activity (Halsell et al. 1996; Norgren 1978; and Norgren and Leonard 1973). The ascending pathway has been the focus of nearly all the investigations of central taste coding, mostly using extracellular recording techniques. Unfortunately, aside from a few notable exceptions (Monroe and Di Lorenzo 1995), there is no information to confirm that these neurons relay taste information along the ascending pathway. It is likely that a subset of these first-order neurons in the taste pathway project to the brain stem taste reflex system, and thus have very little to do with the perception of taste quality.

The brain stem taste reflex system regulates ingestive and digestive behaviors, secretion of saliva, and tongue and facial muscle control. Despite this, very little is known regarding the intra-rNTS circuitry responsible for these reflexive behaviors. Recently it has been demonstrated that rNTS neurons directly innervate preoromotor neurons in the intermediate reticular formation and generate both excitatory and inhibitory responses (Nasse et al. 2008). Unfortunately, this is the extent of our current knowledge regarding rNTS influences on the reticular formation. Since ascending and intramedullary projections arise from distinct populations of neurons (Halsell et al. 1996) that regulate different types of gustatory-evoked behaviors, we hypothesize that intramedullary neurons possess characteristics (e.g., biophysical or morphological) unique to this subset of rNTS neurons. However, intracellular recordings to determine these basic physiological and morphological characteristics have never been attempted on intramedullary rNTS projection neurons. Although a number of rNTS neuron populations have been classified by morphological (Renehan et al. 1994; Whitehead et al. 1993) and physiological (Bradley and Sweaze 1992; King and Bradley 1994; Renehan et al. 1996) criteria, the axonal target of these classes of neurons remains unknown. Recently, ascending projection neurons (Suwabe and Bradley 2009) and a subset of GABAergic interneurons (Wang and Bradley 2010a) have been characterized, revealing that these two populations possess distinct repetitive firing patterns. Whether similar distinctions exist for intramedullary projecting neurons remains to be determined.

The rNTS has been divided into several subdivisions based on afferent connections and morphological properties (Halsell et al. 1996; Whitehead 1988): medial, rostral-central, rostral-lateral, and ventral (Fig. 1, A–C). Investigators report that the rNTS intramedullary projection neurons are likely activated by primary afferents through an intra-rNTS polysynaptic circuit based on the location of the neurons in the ventral subdivision (Halsell et al. 1996) relative to the rostral-central location of the densest afferent terminal field (Corson et al. 2012; Whitehead 1988; Whitehead and Frank 1983). Also, very few taste-responsive neurons have been specifically localized to the ventral subdivision, although multiunit activity (McPheeters et al. 1990) as well as indirect single-unit localization data without explicit subdivision specificity (Travers and Norgren 1995) suggest gustatory influences (mono- or polysynaptic) on neurons in the ventral subdivision.

To begin to elucidate the biophysical, morphological, and synaptic properties of rNTS intramedullary projection neurons,
we used an acute slice preparation to record intracellularly from rNTS neurons prelabeled by focal tracer injection into the reticular formation. Using whole cell recording, solitary tract (ST) stimulation in a novel rNTS slice preparation, and high-resolution neuronal morphology reconstructions, we examined the repetitive firing properties, ionic current expression, primary afferent-driven synaptic responses, and morphological characteristics of rNTS intramedullary projection neurons.

METHODS

Female Sprague-Dawley rats (Charles River Laboratories) were used in this study. All surgical procedures conformed to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University Committee on Use and Care of Animals of the University of Michigan.

Retrograde labeling of rNTS intramedullary projecting neurons. To retrogradely label intramedullary projecting rNTS neurons, rats (22–28 days old) were iontophoretically injected with the neural tracer Fast DiI (1% in ethanol; Life Technologies, Grand Island, NY) into the reticular formation, subjacent to the rNTS. The injection was made via a lateral approach at the rostral-caudal level where the NTS abuts the fourth ventricle to avoid passing through the rNTS and thereby avoid spurious labeling.

Animals were anesthetized with a mixture of ketamine (80 mg/kg ip) and xylazine (5 mg/kg ip), and the head was secured in a stereotaxic apparatus with atraumatic ear bars. Body temperature was maintained with a water-circulating heating pad throughout the surgery. The scalp was incised and retracted, exposing the occipital, parietal, and mastoid bones. A borosilicate glass micropipette (tip diameter 10–20 μm) filled with the DiI solution was advanced through the lateral cerebellum at a 50° lateral-medial angle, 7.6 mm lateral to the midline, 1.5 mm posterior to the junction of the lambdoideal, parietomastoid, and occipitomastoid sutures, and 5.3 mm from the dural surface. This targeted the lateral parvicellular and intermediate zones of the reticular formation previously used by others (Halsell et al. 1996). Small iontophoretic injections were made by passing 0.8-μA, 10-s pulses for 5 min (Suwabe and Bradley 2009). The micropipette remained in place for 10–15 min to allow for tracer absorption. The micropipette was then removed, the craniotomy was covered with bone wax, and the scalp was sutured. Animals recovered on the heating pad until ambulatory and were then returned to their home cage.

Electrophysiological recording. After a 24- to 48-h survival time for tracer transport, in vitro whole cell brain slice recordings were performed with previously detailed techniques (Bradley and Sweazey 1990; Suwabe and Bradley 2009; Wang and Bradley 1995).

Animals were anesthetized with halothane and decapitated, and the brain was rapidly removed and immersed in ice-cold oxygenated sucrose-based artificial cerebrospinal fluid (ACSF) composed of (in mM) 206 sucrose, 2 KCl, 2 MgSO4, 1.25 NaH2PO4, 1 CaCl2, 1 MgCl2, 26 NaHCO3, and 10 dextrose. The solution was gassed with 95% O2-5% CO2 to achieve a solution pH of 7.4. rNTS slices (300–400 μm) were cut with a Vibratome (Technical Products International, St. Louis, MO) in ice-cold sucrose-ACSF in either the coronal plane (for biophysical and morphological analyses) or the parasagittal plane (parallel to the ST to examine the neuron synaptic connections with primary afferents). Slices were then allowed to

Fig. 1. DiI injections in the subjacent reticular formation retrogradely label neurons in rostral nucleus of the solitary tract (rNTS). A: the injection site and micropipette tract are visualized with epifluorescent illumination, and the coronal brain stem section is visualized with differential interference contrast optics. B and C: neurons in both ipsilateral and contralateral rNTS are labeled after 48-h survival. Neurons are most densely localized in the rostral-central and ventral subdivisions, with sparser labeling in the rostral-lateral and medial subdivisions. D and E: labeled neuron somata as well as proximal dendrites were clearly identifiable after DiI labeling. Both multipolar (D) and elongate (E) neurons were retrogradely labeled. RL, rostral-lateral; RC, rostral-central; M, medial; V, ventral. Scale bars: 500 (A), 175 (B and C), and 20 (D and E) μm.
recover in ACSF for at least 1 h prior to recording. ACSF contained (in mM) 124 NaCl, 5 KCl, 1.3 MgSO₄, 2.5 CaCl₂, 1.25 KH₂PO₄, 26 NaHCO₃, and 10 dextrose and was oxygenated with 95% O₂-5% CO₂ to obtain a final pH of 7.4.

After the equilibration period, a slice containing rNTS was transferred to a recording chamber attached to a Nikon E600 microscope, secured with nylon mesh, and superfused with oxygenated ACSF at a rate of 2.0–2.5 ml/min. Intramedullary projection neurons were identified with brief epifluorescent illumination of the DiI label followed by subsequent visualization with infrared-differential interference contrast (IR-DIC) optics with a CCD video camera (IR-1000, Dage-MTI, Michigan City, IN). Retrogradely labeled neurons were recorded in whole cell mode with a patch-clamp amplifier (Axoclamp-2B, Molecular Devices, Sunnyvale, CA). Signals were low-pass filtered at 2 kHz, digitized at 20 kHz (Digidata 1400, Molecular Devices), and acquired with pCLAMP 10 software (Molecular Devices). Patch pipettes were pulled from thin-wall borosilicate capillaries (TW150F-4, World Precision Instruments, Sarasota, FL) with a two-stage puller (PP-83, Narishige) to a 6- to 8-MΩ tip resistance and filled with an internal solution containing (in mM) 124 K-glucuronate, 10 N-2-hydroxyethylpipеразин-Ν-2-ethanesulfonic acid (HEPES), 10 ethylene glycol-bis(β-aminoethyl ether)-Ν,N,N',Ν'-tetraacetic acid (EGTA), 1 MgCl₂, 1 CaCl₂, and 2 ATP. Lucifer yellow (0.1%) was included in the pipette solution to label cells for subsequent morphological analysis. rNTS subdivisions were determined by comparing previously published myelin-staining subdivision delineations (Corson et al. 2012; Whitehead 1988) with the density of fiber labeling visible in IR-DIC images.

Biophysical properties and voltage-gated ionic channel expression of intramedullary projection neurons were investigated with a variety of current-clamp and voltage-clamp protocols previously used to allow for comparison with rNTS neuronal populations previously characterized (Bradley and Sweazey 1992; Suwabe and Bradley 2009; Tell and Bradley 1994).

Synaptic connectivity between primary afferents and intramedullary projection neurons was determined in parasagittal rNTS slices. This slicing plane allowed visualization and recording from intramedullary projection neurons throughout the rostro-caudal and dorsoventral extent of rNTS while stimulating the anterior-most portion of the ST. Brief current pulses (≤500 μA, 0.5-ms duration) were delivered to the ST via a concentric bipolar electrode (125-μm OD; FHC, Bowdoin, ME). Excitatory postsynaptic responses were isolated by holding neurons at the Cl⁻ reversal potential of ~70 mV. Frequency-dependent depression was calculated as the relative change in postsynaptic current amplitude during trains of five pulses delivered at 5, 10, 25, and 50 Hz. Synaptic latency was measured from stimulus artifact onset to the point when the postsynaptic current exceeded background noise. Synaptic jitter was measured as the standard deviation of synaptic latency during the first stimulation of the 10-Hz stimulation over 10 trials. Glutamate receptor composition of primary afferent synapse was determined by superfusing glutamate receptor blockers [10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 μM DL-2-amino-5-phosphonopentanoic acid (APV)] over the slices. All chemicals were purchased from Sigma (St. Louis, MO).

Neuron reconstruction. After whole cell recording, the slices were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) for at least 24 h at 4°C. Slices were then mounted under coverglass with Prolong gold (Life Technologies) and imaged with a Nikon C1 laser scanning confocal microscope at ×60 magnification with a 0.5-μm step size. Multiple z-stacks containing individual neurons were reconstructed and morphometrically analyzed with Neurolucida software (MBF Biosciences, Williston, VT).

Data analysis. Electrophysiological data were analyzed with Clampfit (Molecular Devices) and MATLAB software (MathWorks, Natick, MA). The junction potential due to potassium gluconate (10 mV) was subtracted from the membrane potential values. Statistical analysis was conducted with the SPSS program (IBM, Armonk, NY). Numerical values are given as means ± SE, and statistical significance (P < 0.05) was assessed with Student’s t-test or ANOVA with a Bonferroni’s post hoc test for comparison of mean values.

Chorda tympani nerve label and myelin staining. In a separate group of animals, the chorda tympani nerve was labeled with 3-kDa biotinylated dextran amine (BDA, Life Technologies) with previously published techniques (Corson et al. 2012; May and Hill 2006). After 48-h survival for tracer transport, the animal was deeply anesthetized with an overdose of urethane and transcardially perfused with PBS followed by 4% paraformaldehyde in PBS. Brains were postfixed overnight in the same solution. The NTS was then sectioned in the parasagittal plane at 50 μm and serially collected into individual well plates. Chorda tympani nerve terminal field was visualized by reaction with streptavidin-Allexa Fluor 488 (1:200 in PBS; Life Technologies). Sections were then rinsed in PBS, mounted onto subbed slides, and coverslipped with Prolong (Life Technologies). Fluorescent terminal fields were imaged on a Nikon C-1 laser scanning confocal microscope at ×10. After imaging, coverslips were removed in 37°C PBS and the sections were stained for myelin with the gold hydrochloride method previously described (Corson et al. 2012). Maximum-intensity collapsed z-stacks of chorda tympani nerve terminal field label were then digitally overlaid (in register) onto images of the myelin-stained sections to show the distribution of terminal field within the parasagittal rNTS.

RESULTS

Retrogradely labeled neurons. Injection of DiI into the reticular formation (Fig. 1A) reliably labeled neurons throughout the rostral-central and ventral rNTS subdivisions (Fig. 1, B and C). Sparse labeling was also present in the rostral-lateral subdivision. Labeled neurons were bilaterally observed throughout the rostral-caudal extent of the rNTS. Both multipolar and elongate neurons were labeled (Fig. 1, D and E), although as only the most proximal dendritic segments were visible with DiI labeling, fine comparisons of the cellular morphology between subdivisions were not made. The injection site was clearly visible and separated from the rNTS throughout its rostral-caudal extent, and the ventral subdivision (which is directly adjacent to the reticular formation) did not show global, nonspecific labeling. Thus it is unlikely that the rNTS labeling resulted from tracer leakage into the rNTS rather than actual retrograde labeling of intramedullary projection neurons.

Membrane properties. A total of 40 retrogradely labeled neurons were recorded from 12 animals. To be included in the data set, neurons had to have a resting membrane potential (RMP) of at least ~40 mV, an action potential amplitude of at least 40 mV, action potential afterhyperpolarization overshoot of at least ~10 mV, and an input resistance of at least 100 MΩ. The results are based on neurons with a mean RMP of ~53 ± 1 mV and an input resistance of 669 ± 55 MΩ. No differences were observed between ipsilaterally and contralaterally labeled projection neurons for any of the following analyses, and thus they were pooled for the remainder of the study.

Repetitive firing properties. Intramedullary rNTS projection neurons had heterogeneous repetitive firing patterns (Fig. 2). Most neurons (~63%) responded to a depolarizing current injection with tonic spiking activity throughout the duration of the current injection, with little change in action potential amplitude or threshold (Fig. 2A). The remaining neurons responded to depolarizing current injection with a rapidly adapting burst of action potentials followed by a steady-state depo-
more frequently encountered than those with a delay in firing (14 vs. 3 neurons). Voltage-clamp recording in which the neuron was briefly held at 50 mV below RMP and then depolarized to 20 mV above RMP indicated that this hyperpolarization-induced change in firing pattern was due to a transient outward current, similar to the A-type potassium current ($I_{K_A}$) observed in other rNTS populations (Fig. 3B). Bath application of 4-aminopyridine (4-AP) blocked the transient outward current but left a delayed, steady-state outward current intact (Fig. 3, C–E).

Other hyperpolarization-activated currents were also observed in rNTS intramedullary projection neurons. Neurons were injected with hyperpolarizing current pulses ranging from −20 to −100 pA in 20-pA increments. Upon release of the hyperpolarizing current injection, 65% of the neurons responded with a rebound depolarization that resulted in a single or short burst of action potentials (Fig. 4A). Voltage-clamp recording showed that this was due to a postinhibitory rebound current ($I_{h}$). This transient inward current displayed a fast decay time that occurred immediately after release of hyperpolarization and whose amplitude was proportional to the amplitude of hyperpolarization (Fig. 4B, arrow). In ~60% of the neurons a membrane potential “sag” in response to the hyperpolarization occurred at higher current injections (Fig. 4C). Comparable voltage-clamp recordings with neurons being held at increasingly hyperpolarized membrane potentials revealed that this membrane sag was associated with a slowly decaying inward current (Fig. 4D, arrow). None of these voltage-activated ionic currents was correlated with a specific repetitive firing pattern (tonic or burst) or rNTS subdivision location.

**Primary afferent innervation.** The rNTS is a complex structure consisting of a number of different subdivisions and primary afferent terminal fields that traverse through the rostral-caudal axis of the nucleus. Thus the plane of section examined must be carefully chosen depending on the experiment. Traditionally, the rNTS has been examined in either the coronal or horizontal plane. The coronal plane allows all subdivisions to be sampled but transects the ST. The horizontal plane preserves the majority of the ST but severely limits sampling in the dorsal-ventral plane. To examine the connections of rNTS intramedullary projection neurons with primary afferent fibers relay ing orosensory information, a parasagittal slice preparation was developed (Fig. 5A). This preparation consisted of a single 400-μm slice that included the intramedullary projection neurons in the ventral and rostral-central subdivisions, the rostral ST, and the terminal fields of the primary orosensory afferents (Fig. 5, B and C). It also permitted the placement of a stimulating electrode away from the recording location in the ventral rNTS (something that is not possible in the coronal or horizontal planes previously used), to eliminate the possibility of direct electrical stimulation of the recorded neuron. Although Fig. 5 depicts only the chorda tympani nerve terminal field, it is important to note that the greater superficial petrosal nerve as well as the lingual branch of the glossopharyngeal nerve will also be stimulated. Thus, as with other in vitro examinations of rNTS primary afferent connectivity, the specific identity of the activated nerve is not known.

Neurons were held at −70 mV, and primary afferents were stimulated via a concentric bipolar stimulating electrode placed
in the rostral-most portion of the ST. A total of 19 neurons were tested for synaptic connectivity with primary afferents. Of those, four neurons did not respond to tract stimulation with any synaptic currents and thus could not be included in any analyses involving synaptic latency or jitter. When ST stimulation evoked multiple current peaks, only the first peak was analyzed because latter peaks were likely polysynaptic. Seven neurons responded to ST stimulation with short-latency, low-jitter postsynaptic currents (Fig. 6A). Synaptic responses were classified as monosynaptic if they displayed a jitter value of <300 μs measured from 10 repetitive stimulations (Boxwell et al. 2013; Doyle and Andresen 2001; Wang and Bradley 2010b). Monosynaptic responses also had consistent amplitudes between stimulations and no failures. Synaptic responses with jitter values >300 μs were considered to be polysynaptic. The amplitudes of these responses varied greatly between individual stimulations and also displayed a higher failure rate (Fig. 6B). There was a positive correlation between synaptic latency and jitter values [Pearson’s $r$ (13) = 0.553, $P < 0.05$] (Fig. 6C). However, the use of synaptic latency alone to classify synaptic responses as monosynaptic or polysynaptic would lead to both false positive and false negative conclusions. Postsynaptic latencies as low as 5 ms were observed for neurons with both high and low jitter values. There were no differences between synaptic latency for cells located in different subdivisions. Also, synaptic latency and jitter did not differ for neurons with burst versus tonic repetitive firing patterns.

Primary afferent synaptic responses were further investigated by modulating both the intensity and frequency of ST stimulation. Stimulation intensity modulated between 10 and 400 μA resulted in a corresponding increase in postsynaptic current amplitude (Fig. 7A). Increases in postsynaptic current amplitude did not alter synaptic latency or jitter. Increases in ST stimulation magnitude resulted in an abrupt stepwise response in synaptic response magnitude (Fig. 7B) indicative of unitary synaptic input (Doyle and Andresen 2001; Suwabe and Bradley 2009; Wang and Bradley 2010b). Each successive increase in postsynaptic current likely represents the recruitment of additional primary afferent axons.

Modulation of the stimulation frequency between 5 and 50 Hz resulted in a frequency-dependent depression of the resulting postsynaptic current amplitude (Fig. 8, A and B). Increases in stimulation frequency produced corresponding increases in the depression of subsequent evoked postsynaptic current amplitudes. ST stimulation of 50 Hz resulted in a 63% ± 7% reduction in excitatory postsynaptic current (EPSC) amplitude by the final stimulation (Fig. 8C).

The neurotransmitter receptor composition of ST-evoked postsynaptic responses was investigated by superfusing receptor antagonists and by systematically varying the holding potential to isolate EPSCs and inhibitory postsynaptic currents (IPSCs). Bath application of 10 μM CNQX resulted in an 82.3% ± 6.3% decrease in EPSC amplitude ($n = 6$; Fig. 9A). Addition of 50 μM APV resulted in an additional 5.2% ± 0.8% ($n = 6$) reduction in EPSC amplitude (Fig. 9A), indicating the presence of both AMPA and NMDA receptors at primary afferent synapses with intramedullary projection neurons. Further indicative of AMPA and NMDA sensitivity, calculation of the CNQX- and APV-sensitive components of the EPSC through arithmetic subtraction shows that APV-sensitive currents have
slower rise and decay times than CNQX-sensitive currents (Fig. 9B).

Monosynaptic ST-evoked EPSCs were often followed by a volley of IPSCs (Fig. 10A). This barrage of IPSCs was isolated by increasing the holding potential above the Cl− reversal potential and lasted as long as 50 ms in some neurons. This delayed inhibitory response appeared as multiple peaks suggesting convergence of inhibitory inputs that were all activated by a single ST stimulation (albeit likely from the stimulation of multiple individual primary afferent fibers). The same results plotted in a heat map demonstrate that at depolarized holding potentials the initial inward current is no longer present and the later postsynaptic current reverses into an outward current (Fig. 10B). The strongest outward currents do not occur until 20 ms after stimulation (15 ms after the initial postsynaptic current). Current-voltage (I-V) curves (fitted with the Boltzmann equation) show the reversal potentials of postsynaptic currents at two time points in the traces (6 ms and 8 ms after tract stimulation) in Fig. 10C. This again demonstrates that the initial postsynaptic response does not reverse until membrane potentials are above 0 mV and the delayed portion of the response reverses polarity at approximately −55 mV. These results indicate that the initial portion of the postsynaptic response is purely excitatory, followed by mixed excitatory and inhibitory components.

**Morphological characteristics.** Of the 40 neurons recorded, 32 were morphologically reconstructed (Fig. 11). An example of two neurons recorded in the same rNTS slice is shown in Fig. 11A and reconstructed in Fig. 11B, showing both the axonal and dendritic morphologies. Intramedullary projection neuron somata were relatively homogeneous, with average perimeter of 39.1 ± 1.3 μm, area of 101.8 ± 6.3 μm², maximum diameter of 14.4 ± 0.6 μm, and aspect ratio of 1.5 ± 0.1. Neurons had on average 3...
primary dendrites with $7 \pm 1$ branch points and $1,397.0 \pm 88.2\ \mu m$ of total dendritic length visible within the slice. However, the dendrites were often sectioned during slicing, and thus these morphological measures are likely underestimates of the true dendritic branch points and length. The number of dendritic branch points differed according to the plane of section, with coronal slices containing $6 \pm 1$ branch points per $100\ \mu m$ of dendrite and parasagittal slices containing $4 \pm 0.4$ branch points per $100\ \mu m$ [$t(29) = 3.199, P < 0.01$]. None of the other morphological parameters differed by plane of section, indicating that although intramedullary projection neuron dendrites branch more extensively in the medial-lateral plane, there is no preferred dendritic orientation between the medial-lateral and rostral-caudal planes. Intramedullary projection neurons were markedly spiny ($9 \pm 1$ spines per $100\ \mu m$ dendrite; Fig. 11C). This is in stark contrast to the morphologies of previously examined rNTS neuron populations (Davis 1993; Suwabe and Bradley 2009; Wang and Bradley 2010a; Whitehead 1990) that were reported to have relatively few dendritic spines.

Fig. 5. A parasagittal plane of section was used to examine the synaptic connectivity between primary afferents and intramedullary projection neurons. A: a parasagittal rNTS slice preparation (as depicted by the gray shaded regions) combines the benefits of the horizontal and coronal planes of section. The width of the gray shaded region in both diagrams is $400\ \mu m$ (the thickness of the in vitro preparations). B: myelin staining of a 50-µm parasagittal section reveals that both the solitary tract (arrowheads) as well as the multiple subdivisions can be visualized in the same plane. Rostral-caudal traversing myelinated fiber bundles provide a demarcation for the ventral subdivision, while the dorsal-ventral myelinated bundles and myelin-sparse regions demarcate the rostral-central subdivision. C: fluorescently labeled chorda tympani nerve terminal field photomicrographs were overlaid onto the myelin-stained photomicrograph in B. Note that these images are from the same section (see Methods), allowing for exact registration of terminal field and NTS architecture. Scale bar in B: 1 mm.

Fig. 6. Solitary tract stimulation evoked both monosynaptic and polysynaptic responses in intramedullary projection neurons. A: synaptic responses were classified as monosynaptic if they displayed a jitter value < 300 µs as measured from 10 separate stimulations (10 individual stimulations overlaid here). B: synaptic responses with jitter values > 300 µs were classified as polysynaptic. The amplitudes of these responses varied greatly between individual stimulations and also displayed a high failure rate. C: synaptic latency and jitter were positively correlated ($P < 0.05$), with longer latencies more often displaying increased jitter.
Intra- and extra-rNTS dendritic and axonal branching patterns were examined to determine the extent to which a given neuron may communicate with neighboring regions. Dendritic branching differed by rNTS subdivision location. Cells located in the rostral-central subdivision displayed $5.8 \pm 0.6$ branch points per 100 $\mu$m of dendrite, and cells located in the ventral subdivision displayed $3.8 \pm 0.5$ branch points per 100 $\mu$m of dendrite ($t(23) = 2.400, P < 0.05$). However, neuronal dendritic arborization was not confined to the subdivision containing the soma (Fig. 11, D and E). The subdivisional distribution of dendritic branching is summarized in Table 1. Additionally, axons were reconstructed for 24 neurons. Axons were differentiated from dendrites by their thinner initial diameter, which remained relatively constant throughout the process. Axons also more often branched at right angles, especially noticeable compared with the acute angle branching of dendrites. Axon length ($867.9 \pm 94.1 \mu$m) and branching ($7.6 \pm 1.9$ branch points per 100 $\mu$m of axon) were considerably more variable than for dendrites, although this may be due to the difficulty in reconstructing these fine processes deep into the tissue. The subdivisional distribution of axonal branching is summarized in Table 2. This extensive intra-rNTS axon collateralization and dendritic arborization indicates that intramedullary projection neurons may also have a profound influence on local circuit modulation of incoming sensory information.

Fig. 7. Monosynaptic primary afferent synapses responded to increasing stimulus current intensity with unitary, stepwise increases in postsynaptic current amplitude. A: stimulus current intensity increases from 10 to 400 $\mu$A resulted in corresponding increases in postsynaptic current amplitude. Note that increases in postsynaptic current amplitude did not alter synaptic latency. Arrow marks stimulus artifact. B: stepwise increases in postsynaptic current amplitude can be seen with increasing stimulus current intensities, indicative of unitary synaptic input. Each step increase likely represents the recruitment of additional primary afferent fibers. Postsynaptic currents do not increase beyond 200-$\mu$A stimulating current.

Fig. 8. Primary afferent synapses responded to repetitive stimulation with frequency-dependent depression. A: average traces (over 10 repeated stimulation series) depict the degree of frequency-dependent depression. Timescales have been normalized to align each set of 5 stimulations. B: the first and second stimulations from A have been expanded to show the decrease in amplitude of the second evoked postsynaptic current (PSC) relative to the first. C: increasing the stimulation frequency from 5 to 50 Hz results in a corresponding increase in the magnitude of synaptic depression.
Overall, except for a few notable parameters, neuron morphology did not correlate with the biophysical and synaptic properties of rNTS intramedullary projection neurons (Fig. 12). No significant differences between biophysical or primary afferent synaptic properties were found for somal or axonal parameters. Likewise, dendritic length and branching did not differ among physiological groupings. There were also no differences in spine density between neurons grouped according to their repetitive firing patterns (Fig. 12A). However, spine density was significantly different for neurons displaying hyperpolarization-induced changes in firing patterns (Fig. 12B). Neurons that had a delay in action potential firing caused by the hyperpolarizing prepulse had a significantly greater spine density than those that had no such change in firing pattern \([14 \pm 5 vs. 7 \pm 3 \text{ spines per } 100\mu\text{m of dendrite}; F(3,27) = 5.449; \text{Bonferroni’s post hoc test}, P < 0.05]\). As such, neurons that displayed \(I_{\text{Ca}}\) also had a greater spine density than those that had no such current \([11 \pm 4 vs. 8 \pm 4 \text{ spines per } 100\mu\text{m of dendrite}; t(29) = 2.352, P < 0.05\] (Fig. 12C). There was no

Fig. 9. Primary afferent synaptic connections with intramedullary projection neurons are primarily AMPAergic. \(A\): bath application of the selective AMPA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) blocks the majority of the postsynaptic current, leaving a much smaller current that can be blocked with subsequent addition of the selective NMDA antagonist \(N\)-2-amino-5-phosphonopentanoic acid (APV). Traces are the average of 10 consecutive trials. \(B\): isolation of the CNQX- and APV-sensitive components of the postsynaptic current through arithmetic subtraction reveals that the APV-sensitive component has slower rise and decay time compared with the CNQX-sensitive component, consistent with NMDA receptor kinetics.

Overall, except for a few notable parameters, neuron morphology did not correlate with the biophysical and synaptic properties of rNTS intramedullary projection neurons (Fig. 12). No significant differences between biophysical or primary afferent synaptic properties were found for somal or axonal parameters. Likewise, dendritic length and branching did not differ among physiological groupings. There were also no differences in spine density between neurons grouped according to their repetitive firing patterns (Fig. 12A). However, spine density was significantly different for neurons displaying hyperpolarization-induced changes in firing patterns (Fig. 12B). Neurons that had a delay in action potential firing caused by the hyperpolarizing prepulse had a significantly greater spine density than those that had no such change in firing pattern \([14 \pm 5 vs. 7 \pm 3 \text{ spines per } 100\mu\text{m of dendrite}; F(3,27) = 5.449; \text{Bonferroni’s post hoc test}, P < 0.05]\). As such, neurons that displayed \(I_{\text{Ca}}\) also had a greater spine density than those that had no such current \([11 \pm 4 vs. 8 \pm 4 \text{ spines per } 100\mu\text{m of dendrite}; t(29) = 2.352, P < 0.05\] (Fig. 12C). There was no

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**Fig. 10.** Intramedullary projection neurons respond to solitary tract stimulation with initial excitation followed by inhibition. \(A\): neurons were held at \(-110\text{ mV}\) to \(+20\text{ mV}\) in 10-mV increments while solitary tract-evoked postsynaptic currents were recorded. Holding currents were subtracted to obtain postsynaptic currents relative to a standard baseline. \(B\): the same traces plotted in a heat map demonstrate that at depolarized holding potentials the initial inward current is no longer present and the latter postsynaptic current reverses into an outward current. Each recording trace is separated into 1-ms bins and the data averaged within each bin. The resulting postsynaptic current (relative to a 100-ms baseline obtained prior to stimulation) was color coded by intensity, with warm colors (yellow and red) representing outward currents and cool colors (blues) representing inward currents. The strongest outward currents do not occur until 20 ms after stimulation (15 ms after the initial postsynaptic current). \(C\): Current-voltage (\(I-V\)) curves showing the reversal potentials of postsynaptic currents at 2 time points in the traces (6 ms and 8 ms after tract stimulation). Data are fitted with Boltzmann equation curves. This again demonstrates that the initial postsynaptic response (at 6 ms) does not reverse until membrane potentials above 0 mV, indicative of excitation. The latter portion of the response (at 8 ms) reverses at approximately \(-55\text{ mV}\), indicative of predominantly inhibition.
significant difference in spine density for neurons expressing either $I_h$ or $I_{PIR}$ (Fig. 12, D and E). Spine density also differed with respect to ST-evoked postsynaptic responses. Neurons that responded monosynaptically to ST stimulation had a greater spine density that those that responded polysynaptically [Fig. 12F; 11 ± 1 vs. 7 ± 2 spines per 100 μm of dendrite; $t(10) = -2.243$, $P < 0.05$]. These physiological correlations with spine density suggest that intramedullary projection neurons can be classified on the basis of detailed anatomical parameters, and that these subgroupings may represent distinct neuron subpopulations positioned differently within the rNTS microcircuit.

**DISCUSSION**

Through combinations of voltage-gated ion channels and local circuit interactions, the rNTS transforms incoming sensory information into efferent signals that are conveyed to second-order gustatory structures. These second-order structures are functionally diverse, and the efferent rNTS signals likely parallel this heterogeneity. However, few studies have

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**Table 1. Dendrite length in rNTS subdivisions**

<table>
<thead>
<tr>
<th>Soma Location</th>
<th>Rostral-central</th>
<th>Rostral-lateral</th>
<th>Ventral</th>
<th>Reticular formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rostral-central</td>
<td>869.3 ± 151.1</td>
<td>124.3 ± 66.0</td>
<td>205.8 ± 74.3</td>
<td>85.3 ± 45.1</td>
</tr>
<tr>
<td>Ventral</td>
<td>213.9 ± 107.1</td>
<td>47.1 ± 29.4</td>
<td>981.0 ± 184.7</td>
<td>206.8 ± 118.3</td>
</tr>
</tbody>
</table>

rNTS, rostral nucleus of the solitary tract.

**Table 2. Axon length in rNTS subdivisions**

<table>
<thead>
<tr>
<th>Soma Location</th>
<th>Rostral-central</th>
<th>Rostral-lateral</th>
<th>Ventral</th>
<th>Reticular formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rostral-central</td>
<td>597.5 ± 133.6</td>
<td>53.2 ± 52.2</td>
<td>159.1 ± 75.1</td>
<td>65.3 ± 31.5</td>
</tr>
<tr>
<td>Ventral</td>
<td>23.7 ± 15.5</td>
<td>0</td>
<td>702.1 ± 152.7</td>
<td>76.3 ± 52.2</td>
</tr>
</tbody>
</table>

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examined the rNTS circuitry with respect to these discrete output pathways. This study represents the first investigation into the biophysical and morphological properties of rNTS neurons involved in circuitry governing oromotor reflexive behaviors. These intramedullary projection neurons display both tonic and burst repetitive firing patterns as well as a variable combination of voltage-activated ionic currents. They also possess morphological characteristics (i.e., dendritic spines) that distinguished them from previously studied rNTS neuron populations. Interestingly, the density of dendritic spines was not uniform across neurons studied but displayed high variance with physiological correlates. Neurons that expressed \( I_{\text{Ka}} \) as well as neurons that received monosynaptic primary afferent innervation displayed a denser array of dendritic spines than those that did not express such a current, but there was no difference in spine density for neurons expressing either \( I_h \) or \( I_{\text{PIR}} \) (D and E, respectively). F: neurons that responded to solitary tract stimulation with monosynaptic currents had a greater spine density than those that responded with polysynaptic currents. *\( P < 0.05 \), **\( P < 0.01 \).

Fig. 12. Of all morphological parameters examined, only spine density showed any relationship to the physiological properties. Each box plot shows the mean as the horizontal line, 1 standard deviation above and below the mean in the bars, and data outside of the 95% confidence interval as individual markers. A: there were no differences in spine density between neurons grouped according to their repetitive firing patterns. B: however, neurons that responded to a hyperpolarizing pulse preceding depolarization with a delay in firing had a greater spine density than those that had no change. C: likewise, neurons that expressed \( I_{\text{Ka}} \) had a greater spine density than those that did not express such a current, but there was no difference in spine density for neurons expressing either \( I_h \) or \( I_{\text{PIR}} \) (D and E, respectively). F: neurons that responded to solitary tract stimulation with monosynaptic currents had a greater spine density than those that responded with polysynaptic currents. *\( P < 0.05 \), **\( P < 0.01 \).
lotion morphologically distinct from that examined here (Davis 1993). Developmental alterations in synaptic circuitry for the glossopharyngeal and greater superficial petrosal nerves remain to be determined. Future studies focused on development-ral changes in the circuitry of these primary afferents will be invaluable, because we are currently unable to isolate individual primary afferent populations in vitro.

Another caveat of this study is the use of DiI (a lipophilic tracer) for retrograde neuron labeling rather than other retrograde tracers. The projections from rNTS to the reticular formation are diffuse and widespread over the parvicellular region (Beckman and Whitehead 1991; Streefland and Jansen 1999; Travers 1988). Thus targeted injection of a retrograde tracer that is only picked up by axon terminals in the immediate vicinity would only label a subpopulation of intramedullary projection neurons, biasing the study to those neurons. The possibility of specific intramedullary projection neuron sub-populations may account for some of the heterogeneity found in this study and will be explored in future experiments. However, it is worth noting that the accidental labeling of ascending projection neurons projecting to the parabrachial nucleus is very unlikely. These projections take a rostral-dorsal trajectory out of rNTS en route to the parabrachial nucleus (Streefland and Jansen 1999; Travers 1988). Injections in this study were made ventral to rNTS into the reticular formation at the rNTS-4th ventricle junction, well caudal to the ascending afferents. Thus, although we are unable to define separate subpopulations of intramedullary projections, the neurons investigated in this study are certainly distinct from the previously examined ascending projection neurons (Suwabe and Bradley 2009).

Biophysical properties. The biophysical properties of rNTS neurons have received considerable attention over the past two decades. Three voltage-gated currents ($I_{Ka}$, $I_h$, and $I_{PR}$) have been identified for these neurons, each having a profound effect on the repetitive discharge pattern. Individual rNTS neurons that project to the subjacent reticular formation also possess some combination of these three voltage-gated currents, with spiny neurons more likely to express $I_{Ka}$. $I_{Ka}$ is activated at hyperpolarized states and leads to a delay in repetitive discharge following depolarization. Neurons expressing $I_{Ka}$ in essence have a built-in, temporal filter that is only activated at hyperpolarized states. Thus a temporally acute depolarizing signal [e.g., possibly a barrage of excitatory postsynaptic potentials (EPSPs) from tactile stimulation of the tongue] would be unable to activate these neurons immediately after hyperpolarizing activation of the $I_{Ka}$. However, sensory stimulation resulting in a prolonged barrage of EPSPs (e.g., gustatory stimulation) would likely still generate a train of action potentials in these neurons, albeit delayed. Therefore, it is conceivable that the activation of this current may be able to filter out specific sensory modalities in a subset of neurons. Indeed, most gustatory neurons in rNTS also respond to somatosensory activation of the oral cavity (Travers and Norgren 1995). Even though the sensory response properties of the recorded neurons cannot be examined with an in vitro slice preparation, such dual-modality sensitivity and sensory filtering through $I_{Ka}$ is certainly plausible.

Interestingly, rNTS GABAergic neurons (at least those expressing GAD67) do not express $I_{Ka}$ and thus do not have hyperpolarization-induced changes in repetitive firing (Wang and Bradley 2010a), suggesting that this mode of spike timing modulation may be specific for projection neurons in rNTS (Suwabe and Bradley 2009). GABAergic neurons respond to depolarization with an initial burst of action potentials followed by a steady-state plateau (Wang and Bradley 2010a), and thus their activation will produce only a brief barrage of inhibition on postsynaptic neurons. However, if this inhibition is sufficient to activate $I_{Ka}$ in projection neurons, the suppression of action potential firing has the potential to last beyond the actual inhibitory interneuron activation. It has been suggested that this temporal suppression of action potential firing can serve to correlate activity patterns between groups of neurons with synchronous inputs (Barreiro et al. 2012; Fransen and Tigerholm 2010). It is conceivable that such correlation of activity patterns may be important for the temporal coding of gustatory stimuli (Chen et al. 2011; Di Lorenzo et al. 2009; Rosen and Di Lorenzo 2012). Thus these biophysical properties likely play a significant role in shaping circuit-wide activity in the rNTS and the neural coding of gustatory stimuli. Future examinations into the relationship between local rNTS microcircuitry and the biophysical properties of these rNTS subpopulations can be used to determine the role of voltage-gated current expression and correlated activity patterns.

Synaptic interactions. Primary afferent innervation was observed in approximately half of all intramedullary projection neurons tested, suggestive of a direct control of reflexive oromotor rNTS output by sensory stimulation. These synapses produced reliable, unitary postsynaptic responses with low variability, indicative of monosynaptic innervation (Doyle and Andresen 2001; Suwabe and Bradley 2009). The identification of such connectivity is certainly influenced by slice orientation, in which many connections are inevitably severed during sectioning. As such, the proportion of monosynaptic connections observed in this study is likely an underestimate. Nevertheless, the ability of primary afferents to directly excite at least a subpopulation of intramedullary projection neurons is no longer in question.

Primary afferent excitation of intramedullary projection neurons is temporally filtered by both dynamic release properties and local circuit interactions. Similar to other NTS neuron populations (Doyle and Andresen 2001; Miles 1986; Wang and Bradley 2010b), repetitive primary afferent activation produced a frequency-dependent depression in the postsynaptic response. This can act as a low-pass filter for the incoming input, limiting the action potential train resulting from high levels of sensory activity. In addition to frequency-dependent depression, primary afferent activation also produced a longer-latency barrage of inhibition. This polysynaptic inhibition likely arises from local GABAergic interneurons and as such highlights the strong influence that the intrinsic rNTS circuitry has on shaping efferent signaling. Thus both the local inhibitory circuitry and intrinsic sensory afferent properties further refine the response dynamics of intramedullary projection neurons.

Gustatory primary afferent neurons respond to sensory stimulation of their receptive field with action potential trains in excess of 40 Hz, with increasing stimulus concentrations producing higher-frequency spike trains (Lundy and Contreras 1999; Pfaffmann 1955; Sollars and Hill 2005). Despite the dampening of the primary afferent synapses via the above-mentioned mechanisms, on average rNTS neurons respond to gustatory stimulation with higher spike frequency than primary afferents (Hill et al. 1983).

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However, these maximal response frequencies vary over a wide range, indicating heterogeneity in stimulus response magnitude. Given the strong polysynaptic inhibition following primary afferent activation, it is possible that intramedullary projection neurons respond to gustatory stimulation with a lower action potential frequency than other neuron populations. However, the convergence of multiple primary afferents onto a single projection neuron would also likely result in higher spike frequencies. The stepwise increase in PSC amplitude with increasing stimulation intensities indicates the convergence of multiple primary afferents. Unfortunately, the degree of primary afferent convergence for this neuron population, and whether this convergence would result in the higher firing rates observed in extracellular recording, remain unknown. This discrepancy between sensory afferent and second-order neuron responses may also arise from the intrinsic membrane properties of rNTS neurons (e.g., lower spike threshold) and/or local excitatory rNTS circuitry and feedback projections from higher-order gustatory structures. However, extremely little is known regarding these various rNTS circuits, and as such the functional role and significance that they have in shaping rNTS efferent signaling need further examination.

**Morphological characteristics.** Thus far, neuron morphology has been a poor predictor of physiological properties in the rNTS (King and Bradley 1994) despite its clear relationship in other brain regions such as the cerebral cortex and hippocampus (Connors and Gutnick 1990; Graves et al. 2012; Kawaguchi 1993). This lack of a structure-function relationship in rNTS could be due to differences in neuron diversity between brain regions or possibly to shortcomings in our own understanding of the subtle differences in rNTS neuron morphology and thus our ability to classify such neurons. As with other studies, we found no relationship between repetitive firing characteristics (i.e., burst vs. tonic) and dendritic morphology. However, we did find a significant correlation between spine density and the expression of \( I_{KS} \). Our results demonstrate that neurons expressing \( I_{KS} \) have a greater spine density than neurons not expressing this current. Likewise, neurons receiving monosynaptic innervation from primary afferent fibers had a greater spine density than neurons receiving polysynaptic innervation. This points to the existence of intramedullary projection neuron subtypes, which may be identifiable on the basis of their dendritic spine characteristics as well as their position within the gustatory-oromotor reflex circuitry.

A subset of intramedullary projection neurons examined in this study responded to depolarizing current pulses with a burst of action potentials, similar to the responses previously reported for a subset of GABAergic neurons located primarily in the ventral rNTS subdivision (Wang and Bradley 2010a). While it was assumed that these GABAergic neurons are locally projecting interneurons, it is possible that they also have connections with the subjacent reticular formation and that the bursting neurons recorded in this study are inhibitory projecting neurons. NTS-evoked inhibitory responses in reticular formation preoromotor neurons have been reported previously (Nasse et al. 2008), and the heterogeneity of the neurons sampled in this study certainly suggests the existence of distinct subpopulations. Reports regarding primary afferent innervation onto GABAergic neurons have been somewhat conflicting. Electron microscopy has revealed sparse chorda tympani innervation onto GABAergic dendrites for the rat (Wang et al. 2012). However, recent whole cell physiology has revealed somewhat widespread primary afferent innervation for the mouse (Boxwell et al. 2013). Whether these represent species differences or nerve-specific differences remains to be determined. Also, as previously stated, GABAergic neurons do not express \( I_{KS} \) (Wang and Bradley 2010a). The apsiny neurons recorded in this study share these characteristics and thus may send inhibitory projections to the reticular formation. Additional examination of the neurochemical phenotypes of intramedullary projection neurons will provide valuable information on the complexity of NTS-reticular formation interactions.

Spiny neurons represent a unique morphological class in the rNTS, where the vast majority of neurons possess relatively smooth, simple dendritic trees (Davis and Jang 1988; Whitehead 1988; Whitehead et al. 1993). While this neuron type had been previously identified (Renahan et al. 1994), the axonal target and biophysical properties remained unknown. As no other characterized rNTS neuronal population has such a high spine density, this may represent a unique morphological characteristic of a subset of intramedullary projection neurons. Interestingly, this spiny class of rNTS neurons is also more narrowly tuned to tastes than rNTS neurons with low spine density (Renahan et al. 1996), albeit only the anterior tongue was stimulated and thus only chorda tympani innervation was examined. Similar to gustatory primary afferent fibers (Bradley and Mistretta 1973; Ogawa et al. 1968; Pfaffmann 1955; Sollars and Hill 2005), most rNTS neurons are broadly tuned to a number of different tastants (Hill et al. 1983; Sweazey and Smith 1987). Although the tastant tuning for the recorded neurons cannot be tested in an in vitro preparation, the shared unique morphological properties of these narrowly tuned neurons with the intramedullary projection neurons recorded in this study suggest that they may be from the same population. Such a narrow tuning could relate to the specificity of some oromotor responses for specific tastants (e.g., stereotypical gaping in response to bitter stimulants). However, responses of these spiny neurons to palatal and posterior tongue stimulation remain unknown and are necessary to definitively determine a given neuron’s tastant tuning profile. This is especially important for the posterior tongue, as previous studies have demonstrated that IX preferentially synapses with dendritic spines (May et al. 2007). Given the spiny nature of these intramedullary projection neurons, it is likely that they also have a strong IX innervation. Future studies into the rNTS circuitry, both local and efferent, will continue to increase our understanding of the brain stem’s role in gustatory information processing.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: J.A.C. and R.M.B. conception and design of research; J.A.C. performed experiments; J.A.C. analyzed data; J.A.C. and R.M.B. interpreted results of experiments; J.A.C. prepared figures; J.A.C. drafted manuscript; J.A.C. and R.M.B. edited and revised manuscript; J.A.C. and R.M.B. approved final version of manuscript.
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