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SENSORY AFFERENT AND HYPOXIA-MEDIATED ACTIVATION OF NUCLEUS TRACTUS SOLITARIUS NEURONS THAT PROJECT TO THE ROSTRAL VENTROLATERAL MEDULLA

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Abstract—The nucleus tractus solitarius (nTS) of the brainstem receives sensory afferent inputs, processes that information, and sends projections to a variety of brain regions responsible for influencing autonomic and respiratory output. The nTS sends direct projections to the rostral ventrolateral medulla (RVLM), an area important for cardiorespiratory reflexes and homeostasis. Since the net reflex effect of nTS processing ultimately depends on the properties of output neurons, we determined the characteristics of these RVLM-projecting nTS neurons using electrophysiological and immunohistochemical techniques. RVLM-projecting nTS neurons were identified by retrograde tracers. Patch clamp analysis in the horizontal brainstem nTS slice demonstrated that RVLM-projecting nTS cells exhibit constant latency solitary tract evoked excitatory postsynaptic currents (EPSCs), suggesting they receive strong monosynaptic contacts from visceral afferents. Three distinct patterns of action potential firing, associated with different underlying potassium currents, were observed in RVLM-projecting cells. Following activation of the chemoreflex in conscious animals by 3 h of acute hypoxia, 11.2±1.9% of the RVLM-projecting nTS neurons were activated, as indicated by positive Fos-immunoreactivity. Very few RVLM-projecting nTS cells were catecholaminergic. Taken together, these data suggest that RVLM projecting nTS neurons receive strong monosynaptic inputs from sensory afferents and a subpopulation participates in the chemoreflex pathway.

Key words: cardiovascular, respiration, chemoreflex, retrograde, Fos.

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Abbreviations: aCSF, artificial cerebral spinal fluid; AP, action potential; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CS, calamus scriptorius; CtB, cholera toxin B; CVLM, caudal ventrolateral medulla; DE, delayed excitation; EPSC, excitatory postsynaptic current; FG, fluorogold; I_K, potassium current; IR, immunoreactive; NDS, normal donkey serum; NMDA, N-methyl-D-aspartate; nTS, nucleus tractus solitarius; PVN, paraventricular nucleus; RVLM, rostral ventrolateral medulla; SFA, spike frequency adaptation; TH, tyrosine hydroxylase; TS, tractus solitarius.

The nucleus tractus solitarius (nTS) is the primary site of termination for multiple visceral afferents including those involved in cardiorespiratory reflexes (Andresen and Kunze, 1994; Spyer, 1994; Kline, 2008). This vital brainstem nucleus processes and integrates information from sensory afferents such as the arterial baroreceptors and carotid body chemoreceptors. Projection neurons from the nTS then send this information to numerous brain regions involved in autonomic and respiratory regulation (Andresen and Kunze, 1994). The activity of nTS projection neurons depends on afferent input to the nTS, processing within the nucleus, and the intrinsic properties of the output neurons themselves. Ultimately, the activity of these projection neurons determines the net effect of the nTS on cardiorespiratory reflex responses.

The rostral ventrolateral medulla (RVLM) plays an essential role in basal and reflex regulation of the autonomic nervous system and breathing (Guyenet, 2006). This brain region contains neurons crucial for controlling sympathetic activity in both arterial baroreflex and chemoreflex pathways. Blockade of neuronal activity in the RVLM eliminates baroreflex-mediated changes in sympathetic nervous system activity in response to alterations in arterial pressure (Dampney, 1994a; Guyenet, 2006). Similarly, sympathoexcitatory responses to chemoreceptor stimulation require activation of neurons in the RVLM (Koshiya et al., 1993). Thus, the RVLM is critical in the pathway for cardiorespiratory reflex adjustments in response to changes in afferent input.

The nTS sends monosynaptic projections to the RVLM, including both excitatory and inhibitory inputs (Ross et al., 1985; Hancock, 1988; Van Bockstaele et al., 1989; Aicher et al., 1996; Koshiya and Guyenet, 1996). It has been suggested that excitatory synapses within the RVLM contribute to pressor responses to carotid chemoreflex activation and these may arise from direct nTS projections (Van Bockstaele et al., 1989; Aicher et al., 1996; Koshiya and Guyenet, 1996). However, the fundamental properties of these RVLM-projecting nTS neurons are not known. It is important to determine such characteristics because, in distinct populations of nTS projection neurons, the fundamental properties, including afferent processing, are target specific (Bailey et al., 2006). Moreover, the activity of these projection neurons directly influences cardiorespiratory reflex output.

This study was designed to examine the intrinsic characteristics of nTS neurons that project to the RVLM. We hypothesized that (1) given the importance of the RVLM in cardiorespiratory regulation, inputs from cranial visceral

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afferents to RVLM-projecting nTS neurons are monosynaptic and exhibit a high success rate of synaptic transmission; and (2) a subpopulation of RVLM-projecting nTS neurons is activated by acute hypoxia *in vivo*, as indicated by Fos expression. We used the *in vitro* brainstem slice preparation and Fos immunohistochemistry to test these hypotheses. Retrograde tracers were used to identify nTS neurons that project to the RVLM. Results of these studies indicate that RVLM-projecting nTS neurons exhibit heterogeneous firing properties that are influenced by their compliment of potassium currents. These cells receive strong monosynaptic contacts from visceral afferents and some of these contacts originate from the carotid body. In addition, acute hypoxia activates a population of RVLM-projecting nTS neurons that was not catecholaminergic.

EXPERIMENTAL PROCEDURES

Animals

All procedures were conducted in accordance with the guidelines in the NIH "Guide for the Care and Use of Laboratory Animals" and were approved by the University of Missouri Animal Care and Use Committee. Adult male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA, $n=30$; 280±37 g) were maintained on a 12 h light–dark cycle with food and water provided *ad libitum*. All rats were allowed a minimum of 7 days to acclimate to the surroundings prior to any experimental procedure.

Surgical procedures

Rats were anesthetized with Isoflurane [AErane, Baxter, Deerfield, IL, USA (induction: 5% in 100% O_2, 2 L/min; maintenance: 2–2.5%)]. Using aseptic technique, a catheter was inserted into the aorta via the femoral artery and arterial pressure was monitored. Rats then were placed in a stereotaxic apparatus (Kopf, Tujunga, CA, USA), and the dorsal surface of the medulla exposed via a midline incision. Calamus scriptorius (CS, the caudal most portion of the area postrema) was identified and the head deflected downward so that inter-aural zero was 2.4 mm rostral to CS (Kiely and Gordon, 1993; Moffitt et al., 2002), placing the brainstem on the horizontal plane.

Microinjection procedures

Double-barreled glass pipettes (OD 20–30 μm), with one barrel containing L-glutamate (Glu, 10 mM) and the second filled with retrograde tracer were inserted into the brainstem with the aid of a dissecting microscope. The pipette was advanced to the initial target stereotaxic coordinates for the RVLM (anterior–posterior +0.7–0.8 mm, lateral +1.6–1.8 mm, and ventral −3.6–4.2 mm relative to CS and the dorsal surface of the brain; brainstem positioned horizontally). The RVLM was identified functionally by pressor responses (≥10 mm Hg) to microinjection of L-glutamate (10 mM, 30 nl). The retrograde tracer (30 nl) was then microinjected into the same site through the second barrel of the pipette over ~1 min. Microinjections were performed using a custom-built pressure microinjection system. Injection volumes were quantified by monitoring the movement of the meniscus within a pipette barrel of known diameter using a 150× microscope (Rolyn Optics, Corvina, CA, USA) with a calibrated eyepiece micrometer. The retrograde tracer used varied depending upon the experiment. For immunohistochemistry experiments Fluoro-Gold (FG, 2% in dH_2O, Fluorochrome Inc, Denver, CO, USA) was injected. Because FG was not conducive to whole cell recording in the *in vitro* brainstem slice in our hands, brainstem slice electrophysiology experiments utilized fluorescent Retrobeads (LumaFluor, Naples, FL, USA) which have been extensively used in electrophysiological studies and do not alter the physiological properties of recorded neurons (Katz et al., 1984; Dekin et al., 1987). For experiments in which nTS cell neuronal morphology was assessed, cholera toxin B (CtB, 1%) was used. Although not quantified, the distribution of Retrobead-, Fluoro-Gold-, and CtB-labeled cells did not appear different.

Following microinjection of retrograde tracer, the pipette remained in the medullary tissue for at least 5 min to minimize movement of tracer up the injection tract. The arterial catheter was withdrawn and wounds sutured closed. Rats were treated post operatively with s.c. fluids (3 ml, 0.9% saline) and administered Baytril (0.03 ml i.m., Bayer, Shawnee Mission, KS, USA) and Buprenex (0.6 mg/ml s.c., Reckitt Benckiser Pharmaceuticals, Richmond, VA, USA) to prevent infection and for pain management, respectively. Upon recovery from anesthesia animals were returned to their cages.

In a subset of animals, the carotid body was labeled with anterograde tracer to study RVLM-projecting nTS cells which receive contact from chemoreceptor sensory afferents. Briefly, rats were anesthetized with Isoflurane and a midline incision in the neck was made. The carotid body was located, separated from the surrounding tissue, and labeled with anterograde tracer. For electrophysiological studies ($n=3$), the lipophilic dye DiI (Molecular Probes, Eugene, OR, USA) was placed on the carotid body and sealed in place by Kwik-Sil (WPI). For immunohistochemical studies ($n=3$), CtB was microinjected directly into the carotid body. The neck wound was sutured closed. Microinjection of retrograde tracer in the RVLM was then performed.

Brainstem slice preparation and electrophysiology

Seven to ten days after microinjection of retrograde tracer or 4 weeks following combined retrograde tracer and DiI anterograde tracer, brainstem slices containing the nTS were prepared (Kline et al., 2002). Animals were anesthetized with Isoflurane and decapitated. The brainstem was removed and placed in ice-cold low calcium-high magnesium artificial cerebral spinal fluid (aCSF) containing the following (in mM): 124 NaCl, 3 KCl, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 11 D-glucose, 0.4 L-ascorbic acid, 2 $MgCl_2$ and 1 $CaCl_2$, saturated with 95% O_2–5% CO_2, pH 7.4 (300 mOsm). Horizontal slices (~300 μm) were cut using a vibrating microtome (VT 1000S; Leica, Germany). Tissue sections were placed in a superfusion chamber that contained inlet and outlet ports for aCSF flow. The submerged sections were secured with a nylon mesh and superfused at a flow rate of 3–4 ml/min with standard recording aCSF (in mM: 124 NaCl, 3 KCl, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 11 D-glucose, 0.4 L-ascorbic acid, and 2 $CaCl_2$, saturated with 95% O_2–5% CO_2, pH 7.4, 300 mOsm) at 31–33 °C. All recordings were made from fluorescent Retrobead-identified RVLM projecting cells in the caudal nTS (medial and commissural subnuclei), which receives a high density of carotid body afferent and cardiorespiratory innervation (Andresen and Kunze, 1994; Kline et al., 2002). Caudal nTS neurons were visualized using an Olympus BX-51WI microscope (40× magnification, Tokyo, Japan) equipped with fluorescence, differential interface contrast, and an infrared-sensitive camera. The pipette was guided using a piezoelectric micromanipulator (PCS-6000; Burleigh, Victor, NY, USA). Recording electrodes (8250 glass) were filled with a solution containing the following (in mM): 10 NaCl, 130 K-gluconate, 11 EGTA, 1 $CaCl_2$, 10 HEPES, 1 $MgCl_2$, 2 MgATP and 0.2 NaGTP, pH 7.3 (290–295 mOsm, 2.5–3.5 MΩ). For post-recording cell identification, in some experiments the pipette contained 1 mg/ml Alexa Fluor 594 Hydrazide (Molecular Probes, Carlsbad, CA, USA). Following recordings, sections were incubated in 4% paraformaldehyde (2 h), washed in PBS and mounted on a gelatin-coated slide. Morphology was evaluated using the NeuronJ plugin (ver 1.4.1) in ImageJ.