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ALTERED NEUROBIOLOGICAL FUNCTION OF BRAINSTEM HYPOGLOSSAL NEURONS IN DIGEORGE/22Q11.2 DELETION SYNDROME

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Abstract—DiGeorge/22q11.2 Deletion Syndrome (22q11DS) is a common genetic microdeletion syndrome that underlies several neurodevelopmental disorders including autism, attention deficit/hyperactivity disorder, and schizophrenia. In addition to cognitive impairments, those with 22q11DS have disrupted feeding and swallowing from birth onward. This perinatal dysphagia significantly compromises nutritional status, impairs appropriate weight gain, and can lead to life threatening aspiration-based infections. Appropriately timed excitation and inhibition of brainstem hypoglossal motor neurons, which innervate tongue muscles, is essential for proper feeding and swallowing. In this study we have examined changes in hypoglossal motor neuron function in the *LgDel* mouse model of 22q11DS. Hypoglossal motor neurons from *LgDel* mouse pups have action potentials with afterhyperpolarizations, mediated by a large conductance charybdotoxin-sensitive Ca-activated K current, that are significantly shorter in duration and greater in magnitude than those in wild-type pups. In addition, the amplitude, but not frequency, of glutamatergic excitatory glutamatergic postsynaptic currents (EPSCs) is diminished, and GABAergic, but not glycinergic, neurotransmission to hypoglossal motor neurons was reduced in *LgDel* animals. These observations provide a foundation for understanding the neurological changes in hypoglossal motor neuron function and their contribution to swallowing abnormalities that occur in DiGeorge/22q11.2 Deletion Syndrome.

Key words: pediatric dysphagia, hypoglossal, brainstem circuitry, 22q11.2 Deletion/DiGeorge Syndrome.

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Abbreviations: AHP, afterhyperpolarization; ChTX, charybdotoxin; EPSCs, excitatory glutamatergic postsynaptic currents; IPSCs, inhibitory postsynaptic currents; NTS, nucleus tractus solitarius; WT, wild type.

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INTRODUCTION

Dysphagia, difficulties with feeding and swallowing, is a major complication in infants with a broad range of neurodevelopmental disorders (LaMantia et al., 2016). There is a substantially increased frequency of perinatal and pediatric dysphagia associated with DiGeorge/22q11.2 Deletion Syndrome (22q11DS), a common genetic microdeletion syndrome that includes autism, attention deficit/hyperactivity disorder, and schizophrenia. Disrupted feeding and swallowing from birth onward significantly compromises nutritional status, impairs appropriate weight gain, and can lead to life threatening aspiration-based infections of the nasal sinuses, middle ears, and lung in 22q11DS (LaMantia et al., 2016). Unfortunately, however, there are few, if any, therapeutic approaches to alleviate swallowing difficulties and the accompanying significant health problems. Discovery of potential new treatments for dysphagia in 22q11DS is hindered by the paucity of knowledge on the developmental, neuronal or circuit anomalies that contribute to feeding and swallowing dysfunction.

Feeding and swallowing require a complex series of movements that depends upon sequential activation of proximal motor neurons followed by inhibition of more distal motor neurons to ensure appropriately timed, unidirectional food ingestion and movement. Hypoglossal motor neurons, which innervate tongue muscles, are essential for proper sucking, mastication and deglutition/swallowing. These motor neurons receive both excitatory and inhibitory inputs that normally insure their activity is appropriately timed for tongue movements essential in feeding and swallowing. We have demonstrated that the patterning of the hindbrain, including regions that may contribute to the hypoglossal nucleus, and its inputs as well as the hypoglossal nerve are compromised during early development. These changes could result in divergent neuronal specification leading to altered intrinsic excitable properties or circuit modulation of hypoglossal neurons and the broader feeding and swallowing network.

We have examined changes in hypoglossal motor neuron function in the *LgDel* mouse model of 22q11DS. Infant mice with a heterozygous deletion of 28 contiguous genes, orthologous to the minimal critical deletion in human 22q11DS, apparently have feeding and swallowing difficulties. These include reduced weight compared to age- and sex-matched wild-type (WT) littermates from shortly after birth through early maturity, delayed milk emptying from mouth to

esophagus, and aspiration of milk into the nasal pharynx and lungs (LaMantia et al., 2016). Thus, we compared the electrophysiological properties, synaptic neurotransmission and activation of postsynaptic receptors in hypoglossal motor neurons in *LgDel* and WT mouse pups to determine whether compromised patterning and early cranial nerve development is reflected in altered excitability in hypoglossal motor neurons or neurotransmission to these neurons.

EXPERIMENTAL PROCEDURES

All animal experimental procedures were performed in accordance with NIH and Institutional Animal Care and Use Guidelines. A total of 105 animals (64 WT, 41 LD) were used to study hypoglossal motor neuron intrinsic firing characteristics (41 mice), the effect of Ca^{2+}-dependent potassium channel antagonists on after-hyperpolarization potentials (27 mice), as well as spontaneous and miniature inhibitory and excitatory postsynaptic currents (54 mice). Only one neuron was recorded from each animal.

The minimal critical deletion associated with 22q11DS, a 1.5 MB heterozygous deletion on human Chr. 22, is paralleled in *LgDel* mice by an orthologous deletion on murine Chr. 16 (Maynard et al., 2013). Heterozygous adult *LgDel* males(C57BL6N background) were bred to C57BL6N WT females. Thus, the deletion is inherited paternally in all mice analyzed. The day of birth was noted as postnatal day 0 (P0) based on the presence of a litter seen during a daily check of breeding cages. P7-14 pups were selected for electrophysiological experiments in a double-blinded manner. After experiments were performed and data were analyzed the identities of each animal was revealed based upon PCR genotyping (Meechan et al., 2009).

Acute brainstem slice preparation and electrophysiology patch-clamp techniques

On the day of the experiment, pups were anesthetized with the short acting inhalation anesthetic isoflurane and sacrificed by cervical dislocation. The brains were quickly removed, and placed in cold (2 °C) buffer (in mM): NaCl 140, KCl 5, $CaCl_2$ 2, D-Glucose 5, HEPES 10, pH 7.4, equilibrated with 100% O_2. Slices were prepared by vibratome sectioning. The brains were oriented for sectioning with their caudal ends up and their rostral sides attached to an agar block perpendicular to the plane of the blade. The hypoglossal motor neurons in the dorsomedial brainstem were identified in the *in vitro* slice by their unique location and morphology in a transverse 350 microns thick section (as described previously (Wang et al., 2002); see also Fig. 1).

Cut slices were then moved from the bath solution and incubated in the NMDG recovery solution (in mM) NMDG 93, HCl 93, KCl 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25, HEPES 20, D-Glucose 25, $MgSO_4$10, $CaCl_2$ 0.5 bubbled with 95% O_2/5% CO_2 at 34 °C in a water bath for 10 min. The brainstem slices were then moved to a recording chamber and perfused with standard aCSF solution contained (in mM) NaCl 125, KCl 3, $NaHCO_3$ 25, HEPES 5, D-glucose 5, $MgSO_4$ 1, $CaCl_2$ 2 and continuously bubbled with 95% O_2/5% CO_2 to maintain pH at 7.4 at room temperature (22–24 °C). The electrodes were pulled from thin wall glass capillaries (World Precision Instruments, Inc. FL, USA) with a tip resistance of ~3–4 MΩ. Patch pipettes were filled with a pH 7.3 solution of either KCl (150 mM), $MgCl_2$ (4 mM), EGTA (10 mM), Na-ATP (2 mM), HEPES (10 mM) or K-gluconic acid (150 mM), HEPES (10 mM), EGTA (10 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1 mM) to isolate inhibitory or excitatory currents, respectively. Identified hypoglossal motor neurons were voltage-clamped at a holding potential of −80 mV to examine synaptic events. Gabazine (25 μM) was used to block GABAergic inhibitory neurotransmission, strychnine (1 μM) to block glycinergic inhibitory neurotransmission, and D(-)-2amino-5-phosphopentanoic acid (AP5; 20 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μM) to block glutamatergic excitatory neurotransmission in appropriate combinations to isolate glutamatergic excitatory glutamatergic postsynaptic currents (EPSCs), GABAergic and/or glycinergic inhibitory postsynaptic current (IPSCs). Focal drug application was performed using a PV830 Pneumatic PicoPump pressure delivery system (WPI, Sarasota, FL). Drugs were ejected from a patch pipette positioned within 30 μm from the patched hypoglossal motor neurons. The maximum range of drug application has been previously determined to 100–120 μm from the tip the drug pipette forward, and considerably less behind the drug pipette (Wang et al., 2001). To study the effects of Ca activated potassium channel antagonists on the afterhyperpolarization (AHP), apamin (at doses of 100 nM and 200 nM) and charybdotoxin (at concentrations of 1 nM, 10 nM, and 40 nM) were added to the perfusate 15 min after baseline measurements were recorded and were maintained in the perfusate for an additional 15 min.

Visualization of XII MNs by biocytin injection

To visualize and confirm the identity of the recorded hypoglossal motor neurons (XII MNs), we added 0.5% biocytin into the patch pipette solution. At the end of each electrophysiology experiment the brainstem slices were soaked overnight in 4% paraformaldehyde and were then washed with PBS and incubated in streptavidin Alexa Fluor@647 in PBS containing 0.1% Triton X-100. Slices were mounted and cover slipped with prolong anti-fade mounting medium (Invitrogen, Eugene, OR). The brainstem slices containing biocytin-injected XII MNs were imaged by confocal microscopy (Zeiss LSM 710, Thornwood, NY, USA). Serial optical sections from the entire column of the slice were collected in combination with tile scans using a 25x oil objective in a Zeiss 710 confocal system. Imaris software was used to process the image data.

Data analyses

All electrophysiological data were collected and digitized via Clampex (10.2) and analyzed using Clampfit (10.2).