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Research Paper

Accessory respiratory muscles enhance ventilation in ALS model mice and are activated by excitatory V2a neurons

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ABSTRACT

Inspiratory accessory respiratory muscles (ARMs) enhance ventilation when demands are high, such as during exercise and/or pathological conditions. Despite progressive degeneration of phrenic motor neurons innervating the diaphragm, amyotrophic lateral sclerosis (ALS) patients and rodent models are able to maintain ventilation at early stages of disease. In order to assess the contribution of ARMs to respiratory compensation in ALS, we examined the activity of ARMs and ventilation throughout disease progression in $SOD1^{G93A}$ ALS model mice at rest using a combination of electromyography and unrestrained whole body plethysmography. Increased ARM activity, accompanied by increased ventilation, is observed beginning at the onset of symptoms. However, ARM recruitment fails to occur at rest at late stages of disease, even though the same ARMs are used for other behaviors. Using a chemogenetic approach, we demonstrate that a glutamatergic class of neurons in the brainstem and spinal cord, the V2a class, is sufficient to drive increased ARM activity at rest in healthy mice. Additionally, we reveal pathology in the medial reticular formation of the brainstem of $SOD1^{G93A}$ mice using immunohistochemistry and confocal imaging. Both spinal and brainstem V2a neurons degenerate in ALS model mice, accompanied by regional activation of astrocytes and microglia. These results establish inspiratory ARM recruitment as one of the compensatory mechanisms that maintains breathing at early stages of disease and indicate that V2a neuron degeneration may contribute to ARM failure at late stages of disease.

1. Introduction

Amyotrophic lateral sclerosis (ALS) patients and animal models have the remarkable capacity to maintain relatively normal ventilation, despite progressive denervation and dysfunction of the diaphragm (Barneoud et al., 1997; Cappello et al., 2012; Hegedus et al., 2007; Kennel et al., 1996; Valdez et al., 2012), until late in disease progression when ventilation sharply declines (Hardiman, 2011; Nichols et al., 2013; Tankersley et al., 2007). Respiratory failure is the leading cause of death in ALS patients (Hardiman, 2011) and understanding how the respiratory system compensates - and ultimately fails to compensate - for the decline in motor function will be critical in the development of therapeutic interventions to prolong ventilator independence and improve the duration and quality of life of ALS patients.

Multiple compensatory mechanisms likely cooperate to maintain ventilation in ALS (Johnson and Mitchell, 2013; Nichols et al., 2013). One likely example, and the focus of this study, is the compensatory recruitment of inspiratory accessory respiratory muscles (ARMs). The diaphragm is the main inspiratory muscle in healthy mammals at rest. However, inspiratory ARMs, including the trapezius, scalenes, sternocleidomastoid, and external intercostal muscles are typically active during high ventilatory demand, such as exercise, to expand and stabilize the chest wall and enhance respiratory function (Sieck and Gransee, 2012). ARMs can also be recruited for breathing when diaphragm function is impaired, such as in ALS, spinal cord injury, and muscular dystrophy (Johnson and Mitchell, 2013; Pinto and de Carvalho, 2008; Smittkamp et al., 2010), and are sufficient to fully drive respiration in patients with diaphragmatic paralysis (Bennett et al., 2004). Remarkably, one study showed that ALS patients that recruit ARMs at rest sleep better and survive longer than patients with similar diaphragm dysfunction that do not recruit ARMs (Arnulf et al., 2000). It is not clear when during disease progression resting ARM activity begins or how long it might last. We use a unique combination of electromyography (via implanted telemetry devices) and whole body plethysmography to measure ARM activity and ventilation in

Abbreviations: ALS, Amyotrophic Lateral Sclerosis; ARM, Accessory Respiratory Muscle; EMG, Electromyography; mRF, Medial Reticular Formation; WBP, Whole Body Plethysmography; SOD1, Superoxide Dismutase 1; PIF, Peak Inspiratory Flow; TV, Tidal Volume; MV, Minute Volume.

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non-anesthetized, unrestrained mice in the SOD1^{G93A} model of ALS for which the onset and timing of disease progression is predictable, consistent, and well established.

Despite the importance of ARMs for maintaining breathing in a variety of physiological and pathological states, little is known about the neural circuits that drive ARM activity or how they might be altered by ALS pathology. An important step in understanding the circuits that control ARMs is to identify neuron types capable of driving or regulating inspiratory ARM activity. The V2a class of glutamatergic neuron in the spinal cord and brainstem has direct and indirect influences on respiratory and non-respiratory motor neurons (Azim et al., 2014; Bouvier et al., 2015; Bretzner and Brownstone, 2013; Crone et al., 2008; Crone et al., 2012, 2009; Zhong et al., 2010). V2a neurons comprise the majority of glutamatergic reticulospinal neurons in the medial reticular formation (mRF) (Bouvier et al., 2015; Bretzner and Brownstone, 2013). A subset of these neurons may be driven in a speed dependent manner based on the observation that they can be activated by the mesencephalic locomotor region (Bretzner and Brownstone, 2013). In addition, a population of V2a in the mRF have direct projections to the ventral respiratory column and provide excitatory drive to central respiratory circuits essential for normal frequency and regularity of breathing in neonatal mice (Crone et al., 2012). The potential role of V2a neurons in adult respiratory circuits has not yet been tested. Furthermore, spinal V2a neurons, located in the intermediate laminae, are required for the speed-dependent recruitment of locomotor pattern generator neurons as well as for maintaining consistent frequency and amplitude of motor burst activity during locomotion (Ampatzis et al., 2014; Crone et al., 2008; Crone et al., 2009; Kimura et al., 2013; Ljunggren et al., 2014). Because inspiratory ARM activity normally parallels locomotor activity and V2a neurons are important for speed-dependent activation of motor circuits, we hypothesize that a subset of V2a neurons could provide excitatory drive to coordinate and recruit ARM motor neurons (or premotor neurons) for breathing.

The goals of this study are to: 1) characterize the onset, extent and duration of ARM recruitment at rest during ALS-like disease progression in SOD1^{G93A} mice, 2) examine the potential of V2a neurons to drive ARM activity in healthy mice, 3) determine if V2a neurons are adversely affected in SOD1^{G93A} mice. Understanding how neural circuits control ARM activity will provide the necessary foundation for developing therapies targeting ARM circuits to improve breathing in ALS, spinal cord injury, and other pathological conditions.

2. Methods

2.1. Animals

All procedures were performed according to National Institutes of Health guidelines and approved by Cincinnati Children's Hospital Medical Center Animal Care and Use Committee. Plethysmography and/or electromyography was performed on adult male SOD1^{G93A} transgenic mice (B6.Cg-Tg (SOD1*G93 A)1Gur/J; Stock #004,435 Jackson Laboratory, Bar Harbor, ME), adult male non-transgene carrier controls, and adult V2a-CHRM3 mice. V2a-CHRM3 mice were generated by first breeding ROSA$^{PNP\text{-}tTA/+}$ (Stock #008,600 Jackson Laboratory) to TetO-CHRM3/+ (Stock #014,093 Jackson Laboratory). ROSA$^{PNP\text{-}tTA/+}$; TetO-CHRM3/+ offspring were next bred to Chx10$^{Cre/+}$ mice to generate Chx10$^{Cre/+}$; ROSA$^{PNP\text{-}tTA/+}$; TetO-CHRM3/+ experimental mice. Electrophysiology was performed on spinal cord slices from postnatal 11–12 day V2a-CHRM3/tdTomato mice that also contained the ROSA$^{PNP\text{-}tdTomato}$ reporter allele (Chx10$^{Cre/+}$; ROSA$^{PNP\text{-}tTA/PNP\text{-}tdTomato}$; TetO-CHRM3/+ mice).

All immunohistochemical and imaging analysis was performed on adult SOD1^{G93A} transgenic mice (B6SJL-Tg(SOD1*G93 A)1Gur/J; Stock #002,726 Jackson Laboratory) maintained on a mixed 50% ICR and 50% B6SJL genetic background and on adult non-transgene carrier controls of the same genetic background. Additional experiments were performed on SOD1^{G37R} mice (Stock #003,651, Jackson Laboratory) containing a LoxP flanked allele of the SOD1^{G37R} transgene (but these mice were never crossed to a Cre line so the mutant allele is expressed in all cell types). The SOD1^{G37R} mice have later disease onset and slower disease progression compared to the SOD1^{G93A} mice (Boillee et al., 2006). In addition, two mouse lines were used to visualize V2a neurons. First, Chx10$^{CFP/+}$ mice (Crone et al., 2008) were breed to SOD1^{G93A} mice to generate SOD1^{G93A}; Chx10$^{CFP/+}$ mice. Secondly, ROSA$^{PNP\text{-}tdTomato/PNP\text{-}tdTomato}$ mice (Stock #007,914, Jackson Laboratory) were bred to Chx10$^{Cre/+}$ mice to generate Chx10$^{Cre/+}$; ROSA$^{PNP\text{-}tdTomato/+}$ mice (called V2a-tdTomato mice).

2.2. ALS-like Disease Progression

Hindlimb and neurological deficits were assessed in adult male SOD1^{G93A} transgenic and control mice weekly beginning at 11–12 weeks of age. Mice were staged from 0 to 5 based on established methods and observations (Hatzipetros et al., 2015) (see Table 1). At Stage 3, food pellets were placed on mouse cage bedding and supplemental long sipper water bottles were provided. At Stage 4, special care was taken to ensure proper hydration and comfort of mice by providing Napa Nectar, diet gel and subcutaneous saline injections. Mice were sacrificed upon reaching endstage (Stage 5), defined as being unable to right themselves within 30 s after being placed on their back or side, or unable to reach food and water.

2.3. Surgical Implantation of Telemetry Devices and Electromyography

To study accessory respiratory muscle (ARM) recruitment in non-anesthetized, untethered and freely moving mice, subcutaneous surgical implantation of telemetry devices (F20-EET, Data Sciences International (DSI); St. Paul, MN) was performed in adult mice (>24 g bw). All aseptic surgeries were performed in deeply anesthetized mice, indicated by absent pedal withdrawal and corneal reflex, via isoflurane inhalation (induction 4–5%; maintenance 1–2%, both in 100% O_2) on a heated water blanket to maintain body temperature. A right transverse supraclavicular incision, followed by blunt separation of tissue with #2 forceps was used to expose the brachial plexus and phrenic nerve, landmarks used identify the trapezius and middle scalene. Once the desired muscles were exposed, the sterilized transmitter, with two pairs of stainless steel biopotential leads, was inserted subcutaneously and positioned just posterior of the scapulae. The biopotential lead pairs were tunneled to two different ARMs and implanted approximately 1-2 mm apart using a 25G needle guide. To secure the implanted leads, extra lead insulation was applied with cyanoacrylate adhesive (Gluture) to the distal ends following implantation. Care was taken during implantation to ensure that the biopotential leads were fully imbedded into the muscle, with minimal exposed lead. Carprofen (1.0 mg/kg; S.Q.) was administered to minimize postoperative pain. Mice recovered for at least 7 days before experiments began.

To record electromyography (EMG) signals, mice were placed in individual plethysmography chambers (see Whole Body Plethysmography methods) and positioned on telemetry receivers (Model RPC-1;

Table 1
Neurological scoring of ALS-like disease progression in SOD1^{G93A} mice.

Stage	State	Hindlimb Presentation
0	Pre-symptomatic	No notable differences compared to wildtypes.
1	Disease onset	Hindlimb collapse when mouse is suspended from tail.
2	Paresis	Full or partial hindlimb collapse with appearance of tremor.
3	Paralysis onset	Difficulty walking, toe curling and/or foot dragging.
4	Advanced paralysis	Minimal joint movement, hindlimb not being used for forward motion.
5	Endstage	Mouse unable to right itself from side within 30 seconds.