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Research Paper

Cell-type specific expression of constitutively-active Rheb promotes regeneration of bulbospinal respiratory axons following cervical SCI

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ABSTRACT

Damage to respiratory neural circuitry and consequent loss of diaphragm function is a major cause of morbidity and mortality in individuals suffering from traumatic cervical spinal cord injury (SCI). Repair of CNS axons after SCI remains a therapeutic challenge, despite current efforts. SCI disrupts inspiratory signals originating in the rostral ventral respiratory group (rVRG) of the medulla from their phrenic motor neuron (PhMN) targets, resulting in loss of diaphragm function. Using a rat model of cervical hemisection SCI, we aimed to restore rVRG-PhMN-diaphragm circuitry by stimulating regeneration of injured rVRG axons via targeted induction of Rheb (ras homolog enriched in brain), a signaling molecule that regulates neuronal-intrinsic axon growth potential. Following C2 hemisection, we performed intra-rVRG injection of an adeno-associated virus serotype-2 (AAV2) vector that drives expression of a constitutively-active form of Rheb (cRheb). rVRG neuron-specific cRheb expression robustly increased mTOR pathway activity within the transduced rVRG neuron population ipsilateral to the hemisection, as assessed by levels of phosphorylated ribosomal S6 kinase. By co-injecting our novel AAV2-mCherry/WGA anterograde/trans-synaptic axonal tracer into rVRG, we found that cRheb expression promoted regeneration of injured rVRG axons into the lesion site, while we observed no rVRG axon regrowth with AAV2-GFP control. AAV2-cRheb also significantly reduced rVRG axonal dieback within the intact spinal cord rostral to the lesion. However, cRheb expression did not promote any recovery of ipsilateral hemi-diaphragm function, as assessed by inspiratory electromyography (EMG) burst amplitudes. This lack of functional recovery was likely because regrowing rVRG fibers did not extend back into the caudal spinal cord to synaptically reinnervate PhMNs that we retrogradely-labeled with cholera toxin B from the ipsilateral hemi-diaphragm. Our findings demonstrate that enhancing neuronal-intrinsic axon growth capacity can promote regeneration of injured bulbospinal respiratory axons after SCI, but this strategy may need to be combined with other manipulations to achieve reconnection of damaged neural circuitry and ultimately recovery of diaphragm function.

1. Introduction

Regeneration of central nervous system (CNS) axons after spinal cord injury (SCI) remains a clinical challenge, and while studies have shown that CNS regeneration is possible, reconnection to original secondary neuronal targets remains, as a whole, elusive (Alilain et al., 2011; Cafferty et al., 2008; Mantilla, 2017; Park et al., 2010; Vinit et al., 2006). The lack of a robust axonal regrowth response plays a significant role in persistent functional deficits following SCI. Furthermore, targeted regeneration within the CNS is important for repair of motor, sensory and other functions post-SCI as incorrect targeting of growing axons could not only result in failure to restore damaged circuitry, but

Abbreviations: AAV2, adeno-associated virus serotype-2; C2 (3, 4, 5, etc.), cervical spinal cord level 2 (3, 4, 5, etc.); CMAP, compound muscle action potential; CNS, central nervous system; CTB, cholera toxin subunit B; EMG, electromyography; GAP, GTPase-activating protein; HA, hemagglutinin tag; mTOR, mechanistic target of rapamycin; NMJ, neuromuscular junction; PhMN, phrenic motor neuron; pS6k, phosphorylated ribosomal S6 kinase; PTEN, phosphatase and tensin homolog; rVRG, rostral ventral respiratory group; Rheb, ras homolog enriched in brain; cRheb, constitutively-active Rheb; SCI, spinal cord injury; TSC1/2, tumor sclerosis complex protein 1/2

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also in unwanted side effects (Harel and Strittmatter, 2006). A number of factors, both neuronal-intrinsic and -extrinsic, impede axon regeneration following CNS injury (Busch and Silver, 2007; Cregg et al., 2014; Dell'Anno and Strittmatter, 2017; Fitch and Silver, 2008; Gervasi et al., 2008; Yiu and He, 2006). Low endogenous axonal growth capacity represents a critical impediment inhibiting regeneration of most adult CNS neurons (Lu et al., 2014; Ribas and Costa, 2017). In particular, the mammalian target of rapamycin (mTOR) pathway, a master regulator of cellular growth, protein synthesis and axon development, remains in an inactive state following CNS damage (Liu et al., 2010; Park et al., 2008). Previous studies suggest that altering the expression of molecules within this signaling axis such as genetic knockout of phosphatase and tensin homolog (PTEN) and overexpression of ras homolog enriched in brain (Rheb) can lead to robust regeneration of certain neuronal populations (Danilov and Steward, 2015; Gutilla and Steward, 2016; Liu et al., 2010; Ohtake et al., 2015; Park et al., 2008; Zukor et al., 2013). Rheb directly binds to the mTOR complex, and the active GTP-bound form of Rheb is required for mTOR activation (Durán and Hall, 2012; Long et al., 2005). Additionally, inhibition of this Rheb-mTOR pathway is mediated by the tumor sclerosis complex proteins 1/2 (TSC1/2) (Zhang et al., 2003), which function as GTPase-activating proteins (GAPs) to convert the GTP-bound Rheb to an inactivated GDP-bound form. Altering the expression pattern and function of these molecules within the mTOR pathway has the potential to induce regrowth of damaged axons (including back to their original targets), ultimately resulting in functional restoration.

SCI is a devastating disorder characterized by damage to ascending, descending and intra-segmental neuronal connections. Individuals suffering from cervical injuries often experience difficulty breathing (as well as other pulmonary deficits) due to loss of medullary input to spinal cord, ultimately resulting in partial-to-complete paralysis of diaphragm, a major inspiratory muscle (Vinit and Kastner, 2009). Consequently, many cervical SCI patients must be mechanically ventilated, which leads to enhanced morbidity as well as increased mortality as a result of complications such as respiratory infection (University of Alabama, 2016).

Diaphragm activation is directly controlled by phrenic motor neurons (PhMNs) located within the ventral horn at C3 to C5 levels of the spinal cord (Dobbins and Feldman, 1994). PhMNs receive rhythmic descending glutamatergic input from bulbospinal axons originating in the rostral ventral respiratory group (rVRG) of the medulla; this rVRG-PhMN circuitry is primarily monosynaptic (Lipski et al., 1994). Though some rVRG axons do cross to the contralateral PhMN pool, these connections appear to be latent in intact conditions (Ghali, 2017). Cervical SCI disrupts rVRG axonal input to their PhMN targets and consequently produces loss of hemi-diaphragm function (Vinit et al., 2006). Restoration of this circuitry remains a critical, though elusive, therapeutic goal.

Adeno-associated viruses (AAVs) are powerful tools for altering the expression of specific genes in a defined neuronal population within the CNS. In the current study, we tested the effects on rVRG regeneration of expressing a constitutively-active form of Rheb (AAV2-cRheb) selectively in axotomized rVRG neurons following C2 hemisection SCI. As Rheb is highly unstable in the GDP-bound state, we generated an AAV2 construct to express a mutated GTP-bound active form of the Rheb protein. With this strategy, we aimed to enhance the neuronal-intrinsic capacity of rVRG neurons to repair damaged respiratory circuitry and restore diaphragm function after cervical SCI.

2. Materials and methods

2.1. Animals

Female Sprague-Dawley rats weighing 250–300 g were purchased from Taconic Farms. All animals were housed three animals per cage in temperature, humidity, and light controlled environments with ad libitum access to food and water. Experimental procedures were approved by the Thomas Jefferson University IACUC and conducted in compliance with ARRIVE (*Animal Research: Reporting of In Vivo Experiments*) guidelines.

2.2. C2 hemisection SCI

Rats were deeply anesthetized with an intraperitoneal injection of a cocktail of ketamine HCl (95.0 mg/kg) (Vedco, Saint Joseph, Missouri), xylazine (10.0 mg/kg) (Lloyd Laboratories, Quezon City, Metro Manila, Philippines) and acepromazine (0.075 mg/kg) (Phoenix Pharm Inc., Burlingame, California). Once the rats were completely anesthetized, assessed via loss of toe pinch reflex and orbital reflex, the dorsal surface of the skin was shaved and disinfected with a 70% ethanol solution and topical iodine (Dynarex, Orangeburg, New York). Using a sterile #11 surgical blade (Electron Microscopy Sciences, Hatfield, Pennsylvania), a one-inch midline incision was made on the dorsal surface of skin and muscle starting from the caudal portion of the occipital bone. Retractors were then used to expose the dorsal surface of the C2 and C3 vertebrae. Using rongeurs (Fine Science Tools, Foster City, California), remaining tissue was removed from the vertebrae and a laminectomy was performed to expose the spinal cord. The C2 and C3 dorsal roots were located, and a hemisection was performed at a location just caudal to the C2 root with a dissecting knife (Fine Science Tools, Foster City, California). To ensure a complete hemisection, a 30-gauge needle (BD Biosciences, San Jose, California) was passed through the injury several times. Following complete hemisection, the dorsal muscle layers were sutured with 4–0 silk sutures (Covidien, Minneapolis, Minnesota), and the skin was stapled closed with surgical staples (Braintree Scientific, Braintree, Massachusetts). The surface of the skin was treated with a topical iodine solution. Immediately following the procedure, rats were treated with 5 mL subcutaneous injections of Lactated Ringer's solution (Hospira, San Jose, California), buprenorphine hydrochloride (0.05 mg/kg) (Hospira, San Jose, California) and cefazolin (6 mg) (Hospira, San Jose, California). Rats were then placed in a clean cage on a surgical heating pad set to 37 °C (Gaymar, Orchard Park, New York) and monitored until fully recovered from anesthesia. At both 12 and 24 h after surgery, each rat was given an additional dose of buprenorphine hydrochloride (0.05 mg/kg) and 5 mL of Lactated Ringer's solution and monitored for pain/distress. Rats were subsequently monitored daily for pain, as well as for forepaw and hindpaw autophagy.

2.3. rVRG injection

Rats were anesthetized with a mixture of ketamine/xylazine/acepromazine. Previous surgical staples were removed using a reflex clip remover. The skin was shaved and then disinfected with a 70% ethanol solution and topical iodine. Using a sterile #11 surgical blade, a half-inch incision was made on the dorsal surface of the skin at the occipital bone, and surgical scissors (Fine Science Tools, Foster City, California) were used to separate the superficial muscle, exposing the occipital bone and C1 vertebrae. The skin and muscles were separated with a surgical retractor (Fine Science Tools, Foster City, California), and using a surgical blade and rongeurs, the ligament between the occipital bone and the C1 vertebrae was excised followed by removal of the caudal portion of the occipital bone revealing the obex. The animals were then placed on a stereotaxic frame (Kopf Instruments, Tujunga, California). Using the obex as a starting point, the rVRG was located by the following coordinates: 2.0 mm lateral, 1.0 mm rostral, and 2.6 mm ventral. Once properly placed, an UltraMicroPump (World Precision Instruments, Sarasota, Florida) injection system was used to inject 0.3 μL of total volume using a microsyringe (Hamilton, Reno, Nevada) and a Micro4 Microsyringe Pump Controller (World Precision Instruments, Sarasota, Florida). Immediately following injection, the needle was left in place for 5 min before being slowly removed from the medulla. The retractor was removed, the muscles were sutured with