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Regular Article

Riluzole promotes motor and respiratory recovery associated with enhanced neuronal survival and function following high cervical spinal hemisection

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ABSTRACT

Cervical spinal cord injury (SCI) can result in devastating functional deficits that involve the respiratory and hand function. The mammalian spinal cord has limited ability to regenerate and restore meaningful functional recovery following SCI. Riluzole, 2-amino-6-trifluoromethoxybenzothiazole, an anti-glutamatergic drug has been shown to reduce excitotoxicity and confer neuroprotection at the site of injury following experimental SCI. Based on promising preclinical studies, riluzole is currently under Phase III clinical trial for the treatment of SCI (ClinicalTrials.gov: NCT01597518). Riluzole's anti-glutamatergic role has the potential to regulate neuronal function and provide neuroprotection and influence glutamatergic connections distal to the initial injury leading to enhanced functional recovery following SCI. In order to investigate this novel role of riluzole we used a high cervical hemisection model of SCI, which interrupts all descending input to motoneurons innervating the ipsilateral forelimb and diaphragm muscles. Following C2 spinal cord hemisection, animals were placed into one of two groups: one group received riluzole (8 mg/kg) 1 h after injury and every 12 h thereafter for 7 days at 6 mg/kg, while the second group of injured rats received vehicle solution for the same duration of time. A third group of sham injured rats underwent a C2 laminectomy without hemisection and served as uninjured control rats. Interestingly, this study reports a significant loss of motoneurons within the cervical spinal cord caudal to C2 hemisection injury. Disruption of descending input led to a decrease in glutamatergic synapses and motoneurons caudal to the injury while riluzole treatment significantly limited this decline. Functionally, Hoffmann reflex recordings revealed an increase in the excitability of the remaining ipsilateral cervical motoneurons and significant improvements in skilled and unskilled forelimb function and respiratory motor function in the riluzole-treated animals. In conclusion, using a C2 hemisection injury model, this study provides novel evidence of motoneuron loss caudal to the injury and supports riluzole's capacity to promote neuronal preservation and function of neural network caudal to the SCI resulting in early and sustained functional improvements.

1. Introduction

High cervical spinal cord injury (SCI) disrupts supraspinal connections to lower motoneurons and spinal circuitry leading to significant loss of sensory, motor and respiratory function. Majority of injuries to the spinal cord occur at the cervical level and are incomplete, with spared axonal connections to cervical respiratory and somatic motoneurons from supraspinal centers. The central nervous system shows limited capacity to regenerate following partial injuries (Kerschensteiner et al., 2004; Raineteau et al., 2002), however, spontaneous locomotor and respiratory recovery can occur to a limited extent associated with various forms of anatomical and functional plasticity within the caudal cervical region (Lane et al., 2008). This spontaneous plasticity-mediated recovery in itself is not sufficient and leads to limited functional recovery and, as such, effective therapeutic strategies are needed to promote sensory, motor and autonomic functional recovery following SCI (Dougherty et al., 2012). Further, many strategies to strengthen connectivity have presumed that motoneurons within the cervical spinal cord remain intact after high cervical hemisection injury. The fate of motoneurons within the cervical neural circuitry distal to a left C2 hemisection injury is not known. Synergistic neural tissue protection and promotion of structural and functional plasticity of the spared circuitry caudal to the injury is a promising option for restoring meaningful functional recovery.

Loss of descending excitatory input to motoneurons caudal to the injury site leads to the paralysis of muscles innervated by these unexcitable motoneurons. Based on promising preclinical data, riluzole, the only FDA approved drug for ALS and a glutamatergic modulator, is currently in Phase III clinical trial for the treatment of SCI (Grossman

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et al., 2014; Wilson and Fehlings, 2014). Using rodent models of SCI, riluzole has been demonstrated to increase tissue preservation at the site of injury leading to locomotor functional recovery (Wu et al., 2013; Schwartz and Fehlings, 2001). In the CNS, riluzole inhibits glutamate release and promotes reuptake by astrocytes resulting in improved glutamatergic regulation (Anon., 2003; Frizzo et al., 2004; Heurteaux et al., 1994; Kim et al., 2007; Kniest et al., 2001; Pereira et al., 2014). In addition, riluzole also has the ability to modulate synaptic activation of the ionotropic glutamate receptors N-methyl-D-aspartate (NMDA) and 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid (AMPA). Both NMDA and AMPA receptor modulation of neurons play a key role in synaptic plasticity (Malinow and Malenka, 2002; Mantilla et al., 2012). At the acute stage, in the injured spinal cord, riluzole's role in decreasing intraspinal glutamate levels and excitotoxicity to promote tissue preservation has been demonstrated by our laboratory and others. Tissue preservation can be a significant mediator of functional recovery following SCI as it has been demonstrated that even a small amount of tissue preservation can lead to substantial functional recovery (Fehlings and Tator, 1995). However, riluzole's ability to modulate synaptic glutamate receptors to enhance synaptic strength, amplify motoneuron excitability and protect motoneurons caudal to the injury site has not been explored following SCI. Further examination and understanding of these mechanisms is important to optimize its use in the clinic.

Here, we interrogated the hypothesis that high cervical SCI mediated decrease in synaptic input onto caudal motoneurons leads to a decrease in their excitability and survival. Most importantly, for the first time this study reveals that a sharp hemisection injury to the cervical spinal cord at C2 leads to significant loss of motoneurons within a specified region of the cervical enlargement. Also, this study demonstrates that acute and subacute administration of riluzole following C2 hemisection partially restores respiratory related diaphragmatic function and promotes forelimb motor recovery by preventing this loss of motoneurons and enhancing their glutamate receptor-mediated connectivity.

2. Materials and methods

All experimental procedures were performed on male Wistar rats (2–3 months; 300–325 g; Jackson laboratories). Animal care and handling were conducted with the approval of the animal care committee of the University Health Network in accordance with the policies outlined in the guide on the care and use of experimental animals prepared by the Canadian Council of Animal Care. Rats were housed individually with free access to food and water.

2.1. Experimental design

40 rats were subjected to complete left hemisection of the spinal cord from the midline below the C2 dorsal roots. Injured animals were placed into one of two groups: one group of animals received intraperitoneal (IP) administration of riluzole (Sigma, R116) (8 mg/kg) 1 h post-C2 hemisection injury and every 12 h thereafter for 7 days at 6 mg/kg ($n = 18$). A second group of injured rats served as vehicle treated controls and were administered vehicle solution 1 h post-injury and twice daily (IP) for the same duration of time ($n = 17$). Riluzole was dissolved in 30% (*w*/*v*) of solubilizer 2-hydroxypropyl-β-cyclodextrin (HBC, Sigma, H-107). A third group of sham injured rats underwent a C2 laminectomy without hemisection and served as uninjured control rats ($n = 5$). An investigator blinded to the experimental groups prepared all experimental drugs and the dose of riluzole used in this study was based on our previous work on the pharmacodynamic and kinetic properties of riluzole (Wu et al., 2013). Spontaneous and riluzole-induced changes in skilled and unskilled forelimb function were monitored for 6 weeks post-injury. Terminal electrophysiological experiments were performed at 2 and 6 weeks to evaluate respiratory functional recovery and motoneuron excitability, respectively (Fig. 1).

2.2. Spinal cord injury: C2 hemisection

35 rats were anesthetized with 2% isoflurane in oxygen (1 l/min) and placed in a stereotaxic frame on a heating pad at 37 °C. Under aseptic conditions, following a midline dorsal incision the superficial muscles were retracted to expose the dorsal spinal cord. Following a C2 laminectomy and durotomy, the left side of the spinal cord was hemisected from the midline extending to the lateral spinal cord using microscissors (Kajana and Goshgarian, 2008). The hemisection was just caudal to the dorsal roots of the second cervical segment and the dorsal roots were not injured. A surgical probe was used to ensure anatomical completeness of the hemisection by scraping the floor and walls of the vertebral canal adjacent to the lesion. Following surgery, all animals received analgesic (Buprenorphine 0.5 mg/kg; twice daily for 48 h) and 10.0 ml of saline subcutaneously. Animals were placed under heat lamps in individual cages to recover during post-operative recovery period. The rats were maintained in a 12-h light/dark cycle in a temperature-controlled room at 27 °C for the duration of the experiment. Spinal tissue at the site of injury was stained with hematoxylin and eosin to confirm the completeness of the hemisection injury. In order to verify the loss of cervical motoneurons, a left C2 hemisection injury or sham surgery was performed in a group of ChAT-eGFP transgenic mice that express the green fluorescence protein (GFP) under the control of choline acetyltransferase promotor (Jackson Laboratory B6.Cg-Tg(RP23-268L19-EGFP)2Mik/J). This transgenic mouse line allows for fluorescent visualization of cholinergic motoneurons and cholinergic interneurons in the neonate as well as in adult mice. 6 weeks after the left C2 hemisection or sham surgery, the spinal cord from C2 to T3 was examined for expression of eGFP expressing neurons in the ventral horn.

2.3. Functional assessments

Investigators blinded to treatment groups carried out all of the neurobehavioral assessments and analysis from day one until 6 weeks post-injury.

2.4. Forelimb grip strength assessment

Ipsilateral and contralateral forelimb grip strengths were individually evaluated using a grip strength meter (Grip Strength System, DFM-1d; San Diego Instruments, San Diego, CA) every other day during the first 2 weeks (at days 1, 3, 5, 7, 9, 11, 13, and 15) and once per week during weeks 3, 4, 5 and 6. The animals were held parallel to a series of metal grip bars and allowed to grasp the bar individually with the ipsilateral alone and contralateral forelimb. After grasping the bar, animals were pulled away parallel to the degree they were held at until they released the bar and their grip force was measured. Ipsilateral and contralateral grip strengths were tested five times per session. The average ipsilateral and contralateral grip strength in grams of force was recorded for ipsilateral and contralateral forelimbs of each animal at each assessment.

2.5. Paw placement test

Spontaneous forelimb usage was evaluated during explorative behavior in a transparent cylinder of 20 cm in diameter and 30 cm in height at 2, 4 and 6 weeks after injury. A mirror placed behind the cylinder was used to observe forelimb movements from all angles. The use of the cylinder encouraged vertical exploration using the forelimb and the behavior was recorded and analyzed offline in slow motion. The number of times the animal reached up and placed its ipsilateral forelimb, contralateral forelimb or both forelimbs was recorded. Ipsilateral and contralateral forelimb placements were represented as the percentage of the total number of paw placements [% ipsilateral/contralateral forelimb placement = (ipsilateral/contralateral forelimb placement + both forelimb placement) / total forelimb placements × 100].