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Research Paper

Stimulation-dependent remodeling of the corticospinal tract requires reactivation of growth-promoting developmental signaling pathways

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ABSTRACT

The corticospinal tract (CST) can become damaged after spinal cord injury or stroke, resulting in weakness or paralysis. Repair of the damaged CST is limited because mature CST axons fail to regenerate, which is partly because the intrinsic axon growth capacity is downregulated in maturity. Whereas CST axons sprout after injury, this is insufficient to recover lost functions. Chronic motor cortex (MCX) electrical stimulation is a neuromodulatory strategy to promote CST axon sprouting, leading to functional recovery after CST lesion. Here we examine the molecular mechanisms of stimulation-dependent CST axonal sprouting and synapse formation. MCX stimulation rapidly upregulates mTOR and Jak/Stat signaling in the corticospinal system. Chronic stimulation, which leads to CST sprouting and increased CST presynaptic sites, further enhances mTOR and Jak/Stat activity. Importantly, chronic stimulation shifts the equilibrium of the mTOR repressor PTEN to the inactive phosphorylated form suggesting a molecular transition to an axon growth state. We blocked each signaling pathway selectively to determine potential differential contributions to axonal outgrowth and synapse formation. mTOR blockade prevented stimulation-dependent axon sprouting. Surprisingly, Jak/Stat blockade did not abrogate sprouting, but instead prevented the increase in CST presynaptic sites produced by chronic MCX stimulation. Chronic stimulation increased the number of spinal neurons expressing the neural activity marker cFos. Jak/Stat blockade prevented the increase in cFos-expressing neurons after chronic stimulation, confirming an important role for Jak/Stat signaling in activity-dependent CST synapse formation. MCX stimulation is a neuromodulatory repair strategy that reactivates distinct developmentally-regulated signaling pathways for axonal outgrowth and synapse formation.

1. Introduction

Brain and spinal cord injury (SCI) interrupt neural connections between the motor centers that initiate and regulate movements from the spinal circuits that produce movement, resulting in weakness or paralysis. To abrogate these motor impairments, we must reconnect the brain and spinal cord. The corticospinal tract (CST) is a key motor pathway for controlling movements (for review, see (Lemon, 2008; Porter and Lemon, 1993)) and an important target for repair (Baker et al., 2015; Carmel and Martin, 2014). Adult mammalian neurons have a limited ability to regenerate damaged axons due to the developmental loss of an intrinsic capacity to initiate axonal outgrowth (Jacobson et al., 1986; Park et al., 2008; De-Fraja et al., 1998; Hoffman, 1989; Laub et al., 2005; Sun and He, 2010). And what limited capacity that remains may be further reduced by extrinsic inhibitory factors in the damaged environment (Caroni and Schwab, 1988; Fitch and Silver, 2008; McKerracher et al., 1994; Mukhopadhyay et al., 1994). Although unable to regenerate lost axons, undamaged CST neurons have some capacity for local axonal outgrowth or sprouting. Since brain or spinal cord injury is usually incomplete (Chen et al., 2016; Raineteau and Schwab, 2001), the remaining spared CST axons can grow collaterals in response to the injury and contribute to functional recovery (Brus-Ramer et al., 2007; Thallmair et al., 1998; Weidner et al., 2001). Unfortunately, this innate sprouting capacity also is limited; without further intervention, injury-dependent sprouting is insufficient to restore control after severe injury (Carmel et al., 2010; Maier et al., 2008).

CST axonal outgrowth and synapse formation is regulated by neural activity during development (Friel and Martin, 2007b; Salimi et al.,

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2004; Salimi et al., 2008). Activity can be exploited in the adult brain to promote CST axonal outgrowth. Chronic daily electrical stimulation of the motor cortex (MCX), the principal source of CST neurons (Lemon, 2008), is a neuromodulatory strategy that augments contralateral and ipsilateral CST axon sprouting in intact adult rats and is additive when applied to rats with unilateral pyramidotomy (Brus-Ramer et al., 2007). This stimulation-dependent ipsilateral CST sprouting, as well as sprouting into brain stem nuclei is sufficiently robust following chronic MCX stimulation to restore movements after injury (Carmel et al., 2010; Carmel et al., 2013). Moreover, reversible inactivation of the ipsilateral MCX in chronically-injured animals reinstates the motor impairment (Carmel et al., 2014), showing a key role for the ipsilateral MCX descending projection in motor recovery. Thus, MCX stimulation is a promising neuromodulatory strategy that can be harnessed for CST repair after injury.

In this study we examine the molecular mechanisms of MCX stimulation-dependent CST axonal sprouting and synapse formation. To eliminate the contributions of injury-dependent sprouting, we studied activity-dependent CST axonal sprouting by applying electrical stimulation to the MCX of uninjured adult rats. We focus on the capacity for electrical stimulation to upregulate neuron-intrinsic factors for CST axon sprouting. Whereas there are many targets for enhancing intrinsic factors to augment axonal outgrowth (Blackmore et al., 2012; Moore et al., 2009), we examine the mTOR and Jak/Stat pathways, which are highly active during development and are downregulated in maturity (Dziennis and Alkayed, 2008; Hentges et al., 2001; Lipton and Sahin, 2014; Takeda et al., 1997). Signaling in these pathways is critically involved in injury-driven sprouting and axon regeneration. In maturity mTOR activation by downregulation of the repressor PTEN leads to CST axon regeneration (Geoffroy et al., 2015; Lee et al., 2014; Park et al., 2008; Sun et al., 2011). Stat3 activation in maturity, via SOCS3 deletion, results in optic nerve axon regeneration (Muller et al., 2009; Smith et al., 2009; Sun et al., 2011). We hypothesize that mTOR and Stat3 are upregulated by MCX electrical stimulation and are necessary for the consequent CST axonal sprouting and synapse formation.

We show that stimulation acutely activated the mTOR and Jak/Stat pathways in the corticospinal system. Chronic stimulation, which leads to CST axon sprouting, shifted the equilibrium of the mTOR repressor PTEN to an inactive phosphorylated form, as well as further enhanced mTOR and pStat3 activity. This suggests a molecular transition to an axon growth state. Selective pathway blockade revealed that mTOR activation is necessary for CST activity-dependent sprouting but, surprisingly, that Stat3 is necessary for formation of CST presynaptic sites/synapses, not axon sprouting. MCX stimulation is a neuromodulatory repair strategy that recapitulates key intracellular physiological conditions to activate developmentally-regulated molecules for axonal outgrowth and synapse formation.

2. Materials and method

2.1. Animal groups

All animal experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All animal protocols were approved by the City College Institutional Animal Care and Use Committee. Adult female Sprague Dawley rats weighing between 225 and 275 g were housed under a 12 h light/dark cycle in the institutional vivarium and provided with water and food. All surgeries were performed under general anesthesia (90 mg/kg ketamine, 10 mg/kg xylazine) administered intraperitoneally in aseptic condition.

2.2. Anterograde labeling of CST axons

Rats were anesthetized and placed in a stereotaxic frame (Kopf Instruments). Body temperature was maintained at 37 °C by a heating blanket. Following craniotomy to expose the forelimb primary MCX, 7 pressure injections (300ηL/each) of biotinylated dextran amine (BDA; 10,000 MW; Molecular Probes; 10% in 0.1 M PBS) were made in the forelimb MCX (depth 1.5 mm, lateral 2.5 to 3.5 mm; rostral: 0.5 to 2.5 mm; separated by 0.5 mm each injection site) using a Hamilton syringe (33 gauge needle). Tracer was injected using a micropump (WPI). Animals survived for two weeks.

2.3. Electrode implantation and stimulation

After completing tracer injections, a stainless steel stimulating electrode was placed over the dura covering the forelimb MCX (Brus-Ramer et al., 2007). The electrode wires were bent into an "L" shape with the bottom of the L deinsulated. We placed the angle of the L-shaped electrodes 2 mm and 3.5 mm lateral to bregma, respectively, with the exposed contact extending rostrally over the forelimb M1. To confirm electrode placement, we stimulated the MCX to provoke a motor response. A constant current stimulator (A-M systems) was used to deliver trains of stimuli (0.2 msec duration, 333 Hz, 45 msec, every 2 s). We adjusted the current to the minimal value to evoke a contralateral forelimb movement, which was typically (1.1 to 2 mAmps); no ipsilateral forelimb movement was observed. The electrode was secured using microscrews (Plastics One) and dental acrylic cement.

Rats were stimulated 6 h/day for either one or ten days (Brus-Ramer et al., 2007; Carmel et al., 2010) using the same stimulation parameters described above. Animals were awake for daily stimulation and were attached to the stimulator via a commutator (Plastics One) to prevent the wires from twisting as they moved about the cage. Initial threshold current for stimulation to evoke forelimb movement was determined and stimulation continued at this current for the duration of the 6 h period. For our 10-day stimulation, the threshold current was checked daily. Whereas there were day-to-day fluctuations (e.g., in the range of 0.1–0.3 mAmp), we did not observe a trend to higher or lower thresholds.

2.4. Pathway blockade

The functional requirement of the mTOR and the JAK/STAT pathways in CST sprouting and CST synapse formation were examined by blocking these pathways separately. We used rapamycin (10 mg/kg, IP, every 48 h; LC Labs) to block mTOR signaling (Park et al., 2008) and AG490 (20 mg/kg, IP, daily, LC Labs) to block JAK/STAT signaling (Iwamaru et al., 2007).

2.5. Western blotting

For Western blotting and quantitative PCR, animals were euthanized within 30 min after cessation of stimulation. The stimulated and non-stimulated MCXs were dissected into ice cold HBBS (Hank's balanced salt solution; Invitrogen). The forelimb M1 region was placed in a microtube and homogenized promptly in N-PER buffer (Invitrogen) supplemented with a protease-inhibiting cocktail (Invitrogen). Bradford protein analysis was carried out in order to measure protein concentration and approximately 50 μg of protein was subjected to SDS-acrylamide-bisacrylamide gel electrophoreses along with a protein ladder (Crystalgen) for size comparison. Following electrophoresis, the protein bands were transferred to PVDF membrane (Millipore) and probed against the primary antibodies. An ECL chemiluminescent system (Cell Signaling Technology) was used to develop the Western blots. The following primary antibodies were used: phos-S6 (S235/S236) at 1:1000; total S6 at 1:1000; phos-Stat3 (Y705) at 1:500; total Stat3 at 1:1000 (Cell Signaling Technology); phos-PTEN (S380) at 1:500; total PTEN at 1:1000 (Santa Cruz Biotech); and GAPDH at1:5000 (Sigma Aldrich). Secondary antibodies include anti-mouse and anti-rabbit at 1:10,1000 (Vector Laboratories).