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Inhibition of CRMP2 phosphorylation repairs CNS by regulating neurotrophic and inhibitory responses

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ABSTRACT

Central nervous system (CNS) regeneration is restricted by both the lack of neurotrophic responses and the presence of inhibitory factors. As of yet, a common mediator of these two pathways has not been identified. Microtubule dynamics is responsible for several key processes after CNS injuries: intracellular trafficking of receptors for neurotrophic factors, axonal retraction by inhibitory factors, and secondary tissue damages by inflammation and scarring. Kinases regulating microtubule organization, such as Cdk5 or GSK3β, may play pivotal roles during CNS recovery, but the molecular mechanisms remain to be elucidated. Collapsin response mediator protein 2 (CRMP2) stabilizes cytoskeletal polymerization, while CRMP2 phosphorylation by Cdk5 and GSK3β loses its affinity for cytoskeleton proteins, leading to the inhibition of axonal growth. Here, we characterized CRMP2 phosphorylation as the first crucial factor regulating neurotrophic and inhibitory responses after spinal cord injury (SCI). We found that pharmacological inhibition of GSK3β enhanced brain-derived neurotrophic factor (BDNF)-induced axonal growth response in cultured dorsal root ganglion (DRG) neurons. DRG neurons from CRMP2 knock-in (*Crmp2*KI/KI) mice, where CRMP2 phosphorylation was eliminated, showed elevated sensitivity to BDNF as well. Additionally, cultured *Crmp2*KI/KI neurons exhibited suppressed axonal growth inhibition by chondroitin sulfate proteoglycan (CSPG). These data suggest a couple of new molecular insights: the BDNF/GSK3β/CRMP2 and CSPG/GSK3β/CRMP2 pathways. Next, we tested the significance of CRMP2 phosphorylation after CNS injury in vivo. The phosphorylation level of CRMP2 was enhanced in the injured spinal cord. *Crmp2*KI/KI mice exhibited prominent recovery of locomotive and nociceptive functions after SCI, which correlated with the enhanced axonal growth of the motor and sensory neurons. Neuroprotective effects against SCI, such as microtubule stabilization, reduced inflammation, and suppressed scarring were also observed by inhibiting CRMP2 phosphorylation. Therefore, inhibition of CRMP2 phosphorylation demonstrates the unique potential to repair SCI by both enhancing sensitivity to BDNF and reducing inhibitory responses.

1. Introduction

The lack of neurotrophic responses and the presence of inhibitory molecules are two main obstacles limiting functional recovery after injury in the adult mammalian central nervous system (CNS). In recent decades, a number of inhibitory molecules for CNS regeneration have been suggested, including chondroitin sulfate proteoglycans (CSPGs) (Bradbury et al., 2002; Silver and Miller, 2004), and Semaphorin 3A (Sema3A) (Kaneko et al., 2006) derived from scar tissue surrounding the injury site, and myelin-associated inhibitors (MAIs) within CNS myelin (Yiu and He, 2006). In contrast, neurotrophins, e.g., brain-derived neurotrophic factor (BDNF), have been suggested to support the growth-supporting properties of axonal growth after injury (Romero et al., 2001; Thuret et al., 2006). Although combinatorial treatments have induced remarkable recovery after CNS injury in rodents (Alilain et al., 2011; Lu et al., 2012), clinical translation will be difficult due to the multiple drugs, enzymes, and interventions involved.

The lack of appropriate growth factor receptors in injured axons causes insufficiency in neurotrophic responses (Thuret et al., 2006), and receptor trafficking is strictly regulated by microtubule-dependent exocytic vesicle transport (Segal, 2003). Inhibitory responses after injury induce microtubule destabilization to inhibit CNS regeneration (Ruschel et al., 2015; Ertürk et al., 2007). Thus, a possible common mediator that controls both inhibitory and growth responses

Abbreviations: ANOVA, analysis of variance; BMS, Basso Mouse Scale; BDNF, brain-derived neurotrophic factor; CGRP, calcitonin gene-related peptide; CNS, central nervous system; CRMP, collapsin response mediator protein; CSPG, chondroitin sulfate proteoglycan; CST, corticospinal tract; Cdk5, cyclin-dependent kinase 5; DRG, dorsal root ganglion; GAP43, growth-associated protein 43; GFAP, glial fibrillary acidic protein; GSK3β, glycogen synthase kinase 3 beta; O/N, overnight; RT, room temperature; S.E.M., standard error of the mean; SCI, spinal cord injury; YFP, yellow fluorescent protein.

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in the injured CNS could be a regulator of microtubule dynamics. Kinases such as GSK3β and Cdk5 are key subcellular molecules that regulates cytoskeletal organization for CNS repair (Liz et al., 2014; Dill et al., 2008; Renault-Mihara et al., 2011), but its molecular mechanisms remain to be identified.

The CRMP family of proteins is highly expressed in the developing and adult nervous system, and regulates aspects of neurite growth by binding to cytoskeletal proteins (Goshima et al., 1995; Wang and Strittmatter, 1996). Sema3A-induced signaling activates phosphorylation of CRMP2 by Cdk5 at the Ser-522 residue, which primes CRMP2 for subsequent phosphorylation by GSK3β at the Ser-518/Thr-514/Thr-509 residues (Uchida et al., 2005; Yamashita and Goshima, 2012); this reduces CRMP2's binding affinity for tubulin heterodimers (Fukata et al., 2002), resulting in microtubule depolymerization. CRMP2 has also been reported to participate in anterograde microtubule transport of exocytic vesicles containing TrkB, the receptor for BDNF (Arimura et al., 2009). Additionally, elevated levels of CRMP2 phosphorylation have been observed during CNS injury in vivo (Petratos et al., 2012; Gögel et al., 2010; Mimura et al., 2006). The inhibition of CRMP2 phosphorylation at the Thr-514 residue (pCRMP2T514), a phosphorylation site for GSK3β, delayed Wallerian degeneration in vitro, and after optic nerve injury (Wakatsuki et al., 2011). However, the role of CRMP2 phosphorylation in functional recovery after CNS injury remains largely unknown.

In this study, we identified CRMP2 phosphorylation to be a critical mediator of the neurotrophic response, as well as, the inhibitory response of axonal growth after spinal cord injury (SCI). First, we found that the sensitivity to BDNF was elevated by a GSK3β inhibitor in cultured dorsal root ganglion (DRG) neurons. We harvested DRG neurons from *Crmp2*KI/KI mice where CRMP2 phosphorylation sites for Cdk5 and GSK3β were eliminated by replacing Ser-522 with an inactive Ala residue (Yamashita et al., 2012), and found that *Crmp2*KI/KI neurons showed elevated sensitivity to BDNF. Likewise, as shown through GSK3β inactivation (Dill et al., 2008), CSPG-induced axonal outgrowth inhibition was suppressed in cultured *Crmp2*KI/KI neurons. Next, we found an increase in phosphorylated CRMP2 in injured spinal cords. We then investigated the significance of CRMP2 phosphorylation after SCI. Locomotive and nociceptive recovery after SCI was correlated with enhanced axonal growth in motor (5-HT-positive raphespinal and cortico-spinal tract) axons and sensory (CSPG-positive and dorsal column) axons in the injured spinal cord. In the injured *Crmp2*KI/KI mice, a more permissive environment for axonal growth is provided by the reduction of microtubule destabilization in white matter axons, inflammation, and scarring. Our results suggest that the inhibition of CRMP2 phosphorylation has great potential to regulate both neurotrophic and inhibitory responses, and to overcome the two main obstacles to recovery after CNS injuries.

2. Materials and methods

2.1. Animals

All the experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at Waseda University. The *Crmp2*KI/KI mice (Yamashita et al., 2012) and control *Crmp2*+/+ mice were obtained by intercrossing *Crmp2*KI/+ mice and their offspring. YFP-H mice (Feng et al., 2000) were crossed with them for analyses of YFP-positive axons.

2.2. Surgical procedures

A 1.5-mm-deep near-complete dorsal transection (Nagai et al., 2015; Hill et al., 2009) was performed at T7-T8 of mice (6–9 weeks old), followed by manual bladder evacuation once per day. There was no difficulty in accessing water and foods. Mice that showed 0–1 points of BMS score at 1 day after SCI were used for the following experiments and analyses. Mice were given an intraperitoneal (i.p.) injection of either 8 mg/kg AR or DMSO just after and 12 h after transection, and then sacrificed 24 h after injury for immunohistochemical analysis.

2.3. Behavioral analysis

Hindlimb motor function was evaluated using Basso Mouse Scale (BMS), as previously described (Basso et al., 2006). A team of two experienced examiners evaluated each animal for 3–5 min and assigned a score based on a defined method, wherein performance of the left and right hind limbs were averaged. Nociceptive function of the hindlimbs was analyzed using the hot plate test (maintained at a temperature of 52.0 ± 0.2 °C), as previously described (Devilliers et al., 2013). The latencies of the first response of avoidance behavior of the mice on the hot plate (licking, shaking hindlimbs or jumping) were measured twice and averaged. An arbitrary cutoff time of 60 s was adopted.

2.4. Neuronal culture and CSPG and BDNF assays

The dissociated and explant culture of DRG neurons from 6 to 9-week-old mice was performed as described previously (Nagai et al., 2012). For the BDNF assay, 100 ng/mL BDNF or PBS was administrated into the culture and incubated for 24 h. 20 μM AR-A01448 (AR, Abcam, ab141295), a potent GSK3β inhibitor, or dimethyl sulfoxide (DMSO, SIGMA, D2650) was added into the culture 4 h before BDNF or PBS administration. To examine the phosphorylation level of Erk and Trk, DRG explants were stimulated with 100 ng/mL or 20 ng/mL BDNF or PBS for 10–15 min. The area of DRG halo, the length of axon growth, and the immunoreactivity of pTrk were measured by ImageJ software (Schneider et al., 2012). For the CSPG assay, glass coverslips on the culture dishes were coated with 1.5 μg/mL CSPG or bovine serum albumin (BSA) for 5 h. The lengths of axon growth were measured using MetaMorph image analysis (Universal Imaging Systems).

2.5. Immunoblotting

The Western blotting was performed as previously described (Ohshima et al., 2007; Nagai et al., 2015). A spinal cord tissue block of 3-mm length centered at the injury site was dissected out at indicated time points. The blots were incubated with anti-GAP43 (rabbit IgG, 1:1000, ab16053, Abcam) or anti-β-tubulin (rabbit IgG, 1:1000, T8328, Sigma-Aldrich), followed by reactions with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:10,000, sc-2005 or sc-2004, Santa Cruz Biotechnology) and a color substrate (Pierce® Western blotting Substrate Plus, NCI32132JP, Thermo Fisher Scientific). The protein levels were quantified and normalized relative to tubulin level for each sample using the ImageJ software (Schneider et al., 2012).

2.6. Immunohistochemistry

The 30-μm frozen sections from mice fixed with 4% PFA were stained as previously described (Ohshima et al., 2007; Nagai et al., 2015) with the following primary antibodies; anti-phosphorylated CRMP2 at Thr-509 (rabbit IgG, 1:200; Yamashita et al., 2012), anti-MAP2 (mouse IgG, 1:200, MAB3418, Millipore), anti-detyrosinated α-tubulin (Glu-tubulin) (mouse IgG, 1:500, ab24622, Abcam), anti-neuron-specific class III β-tubulin (Tuj1, rabbit IgG, 1:1000, MMS-435P, Covance), anti-GFAP (mouse IgG, 1:400, G3893, Sigma-Aldrich), anti-CD11b (rat IgG, 1:100, Serotec), anti-Iba1 (rabbit IgG, 1:500, 019-19741, Wako) and anti-GFAP (rabbit IgG, 1:500, Z0334, Dako) antibody. The manufacturer's protocol was used for immunostaining with anti-GAP43 (rabbit IgG, 1:200, ab16053, Abcam), or anti-collagen IV antibody (1:200, ab19808, Abcam). For immunostaining with anti-5-HT (rabbit IgG, 1:4000, 20,080, Immunostar), a high-salt buffer (HSB; 500 mM NaCl, 9.2 mM NaH_2PO_4, 12.5 mM Na_2HPO_4) was used instead of PBS.

To analyze Glu-tubulin distribution in the axons, we measured the length of Glu-tubulin-positive and Tuj1-positive fibers in each section