Disorders of the Nervous System

CCP1, a Tubulin Deglutamylase, Increases Survival of Rodent Spinal Cord Neurons following Glutamate-Induced Excitotoxicity

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Abstract

Microtubules (MTs) are cytoskeletal elements that provide structural support and act as roadways for intracellular transport in cells. MTs are also needed for neurons to extend and maintain long axons and dendrites that establish connectivity to transmit information through the nervous system. Therefore, in neurons, the ability to independently regulate cytoskeletal stability and MT-based transport in different cellular compartments is essential. Posttranslational modification of MTs is one mechanism by which neurons regulate the cytoskeleton. The carboxypeptidase CCP1 negatively regulates posttranslational polyglutamylation of MTs. In mammals, loss of CCP1, and the resulting hyper-glutamylation of MTs, causes neurodegeneration. It has also long been known that CCP1 expression is activated by neuronal injury; however, whether CCP1 plays a neuroprotective role after injury is unknown. Using shRNA-mediated knock-down of CCP1 in embryonic rat spinal cord cultures, we demonstrate that CCP1 protects spinal cord neurons from excitotoxic death. Unexpectedly, excitotoxic injury reduced CCP1 expression in our system. We previously demonstrated that the CCP1 homolog in Caenorhabditis elegans is important for maintenance of neuronal cilia. Although cilia enhance neuronal survival in some contexts, it is not yet clear whether CCP1 maintains cilia in mammalian spinal cord neurons. We found that knock-down of CCP1 did not result in loss or shortening of cilia in cultured spinal cord neurons, suggesting that its effect on survival of excitotoxicity is independent of cilia. Our results support the idea that enzyme regulators of MT polyglutamylation might be therapeutically targeted to prevent excitotoxic death after spinal cord injuries.

Key words: cilia; excitotoxicity; neuronal injury; neuroprotection; polyglutamylation; spinal cord

Significance Statement

Combining an in vitro model of the secondary phase of spinal cord injury with shRNA knock-down, we demonstrate that the deglutamylase CCP1 protects neurons from excitotoxic death. Excitotoxicity plays a role in the secondary phase of neuronal injuries, contributing to neurodegeneration. CCP1 function was previously known to be associated with cilia. We provide the first demonstration (to our knowledge) that spinal cord interneurons are ciliated. However, our data suggest that neuroprotection by CCP1 may be independent of cilia in spinal neurons. Our work supports the idea that targeting enzymes that modify tubulins, such as glutamylases and deglutamylases, might be an avenue of treatment for nervous system injuries.

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**Introduction**

The development, function, and survival of neurons rely heavily on the function of the microtubule (MT) cytoskeleton (Witte and Bradke, 2008; Varidaki et al., 2018). MTs are hollow cylinders formed by polymerization of α and β tubulin subunits (Pellegrini et al., 2017). Tubulins are highly conserved, differing little in sequence and structure (Gadadhar et al., 2017); however, MTs in different neuronal compartments, such as axons, dendrites, or growth cones, display differences in function and dynamics (Witte and Bradke, 2008). The tubulin code model proposes that in addition to heterogeneity of tubulin isoform composition, post-translational modification of tubulins can specialize the stability, form, and function of MTs (Verhey and Gaertig, 2007; Gadadhar et al., 2017). Tubulin code modifications are proposed to endow specific MTs with particular properties to play essential roles in the function of axons and dendrites as well as directional trafficking, plasticity, and survival (Verhey and Gaertig, 2007; Gadadhar et al., 2017).

Glutamylation, one component of the tubulin code, consists of side-chains of the amino acid glutamate that are posttranslationally added to the carboxy terminal tails of tubulins when assembled into MTs (Eddé et al., 1990; Bre et al., 1994). Glutamylation often decorates MTs in both neurons and cilia (Gadadhar et al., 2017).

The primary cilium, a non-motile sensory organelle that protrudes from most non-dividing cells in the human body, is also a region of MT specialization (Werner et al., 2017). Conserved over eukaryotic evolution from algae to vertebrates, the architecture of cilia consists of a MT cytoskeleton, called the axoneme, in which a ring of nine MT doublets extends along cilia immediately beneath the membrane (Werner et al., 2017). Vertebrate cilia function in processes such as kidney function, olfaction, vision, and development of left-right asymmetry (Youn and Han, 2018). Cilia also play a role in nervous system development because of their function as an essential hub for signaling pathways (Lee and Gleson, 2011; Bay and Caspary, 2012).

Recently, primary cilia that protrude from mammalian neurons have been proposed to play an essential role in maintaining neuronal viability and connectivity (Bowie and Goetz, 2020). Loss of neuronal cilia in the mammalian brain causes neurodegeneration and synapse loss and may be a primary cause of motor coordination defects in spinal cerebellar ataxia (SCA; Bowie and Goetz, 2020). Although neuronal injury can cause ablation of cilia, cilia exert a neuroprotective effect (Choi et al., 2019).

Glutamylation regulates the structure and function of cilia (Ikegami and Setou, 2010; O’Hagan et al., 2011, 2017). M14D carboxypeptidases, such as CCP1 (Rodríguez de la Vega et al., 2007), remove or reduce the length of glutamate side-chains (Rogowski et al., 2010). When deglutamylation function is lost, hyperglutamylation affects ciliary motor transport and causes degeneration of some types of neuronal sensory cilia in Caenorhabditis elegans (O’Hagan et al., 2011; Power et al., 2020). In mice, loss of CCP1 leads to the degeneration of retinal photoreceptors and sperm defects (Fernandez-Gonzalez et al., 2002). These phenotypes are reminiscent of the symptoms of diseases caused by ciliary dysfunction or “ciliopathies” (Mitchison and Valente, 2017).

Glutamylation also occurs on non-ciliary neuronal MTs (Fukushima et al., 2009). Loss of CCP1 perturbs neuronal transport in mice and humans (Fernandez-Gonzalez et al., 2002; Ikegami et al., 2007; Magiera et al., 2018) and leads to infantile hereditary neurodegeneration and cerebellar atrophy in humans (Shashi et al., 2018). In mammals, expression of CCP1 de glutamylation is upregulated in response to transaction or crush injury of the sciatic nerve, suggesting that its function may be required for neuroregeneration (Harris et al., 2000). Loss of de glutamylation activity diminishes regrowth of laser-severed neurons in C. elegans, supporting a possible conserved role in neuroregeneration (Ghosh-Roy et al., 2012).

Questions about the function of CCP1 remain unanswered. Is CCP1 neuroprotective or does it play a pathologic role after neuronal injury? Does CCP1 expression increase in injured neurons of the CNS as it does in the sciatic nerve in the peripheral nervous system (PNS)? Is the role of CCP1 in ciliary maintenance also important in injured neurons?

Here, using an in vitro model of the secondary phase of spinal cord injury (Du et al., 2007), we find that knock-down of CCP1 decreases the survival of spinal cord neurons subjected to excitotoxic glutamate treatment. In contrast to the reported upregulation of CCP1 in response to injury of the sciatic nerve, our analysis showed that glutamate-induced excitotoxic injury reduces CCP1 expression in spinal cord neurons. However, shRNA CCP1 knock-down in cells subjected to excitotoxic glutamate did not reduce CCP1 expression to lower levels than excitotoxic glutamate alone.

We also used shRNA to knock down CCP1 expression to test whether CCP1 activity affects neuronal cilia in embryonic rat spinal cord cultures. Using immunofluorescence-based detection of the ciliary marker ARL13B, we found that a primary cilium protrudes from the majority of neurons in our spinal cord cultures. Unexpectedly, CCP1 knock-down did not decrease the percentage of ciliated neurons or ciliary length. Our data suggest that CCP1 activity is protective for neurons and support the idea that targeting regulators of MT glutamylation may offer a new option for treatment of excitotoxic damage resulting from nervous system injury.

**Materials and Methods**

All animal experiments were conducted in accordance with the National Institutes of Health (NIH) Guide for the
Spinal cord cultures
Our method was similar to that used by Du et al. (2007). Briefly, spinal cords were dissected from Sprague Dawley rat embryos at gestational day 15 [embryonic day (E)15] by removing meninges and attached dorsal root ganglia. Cords were then gently triturated to dissociate a mixture of neurons, astrocytes, and microglia from the tissue. Cells were plated at a density of ~523 cells/mm², or 100,000 cells per well, in a 24-well plate containing coverslips coated with 0.01% solution of poly-D-lysine (Sigma) dissolved in 0.1 M borate buffer (sodium tetraborate, boric acid) for cell adhesion. Mixed cultures were grown in DMEM (Invitrogen) supplemented with 10% horse serum (Invitrogen) for 7 d before glutamate treatment.

Lentiviral production and infection
Lentiviruses were produced as previously described (Patel et al., 2019). In summary, HEK293T cells, plated at a density of 6.5 × 10⁶ cells/T75 flask were grown for 1 d and then transfected on day in vitro (DIV)2 with lentiviral plasmids (VectorBuilder) carrying scrambled shRNA (target sequence: CCTAAGGTAAAGTCCCTCG; vector ID: VB170329-1128paq), CCP1 knock-down shRNA (target sequence: CCACCTCCAGTGTCCCTCG; vector ID: VB170111-1192pab), or CCP1 cDNA (RGD ID: 1306307; AGTPBP1 also known as CCP1) and GFP or RFP fluorescent markers. VSV and PAX2 packaging vectors and Lipofectamine 2000 (Invitrogen) were used according to the manufacturer’s protocol to mediate transfection. After 3 d, the media were collected and centrifuged at 1500 × g for 5 min to pellet dead cells and debris. The supernatant was then collected and incubated with PEG-it (System Biosciences) in a 5°C solution at 4°C for 2 d to precipitate the viruses. On day 7, the solution was centrifuged at 1500 × g for 30 min at 4°C to pellet the viruses. The supernatant was removed and discarded, and the virus pellet was resuspended in 150 μl of sterile 1× PBS and frozen (at ~8°C) in 5 μl aliquots until use.

All spinal cord culture infections were performed on DIV2 at a 1:5000 dilution by replacing one-fourth of the medium in the well with a 1:1250 dilution of virus in fresh DMEM + 10% horse serum.

Glutamate treatment
Glutamate-induced excitotoxicity was performed as previously described (Du et al., 2007). Glutamate (L-glutamic acid, Sigma Life Sciences) dilutions were made from a 1 mM stock solution dissolved in DMEM + 10% horse serum warmed to 37°C. During treatment on DIV7, medium was collected from each well and replaced with varying concentrations of glutamate-containing medium as described in results for 1 h at 37°C and 5% CO₂. Conditioned medium (collected before treatment) was combined with an equal volume of fresh medium (1:1 solution) and used as recovery medium following glutamate treatment. Cells were allowed to recover for 24 h at 37°C and 5% CO₂ before fixation.

Immunocytochemistry
Cells were fixed by incubation in 4% paraformaldehyde in 1× PBS for 15 min. Following fixation, cells were washed three times with 1× PBS and incubated in a blocking solution (2% normal goat serum, 0.1% Triton X-100, 0.02% NaNO₃ diluted in 1× PBS) for 1 h. Cells were incubated in primary antibody solution (1:500) overnight (~18 h), washed three times in 1× PBS, and incubated with appropriate secondary antibody solutions (1:1000) for 1 h the next day, followed by another three washes in 1× PBS. Coverslips were then removed and mounted on glass slides for imaging.

Anti-MT-associated protein 2 (MAP2) antibody (ThermoFisher) was used to identify neurons. Anti-GFAP (glial fibrillary acidic protein; Millipore, rabbit) was used to label astrocytes, and anti-IBA1 (ionized calcium binding adaptor molecule 1; Proteintech, mouse) was used to label microglia. Cilia were immunolabeled using a monoclonal anti-ARL13B antibody (Proteintech, mouse). PolyE antibody (Adipogen catalog #AG-25B-0030-C050), a polyclonal antibody which only binds polyglutamylated substrates (three or more glutamate residues; Rogowski et al., 2010), was used to visualize polyglutamylated MTs. Anti-choline acetyltransferase (CHAT; Millipore Sigma, goat) antibody was used to identify motorneurons. Alexa Fluor-conjugated secondary antibodies (488, 568, 647) were used to visualize cell-specific markers. Nuclei were labeled using NucBlue Live Ready Probes reagent (ThermoFisher), which was excited at 350 nm.

Imaging and microscopy
Image Z-stacks were acquired with MetaMorph software (Molecular Devices) using a Zeiss Axioplan2 microscope with 10×, 63× (NA 1.4), and 100× (NA 1.4) oil-immersion objectives, equipped with a Hamamatsu C11440-42U ORCA-Flash4.0 LT Digital CMOS camera (Hamamatsu). Images were uploaded into FIJI/Image J 2.0 to create optical Z-stack projections, add scale bars, and adjust brightness/contrast. The cell counter plugin was used to count MAP2-immunopositive and GFP-positive cells. Images were then exported as PNG files for assembly into figures in Adobe Illustrator CS.

Scoring neuronal survival
To assess neuronal survival following glutamate treatment, neurons identified by MAP2 immunostaining were counted from five 1.4 × 1.4 μm regions imaged from each coverslip using the 100× objective and analyzed as ratios of neurons remaining following glutamate treatment/neurons present in the absence of a glutamate treatment.

Scoring neuronal ciliation and cilia length
Neurons were identified using anti-MAP2 immunofluorescence, and viral infection with shRNA vectors (scrambled or anti-CCP1) was scored by expression of GFP. For ciliation, the presence or absence of a cilium immunolabeled by ARL13B on the cell body of each neuron in 5 randomly chosen areas was scored and analyzed from Z-stacks of
images taken using the 100× objective on the Zeiss Axioplan2 microscope. Z-stacks were uploaded into FIJI/ImageJ, Z-projected, and neuronal cilia were counted using the Cell Counter plugin.

For cilia length, only neurons with “horizontally-projecting” cilia (the entire length of the cilium was visible in a single focal plane of a Z-stack) were scored from images taken with the 100× objective on the Zeiss Axioplan2 microscope. Z-stacks were uploaded into FIJI/ImageJ, Z-projected, and measured for pixel length using the tracing tool. Pixels were converted to microns (at 100×, 0.6566 μm = 1 pixel) before graphing and statistical analysis.

**Western blot analysis**

For Western blot assays, spinal cord cultures were grown at 1 million cells per well in a six-well plate and infected with viruses and/or treated with glutamate at similar concentrations as described above. On DIV8, cells were homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% deoxycholate, 1% NP-40, 0.1% SDS, and 1 mM EDTA, pH 7.4) by scraping the cells into the buffer and sonication. Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Scientific) according to the manufacturer’s protocol. Proteins (30 μg/lane) were resolved on a 10% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% bovine serum albumin (BSA) in 1× TBST (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 h. Blots were incubated in primary antibody solutions (1:1000) overnight and then washed and incubated in appropriate horseradish peroxidase-conjugated secondary antibody solutions (1:5000) for 1 h the next day. Blots were probed with the following antibodies: anti-CCP1 (Proteintech, rabbit) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; EMD Millipore, mouse), which served as a loading control. Bands were visualized using the LI-COR Biosciences digital imaging system, and pixel intensity was analyzed using ImageJ (NIH). CCP1 band intensities were normalized to corresponding GAPDH band intensities.

**Experimental design and statistical analyses**

All statistical analyses were performed using a combination of GraphPad Prism (version 5.01, GraphPad Software) and Microsoft Excel (Version 14.0.7106, 32-Bit, Microsoft Corporation).

**Results**

**Knock-down of CCP1 decreases neuronal survival of excitotoxic challenge**

Loss of CCP1 has long been known to lead to severe neuronal degeneration in the murine CNS (Fernandez-Gonzalez et al., 2002). More recently, it was reported that loss of CCP1 in humans results in degeneration of cerebellar neurons and spinal cord neurons (Shashi et al., 2018). Therefore, we sought to test how CCP1 function affects the survival of spinal cord neurons.

We isolated and dissociated embryonic rat spinal cords (Fig. 1A; Extended Data Fig. 1-1A) from pregnant mothers at E15 and grew mixed cultures of astrocytes, microglia, and neurons (Fig. 1B; Extended Data Fig. 1-1B). We subjected our cultures to glutamate-induced excitotoxicity, which is characteristic of the secondary phase of spinal cord injury (Du et al., 2007), by incubating the cultures with concentrations of glutamate ranging from 200 μM to 1 mM for 1 h on DIV7 (Fig. 1C). After injury, cultures were allowed to recover for 24 h before fixation on DIV8 and subsequent immunostaining for the neuronal marker MAP2. MAP2-immunopositive surviving neurons were counted, and as expected, glutamate exposure caused neuronal death in a dose-dependent manner (Fig. 1B,C). Approximately 60% of neurons survived following exposure to 1 mM glutamate for 1 h.

To determine whether CCP1 is required for neuronal survival following glutamate-induced excitotoxicity, we used a lentivirus containing an shRNA sequence targeting the 3’ untranslated region (UTR) of CCP1 to knock down CCP1 expression. This sequence was found to be the most efficient at knocking down CCP1, as other viruses containing shRNA targeting the 5’ UTR or the coding region resulted in a lower transduction efficiency (data not shown). Cultures in which CCP1 levels were knocked down by lentiviral shRNA treatment were subjected to varying concentrations of glutamate. Neuronal survival following glutamate exposure was significantly reduced in cultures transduced with shRNA CCP1 knock-down at all glutamate concentrations tested (Fig. 1A,B,D).

To confirm that the knock-down of CCP1 is responsible for decreased neuronal survival following glutamate-induced excitotoxicity, we used Western blot analysis to show that CCP1 levels were reduced by ~40-50% by shRNA expression (Fig. 1E,F). Additionally, we co-infected cultures with two lentiviruses carrying CCP1 shRNA and an shRNA-resistant CCP1 cDNA construct and found that CCP1 levels were rescued to levels similar to controls in these cultures (Fig. 1E,F). Cultures in which levels of CCP1 were rescued exhibited neuronal survival after excitotoxic challenge comparable to control cultures (Fig. 1G). As expected, the lentivirus containing shRNA specific to CCP1 selectively infected more MAP2-positive neurons than non-neuronal cells, such as glia (Extended Data Fig. 1-1C,D). Therefore, the shRNA-mediated decrease in CCP1 levels in these spinal cord cultures is most likely because of knock-down of CCP1 in neurons.

We conclude that the presence of CCP1 in neurons is necessary for protection from excitotoxicity.

**CCP1 levels decrease within 24 h after excitotoxic injury**

We next sought to determine whether glutamate-induced excitotoxicity affects protein levels of CCP1. Western blot analysis indicates that 24 h after excitotoxic injury, the level of CCP1 decreased to ~60% of basal levels in uninjured cells (Fig. 2A,B; Extended Data Fig. 2-1A). Cultures treated with lentiviral CCP1 shRNA knock-down did not show an additional decrease in CCP1 levels after glutamate exposure. Because lentiviral infection was
Figure 1. Knock-down of CCP1 decreases neuron survival in an in vitro model of glutamate-induced excitotoxicity. A, Schematic of treatment of spinal cord cultures with excitotoxic concentrations of glutamate. Primary spinal cord cultures containing neurons and glia were treated with varying concentrations of glutamate for 1 h on DIV7 and allowed to recover for 24 h before fixation (see also Extended Data Fig. 1-1A,B). B, Control and injured spinal cord cultures were immunostained for MAP2 to identify neurons. Glutamate-induced excitotoxic injury leads to loss of neurons and retracted neuronal processes compared with that of uninjured neurons. Also shown are cultures infected with a lentivirus expressing CCP1 shRNA knock-down, and cultures subjected to CCP1 knock-down and shRNA resistant CCP1 cDNA rescue, which ameliorates neuronal death and retraction of processes after injury. Scale bars: 100 μm (see also Extended Data Fig. 1-1C,D). C, Percent neuronal survival after excitotoxic injury decreases with increasing glutamate concentration. Error bars represent SEM; ***p < 0.001, ****p < 0.0001 versus 0 μM glutamate as determined by one-way ANOVA and Dunnett’s multiple comparison test. D, Quantification of percentage of neuronal survival following treatment with concentrations of glutamate ranging from 200 μM to 1 mM. At every concentration, CCP1 knock-down cultures show significantly reduced neuronal survival compared with those expressing scrambled shRNA control as determined by two-way ANOVA followed by Bonferroni post hoc test analysis; *p < 0.05, **p < 0.01, ***p < 0.001 (percentage neuronal survival of scramble shRNA control groups was not significantly different from controls shown in C). E, Western blotting showing CCP1 in scramble, knock-down, and rescued cultures. GAPDH loading controls are also shown. F, Quantification of Western blotting revealed that shRNA-resistant cDNA expression in CCP1 knock-down cells rescues CCP1 to levels similar to controls; *p < 0.05, **p < 0.01, ***p < 0.001 (percentage neuronal survival of scramble shRNA control groups was not significantly different from controls shown in C). G, Quantification of percentage neuronal survival treated with 1 mM glutamate. Neuronal survival of rescue cultures and control cultures are not significantly different as determined by one-way ANOVA followed by Tukey’s multiple comparison test; **p < 0.01, ****p < 0.0001. ns, not significant.
more efficient in neurons than in glial cells (Extended Data Fig. 1C,D), our results suggest that the levels of CCP1 protein are downregulated in neurons after excitotoxic injury.

Mammalian spinal cord interneurons in culture are ciliated

Primary cilia can play a neuroprotective role in the rodent CNS (Choi et al., 2019) and are important for reception of cellular signaling that is essential for development of the mammalian nervous system (Louvi and Grove, 2011; Bay and Caspary, 2012; Youn and Han, 2018). Furthermore, the loss of primary cilia causes neurodegeneration in rodents and humans (Campbell et al., 2002; Fernandez-Gonzalez et al., 2002; Chakrabarti et al., 2010; O’Hagan et al., 2011; Kubo et al., 2015; Branham et al., 2016; Power et al., 2020), we hypothesized that the neuroprotective effects of CCP1 arise from ciliation of spinal cord neurons. However, cilia of spinal cord neurons have not been well-documented. To our knowledge, only the CSF-contacting neurons (Djenoune et al., 2014; Orts-Del’Immagine et al., 2014; Böhm et al., 2016; Stemberg et al., 2018), neuronal precursors, and ependymal cells that line the central canal (Meletis et al., 2008) and motor neurons of the lumbar spinal cord (Ma et al., 2011) are ciliated. Therefore, we first assessed whether spinal cord neurons from cultured rat embryonic spinal cord are ciliated. Indirect immunofluorescence, using an antibody specific to the ciliary membrane protein ARL13B (Higginbotham et al., 2012), demonstrates that 57% of MAP2-immunostained spinal cord neurons in our cultures are ciliated at DIV8 (Fig. 3A,B). Because the dorsal root ganglia were removed from spinal cords before dissociating the cells, primary sensory neurons are not present in our embryonic spinal cord cultures. Interneurons are reported to make up ~97% of all neurons in the spinal cord (Hochman, 2007). Moreover, motor neurons do not survive in embryonic spinal cord cultures under similar culture conditions (Kushima and Hatanaka, 1992), and no neurons in our cultures were positive for immunostaining for the cholinergic motor neuron marker ChAT (Extended Data Fig. 2-1B). Therefore, ciliated MAP2-positive cells in our cultures are interneurons.

Knock-down of the deglutamylase CCP1 increases the presence of cilia on spinal cord neurons but does not significantly affect ciliary length

We hypothesized that if CCP1 functions to maintain cilia in mammalian spinal cord neurons, as it does in C. elegans, then loss of CCP1 function would cause ciliary degeneration (O’Hagan et al., 2011). We counted cilia present on neurons under baseline conditions and with CCP1 knocked down and measured their lengths. Surprisingly, we found that shRNA-mediated knock-down of CCP1 increases the frequency of ciliated neurons (Fig. 3A,B). However, neither CCP1 knock-down nor excitotoxic injury with 500 μM glutamate affected ciliary length (Fig. 3C). Thus, our data suggest that reduction of CCP1 does not lead to degeneration of primary cilia in the murine spinal cord, as it does in C. elegans sensory neurons, at least on a time scale of several days.

Presence of neuronal cilia is not correlated with survival of excitotoxic injury

Cilia can play a neuroprotective role in the CNS as an antagonist to the cell cycle by inhibiting cell division and preventing apoptosis (Choi et al., 2019). To assess whether the presence of cilia plays a role in neuronal survival after glutamate-induced excitotoxicity, we compared the fraction of ciliated neurons after treatment with 500 μM glutamate to uninjured neurons. We hypothesized that if ciliation is neuroprotective, the frequency of cilia would increase in the neurons remaining after excitotoxic treatment, because of a decreased likelihood of survival of neurons without cilia. We found that in injured cultures,
**Figure 3.** Interneurons in spinal cord cultures are ciliated. **A,** Primary spinal cord cultures immunostained for MAP2 and ARL13B, showing ciliated uninjured neurons and ciliated neurons following treatment with 500 µM glutamate. Spinal cord cultures do not contain motoneurons (see also Extended Data Fig. 2-1B). **B,** The percentage of neurons that are ciliated (mean ± SEM) show no significant differences with shRNA treatments or injury (ANOVA). **C,** Average lengths of cilia (mean ± SEM) show no significant differences with shRNA treatments or injury (ANOVA). **D,** Immunofluorescent detection of polyglutamylation (polyE) suggests that neuronal MTs, except those in cilia, are polyglutamylated in spinal cord cultures. No polyE
the percentage of neurons that were ciliated in CCP1 knock-down-treated cultures was ~69% versus 64% in scramble shRNA-treated controls (Fig. 3A,B), a not statistically significant difference (Fig. 3B). The length of neuronal cilia was also not significantly different between CCP1 knock-down and scramble shRNA-treated glutamate-injured cultures (Fig. 3C). Therefore, our data suggest that cilia are not needed for CCP1 to protect sensory spinal cord neurons from excitotoxic death. Because CCP1 reduces the length of polyglutamylation side-chains on α and β tubulins (Rogowski et al., 2010), we tested whether the cilia that decorate spinal cord interneurons in our cultures have long polyglutamylation side-chains. We performed indirect immunofluorescence using a polyE antibody, which detects glutamate side-chains of three or more glutamates (Rogowski et al., 2010). The polyE antibody strongly immunostained neuronal cell bodies and neurites, but not ARL13B-positive cilia (Fig. 3D). Thus, ciliary MTs in spinal cord interneurons may have few or no polyglutamylation side-chains.

**Discussion**

Several lines of evidence support the idea that glutamylation acts as a component of the tubulin code to regulate the MT cytoskeleton and MT-based motors. Glutamylation is a posttranslational modification found on stable MTs (Fukushima et al., 2009; Janke and Bulinski, 2011; Wloga et al., 2017). MT glutamylation regulates MT-based motor trafficking in neurons of *C. elegans* and mice (Ikegami et al., 2009; O'Hagan et al., 2011, 2017; Magiera et al., 2018) and in *in vitro* studies using purified tubulins and motors (Sirajuddin et al., 2014). Defects in tubulin modifications, MT stability, and neuronal trafficking are linked to neurodegenerative diseases, such as Parkinson’s, Huntington’s, and Alzheimer’s diseases (Baas et al., 2016; Matamoros and Baas, 2016; Brady and Morfini, 2017; Vu et al., 2017). Additionally, in humans, loss of the deglutamylase CCP1 (and resulting hyperglutamylation of MTs) leads to fatal infantile neurodegeneration in the spinal cord, cerebellum, and peripheral nerves (Shashi et al., 2018).

Neuronal expression of CCP1 (also known as NNA1 or AGTTPB1) is upregulated after injury of the sciatic nerve, and elevated expression is maintained during target reinnervation (Harris et al., 2000), suggesting that CCP1 plays a role in neuroregeneration and axonal regrowth after injury. Therefore, control of MT glutamylation could represent an important survival factor in the context of both neurodegenerative disease and neuronal injury. In order for regeneration after injury to occur, neurons must also survive (Dusart et al., 2005; Hollis et al., 2009). In this work, by combining lentivirally-delivered shRNA knockdown and a previously established *in vitro* model of the secondary phase of spinal cord injury (Du et al., 2007), we show that CCP1 promotes survival of neurons challenged with excitotoxic levels of glutamate.

We also hypothesized that CCP1 functions in cilia to promote survival of neurons exposed to excitotoxic injury. Cilia can play a neuroprotective role in the rodent CNS (Choi et al., 2019) and are important for reception of cellular signaling that is essential for development of the mammalian nervous system (Louvi and Grove, 2011; Bay and Caspary, 2012; Youn and Han, 2018). Loss of neuronal cilia, caused by mutation of TTBK2 or by conditional knock-out of the ciliary intraflagellar transport protein Ift88, causes degeneration of Purkinje neurons of the cerebellum (Bowie and Goetz, 2020). Motile cilia on ependymal cells, such as those that line the central canal, are essential for spinal cord morphogenesis (Grimes et al., 2016). Cerebrospinal fluid-contacting neurons, which also have a motile cilium, extend an apical projection into the central canal and are proposed to relay cerebrospinal fluid flow and pH information spinal circuits for normal development and function of spinal cord nerves (Böhm et al., 2016; Djenoune et al., 2017; Sternberg et al., 2018). Spinal cord injury can cause degeneration of motile ependymal cilia or ependymal cells, which create cerebrospinal fluid flow in the central canal and neurons (Radojicic et al., 2007). This could result in toxic buildup of CNS waste, possibly preventing regrowth and exacerbating the chronic degeneration of spinal tissues after SCI (Radojicic et al., 2007). CCP1 homologs have been found to regulate the integrity and structure of MTs in cilia, the function of ciliary motors (Suryavanshi et al., 2010; O'Hagan et al., 2011, 2017; Kubo et al., 2015; Hong et al., 2018), and ciliary length (Kim et al., 2010).

To our knowledge, no previous reports have demonstrated that spinal cord interneurons are ciliated. Spinal cord motoneurons have been shown to be ciliated *in vitro* (Ma et al., 2011). We used immunofluorescence to detect the ciliary protein ARL13B and found that most neurons in embryonic spinal cord cultures are ciliated. Importantly, under the culture conditions we used, spinal cord cultures lack sensory neurons and motoneurons (Extended Data Fig. 2-1B; Kushima and Hatanaka, 1992). Therefore, our data provide the first evidence (to our knowledge) that spinal cord interneurons are ciliated.

Our data suggest that expression of CCP1 may not strongly regulate ciliation in spinal cord neurons. This was surprising, as we had previously demonstrated that the lack of a CCP1 homolog in nematodes causes the degeneration ciliary MTs and of neuronal cilia (O’Hagan et al., 2011). An siRNA screen in immortalized human retinal pigmented epithelial cells had also found that CCP1 can positively regulate cilia length (Kim et al., 2010). Our result may be explained by the fact that the tubulin code, and glutamylation in particular, can result in cell-specific effects (O’Hagan et al., 2011, 2017), likely because of differences in expression of genes that read, write, or interpret the tubulin code modifications. Even within a single cell, different populations of MTs could have different glutamylation states, endowing them with specific properties. The
fact that loss of CCP1 function in humans, mice, and
sheep results in degeneration of particular neurons, such
as cerebellar Purkinje cells and spinal cord motor neurons
(Fernandez-Gonzalez et al., 2002; Zhao et al., 2012;
Shashi et al., 2018), rather than all neurons, may reflect
such differences in glutamylation in different neuronal
types. In our cultures, we observed that the polyE antici-
body detected polyglutamylation throughout neurons but
not in cilia. This could explain why CCP1-knock-down re-
cduced neuronal survival but did not result in degeneration
of neuronal cilia in our cultures: ciliary MTs with minimal
polyglutamylation might not require activity of CCP1, which
functions to reduce the length of glutamate side-
chains. Therefore, CCP1 might affect neuronal survival by
reducing polyglutamylation on MTs in compartments
other than cilia, such as axons or dendrites. However,
using our in vitro system, we cannot address how CCP1
might function in cilia of ependymal cells, cerebrospinal
fluid-contacting neurons, or motor neurons in response to
nerve injury.

Because CCP1 reduces the length of polyglutamylation
side-chains on MTs (Rogowski et al., 2010) and polygluta-
mylation regulates MT severing (Janke and Magiera,
2020), we envision that regulation of CCP1 expression
and function is necessary to appropriately reorganize MT
dynamics following neuronal injury to facilitate axonal re-
generation and recovery. This notion is supported by the
previous finding that CCP1 homologs in the invertebrate C.
elegans are necessary for normal neuronal outgrowth
following axotomy of touch receptor neurons (Ghosh-Roy
et al., 2012). When CNS axons fail to regenerate, MTs are
disorganized after injury, whereas regenerating PNS
axons form organized MT networks in their growth cones
(Ertürk et al., 2007), suggesting that one difference in the
regenerative potential of CNS versus PNS neurons is the
capability of reorganizing the cytoskeleton for regrowth.
Additionally, MT defects are proposed to underlie neuro-
degeneration after traumatic brain injury (Tang-Schomer
et al., 2010).

Our results suggest that CCP1 function is needed to im-
prove neuronal survival of excitotoxic injury. We found
that CCP1 expression significantly decreased following gluta-
tamate-induced excitotoxicity in spinal cord neurons. When
CCP1 was knocked down before injury, excitotoxic ity
did not further reduce CCP1 expression. Our observa-
tions differ from a previous study (Harris et al., 2000),
which reported upregulation of CCP1 following trans-
section or nerve crush injury of the sciatic nerve in the PNS.
There are at least two possible explanations for the ob-
served difference in CCP1 levels. Acute injury that severs
or damages the axonal cytoskeleton might increase
CCP1 expression, while chronic (excitotoxic) injury might
decrease CCP1 expression. Alternatively, injury of PNS
neurons might upregulate CCP1 expression, while injury of
the CNS might downregulate of CCP1 expression. In
this case, differences in the function or structure of the
MT cytoskeleton, mediated at least in part by CCP1,
might explain the poor regenerative potential in the CNS
and robust regeneration in the PNS.

Pharmacological targeting of tubulin-modifying en-
zymes has been proposed as a new approach to treating
neurodegenerative disease (Rogowski et al., 2021). We
suggest that drugs that target enzyme regulators of post-
translational MT modifications might reduce cumulative
neuronal damage from excitotoxicity during the second-
ary phase of CNS injury. Our data support the idea that di-
rect or indirect activation of CCP1, or prevention of its
downregulation by excitotoxicity, might protect injured
neurons. Future investigations could include methods to
inactivate TTLL glutamylase enzymes that oppose CCP1
by initiating or elongating polyglutamylation side-chains
(van Dijk et al., 2007; Mahalingan et al., 2020), which
might also provide a neuroprotective effect by reducing
polyglutamylation of MTs.

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