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INVITED REVIEW

Collapsin response mediator protein-2 plays a major protective role in acute axonal degeneration

Jian-Nan Zhang[1, 2], **Jan C. Koch**[1, 3, *]
1 Department of Neurology, University Medicine Göttingen, Göttingen, Germany
2 Department of Neurobiology, Beijing Institute for Brain Disorders and Key Laboratory for Neurodegenerative Disease of the Ministry of Education, Capital Medical University, Beijing, China
3 Center for Nanoscale Microscopy and Molecular Physiology of the Brain (CNMPB), Göttingen, Germany

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Abstract

Axonal degeneration is a key pathological feature in many neurological diseases. It often leads to persistent deficits due to the inability of axons to regenerate in the central nervous system. Therefore therapeutic approaches should optimally both attenuate axonal degeneration and foster axonal regeneration. Compelling evidence suggests that collapsin response mediator protein-2 (CRMP2) might be a molecular target fulfilling these requirements. In this mini-review, we give a compact overview of the known functions of CRMP2 and its molecular interactors in neurite outgrowth and in neurodegenerative conditions. Moreover, we discuss in detail our recent findings on the role of CRMP2 in acute axonal degeneration in the optic nerve. We found that the calcium influx induced by the lesion activates the protease calpain which cleaves CRMP2, leading to impairment of axonal transport. Both calpain inhibition and CRMP2 overexpression effectively protected the proximal axons against acute axonal degeneration. Taken together, CRMP2 is further characterized as a central molecular player in acute axonal degeneration and thus evolves as a promising therapeutic target to both counteract axonal degeneration and foster axonal regeneration in neurodegenerative and neurotraumatic diseases.

Key Words: *collapsin response mediator protein-2; CRMP2; axonal regeneration; optic nerve cruch; axonal degeneration; calpain; axonal transport*

Correspondence to:
Jan C. Koch, M.D.,
jkoch@med.uni-goettingen.de.

orcid:
0000-0002-3778-0683
(Jan C. Koch)

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Introduction

The degeneration of axons is a core pathological feature in many neurological diseases including Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, multiple sclerosis and spinal cord lesion. It often precedes the death of the neuronal cell body and results in impaired connectivity of the affected regions of the central nervous system (CNS) with high clinical relevance. The deficits are most often persistent due to the inability of CNS axons to regenerate, which is caused by inhibitory molecular cues of the glial environment in the adult CNS, the lack of neurotrophic responses and the low intrinsic regenerative ability of differentiated CNS neurons. Thus, therapeutic approaches to tackle neurodegenerative diseases should counteract axonal degeneration and foster regeneration of already lesioned axons in the inhibitory CNS environment. Interestingly, increasing evidence suggests that collapsin response mediator protein-2 (CRMP2) (also known as dihydropyrimidinase-related protein 2 (DPYSL2)) is a multifunctional molecule that mediates both axonal re- and degeneration and could thus be a very promising drug target in these diseases.

CRMP2 Promotes Neurite Outgrowth

CRMP2 is a cytoplasmic protein that is highly expressed in neurons and oligodendrocytes in the developing and adult CNS. It has been implicated in several neuronal processes, mainly neurite outgrowth, but also in axon/dendrite specification, neuronal polarity, kinesin-dependent axonal transport, microtubule dynamics, synaptic assembly, neurotransmitter release and calcium channel regulation (Hensley et al., 2011). Downstream molecular targets of CRMP2 comprise the cytoskeletal proteins tubulin, actin and vimentin, the motor protein kinesin, the calcium binding protein calmodulin and the N-type voltage-gated calcium channel CaV2.2. It has been shown that CRMP2 binds to α–β-tubulin dimers to promote microtubule polymerization and stabilization resulting in increased neurite outgrowth. Several upstream post-translational modifications regulate CRMP2 function, including glycosylation, oxidation, proteolysis and phosphorylation. Especially phosphorylation of CRMP2 has been studied extensively. Among the kinases that have been shown to phosphorylate CRMP2 at specific sites are glycogen synthase kinase (GSK)-3β, cell division protein kinase (Cdk)-5, Ca^{2+}/calmodulin-dependent protein kinase (CaMK)-II, Rho-Kinase (ROCK), and the Src family kinase Fyn. The majority of these phosphorylations inhibit CRMP2 function resulting in neurite retraction, growth cone collapse and failure of regeneration when these kinases are over-acti-

vated after axonal injury, whereas basic physiological levels of CRMP2 phosphorylation, *e.g.*, by GSK-3β or CDK-5, do not affect neurite outgrowth. Therefore, dephosphorylation or inhibition of excessive CRMP2 phosphorylation fosters axonal regeneration, enhances sensitivity to the neurotropic factor BDNF, and counteracts the inhibitory effect on axonal regeneration induced by chondroitin sulfate proteoglycan (CSPG) and myelin-associated glycoprotein (MAG) *in vitro* (Mimura et al., 2006; Nagai et al., 2016). After spinal cord injury in mice *in vivo*, inhibition of CRMP2 phosphorylation improved the recovery of motor and sensory functions and inhibited scaring (Nagai et al., 2016).

CRMP2 Affects Axonal Degeneration

Beside this compelling evidence that CRMP2 is crucial for axonal regeneration, several studies have demonstrated a major role of CRMP2 in axonal degeneration including neurodegenerative conditions like Alzheimer's disease and amyotrophic lateral sclerosis (Hensley et al., 2011). However, the underlying molecular mechanisms are still not understood in detail. In mouse dorsal root ganglia neurons *in vitro*, increased levels of the phosphorylated form p-CRMP2-T514 induced by activated GSK-3β were detected in neurites 6 hours after the initiation of Wallerian degeneration, while overexpression of non-phosphorylated CRMP2 significantly delayed Wallerian degeneration (Wakatsuki et al., 2011). Similarly, levels of the phosphorylated form p-CRMP-2-Thr555 that is induced by ROCK, were increased in degenerating axons in a mouse model of multiple sclerosis *in vivo*, while lower levels of p-CRMP-2-Thr555 were associated with more limited signs of axonal degeneration (Petratos et al., 2012). In mouse superior cervical ganglia cells after NGF deprivation *in vitro*, increased levels of the cleaved form of CRMP2 were observed in swellings of degenerating neurites (Touma et al., 2007), whereas overexpression of CRMP2 reduced the number of varicosities in neurites of cortical neurons after glutamate excitotoxicity (Hou et al., 2009). These results suggest a protective role of the physiological, non-phosphorylated CRMP2-isoform and a detrimental role of the phosphorylated and the cleaved form of CRMP2 in axonal degeneration. However, further studies are urgently needed to decipher the exact functions of different isoforms and phosphorylation states of CRMP2 and the involved molecular downstream targets in axonal degeneration.

Acute Axonal Degeneration in the Optic Nerve

We have previously analyzed the molecular mechanisms of acute axonal degeneration (AAD) in the optic nerve crush (ONC) lesion model in rat. AAD is the earliest form of axonal degeneration and is characterized by a rapid fragmentation of the adjacent proximal and distal axonal stump on both sides of the lesion within several hours after a focal axonal lesion. It is followed by Wallerian degeneration (WD) starting at 24–72 hours after lesion, which results in the complete fragmentation of the distal part of the axon. In the rat optic nerve *in vivo*, we previously demonstrated that a crush lesion leads to a strong transient calcium influx into the axon resulting in activation of autophagy, cytoskeletal abnormalities, dysmorphic mitochondria, bulb formation and finally axonal disintegration. The molecular mechanisms connecting these pathological features, however, remain unknown. Therefore we used the established rat ONC to further elucidate the molecular cascade of acute axonal degeneration (Zhang et al., 2016). Optic nerve injury is an excellent model system to study axonal degeneration, as it affects a well-defined axonal tract of the CNS that is surgically easily accessible. Moreover, axonal degeneration of the optic nerve is a critical event in several clinically relevant human disorders, including optic neuritis, glaucoma and hereditary optic atrophy.

Calpain is Activated Early in Acute Axonal Degeneration

Based on data from other studies, *e.g.*, in the spinal cord, we first checked whether the calcium-dependent protease calpain was activated after ONC following calcium influx. Calpain activation was evaluated by analyzing the expression levels of the 145 kDa breakdown product (BDP) of spectrin (α-fodrin), which is a specific cleavage product of calpain. The levels of cleaved spectrin started to increase already at 5 minutes after ONC, and continued to rise up to 6 hours, while the levels of intact spectrin stayed stable, probably due to a rapid reproduction of the protein. Calpain activation was further demonstrated directly by analysis of both its pro-enzymatic and active isoform (76 kDa). The levels of the active isoform of calpain were increased in relation to its pro-enzymatic isoform at 6 hours after ONC, while the total protein levels of calpain were not changed. Interestingly, calpain activity increased slower on the distal side than on the proximal side of the crush site, which might relate to the subsequently distinct morphological fates of the proximal and distal stumps. Immunofluorescence analysis of the 145 kDa spectrin BDP revealed that calpain activation was specifically localized in the axons in the region morphologically affected by AAD. This local confinement of calpain activation further supports its importance in mediating AAD. Finally, the role of calpain inhibition on AAD was evaluated by *in vivo* live imaging, which allows the visualization of axonal changes in the anesthetized living rat. We found that pharmacological calpain inhibition with intravitreally injected calpeptin stabilizes the axons on the proximal side up to 6 hours after lesion while its protective effect was less pronounced on the distal side. These results establish calpain as the central molecular mediator of AAD downstream of calcium after ONC.

Cleavage of CRMP2 by Calpain Mediates Axonal Degeneration

In order to discover further molecular targets downstream of calpain, we checked expression levels of several previously described or predicted target proteins of the protease calpain after ONC with or without calpain inhibition with calpeptin. The protein that was regulated most was CRMP2. It has been shown before that calpain cleaves CRMP2 after traumatic

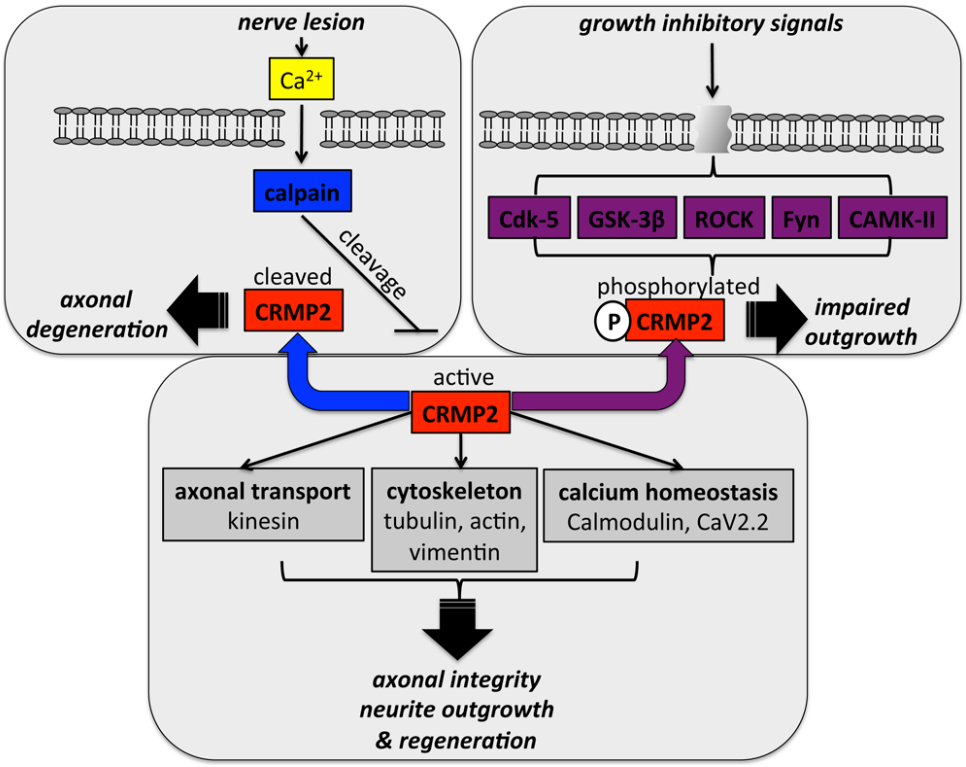

Figure 1 Role of collapsin response mediator protein-2 (CRMP2) in the axon under physiological conditions, after nerve lesion and in neurite outgrowth.
Under physiological conditions, active CRMP2 contributes to axonal integrity and neurite outgrowth through modulations of axonal transport, cytoskeleton assembly and calcium homeostastis (lower box). After a nerve lesion, calcium influx results in an activation of calpain which cleaves CRMP2 (upper left box). Cleaved CRMP2 is not capable of sustaining axonal transport and integrity anymore, thus leading to axonal degeneration. Another way of CRMP2 inactivation is its excessive phosphorylation by several kinases that results in impaired neurite outgrowth (upper right box). Some of these kinases are activated by extracellular inhibitory cues that bind to neuronal transmembrane receptors.

brain injury (Zhang et al., 2007). We found that there was a significant increase in cleaved CRMP2 during AAD and that the cleavage of CRMP2 after ONC was suppressed by calpain inhibition *in vivo*, which was further confirmed at 6 hours after scratch of cortical neurons *in vitro*. Thus, the cleavage of CRMP2 in AAD is mediated by calpain activation. Although our data clearly indicate that enhanced calpain-activation and subsequent CRMP2-cleavage is located primarily in the axonal compartment of the optic nerve during AAD, it is possible that similar molecular mechanisms also occur in the surrounding glial cells (including oligodendrocytes and astrocytes) and might contribute to axonal degeneration at later time points. This should be analyzed in greater detail in the future.

Considering that CRMP2 cleavage was suppressed by calpain inhibition correlating with axonal degeneration, we thus evaluated the role of intact CRMP2 in AAD using adeno-associated viral vectors (AAV) overexpressing CRMP2. *In vitro*, we modeled AAD using a microfluidic chamber system, which allowed the specific analysis of the axonal compartment. We found that CRMP2 overexpression delayed the formation of axonal bulbs after axotomy of cortical neurons *in vitro*. This effect of CRMP2 on bulb formation was sustained over 8 hours after axotomy although some bulbs still developed over time. This finding suggests a crucial role of CRMP2 for the early formation of axonal bulbs during AAD. After ONC *in vivo*, CRMP2 overexpression almost completely prevented axonal fragmentation on the proximal side up to 6 hours after the lesion while the effect on AAD was less pronounced on the distal side. The magnitude of its axon-stabilizing effect on the proximal side of the lesion was similar to the one achieved by calpain inhibition, suggesting that CRMP2 is a major target of calpain in mediating AAD.

We observed no changes in the levels of cleaved CRMP2 despite overexpression of intact CRMP2, indicating that axonal degeneration is not mediated by a toxic mechanism of the cleaved CRMP2 fragment but rather by a loss of functional CRMP2 that is physiologically stabilizing axonal integrity. However, further experiments need to verify this hypothesis.

Interestingly, both calpain inhibition and CRMP2 overexpression exerted much stronger anti-degenerative effects on the proximal than on the distal side of the injury. Similar results were obtained earlier with ROCK2 downregulation. This suggests that axonal degeneration on the distal side is sustained more strongly and is harder to be counteracted at least by these interventions. While axonal transport and energy supply are most probably less severely affected on the proximal side, another possible reason for these findings is the rapid decrease of the axon protective factor nicotinamide mononucleotide ade-

nylyltransferase (NMNAT) in the distal axon that was shown before to occur within 2–3 hours after axonal transection. Decreased levels of NMNAT are one of the major factors driving Wallerian degeneration and seem not to be affected by CRMP2 overexpression or calpain inhibition. However, this needs to be analyzed in more detail in the future.

CRMP2 Controls Bulb Formation and Axonal Transport during Axonal Degeneration

We next assessed the mechanisms underlying AAD downstream of CRMP2. Using live imaging of axonal mitochondria transport in cortical neurons in microfluidic chambers, we found that the percentage of motile mitochondria in axons was significantly decreased within 30 minutes after axotomy, returning to normal levels at later time points. These observations visualize an early transient impairment of axonal mitochondria transport in AAD that might be crucial for the development of axonal degeneration bulbs, which contain accumulations of mitochondria, and subsequent axonal fragmentation. CRMP2 overexpression, on the other hand, inhibited the observed impairment of axonal mitochondrial transport at 30 minutes after axotomy. Interestingly, the most pronounced effect of CRMP2 overexpression on bulb formation also occurs at 30 minutes after axotomy, coinciding with the peak of axonal transport impairment. It has been shown that CRMP2 interacts with the motor proteins kinesin and dynein, which drive antero- and retrograde axonal transport, respectively (Kimura et al., 2005; Arimura et al., 2009). It is thus very likely that the cleavage of CRMP2 affects its binding to these motor proteins, thereby resulting in the impairment of axonal transport.

Proteomics Analysis of the CRMP2 Interactome in Acute Axonal Degeneration

Next, we performed proteomics of optic nerve lysates and compared the proteome 6 hours after ONC to the unlesioned nerve. We found an early regulation of several proteins that are known to interact with CRMP2. Among other proteins, levels of the kinesin-like protein KIF1 were decreased at 6 hours after ONC. It has been shown that CRMP2 binds to KIF1 to regulate tubulin trafficking, the decreased levels of KIF1 may thus exacerbate the negative impact on axonal transport by cleavage of CRMP2. On the other hand, expression levels of several cytoskeletal proteins including spectrin and actinin as well as the mitochondrial protein MDH2 were increased, possibly reflecting the accumulation of cytoskeletal proteins and mitochondria due to impairment of axonal transport. We also found a down-regulation of PEA-15 which plays a role in the ERK/MAPK signaling pathway regulating neuronal cell survival, as well as GOT1, NIT2 and GNB1 which are involved in glutamate metabolism and signal transduction. Thus, the role of CRMP2 on AAD might be also mediated by interacting with these binding partners. Out of all these CRMP2 interactors, KIF1 might be the most interesting one, which should be followed up in the future.

In conclusion, our study further elucidates the molecular cascade underlying AAD assigning a central role to CRMP2 (**Figure 1**). After the initial transient calcium influx caused by the lesion, the protease calpain is rapidly activated in close proximity to the lesion site. Its localization defines the area of AAD. Calpain then cleaves CRMP2 leading to an impairment of axonal transportand formation of degeneration bulbs. Overexpression of CRMP2 attenuated axonal degeneration of the proximal axon.

Together with the well-established positive effects on regeneration our findings on its central role in axonal degeneration further support the favorable characteristics of CRMP2 as a promising drug target in neurodegenerative and neurotraumatic diseases that can positively mediate both axonal de- and regeneration.

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