Review

Intrinsic regenerative mechanisms of central nervous system neurons

Rieko Muramatsu[1,2,*], Masaki Ueno[1], Toshihide Yamashita[1,*]

[1] *Department of Molecular Neuroscience, Graduate School of Medicine, Osaka University, Osaka, Japan;*
[2] *Department of Mental Health and Environmental Effects Research, The Research Center for Child Mental Development, Osaka University Graduate School of Medicine, Osaka, Japan.*

Summary **Injuries to the adult central nervous system (CNS), such as spinal cord injury and brain contusion, can cause permanent functional deficits if axonal connections are broken. Spontaneous functional recovery rarely occurs. It has been widely accepted that the extracellular environment of the CNS inhibits neuronal regeneration. However, it should be noted that another reason for injured neurons failing to regenerate is their weak intrinsic ability to do so. The regeneration of injured neurons is a process involving many intracellular phenomena, including cytoskeletal changes, gene and protein expression, and changes in the responsiveness to extracellular cues. The capacity of injured neurons to regenerate is modulated to some extent by changes in the expression of intracellular signaling molecules such as glycogen synthase kinase-3β and cyclic adenosine 3′,5′-monophosphate. Knowledge of these effects has guided the development of animal models for regenerative therapies of CNS injury. Enhancing the intrinsic regenerative machinery of injured axons in the adult CNS is a potentially powerful strategy for treating patients with a CNS injury.**

Keywords: Axon, CNS, spinal cord injury, GSK-3β, cAMP

1. Introduction

Lesions of the adult central nervous system (CNS), such as brain contusion and spinal cord injury lead to functional deficits due to neuronal cell death and a loss of axonal connections. Many strategies, including cell transplantation, activation of neurogenesis of neural stem/progenitor cells, and use of undamaged neurons after injury, have been attempted to develop new therapies for recovering the neuronal dysfunction. Importantly, in each approach, axonal regrowth is essential to restore damaged neuronal connections. Unlike embryonic or peripheral nervous system neurons, however, neurons in the adult mammalian brain and spinal cord do not spontaneously regenerate. This prevents neurological symptoms from improving after an injury.

The main reason for injured adult neurons failing to regrow is the inhibitory nature of their extracellular environment. Indeed, providing CNS neurons with a permissive environment, such as peripheral nerve environment, can promote regrowth. For example, the transected axons grow into a transplanted segment of sciatic nerve in the injured spinal cord (*1*,*2*). In dorsal root ganglia (DRG) neuron that sends one axon into peripheral target tissue and the other axon into CNS, the axon can regenerate into peripheral target, but not into CNS after injury (*3*). These findings can be considered as evidence of extracellular mechanisms that inhibit axon growth in the adult CNS. Several types of molecules are implicated in these inhibitory mechanisms, including proteins that are derived from either myelin, reactive astrocytes or those that are

**Address correspondence to:*
Dr. Rieko Muramatsu (e-mail: muramatsu@molneu.med.osaka-u.ac.jp) or Dr. Toshihide Yamashita (e-mail: yamashita@molneu.med.osaka-u.ac.jp), Department of Molecular Neuroscience, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan.

known to repulse axonal extension during ontogenesis (*4-6*).

Despite the inhibitory environment of the adult CNS, spontaneous regeneration and reorganization of axons are also found in animal models of CNS injury. For example, spontaneous axon sprouting of the corticospinal tract, one of the most important descending motor pathways for skilled movements in all mammalian species, was observed at the proximal sites of injuries in the spinal cord injury (SCI) (*5*). It was also observed that a few damaged corticospinal axons regrew beyond the lesion site in SCI mice (*7*). Importantly, these reorganization and regeneration of axonal network have been considered to contribute to a partial recovery of locomotor function in animal models of SCI. In addition, it suggests that adult CNS neurons may have an intrinsic capacity to evoke mechanisms that promote spontaneous axonal regeneration after CNS injury. This has led us to focus on the intrinsic regenerative capacity of adult CNS neurons.

Neutralizing the extracellular factors that inhibit regrowth promotes axonal regeneration to some extent *in vivo*. However, it is expected that the regrowth of axons in the adult CNS can be further improved by enhancing their intrinsic capacity to regenerate. The capacity of adult CNS neurons to regenerate is low compared to that of neurons during earlier stages of development. The growth of axons during development is sustained primarily by (i) cytoskeletal changes, (ii) gene expression and protein synthesis, and (iii) changes in the response to extracellular cues. Thus, regulation of these phenomena in adult CNS neurons may be a powerful strategy for re-establishing neuronal networks after injuries. In consideration of this, our review focuses on the intrinsic molecular mechanisms that promote axon regeneration in the CNS.

2. Molecular mechanisms of modulation in cytoskeletal assembly

Axon elongation is the product of intracellular machinery that assembles cytoskeletal elements when activated by intrinsic signals or extracellular factors. Of particular relevance to the growth of an axon is the assembly of microtubules and actin filaments.

Glycogen synthase kinase-3β (GSK-3β) is an inhibitory molecule to the processes involved in microtubule assembly, and thus prevents axon growth. Treatment with a GSK-3β inhibitor to SCI rats promotes the growth of corticospinal and serotonergic axons in the spinal cord, and thereby promotes functional recovery (*8*). GSK-3β phosphorylates and inactivates collapsin response mediator protein 2 (CRMP-2). It is known that CRMP-2 binds to tubulin heterodimers and actin filaments, thus promoting neurite elongation. During normal neural development, the active form of CRMP-2 has been found to counteract the inhibition of axon elongation produced by constitutively active GSK-3β (*9*). In adult CNS, increased expression of a non-active form of CRMP-2 was observed in spinal cord neurons that failed to regenerate after SCI (*10*). These findings suggest that, because of its effects on CRMP-2 activity, inhibition of GSK-3β may be an appropriate target in the treatment of CNS injuries.

Besides CRMP-2, GSK-3β has other physiological substrates in growing axons; adenomatous polyposis coli (APC) and microtubule binding protein 1B (MAP1B). APC appears to have roles in cytoskeletal integrity of actin and microtubule dynamics, and in promoting neurite elongation. As in the case of CRMP-2, phosphorylation of APC by GSK-3β inhibits neurite elongation by blocking its ability to bind to microtubules (*11*). Similarly, phosphorylation of MAP1B by GSK-3β increases the population of unstable microtubules, thus negatively regulates axon growth (*12*). Targeting the APC, MAP1B, or CRMP may be effective strategies to treat patients with a CNS injury.

In contrast to the beneficial effects of CRMP activation on neurite outgrowth, injury-induced proteolytic cleavage of CRMP seems to have negative effects on it. Calpain, which is a calcium-dependent protease, cleaves a large number of substrates including CRMPs. Interestingly, calpain activity is increased in the focal cerebral ischemic brain, and cleavage of CRMP also occurs following cerebral ischemia (*13*). The expression of cleaved product of CRMP is correlated with neuronal death during cerebral ischemia. Indeed, overexpression of cleaved products of CRMP into cultured cerebellar granule neurons causes neuronal death (*14*). These researches indicate that truncated CRMP plays a key role in neuronal survival. In parallel, the truncated CRMP also seems to regulate neurite outgrowth and degeneration. Short isoform of CRMP-2, identified as a C-terminal processed form by calpain, inhibits neurite outgrowth in cultured cortical neurons (*15*). This finding suggests that truncated CRMPs may negatively regulate the axon elongation. The role of CRMP cleavage on neurite degeneration have also become clear in the researches using *Wld*^{S} (Wallerian degeneration slow) mice, in which neurite degeneration is delayed. The neurites of cultured suprior cervical ganglias (SCGs) are degenerated by NGF deprivation. CRMP proteins are cleaved during neurite degeneration in NGF-deprived neurite of wild-type but not *Wld*^{S} mutant mice. Increase in truncated form of CRMP parallels the beading formation in degenerating neurites in SCGs (*16*). Thus, it is possible that CRMP cleavage is also involved in the process of neurite degeneration, and in turn, inhibits regeneration due to the loss of active form of CRMP. These findings suggest that injury-induced proteolytic cleavage of CRMP may act negatively on axonal regeneration. Hence, in order to explain the regenerative effects of CRMP, we must

focus on both activation (positive effect) and protein cleavage (negative effect) of CRMP.

The second messenger cyclic adenosine 3′,5′-monophosphate (cAMP) is another molecule involved in cytoskeletal rearrangement, and contributes to axon regeneration. Rolipram is a selective cAMP phosphodiesterase type IV inhibitor, and elevates the intracellular cAMP levels. It was shown that treatment with rolipram promotes both the regeneration of raphespinal tract neurons and functional recovery in SCI rats (*17*). In the downstream cascades, cAMP activates protein kinase A (PKA), which, in turn, restricts the inhibition of axonal growth by inactivating members of the Rho family of small GTPase proteins. RhoA and Rho kinase have emerged as negative regulators of the actin and tubulin polymerizations by a mechanism dependent on CRMP-2 inactivation (*6*,*10*). Treatment with C3 transferase, a Rho inhibitor, or the Rho kinase inhibitor, Y27632, causes axon regeneration in the injured spinal cord and promotes recovery of motor function (*18*). Thus, inhibition of Rho-Rho kinase pathway by the activation of cAMP-PKA signaling may also be useful to induce cytoskeletal changes that facilitate axon regeneration.

3. Molecular mechanism of gene expression and protein synthesis

A lack of gene expression and protein synthesis is thought to be responsible for the poor regeneration of neurons in the adult CNS. It is thus likely that transcriptional activation can be targeted so as to beneficially affect axon growth and functional recovery.

The cAMP-CREB-arginase 1 (Arg1) system is the most promising candidate target for approaches to promote axon regeneration by gene expression. A decrease in endogenous cAMP level has been found to coincide with a loss of the capacity of neurons to regenerate (*19*). This suggests that cAMP signaling may enhance the intrinsic regenerative capacity of adult CNS neurons. One of the downstream molecules of cAMP is the cAMP response element binding protein (CREB). It has been shown in cultured DRG neurons that the expression of constitutively active CREB overcomes the inhibitory effect of myelin-associated glycoprotein (MAG) on neurite growth (*20*). Moreover, in SCI rats, CREB activation is reported to promote the growth of dorsal column axons through the lesion site (*20*). It thus seems that cAMP-CREB signaling makes an important contribution to axon regeneration of the adult CNS. Activated CREB further regulates the transcription of many growth factor genes and facilitates an increased expression of the enzyme Arg1. Overexpression of Arg1 in cerebellar neurons can overcome the inhibition of axon growth by MAG and myelin, without elevation of cAMP (*21*). An increase in Arg1 expression (as induced by CREB) results in the synthesis of the polyamine putrescine, which is converted to spermidine. Administration of spermidine also promotes nerve regeneration after optic nerve crush (*22*). Thus, promotion of polyamine synthesis may be able to contribute to the axon regeneration. In addition, Arg1 levels in developing DRG neurons gradually decrease, that correlates with a decrease in endogenous cAMP level and the capacity for regeneration (*21*). From these findings, it appears that cAMP-CREB-Arg1 signal pathway and resulting polyamine synthesis are attractive targets to overcome factors that inhibit axon elongation.

It is thought that the GSK-3β pathway is also closely related to transcriptional system that enhances axonal growth. GSK-3β is inactivated by phosphatydilinositol 3-kinase (PI3 kinase) and extracellular signal-regulated kinase (ERK), both of which can increase protein systhesis to promote axonal growth. The deficiency of phosphatase and tensin homolog (PTEN), a positive regulator of PI3 kinase/mTOR (the mammalian target of rapamycin) pathway, enhances protein translation and promotes the regeneration of retinal ganglion cell (RGC) axons (*23*). Activation of ERK, which is involved in protein synthesis, induces the intrinsic growth state of adult corticospinal tract, thus promotes axon regeneration into subcortical lesion site (*24*). Although there seems to be few evidences, it might be possible that PI3kinase and ERK activations promote axon regeneration through inactivation of GSK-3β followed by protein synthesis. Indeed, it is reported that activated GSK-3β inhibits the phosphorylation and DNA-binding ability of CREB, a major promoter of protein synthesis (*25*). Thus, it is possible that activated GSK-3β limits the ability of CNS axons to regenerate by suppressing gene expression and protein synthesis.

4. Changes in the responsiveness to extracellular cues

Another aspect that should be investigated is the changes in the responses to the inhibitory environment, which is influenced by intracellular condition. The effects of environmental cues involved in axonal guidance are thought to be influenced by the intracellular levels of second messengers like cAMP and cyclic guanosine monophosphate (cGMP). Along these lines, several studies, which mainly focus on axonal growth in development, have investigated relationships between the responsiveness and cyclic nucleotide levels.

It has been shown that the repulsive response to recombinant proteins consisting of the extracellular domain of MAG can be elevated by cAMP level in *Xenopus* spinal axons (*26*), suggesting that cAMP elevations may modulate the response of neurons to environmental cues. Some of the most defined studies have focused on netrin-1, an axon guidance factor during development that promotes neurite elongation *via* deleted in colorectal cancer (DCC) receptor. Activation

of protein kinase A (PKA), a major downstream signal molecule of cAMP, enhances the DCC mobilization to the plasma membrane and increases axon extension in response to netrin-1 in cultured commissural neurons (*27*). Inhibition of RhoA that is inactivated by cAMP elevation also leads to the recruitment of additional DCC to the plasma membrane on the neurites of cultured explants of embryonic spinal cord (*28*). These findings implicate that elevation of cAMP level enhances the attractive responsiveness to extracellular cue, such as netrin-1, thus promotes neurite elongation. Hence, targeting of molecular mechanism that modulates the expression of guidance receptors may be an important approach for promoting the spontaneous regeneration of neurons.

cGMP level also affects the responses to extracellular cues. Sema3A, a guidance molecule, triggers the repulsive effect to cultured sensory neurons, and the repulsion can be converted to attraction by pharmacological activation of cGMP pathway (*26*). One major downstream targets of cGMP is cGMP-dependent protein kinase 1 (cGK1), which is activated by elevation of cGMP level. It was found in DRG explants of cGK1-deficient mice that raising cytosolic cGMP levels did not prevent the inhibitory effect of Sema3 (*29*), consistent with cGMP-cGK1 signaling being a main pathway that contribute to the change of responsiveness of neurons to extracellular cues. Although the role of cGMP in functional recovery after CNS injury in mammals has not yet been investigated, it has also been proposed that cGMP signaling is thought to play a key role in the regeneration of optic nerves in the goldfish (*30*). Further research on the role of cGMP in the response to inhibitory molecule would clarify the possible involvement of axon regeneration.

The results of several studies suggest that it is not simply their individual levels, but the ratio of cAMP to cGMP is also important for determining the response of extending axons to guidance molecules (*31*). This supports the likelihood of a variety of second messengers having crucial roles in the regulation the responses of the neuronal growth cones to extracellular cues. Although little is known about the molecular mechanisms that modulate the intrinsic response to extracellular cues by second messengers, research concerning the molecular mechanisms of intrinsic responsiveness of adult CNS neurons to extracellular cues may lead to the development of new therapies for CNS injuries.

5. Conclusions

While the extracellular environment of the adult CNS does not support axonal regeneration, a spontaneous partial regeneration of axons after CNS injury has been observed. This suggests that adult CNS neurons may have an intrinsic capacity to evoke mechanisms that promote spontaneous axonal regeneration after CNS injury. Adult neurons need appropriate regulation of cytoskeletal reorganization and protein synthesis to regenerate. In addition, changing the responsiveness of injured axon to chemoattractants may facilitate their regrowth. Extensive researches have revealed the intrinsic molecular mechanisms for axonal outgrowth. An understanding of how these powers are enhanced will contribute to the development of effective therapies for CNS injuries.

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