Review Article

Concept and molecular basis of axonal regeneration after central nervous system injury

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ABSTRACT

Damage to the central nervous system (CNS) leads to the disruption of the axonal network and causes neurological dysfunction. Recovery of neurological functions requires restoration of the axonal network; however, injured axons in the adult mammalian CNS rarely regenerate after injury. Failure of the injured axon to regenerate is attributed at least partly to the inhibitory molecules of the CNS: several proteins have been identified in the CNS that inhibit axonal regeneration. In addition, the molecular mechanisms underlying the manner via which these inhibitors prevent axonal regeneration have been clarified. The neutralization of nonpermissive substrate properties of the CNS has been shown to promote axonal regeneration in an animal model of CNS injury. Drugs that promote axonal regeneration, some of which have undergone clinical trials, have been developed by pharmaceutical companies. However, spontaneous functional recovery occurs sometimes after CNS injury. This review will describe the new concept of the molecular mechanism of restoration of the neuronal network, with a special focus on our recent reports.

Keywords:

P75^{NTR}

RhoA

RGM-a

SHP-1/2

BDNF

Prostacyclin

Introduction

Axonal degeneration is directly triggered by wide range of insults, including injury, inflammation, and genetic defects. Disruption of the neuronal network causes neurological dysfunctions, such as motor, sensory, cognitive, and other deficits. The restoration of neuronal function requires the rebuilding of the neural network based on the regeneration of axons that belong to the neurons that survive the initial effects of injury (Harel and Strittmatter, 2006). However, central nervous system (CNS) axons fail to regenerate beyond the lesion site, which is in contrast with that observed in the developmental nervous system. The reasons underlying the failure of axonal regeneration have been mainly interpreted as environmental inhibitors that are inherent to the adult CNS (Yiu and He, 2006). A rapidly growing number of reports have recently identified molecules that impede the regeneration of damaged nerve fibers.

However, increased knowledge regarding the underlying power of environmental inhibitory factors on axonal regeneration has revealed that extrinsic factors are not sufficient to explain the regenerating failure. This emerging information demands a consideration of the developmental decline in regenerative capacity, which has been pointed out as another cause of regeneration failure (Rossi et al., 2007; Muramatsu et al., 2008). Moreover, the interruption of the regenerative mechanism has been changing over the course of time. The gain of regenerative ability, which is attributed to cell–cell communication in the CNS, is beginning to be targeted as the new strategy for achieving axonal regeneration. This review summarizes our current studies regarding the development of strategies to promote axonal regeneration and functional recovery after adult CNS injury.

The role of myelin-associated inhibitors

Early studies have demonstrated that CNS myelin, but not peripheral nervous system

(PNS) myelin, contains inhibitors of axonal regeneration (Schwab and Thoenen, 1985). The isolation of inhibitory molecules was first attempted using an inhibitory fraction of myelin, termed IN-1, which was isolated for its ability to neutralize myelin inhibition in vitro (Caroni and Schwab, 1988; Schnell & Schwab, 1990). A putative antigen for IN-1 led to the discovery of the protein Nogo, which is a member of the reticulon family. Nogo exists in three isoforms derived from the usage of alternate promoters and splice sites. Nogo-A is the best characterized among these proteins and is the only one that is highly expressed in oligodendrocytes (Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000), which synthesize CNS myelin.

Myelin-associated glycoprotein (MAG) was identified from another myelin compartment that has potent inhibitory activity in vitro (Mukhopadhyay et al., 1994; McKerracher et al., 1994). MAG is a transmembrane protein that has 5 immunoglobulin-like domains in its extracellular region. In addition to Nogo-A and MAG, the glycosylphosphatidylinositol (GPI)-linked oligodendrocyte myelin glycoprotein (OMgp) has been described as an inhibitory protein that is expressed in CNS myelin (Wang et al., 2002a; Kottis et al., 2002). OMgp has five leucine-rich repeats and an N-terminal flanking region. Although no structural similarities have been detected among these inhibitors, they all exhibit high binding affinity to the Nogo receptor (NgR). NgR contains eight consecutive leucine-rich repeat (LRR) domains at its N-terminal region and a C-terminal LRR, which are sufficient to bind each inhibitor (Fournier et al., 2001; He et al., 2003). However, NgR lacks a cytoplasmic domain, suggesting that another receptor is required for the transduction of this signal in neurons. p75^{NTR} was initially identified as a low-affinity neurotrophin receptor that is expressed in many developing neurons. The first hint of the role of p75^{NTR} was provided by research that revealed that neurons from p75^{NTR} mutant mice do not exhibit MAG-mediated inhibition (Yamashita et al., 2002). The subsequent discovery of MAG binding to NgR led to conclusion that p75^{NTR} forms a physical receptor complex with NgR and is involved in inhibitor-mediated axonal growth inhibition (Wang et al., 2002b). In addition, Lingo-1 has been described as a component of the NgR/p75^{NTR} signaling

complex (Mi et al., 2004). Currently, it is well accepted that myelin-associated inhibitors bind to a receptor complex that consists of NgR, p75^{NTR}, and Lingo-1. Furthermore, it is well accepted that p75^{NTR} is a key receptor in the mediation of signal transduction by myelin-associated inhibitors.

The most obvious feature of the signaling cascade in which p75^{NTR} participates is the activation of RhoA, which is a member of the Rho family of small GTPases. A yeast two-hybrid screen and co-immunoprecipitation revealed that p75 binds to RhoA, and that p75 overexpression stimulates RhoA activity (Yamashita, et al., 1999). RhoA interacts with the Rho GDP-dissociation inhibitor (GDI) and is kept in an inactive state via its sequestering in the cytoplasm. The association between p75^{NTR} and Rho-GDI leads to the release of RhoA via the binding of GDI and subsequent activation of RhoA by Rho guanine nucleotide exchange factors (GEFs) (Yamashita and Tohyama, 2003). RhoA activation changes the organization and dynamics of actin and microtubules, and has been shown to correlate with the signals that induce growth-cone collapse and reposition of axon guidance (Etienne-Manneville and Hall, 2002). These observations corroborate the hypothesis that RhoA activation plays a primary role in myelin-associated inhibitor-mediated axonal growth inhibition. To determine the applicability of Rho activation to axonal regeneration in vivo, an assessment of therapeutic efficacy was performed using C3 transferase, which is a specific inhibitor of Rho GTPase. This treatment promoted significant regeneration of corticospinal tract (CST) and improved recovery of hindlimb function in an animal model of spinal cord injury (SCI) (Dergham, et al., 2002). Rho-associated kinase (ROCK) functions as one of the downstream effectors of Rho.

Pharmacological inhibition of ROCK signaling also promotes regeneration of CST and motor recovery after SCI (Dergham et al., 2002). To identify the signaling pathway that leads to the inhibition of axonal regeneration, some pharmaceutical companies have attempted to develop drugs that target molecules involved in the inhibition of axonal regeneration. The humanized anti-Nogo antibody ATI-355 has yielded functional recovery after SCI in animal models,

including primates, and is in an on-going Phase 1 of a clinical trial. BA-210 (trademarked as Cethrin®) is another potent agent that has been developed as an antagonist of recombinant Rho GTPase and has reached Phase 2b in a clinical trial aimed to assess its immune responses and efficacy. These elegantly elucidated molecular mechanisms mediated by myelin-associated proteins have reached the stage of practical realization regarding their clinical application (Fig. 1).

Developmental guidance factors

Genetic studies performed in mice have provided a more crucial assessment of the role of myelin-associated inhibitors in axonal regeneration (Zheng et al., 2006). However, inconsistent results regarding axonal regeneration have been reported in mice lacking each of the 3 myelin inhibitors. The development of the Nogo-, MAG-, and OMgp-deficient mouse allowed testing the hypothesis that the 3 myelin-associated inhibitors play a central role in axonal regeneration failure. Contrary to expectations, these triple-mutant mice failed to exhibit enhanced regeneration of **CST and raphespinal tract** after SCI (Lee et al., 2010). These studies prompted the consideration of the role of other molecules in the inhibition of axonal regeneration in the adult CNS.

We focused on the role of the repulsive guidance molecule-a (RGM-a), which was identified as an inhibitory guidance cue for developmental retinal axons (Monnier et al., 2002). RGM-a is a type of GPI-anchored membrane protein with no significant homology to any guidance molecule. Results from both rodent- and human-based studies provided evidence that the expression of RGM-a is increased in the damaged CNS (Schwab et al., 2005a, b). Our in vitro assay showed that RGM-a inhibits neurite outgrowth in cultured cerebellar neurons (Hata et al., 2006). We examined whether RGM-a inhibition promoted axonal reorganization and the restoration of neuronal function in vivo. Intrathecal injection of neutralizing antibodies against RGM-a promoted the regeneration of CST axons, as well as recovery of **hindlimb motor**

function, after SCI (Hata et al., 2006). In addition, our recent study revealed the mediation of outgrowth inhibition by RGM-a. RGM-a binds to neogenin, which is a well-known receptor of RGM-a, and this binding promotes the association of UNC5B with LARG, which mediates RhoA activation (Hata et al., 2009).

Another study identified several inhibitory components, including transmembrane semaphorin 4D (Sema4D/CD100), Sema 3A, and ephrin B3. Sema4D is expressed on mature oligodendrocytes and induces growth cone collapse (Moreau-Fauvarque et al., 2003). Sema3A is expressed at the lesion site after SCI. A selective Sema3A inhibitor enhances the regeneration of **raphespinal tract** and promotes recovery of **hindlimb movement** after SCI (Kaneko et al., 2006). Ephrin B3 is expressed in postnatal myelinating oligodendrocytes and acts as a repellent during CST projection (Benson et al., 2005). The signal transduction pathway modulated by the binding of these guidance factors to each receptor promotes cytoskeletal changes via Rho family GTPase activation (Swiercz et al., 2002; Iwasato et al., 2007). Therefore, the results described above suggest that blockage of inhibitory guidance cues represents a therapeutic potential for CNS injury. Given the potential application of the molecular mechanisms involved in inhibitory guidance cues to clinical studies, it will be important to explore further the adverse effects associated with each treatment.

Necessity of the **positive factors for axonal growth**

A recent study found that the paired immunoglobulin-like receptor-B (PIR-B), which is a major histocompatibility complex (MHC) class I receptor, is a functional receptor of myelin-associated inhibitors of axonal regeneration (Atwal et al., 2008). We revealed that the binding of MAG to PIR-B leads to the recruitment of the SH2 domain-containing protein tyrosine phosphatase (SHP-1/2) to PIR-B (Fujita et al., 2012). The recruitment of the SHP protein dephosphorylates the Trk receptor, thereby inhibiting axonal growth. The relative

success of targeting SHP and PIR-B in vitro has led to much anticipation regarding the promotion of regeneration and functional recovery after CNS injury in vivo. Using a model of crush injury of the mouse optic nerve, we first asked whether SHP knockdown promoted axonal regeneration. In vivo knockdown of SHP-1 or SHP-2 in the mouse retina resulted in enhance axon regeneration **in the optic nerve**. Surprisingly, however, axonal regeneration failed in a mouse model with genetic deletion of PIR-B. These observations suggest that another mechanism is required for axonal regeneration, in addition to the blockade of inhibitory cues.

Failure of axonal regeneration in the adult CNS might partly be attributed to the age-associated decline of the capacity for axonal regrowth (Rossi et al., 2007). There is ample evidence suggesting that the brain-derived neurotrophic factor (BDNF) is a pivotal neurotrophic factor involved in axonal regeneration (Gorden, 2009). Thus, we wondered whether the activation of the growth-promoting pathway is required for adequate axonal regeneration after crush injury to the optic nerve in PIR-B-deficient mice. As expected, we confirmed that BDNF treatment increased the number of regenerative fibers in PIR-B knockout mice compared with wild-type mice (Fujita et al., 2012). This result established a new strategy based on the observation that the enhancement of intrinsic capacity is essential for regenerative axonal outgrowth, **although it should be noted that the axonal regeneration of the injured optic nerve does not occur spontaneously.**

The role of neuronal communication in regeneration

Throughout the past decade, axons have been generally believed to be incapable of regeneration in the adult CNS, as mentioned above. However, recent studies have provided evidence that some injured axons form new collaterals and connect to secondary neurons, contributing to functional recovery after CNS injury. During the development of the nervous system, axons are guided to their target by extracellular cues (Tessier-Lavigne and Goodman,

1996). Thus, it has been hypothesized that target-neuron-derived support is important in spontaneous rewiring in the adult CNS.

To demonstrate the notion of target-derived support, we used a mouse model of cortical injury. In traumatic brain injury of the unilateral sensorimotor cortex, motor function deficits persist because of the loss of axons along the CST. However, these deficits are frequently followed by partial recovery, which is associated with spontaneous remodeling of the CST network (Harel and Strittmatter, 2006). We found that this reorganization of the CST circuit is induced by BDNF secretion from target interneurons. Damaged CST axons established new contacts with spinal interneurons, which contributed to recover **of forelimb function** after injury. Knockdown of BDNF in spinal neurons abrogates the formation of the CST branches that form the new network (Ueno et al., 2012). There is increasing evidence that plastic changes occur and brain maps change after cortical injury (Murphy and Corbett, 2009). Our study showed that some factors, **which are known as extrinsic trophic factors, are expressed in target neurons and also** exert a positive effect on axonal remodeling and functional recovery in the mature CNS (Fig. 2).

The role of heterocellular communication in regeneration

The neuronal network formed after CNS injury sometimes differs from the developmentally formed one, suggesting that the mechanisms involved in these processes may also differ. We focused on angiogenesis, which is an outstanding and common feature of many CNS disorders (Fokman and Brem, 2009). Because neovascularization is thought contribute to pathologically to the development of inflammation (Costa et al., 2007), targeting angiogenesis has been considered beneficial in delaying the course of disease and as playing a role in wound healing in chronic inflammation (Xiong et al., 2010). Furthermore, the vascular niche is well exemplified by experiments in which the fate of neuronal progenitors is regulated by trophic

support from factors derived from endothelial cells (Carmeliet, 2003). These findings prompted us to investigate whether neovessels formed after CNS injury promote axonal rewiring.

To test this hypothesis, we used experimental autoimmune encephalomyelitis (EAE), which is an animal model of multiple sclerosis. We produced localized EAE in the dorsal thoracic spinal cord, which resulted in the disappearance of the CSTs below the level of the lesion. Severe hindlimb paresis was followed by partial spontaneous recovery after EAE induction because of the spontaneous regeneration of damaged CSTs (Kerschensteiner et al., 2004). We found that robust angiogenesis preceded the onset of CST rewiring at the EAE lesion, suggesting that vascular endothelial cells play a potential role in axonal regeneration (Fig. 3). As we searched for the pivotal factor that mediated axonal growth, we found that prostacyclin derived from vascular endothelial cells is involved in axonal regrowth via a mechanism dependent on the formation of adenosine 3',5'-cyclic monophosphate (cAMP), which is a key messenger in axonal regeneration (Snider et al., 2002; Hannila and Filbin, 2008). To assess whether prostacyclin released from vascular endothelial cells promotes axonal rewiring in EAE mice, we knocked down prostacyclin synthase (PGIS) in vivo by transfecting vascular endothelial cells of the brain with PGIS siRNA in EAE mice. In vivo transfection of PGIS siRNA decrease the formation of CST collaterals in response to localized EAE compared with that observed in control siRNA transfection. Furthermore, spontaneous motor recovery from EAE-induced paresis was delayed by the transfection of PGIS siRNA, suggesting that the vascular niche provides trophic support to regenerating neurons in the adult CNS (Muramatsu et al., 2012). Therefore, axonal regrowth after CNS inflammation is potentially assisted by the specific pathological features of CNS disorders, including marked angiogenesis (Fig. 3).

Conclusions

Research advances reported in recent decades have led to the identification of many

inhibitor molecules in the adult CNS that might be the cause of regeneration failure after injury. These inhibitors are largely classified as myelin-associated factors and guidance cues that are associated with nerve development. In addition, recent studies have captured the factors that are involved in the dynamics of axonal rewiring based on cell–cell communications under pathological conditions (Gensel et al., 2012). The evidence of the significant efficacy of the enhancement of trophic factors in CNS injury will continue to increase in the next decades. Moreover, inflammation at the lesion site is a noteworthy feature in CNS diseases and have a possibility to contribute to plastic changes in the adult CNS. One convincing candidate is resident and/or infiltrating inflammatory cells, which accumulate in both acute and chronic inflammatory lesions in the CNS (Schwartz and Ziv, 2008). More recently, we revealed that factors released from microglia support CST development (Ueno et al., 2013). This finding agrees with the importance of heterocellular communication in the formation of CNS networks. Exploring the biological system underlying the rewiring of CNS axons in a pathological environment may help rebuild the disrupted axonal network and contribute to the development of new therapies for CNS disease.

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Figure Captions

Fig. 1. Molecular mechanisms underlying the action of environmental inhibitory cues in axonal regeneration. The adult CNS environment contains specific molecules that inhibit axonal growth. Myelin-associated inhibitors, including myelin-associated glycoprotein (MAG), Nogo-A, and oligodendrocyte myelin glycoprotein (OMgp), were identified in intact oligodendrocytes and myelin. These myelin-associated inhibitors bind to NgR/p75^{NTR} receptor complex and activate RhoA, leading to growth cone collapse and neurite extension inhibition.

Fig. 2. Schematic picture showing reorganization of CST axons after axonal injury. Injured CST axons lose connection with their target neurons. However, damaged CST axons subsequently create compensatory neural circuits, which promote motor recover after injury.

Fig. 3. Temporal difference in the formation of neovessels and axon collaterals at the lesion site of spinal cords obtained from mice after the induction of localized EAE. Representative images of cross-sections of the thoracic spinal cord showing biotinylated dextran amine (BDA)-labeled hindlimb CST fibers (green) and CD105-positive neovessels (red). (a) Control. (b) Seven days after EAE induction. (c) Twenty-eight days after EAE induction. Axonal sprouting in injured CSTs is massively induced after neovessel formation. Figure reproduced from Muramatsu et al., *Nature Medicine*, 2012. (d) Role of vascular endothelial cells in axonal regeneration. Prostacyclin derived from neovessel formation through CNS inflammation stimulates cAMP synthesis in corticospinal neurons, thus contributing to axonal rewiring after the induction of EAE.

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Figure 1

Figure 2

Figure 2

Figure 3