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Signaling regulations of neuronal regenerative ability

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Abstract

Different from physiological axon growth during development, a major limiting factor for successful axon regeneration is the poor intrinsic regenerative capacity in mature neurons in the adult mammalian central nervous system (CNS). Recent studies identified several molecular pathways, including PTEN/mTOR, Jak/STAT, DLK/JNK, providing important probes in investigating the mechanisms by which the regenerative ability is regulated. This review will summarize these recent findings and speculate their implications.

Introduction

Similar to axon growth during development, axon regeneration requires the axonal extension guided by growth cone structures. This led to the hypothesis that similar principles and molecular players might operate these different processes. Ample evidence suggests that during development the extrinsic environmental cues largely determine the projection of axon growth, although the intrinsic states of responding neurons also modulate axonal responses (1). For axon regeneration, dissecting the relative contributions of such extrinsic and intrinsic mechanisms has been a major focus in the past decades (2–8). Early studies showed that reconstituting a permissive environment by transplanting peripheral nerve grafts allows the regrowth of some injured axons in the adult CNS, even though their numbers are limited (9, 10). These observations have been further supported by elegant in vivo imaging-based analysis studies (11**, 12). Canty et al employed a focused laser method to transect individual axons, with minimal glial scar formation, in the gray matter of the adult mouse brain. Ylera et al also demonstrated that central sensory axon lesion produced by two-photon laser in the spinal cord has minimal scar formation (12). Again, while some types of axons in the brain could regenerate, the majority of injured axons fail to regrow even when visualized for periods of up to a year (11**). These observations further substantiate the notion that the majority of mature neurons in the adult CNS have diminished intrinsic growth ability.

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For a successful regeneration to occur, injured axonal terminals need to re-seal quickly and reform growth cone-like structure which will explore the extracellular environment, determine the direction of growth, and then guide the extension of the axon to the direction of their appropriate targets (13–15). Presumably, the ability of injured axons to regenerate their growth cone structures and extend in injury-disturbed environments should represent the rate-limiting steps of axon regeneration. Recent genetic studies indicated that manipulating several signaling pathways could allow certain populations of mature CNS neurons to mount regenerative growth after injury and provide valuable molecular probes to explore the inner mechanisms of axon regeneration control in mammalian CNS neurons.

PTEN/mTOR: a general pathway of regulating axon regeneration?

All cell types possess certain molecular mechanisms that prevent cellular overgrowth, and many of these pathways have been implicated as tumor suppressors. In an effort to assess whether these growth suppressors play a role in limiting the intrinsic axon regenerative ability, Park et al used an optic nerve injury model and discovered robust long-distance axon regenerations in adult mice with conditional deletion of the phosphatase and tensin homolog (PTEN) gene in retinal ganglion cells (RGCs) (16). Since no manipulation was made in the lesion site, the observed regeneration phenotypes are likely due to the altered intrinsic regenerative ability in the RGCs (16).

The PTEN protein is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase. It contains a tensin-like domain and a catalytic domain similar to that of the dual specificity protein tyrosine phosphatases. Unlike most of the protein tyrosine phosphatases, this protein preferentially dephosphorylates phosphoinositide substrates. It negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate and the activity of the Akt/PKB signaling pathway (17–19). Among multiple down-stream targets in this pathway, mTOR appears to be a critical effector, because administration of rapamycin, an mTOR inhibitor, abolishes the regeneration effect of PTEN deletion (16). Further, the mTOR activation by genetic deletion of TSC1, a specific negative regulator of the mTOR, also promotes axon regeneration (16).

In addition to RGCs, the activation of mTOR activity has been shown to promote the regenerative growth of other types of CNS axons. For example, the adult mice with PTEN inhibition, by either genetic deletion (20) or shRNA-mediated inhibition (21*), in the cortical neurons from the neonatal ages, showed robust regrowth of injured corticospinal tract axons, which are known to be refractory to regeneration (22–24). Similarly, over-expression of constitutively active forms of the kinase Akt and the GTPase Ras homolog enriched in brain (Rheb) induces the regrowth of axons from dopaminergic neurons to their target, the striatum (25). Moreover, a recent study showed that newly differentiated neurons from transplanted neuronal stem cells are able to extend their axons from the spinal cord lesion sites to both sides of host tissues and such axonal growth could be partially blocked by an mTOR inhibitor (*26). However, it remains unknown whether the manipulation of the PTEN/Akt/mTOR pathway could promote the regeneration of all types of axons in the adult CNS.

In contrast to the adult mammalian CNS, injured axons in the peripheral nervous system (PNS) are able to regenerate spontaneously. Activating PI3K or inhibiting PTEN in dorsal root ganglion (DRG) neurons increases the neurite growth (27–32). However, inhibiting mTOR activity by rapamycin fails to block the neurite growth of DRG neurons (28, *31), suggesting that mTOR-independent mechanisms mediate the regeneration of peripheral sensory axons. Instead, Saijilafu et al found that glycogen synthase kinase 3 (GSK3), another down-stream target of PI3K (32), mediates PI3K-dependent augmentation of the growth potential in the PNS (*31). Furthermore, instead of the known role of GSK-3 in regulating microtubule re-organization (32, 33), PI3K-GSK3 signal may induce a transcription factor Smad1, which has been suggested to enhance sensory axon regeneration (34–36). Thus, it appears that different mechanisms mediate the effects of the PTEN/PI3K pathway in axon regeneration of PNS and CNS neurons.

Interestingly, recent studies indicated that the regulation of axon regeneration by the PTEN/mTOR pathway is not limited to mammalian neurons. Inhibiting PTEN or activating Akt in Drosophila class IV da neurons enhances their regenerative responses to axotomy and dendriotomy, suggesting this as an evolutionally conserved growth program that regulates neuronal regenerative ability (37). Although little is known about the regeneration of dendrites in mammals, these observations suggested a possibility that common mechanisms regulate the regeneration of axons and dendrites. However, activation of the Akt pathway is insufficient to confer a regenerative ability in regeneration-incompetent Class I da neurons (38). Nevertheless, these results suggested that PTEN-dependent pathways might be evolutionally conserved in regulating neuronal regenerative responses in a neuron type-specific manner (37, 38).

mTOR as an indicator of regenerative competence?

The precise mechanisms by which PTEN/mTOR controls axon regeneration remain to be elucidated. Like other resting cells, intact mature neurons produce ATP mainly through catabolic processes to fuel the maintenance of energy-costly homoeostatic processes, such as cytoskeletal functions and ion and nutrient transport. However, for an injured neuron to initiate a regenerative growth, it has to shift towards anabolic processes, allowing for the *de novo* synthesis of macromolecules from available nutrients (39). For this, there must be molecular links between the pathways sensing cellular growth signals and those controlling the metabolic networks underlying cell growth. In this regard, mTOR might be a good candidate for such tasks. It is known that in all cell types, mTOR is able to alter cellular metabolism to drive the biosynthesis of building blocks and macromolecules essential for cell growth (17–19). In addition to its well-known effects on cap-dependent protein translation (19), mTOR has been shown to regulate the synthesis of other cellular building blocks such as lipids and nucleotides (40) and regulate mitochondrial oxidative function (41, 42).

On the other hand, mTOR is activated by nutrients, growth factors and certain hormones and is tightly regulated in vivo (17–19). In mammalian RGCs and cortical neurons, the mTOR activity undergoes a development-dependent down-regulation (16, 20). Axotomy further inhibits the neuronal mTOR activity, presumably as a result of injury-triggered stress

responses (16, 20). The detailed molecular mechanisms for such mTOR regulations in neurons are still unknown, although many mTOR regulators have been identified in non-neuronal cells (17–19). A recent study showed that the microRNA (miRNA) bantam (ban) regulates neuronal Akt activity and regenerative ability in Drosophila (37). Instead of acting in the neurons, this miRNA appears to function in their target cells, resulting in a down-regulation of Akt expression in neurons during development (37). Such cell non-autonomous regulation by a miRNA is reminiscent of the role of miR-206, a skeletal muscle-specific miRNA, in regulating the regeneration of the neuromuscular synapses in mice (43). mTOR inactivation has also been documented under cellular stress conditions, such as hypoxia or DNA damage, and many molecules, such as REDD1 (43–45) and sestrin (46), have been implicated in such stress responses. But their relevance to axotomy-induced mTOR regulation remains to be studied. Elucidating the mechanisms of mTOR regulation during development and after injury should reveal new insights into the regulatory mechanisms of axon regeneration.

Although most of studies on PTEN have been focused on its cytosolic lipid phosphatase activity, recent studies suggested that PTEN might also function in the nucleus (47–49). Recently, an intriguing study by Zhang et al demonstrated that nuclear translocation of PTEN might be a step causatively leading to excitotoxic (*in vitro*) and ischemic (*in vivo*) neuronal loss (50). Since PTEN deletion impact both neuronal survival and axon regeneration, it would be interesting to assess the contribution of nuclear vs cytosolic PTEN after axotomy.

Injury signals from the lesioned axons to neuronal soma

As neuronal networks are formed in the developing nervous system, axons progressively cease growing. Even in the adult CNS, transient sprouting could occur in the terminals of injured axons, likely as the result of local cytoskeleton rearrangements. To convert such abortive local events to sustained axon extension, a set of injury signals generated locally need to be retrogradely transported to the cell body and initiate injury responses (Fig. 1). An important topic in the field is to determine the molecular signatures of such injury signals.

As a drastic stress condition, axotomy leads to a variety of changes in axotomized neurons. For example, axotomy triggers a rapid depolarization and leads to an increase of local calcium concentrations that may further propagate towards other neuronal compartments (51). At least in Aplysia, such calcium increase is important for initiating axonal regrowth program (13–15). A recent study suggested that in DRG neurons such back-propagating calcium wave causes nuclear export of histone deacetylase 5(HDAC5), which subsequently activates a regenerative program (52, *53).

In addition, a number of other molecules have been implicated as possible carriers of injury signals. An emerging common theme is that following axotomy different transcription factors undergo certain modifications, which lead to the alteration of their subcellular localizations (*54–58). A well-documented example is that an injury in the peripheral axons of DRG neurons led to the nuclear accumulation of phosphorylated STAT3 (*54,50,60), presumably due to the activation of Jak2 kinase.

In cultured DRG neurons, an inhibitor of Jak2 blocks the neurite growth (61). In vivo perineural infusion of Jak2 inhibitor also attenuates dorsal column axonal regeneation (60). In addition, motorneurons from STAT3 conditional knockout mice showed decreased survival after nerve lesion but it can be rescued by addition neurotrophic factors, including CNTF (59). STAT3 is activated upon injury and retrogradely transported to modulate survival of the host neurons (54), suggesting a role of the Jak/STAT pathway in regulating axon regeneration and survival in these neurons. Cytokines such as CNTF have also been suggested to regulate the survival and axon regeneration of CNS neurons (62–64). However, several studies showed that exogenously delivered cytokines have only limited effects on promoting survival and regeneration following optic nerve injury (65, 66) or spinal cord injury (67). This might be due to the SOCS3-mediated negative feedback (68), thus limiting the activation of the Jak/STAT pathway in responding neurons, because increased neuronal survival and axon regeneration are seen in the adult mice with conditional deletion of SOCS3, a negative regulator of Jak/STAT pathway (69). The co-deletion of SOCS3 and STAT3 in RGCs abolishes the regeneration phenotypes, suggesting that the regeneration phenotype of SOCS3 deletion is dependent on the STAT3-activated gene expression program.

The next question is what are the endogenous sources of cytokines after injury. After peripheral nerve injury, the cells in the lesion, such as Schwann cells, up-regulate expression of interleukin 6 (IL-6) and perhaps other cytokines (70–73). After optic nerve injury, the cells in the retinal ganglion layers, likely to be astrocytes, also showed increased expression of CNTF and LIF (62–65). It is also known that inflammatory stimulations, such as lens injury, could result in enhanced optic nerve regeneration (75). Although multiple molecular players have been implicated (76), genetic studies suggested that neuronal STAT3 is essential for the axon regeneration after such inflammatory stimulations (64, 77).

Compelling evidence suggests the existence of other important molecular carriers of injury signals. For example, dual lencine zipper kinase (DLK) is a component of a conserved MAPK cascade that includes the MAP kinase kinase MKK-4 and the p38 MAP kinase PMK-3. Loss-of-function mutations of the dlk-1, mkk-4 or pmk-3 gene result in axon regeneration defects (78, 79), suggesting that this entire signaling pathway is required for axon regeneration in C. elegans. Importantly, the involvement of this pathway in axon regeneration control is conserved in other species (80–*82). Deletion of DLK in DRG neurons blocks their axon regeneration (81). After optic nerve injury, deletion of DLK increases the survival of injured RGCs but blocks the axon regeneration induced by PTEN deletion (*82). Mechanistically, DLK protein is present in axons, and protein levels are increased in response to axonal injury (80). In C. elegans DLK is activated by a Ca^{++}-dependent switch from inactive heteromeric to active homomeric protein complexes (83). Further, it interacts with the scaffolding protein JNK-interacting protein 3 (JIP3), a component of axonal transport (84–85). As positive and negative retrograde signals have been proposed (86), it will be interesting to examine whether different transcription factors mediate the effects of DLK on neuronal survival and axon regeneration. DLK interacts with JNK1 and is implicated in regulation of microtubule stability (87). It has been demonstrated that microtubule stabilization is an important component of axon regeneration after spinal cord injury (88).

Signaling networks for integrated regenerative programs?

Differential injury responses in axotomized neurons should be the results of the interactions between the intrinsic neuronal state and the injury-triggered signals. As discussed above, JAK/STATs and DLK might participate in axotomy-triggered injury signal generation and delivery, and PTEN/mTOR might be a potential determinant of neuronal competence for axon regeneration (Fig. 1). Recent studies start to reveal interactive mechanisms of these and other molecular pathways.

Kruppel-like factors (KLFs), a subclass of the zinc-finger transcription factors, have been implicated in axon growth control (89–91). In zebrafish, KLF6 and KLF7 were identified among the group of up-regulated genes in regenerating RGCs (89). In mice, different KLFs differ in their expression levels over the course of development: while KLF6/7 are down-regulated, KLF4/9 are up-regulated in adult RGCs (90). Knockout of KLF4 in RGCs promotes axon regeneration after an optic nerve injury (90). Forced expression of KLF7 promotes the regenerative growth of injured corticospinal tract axons (91). In a recent study, Qin et al. showed that KLF4 physically interacts with STAT3 upon cytokine-induced phosphorylation of tyrosine 705 (Y705) on STAT3, resulting in the suppression of STAT3-dependent gene expression by blocking its DNA-binding activity (*92). These findings suggested a possible mechanism by which KLFs impact on the neuronal regenerative ability is by regulating the activity of STAT3.

The cross-talks between these pathways are also indicated from the synergistic effects of PTEN deletion and other treatments, such as inflammatory stimulation (*93) or SOCS3 deletion (**94), on promoting optic nerve regeneration. An obvious explanation is the coordination of increased protein translation and gene transcription under these treatments respectively, resulting in the higher and more sustained activation of regeneration-initiating programs. This could be important considering ample evidence for drastic changes of gene expression in injured neurons. In addition, the gene expression profiling studies indicated that injured RGCs with PTEN and SOCS3 double deletion showed increased expression of mTOR activators, such as small GTPase Rheb and Insulin-like growth factor 1 (IGF1), suggesting that such a positive feedback regulation of the mTOR activity may contribute to the enhanced and sustained axon regeneration (*94). Further studies are needed to dissect the contributions of individual pathways and develop optimized combinatorial treatments with other manipulations.

Perspectives

While these new studies demonstrated exciting possibilities of promoting the regeneration of injured axons in the adult CNS, many challenges remain towards translating these findings to therapeutic strategies. With dramatically increased body size in the adult, regenerating axons usually need to carry out *de novo* growth over relatively vast distances to reach their targets (95). Even with these newly developed strategies of promoting axon regeneration, it is unclear what might be maximal distances these regenerating axons can grow in the adult mammalian CNS. Another key question concerns whether these regenerating axons are able to follow their original projection paths and find their appropriate targets. A recent study

suggested that after a combinatorial treatment of PTEN deletion, inflammatory stimulation and cAMP elevation, regenerating optic nerve axons could follow their original paths and resulted in functional recovery (96). However, others studies failed to reproduce these findings and instead showed that regenerating axons did not follow their original trajectories (97, 98). This might not be completely unexpected, since even in the PNS system, regenerating axons often project ectopically initially and activity-dependent processes later drive the refinement of such regenerating axons. On the other hand, it is not known whether it is necessary for regenerating axons to make direct connections with their original targets for functional recovery. Recent studies suggested that indirect relay connections, such as the axons from differentiated neurons from transplanted neuronal stem cells (*26, 99) or from the rearrangements of local circuits (100, 101), might allow some degree of functional recovery after spinal cord injury. Addressing these and other questions might pave the paths for developing strategies of rebuilding neuronal circuits for functional recovery after damage.

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Highlights

- Differential intrinsic regenerative ability of adult cortical neurons revealed by In vivo imaging analysis
- Evolutionarily conserved pathways in regulating axon regeneration
- Functional interactions among different pathways in regulating axon regeneration

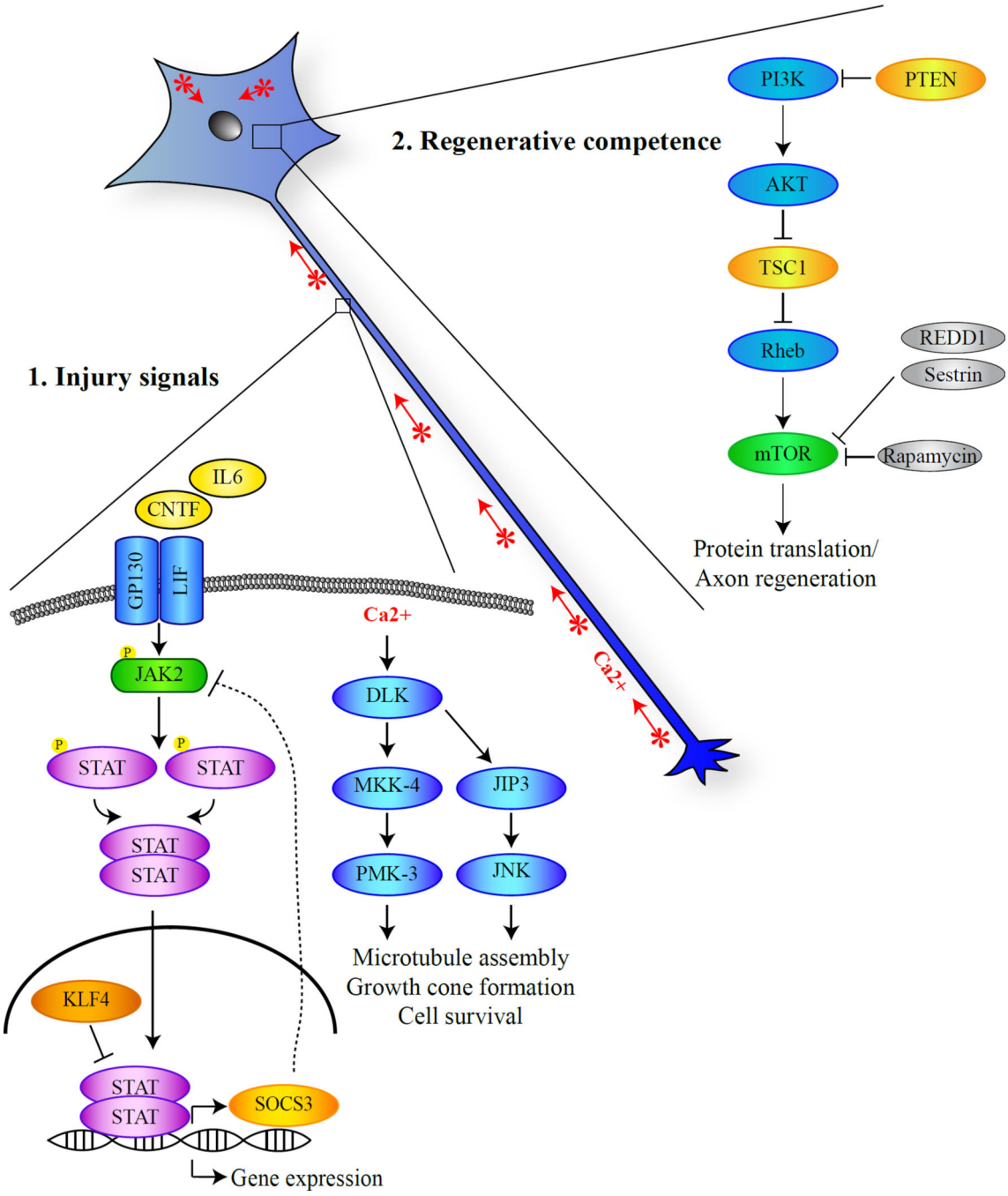

Figure 1. A working model of the mechanisms that regulate the intrinsic regenerative ability of axon regeneration

(1) To respond to axotomy, the neuronal cell bodies need to be informed by retrogradely transported injury signals. In addition to acute axotomy-induced changes such as ion influx, cytokines such as IL-6 and CNTF are up-regulated at the lesion site and/or around the cell body. As a result, DLK and Jak/STAT pathways are activated, resulting in the generation and transport of the injury signals. (2). How injured neurons respond to injury signals is

dependent on their regenerative competence. An important determinant of the competence might be the mTOR activity but other molecular pathways likely exist.