Published in final edited form as:

Wiley Interdiscip Rev Dev Biol. 2018 March ; 7(2): . doi:10.1002/wdev.305.

Timing of Neuronal Plasticity in Development and Aging

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Abstract

Molecular oscillators are well known for their roles in temporal control of some biological processes like cell proliferation but molecular mechanisms that provide temporal control of differentiation and post-differentiation events in cells are less understood. In the nervous system, establishment of neuronal connectivity during development and decline in neuronal plasticity during aging are regulated with temporal precision, but the timing mechanisms are largely unknown. *C. elegans* has been a preferred model for aging research and recently emerges as a new model for the study of developmental and post-developmental plasticity in neurons. In this review we discuss the emerging mechanisms in timing of developmental lineage progression, axon growth and pathfinding, synapse formation and reorganization, and neuronal plasticity in development and aging. We also provide a current view on the conserved core axon regeneration molecules with the intention to point out potential regulatory points of temporal controls. We highlight recent progress in understanding timing mechanisms that regulate decline in regenerative capacity, including progressive changes of intrinsic timers and co-opting the aging pathway molecules.

Graphical abstract

Introduction

In the nervous system, temporal regulation is crucial for the establishment of neuronal connectivity during development and age-dependent decline in neuronal plasticity^{1–5}. Many previous studies have focused on the temporal control of developmental and physiological processes by networks of molecular oscillators, or molecules whose levels of expression or activity signal the progression to the next phase of the cycle. Due to the importance of molecular oscillators in temporal regulation of other processes, this concept will be explored in more depth at the beginning of the next section before we discuss current views on timing of neuronal plasticity in development and aging.

Timing mechanisms involved in the establishment of neuronal connectivity and age-related decline in neuronal regeneration are largely unknown. However, it is well understood that as animals age, there is an intrinsic loss of regenerative potential^{1–5}, which cannot be explained solely through system fatigue. This loss of regenerative potential becomes even more drastic when comparing older vertebrates that show a clear dichotomy in the regeneration of peripheral versus central nervous systems. Mounting studies on the topic of neuroregeneration and its decline with age have started to highlight the importance of both canonical and newly discovered pathways that will be presented in this review.

Caenorhabditis elegans (*C. elegans*) has emerged as a model that is uniquely suited to the study of timing mechanisms of neuronal plasticity in development and aging and has led to the discovery of miRNA as a major temporal regulator of neurons¹. The average lifespan of *C. elegans* is 20 days, and the animal reaches maturity approximately 48 hours after hatching⁶. Additionally, the animal's developmental transitions are well characterized and each of these developmental stages, L1 through L4 between egg hatching and young adulthood, has a distinct appearance. This predictability is particularly useful in the study of timing mechanisms of neuronal plasticity in development and aging. Experiments can be easily carried out at defined stages of the animal's life and the effects of aging can be analyzed accordingly.

The entire nervous system of the animal is comprised of 302 neurons with each connectivity mapped⁷. The transparency of the worm allows targeted visualization of neurons and provides a platform for studying neuronal regeneration after injury⁸. The implementation of laser surgery⁹ has allowed for a proliferation of research on the mechanisms that are involved in axon regeneration. This technique has been used to identify the roles of kinase cascades and cytoskeletal proteins as well as the insulin/IGF pathway on regeneration^{4,5}. The temporal regulation by miRNAs in the age-dependent inhibition of regenerative capacity was also investigated using this method¹. Finally, the robust conservation of genetic programs between *C. elegans* and vertebrates implies that molecular and mechanistic insights gained in this model system will offer fundamental principles that can be applied universally.

Overview of Molecular Oscillators

Studies on temporal control of development in a variety of tissues have frequently converged on the concept of biological oscillators. Biological oscillators are systems of molecules of various levels of expression and activity that act as molecular “clocks” that determine biological rhythms resilient to changes in external environments. Molecular oscillators control a number of critical developmental, metabolic, and behavioral processes, and thus the various biological oscillator systems are named according to their main function, ie. cell cycle clock, circadian clock, and segmentation clock.

Cell cycle clock

Cell cycle progression of any dividing cell depends on the oscillatory network of cyclin dependent kinase (Cdk) activity¹⁰. The activation of one Cdk by specific cyclins leads to the phosphorylation the effector molecules by the Cdk. This causes the accumulation of the following phase's cyclins that can both interact with the Cdk and lead to the disengagement of the Cdk's inhibitors until a threshold level of Cdk activity is reached. At this threshold, there is a switch to a different phase of the cell cycle that initiates degradation of the previous phase's cyclins¹⁰, providing a basis for the cyclical nature of the cycle clock. Although all the required molecules for each phase of the cell cycle clock are not yet determined, the characterized reliance of this system on activator molecules, phosphorylation of key residues on effector molecules, and regulatory feedback loops gives a glimpse into the mechanism of timing control for the cyclical process of cell division.

Circadian clock

Circadian clocks are perhaps the best known biological oscillator model and have been evolutionarily conserved from even the simplest organisms like cyanobacteria¹¹. Although biological oscillators are unlikely to describe the temporal process of neural development, the central nervous system (CNS) relies on molecular oscillators to pattern behavior and metabolism of an organism. The population of cells showing oscillatory gene expression patterns comprising the Suprachiasmatic Nucleus (SCN) of the hypothalamus are known as the master clock and are critical for the continued 24 hour circadian rhythm of other CNS regions and of peripheral oscillators expressed in peripheral tissues spread throughout the organism¹². Briefly, the circadian clock is regulated via transcriptional and post-translational mechanisms. At the center of the oscillatory mechanism in both central and peripheral tissues are the heterodimeric transcription factor CLOCK-BMAL1 that is constitutively bound to the E box enhancers of three main transcriptional targets: *mPer* (*period* 1–3), *mCry* (*cryptochrome* 1–2), and *Rev-Erba* orphan nuclear receptor. The transcriptional targets of CLOCK-BMAL1, especially *mCry*1, are negative regulators of CLOCK-BMAL1 activity. Post-translational control such as phosphorylation of the CLOCK-BMAL1 target proteins can control access of the translated targets to the nucleus and interaction with the CLOCK-BMAL1 transcription factor^{12,13}. The levels of the oscillatory molecules in the SCN are communicated to the rest of the brain as action potentials and as hormonal, autonomic, and behavioral signals that regulate the activity of disperse target organs like the cornea, lung, and liver^{12,14}.

Segmentation clock

The segmentation clock, which determines somitogenesis or the sequential development of a set number of discrete somite blocks from the pre-somitic mesoderm (PSM), is a critical mechanism for the formation of proper bone, muscle, and skin segmentation patterns of an elongating vertebrate¹⁵. Although the temporal control of development by the segmentation clock also relies on the principle of autonomous cells' expression of oscillating levels of key regulatory molecules seen in other types of biological clocks, it also demonstrates a reliance on intercellular communication and spatial gradients of molecules. One of the most popular models to describe the process of somitogenesis is the "clock and wavefront" model, originally described on a more theoretical and mathematical basis by Cooke and Zeeman¹⁶. The clock component relies on the cyclical patterns of expression of transcriptional repressors *Hes/Her* (hairy and enhancer of split/hairy related)^{17,18}. The second component of this model—the wavefront, describes the timed freezing of cellular oscillations which moves from anterior to posterior PSM, such that anterior cells of the PSM determine the boundaries of a somite once they stop genetic oscillation and prior to the arrest of oscillations in the next posteriorly-located somite¹⁵. The likely regulators of this process fall into three major pathways: Delta-Notch, Wnt and FGF/MAPK/ERK¹⁹.

Similarly to the control of circadian clocks, *Hes/Her* genes also display a property of auto-regulation that makes them likely candidates responsible for oscillation of autonomous PSM neurons. For example, *Hes/Her* proteins are able to dimerize and bind to their own promoter to repress their own transcription until these proteins decay, at which point the products can be expressed again^{20,21}. The involvement of Notch in the segmentation clock primarily controls the synchronized oscillation of the individual cells in the PSM²² by intercellular coupling and interaction with *Hes/Her*¹⁷. The other three key players in the vertebrate segmentation clock, namely Wnt, FGF, and retinoic acid (RA) pathways, are all expressed in gradients along the anteroposterior axis of PSM¹⁵. The combined action of these molecules may determine the wavefront velocity, as increased posterior FGF and Wnt signaling leads to decreased length of somite blocks, whereas increases in RA lead to elongation of somites. It is thought that a ratio of the pro-oscillatory FGF and Wnt expression over the antagonistic RA expression defines a threshold below which oscillatory genetic programs stop and a somite is determined¹⁵.

The patterns of regulation of molecular oscillators are not unique to these molecules. Negative and positive auto-regulation loops, phosphorylation, the importance of cell-to-cell communication, and reliance on gradients of antagonistic pairs of molecules are important principles that extend in significance beyond molecular oscillator networks.

Timing mechanisms in developmental lineage progression

The first insights into heterochronic genes in developmental timing came from a forward genetic screen performed in 1984, which led to the discovery of genes that regulated developmental transitions including the first miRNA, *lin-4*^{23–26}. miRNAs are short non-coding RNAs that form a small hairpin loop whose most characterized function is post-transcriptional gene silencing. This downregulation is achieved by the binding of miRNAs to

the 3' untranslated region of their target mRNA^{25,26}. Since their discovery, miRNAs have been shown to play a crucial role in temporal regulation during development. The persistent theme observed in miRNA-mediated regulation revolves around the downregulation of key factors at a specific time by miRNAs^{27,28}.

In early development, there are three phases of miRNA-mediated temporal control in developmental stage transitions: the L1 to L2 transition regulated by the *lin-4-lin-14* pathway, the L2 stage specification via the repression of key regulators LIN-28 and HBL-1^{29–31}, and the L4 to adult stage transition due to the activation of LIN-29 by the *let-7-lin-41* pathway^{32–34}. Interestingly, activation of nicotinic receptors delays developmental lineage progression during the L2 stage in *C. elegans*, suggesting a role for the nicotinic receptors in timing of cell cycle progression³⁵. Besides governing developmental lineage progression, miRNAs also confer temporal control in neurodevelopment and plasticity (Table 1)^{1,36–39}.

Timing mechanisms in axon outgrowth and guidance

lin-4 is a miRNA that is homologous to vertebrate *mir-125* miRNA and whose targets include transcriptional regulators LIN-14, HBL-1, and LIN-28. In *C. elegans* hermaphrodite specific neurons (HSNs), *lin-4*-mediated downregulation of LIN-14 and LIN-28 was found to be necessary for initiating axon elongation during the L3/L4 stages (Table 1)³⁹. In the HSNs, *lin-4* acts cell autonomously and regulates axon outgrowth independently of guidance receptors³⁹. In contrast, in the anterior ventral microtubule (AVM) neuron, the activity of *lin-4* in regulating accurate axon guidance was shown to be closely linked to its modulation of guidance receptors³⁸. In the L1 stage, the axon of the AVM neuron responds to both UNC-6 (Netrin) attractive and SLT-1 repulsive cues to reach the ventral midline⁴⁰. The ability of the growth cones to respond to the attractive UNC-6 (Netrin) cue requires the UNC-40 (DCC) receptor and the MADD-2 cofactor to signal through the UNC-34 (Ena) and CED-10 (Rac1) signaling complexes^{41,42}. During AVM ventral guidance, *lin-4* acts to repress *lin-14* at the end of the L1 stage, which results in the downregulation of UNC-6 (Netrin) signaling (Table 1)³⁸. These two events showcase that the same miRNA can temporally control axon elongation and guidance in two distinct neuron types at different larval stages. In addition, *lin-14* has been shown to temporally regulate expression of the axon maintenance factor *zig4* during *C. elegans* ventral nerve cord development⁴³.

Timing mechanisms in synapse formation and remodeling

Temporal control of synaptic reorganization is imperative for the precise rewiring of functional neuronal networks. For neuronal networks to be functionally established, the neurons must form correct synaptic connections within a specified time frame. The failure of synaptogenesis or remodeling in the appropriate time frame has been linked to disease and neural dysfunction^{44–47}.

The *lin-4-lin-14* pathway has been shown to temporally control multiple aspects of synapse formation and remodeling. In dorsal D-type (DD) motor neurons, LIN-14 controls synaptic remodeling³⁶. Recently, it was found that LIN-14 coordinates with transcription factor

UNC-30 to temporally regulate the expression of *oig-1*, an immunoglobulin domain containing protein, to prevent precocious synapse rewiring. The reduction of LIN-14 in the L2 stage and onwards contributes to the reduction of *oig-1* and leads to synaptic rewiring. This temporal control is abolished when *lin-4* is removed³⁷. The *lin-4-lin-14* pathway has also been suggested to regulate the timely transition from axon pathfinding to synapse formation in AVM neurons^{38,48}.

In addition to the *lin-4-lin-14* pathway, *mir-84*, a paralog of *let-7* miRNA, has been found to control the timing of DD neuron synaptic remodeling through its repression of transcription factor HBL-1⁴⁹. This finding implies that multiple heterochronic genes are involved in different aspects of developmental synaptic plasticity, highlighting the extent of temporal regulation that is required in synaptic development.

Neuronal aging and its effects on plasticity

Aging is inevitable and like any other organism, *C. elegans* demonstrates age-related physiological changes and decline in regeneration ability. Morphological and structural changes such as ectopic neurite growth, axon beading, synaptic alterations, and changes to the soma were observed in a variety of mechanosensory neurons as the worm ages^{50–53}. There are also subcellular alterations associated with aging, which include but are not limited to changes in mitochondrial function, protein quality control, and cytoskeletal integrity⁵⁴. In addition, aging neurons in *C. elegans* have recently been reported to eject misfolded proteins and mitochondria in exophers. These exophers were shown to contain mitochondria with increased matrix oxidation and may be linked to the age-associated functional decline in neurons⁵⁵.

After neurons reach maturity, the axon regrowth ability present in development declines. This intrinsic loss of axon regrowth ability may be due to external factors that cue neurons to irreversibly dampen their growth ability, which will also be discussed in the vertebrate section of this review. Other studies have shown that intrinsic factors also play a critical role in the loss of axon regrowth ability as neurons age^{1,4}.

Pathways for axon regeneration after injury in *C. elegans*

To add further complexity, axon regenerative capacity also changes between different neuronal cell types and at different developmental stages⁵⁶. Here, we recognize the key players that have been implicated in axon regeneration after injury in *C. elegans* (Table 1). Some axon guidance and growth factors that are involved in early development are reused in regeneration (Table 1). These underlying mechanisms and intrinsic factors could provide insights into understanding the failure of CNS regeneration in vertebrates and provide potential targets for therapeutic intervention.

Kinase cascades

Following neuronal injury, two interrelated stress-responsive MAPK cascades are activated: the DLK and JNK signaling pathways⁵⁷. The mixed lineage dual leucine zipper kinase, DLK-1, has emerged as a critical regulator in axonal regeneration as well as apoptosis after

injury^{2,58,59}. Laser axotomy of mature neurons has shown that DLK-1 and p-38 kinase PMK-3 are crucial for the regenerative growth of axons by stabilizing mRNA of CEBP-1, a bZip protein^{2,59}. In *C. elegans*, overexpression of RPM-1 and FSN-1, both negative regulators of DLK-1, resulted in reduced regeneration of injured neurons^{2,60}. Furthermore, *rpm-1* mutant animals displayed enhanced initiation of axon regeneration. In addition to axon regeneration, RPM-1 also regulates neuronal development by binding to PPM-2, a PP2C phosphatase, and inhibiting the DLK-1 signaling pathway⁶¹. These results demonstrated that changes in DLK-1 levels affect axon regenerative capability and that RPM-1 limits DLK-1 signaling^{2,60}.

The JNK signaling pathway was also identified as essential for axon regeneration^{3,62}. In GABAergic motor neurons, overexpression of *mlk-1*, a component of the JNK pathway, improved regeneration while loss-of-function mutations in either *mlk-1* or its downstream kinases compromised regeneration³. It has been further shown that the loss of function in one MAP kinase pathway was promptly compensated by the other^{3,62} and that the DLK-1 and JNK signaling pathways communicate through common targets at the time of regrowth and response to injury. Similar to DLK-1, inhibition of the JNK pathway by RPM-1 also regulates synapse formation in neuronal development⁶³.

Calcium and cAMP

Ca²⁺ and cyclic AMP (cAMP) signaling in response to injury has been shown to influence regenerative potential. Following the axotomy of cultured *Aplysia californica* neurons, a transient increase of intracellular Ca²⁺ rapidly occurs followed by the formation of a new growth cone on severed axons⁶⁴. Mutations of *unc-68*, which encodes for the ryanodine receptor that allows the release of sequestered calcium from the endoplasmic reticulum, lead to stunted regeneration of neurons in *C. elegans* and inappropriate axon growth cone guidance⁶⁵. It was recently shown that elevation of Ca²⁺ and cAMP signaling promotes axon regeneration with the requirement of DLK-1 MAPKKK cascade in *C. elegans*⁶⁶. The transient increase of Ca²⁺, a characteristic event occurring after injury, allows the activation of the DLK-1-mediated axonal regeneration pathway⁶⁷. Surprisingly, the calcium-activated CED-3 caspase, an essential apoptotic molecule in *C. elegans*, has been implicated as an upstream molecule of DLK-1 in axon regeneration⁶⁸. A deeper understanding of the relationship between Ca²⁺, cAMP, and the DLK-1 pathway could provide further insight into inducible axon regeneration.

Insulin/IGF pathways

The insulin/IGF pathway has long been implicated in organismal aging as mutations in *daf-2*, which encodes the insulin/IGF receptor, were shown to double the lifespan of worms⁶⁹. In the context of neuronal regeneration, aged *daf-2* mutants displayed enhanced axon regeneration in GABA motor neurons in a *daf-16* (forkhead transcription factor) and *dlk-1*-dependent manner (Fig. 1B; Table 1)⁴. *daf-2* inhibits axon regeneration in aged neurons is independent of PTEN and TOR (target of Rapamycin)⁴. Recently it was shown that the ability of *daf-2* mutations to increase axon regeneration in aged neurons required the activity of an adult-enriched forkhead transcription factor FKH-9 (Fig. 1B; Table 1)⁵.

Interestingly, UNC-104 (KIF1A), a neuronal kinesin, was identified as a downstream effector of DAF-2 and DAF-16 in the regulation of age-related synaptic decline and memory formation⁵².

Cytoskeletal proteins

Axonal microtubule (MT) dynamics have emerged as an essential determinant of axon regeneration capacity after injury⁷⁰. Genetic screens identified EFA-6, a highly conserved guanine nucleotide exchange factor, as an inhibitor of axon regeneration. EFA-6 rapidly localizes to the ends of MT filaments and blocks modification of axonal MT dynamics upon axon injury^{70,71}. EFA-6 inhibits axon regeneration whereas DLK-1 promotes axon regeneration, in part via MT dynamics.^{70,71}. Although EFA-6 and DLK-1 have been recognized as important regulators of MT dynamics, the nature by which these pathways interact remains unclear.

RNA-binding proteins

A new class of proteins with the unusual RNA binding property, has been recently described in the context of developmental and age-related decline in neuroregeneration. Interestingly, one of these mRNA-interacting proteins, LIN-41, is the direct target of the *let-7* miRNA that has already been tied to the decline in axonal regeneration¹. *let-7* miRNA is expressed in AVM neurons starting in the L3 stage of *C. elegans* development and binds to *lin-41* 3'UTR, which correlates with a decline in the developmental regenerative capacity of the AVM neuron. In contrast to wild types that have limited regeneration potential in the adult stage, *let-7* loss-of-function mutants or *lin-41* overexpression both show increased regeneration potential following axotomy of AVM in the adult stage¹. LIN-41 is a TRIM (tripartite motif) protein that has been recently shown to bind directly to four target mRNAs and silence their expression via two distinct post-transcriptional mechanisms⁷². The target mRNAs of *mab-10*, *mab-3*, and *dmd-3* are degraded as the result of LIN-41 binding to the transcripts' 3'UTR, while *lin-29A* expression is diminished due to LIN-41 binding to the *lin-29A* 5'UTR, causing a translation repression. LIN-29 is a transcription factor whose attenuation has been shown to enhance axonal regeneration¹. These findings demonstrate that the temporal regulation of decreased regenerative capacity of older neurons involves not only the miRNA regulatory mechanisms but also the unique post-transcriptional mechanisms regulated by a RNA binding protein. The implication of this unusual class of proteins in timing of regeneration capacity extends past the *C. elegans* nervous system. LIN-41 has the homolog TRIM71, which displays the same mRNA binding capacity in mammals and is important for stem cell function⁷³.

The RNA-binding protein Rtca has been demonstrated to limit regeneration in *D. melanogaster*. Rtca is a RNA 3' terminal phosphate cyclase that works within the RNA repair and splicing pathway to slow down repair of stress-altered RNA and may lead to splice variants that act as repressors of axonal regeneration⁷⁴. Rtca knockouts are able to augment PNS regeneration and allow for regeneration of CNS axons not only in *D. melanogaster* but also in mouse RGC axons following optic nerve crush. The Rtca pathway likely involves Xbp1 splice variants, a molecular marker for cellular stress⁷⁴, as well as the

canonical PTEN and Rac1 pathways, which appear to also play a role in axonal regeneration in *C. elegans* as was mentioned in the previous section^{4,56}. It remains to be seen how the RNA-binding RtcA inhibits regenerative potential and whether this protein relies on a regulatory loop containing a miRNA.

In summary, studies on *C. elegans* have demonstrated age-related decline in neuronal regeneration following injury^{1–5}. However, the regenerative capacity can be restored by modulation of a few key regulators. It is currently known that MAPK pathway components such as DLK and JNK are critical effectors of axonal regeneration. The DLK pathway, in particular, depends on calcium and cAMP signaling and likely causes changes in the microtubule organization of axons, which has been known to be a key mediator of regenerative potential. Microtubule assembly also links another implicated pathway in axon regeneration, the insulin/IGF pathway, to the control of axonal regenerative potential. RNA interactions have recently emerged as important temporal regulators of regenerative capacity. Both miRNAs and RNA binding proteins have now been implicated in the control of axonal regeneration following injury in adulthood and beyond. Although many key players and regulators are now identified, further studies are needed to tie together the networks of classical signaling pathways and the newly identified RNA-interacting pathways to the control of regeneration. In particular, it will be intriguing to explore miRNA and RNA-binding protein interactions as temporal regulators controlling the progression from high to low regenerative capacity with age. Due to the overlap in pathways controlling neuronal regeneration between *C. elegans* and vertebrate models, exploration of the mechanisms behind the neuronal regenerative capacity in *C. elegans* is likely to reveal useful molecular targets for the restoration of regenerative potential in aging vertebrates.

Axon regeneration in vertebrates: Zebrafish and *Xenopus laevis*

Zebrafish show a variable amount of regeneration following injury. Some neurons display high regenerative capacity, such as Retinal Ganglion Cells (RGCs) and subsets of neurons comprising brainstem-spinal cord projections, while others show low regenerative capacity, including Mauthner neurons, ascending dorsal root axons, and ascending axons of intraspinal neurons⁷⁵. This dichotomous regenerative capacity is reminiscent of mammalian regeneration of peripheral versus central nervous systems (PNS and CNS). The RGCs, which are highly regenerative in zebrafish unlike their mammalian counterparts, show increased expression of GAP-43, L1-related proteins, and alpha-1 tubulin when grown in explant culture^{75–77}. These proteins are also expressed during development, providing evidence that vertebrates can also reuse developmentally important molecules for regeneration in adulthood. The environment surrounding the regenerating axon is also critical to zebrafish axon regeneration. For example, chondroitin sulfate proteoglycans and tenascin-R play a repellent role in regenerating axons of adult zebrafish in vivo and in vitro, and repulsive molecules such as ephrin are upregulated following injury in zebrafish^{75,78}. As will be explored in the next section, similar molecules are implicated in the inhibitory environment of adult CNS in mammals.

Xenopus laevis displays age-related decline in neuronal regeneration following spinal cord injury⁷⁹. In addition, similarly to zebrafish, the RGCs in *Xenopus* robustly regenerate

following crush injury and upregulate tubulin and GAP-43 (growth associated protein-43)⁸⁰. It has been demonstrated that the expression of GAP-43 together with L1 cell adhesion molecules is able to switch the non-regenerative mouse CNS Purkinje neurons to axonal regeneration when guided by predegenerated peripheral nerve grafts⁸¹. Additionally, *Xenopus* studies have highlighted the importance of kruppel-like family (klf) of transcription factors⁸⁰, whose levels become altered in injured axons of rodents as well⁸².

Mammals

Axon regenerative capacity in the mammalian central nervous system is greatly limited or entirely absent. However, the peripheral nervous system shows regenerative capacity and is able to restore fully functional nerves^{83–85}. This difference in regenerative potential between CNS and PNS may be due to two major factors: the presence of an inhibitory environment in the CNS and different intrinsic genetic programs engaged upon injury in the two nervous systems.

Astrocytic glial scars formed after damage to the CNS present a physical barrier to regeneration^{86,87}. Other supportive cells also provide an inhibitory environment for neuronal regeneration, like amacrine cells in the case of retinal ganglion cells in post embryonic rats⁸⁸ and oligodendrocytes in the CNS generally. It is now known that oligodendrocytes promote an inhibitory environment for axonal regeneration due to the secretion of an inhibitory class of molecules called Myelin Associated Inhibitors (MAIs). These MAIs include ligands of the neuronal Nogo-66 receptor 1 (NgR1), including Nogo-A, MAG, OMgp, as well as repulsive guidance molecules like RGM, ephrin B, and semaphorins, in addition to components of the extracellular matrix like chondroitin sulfate proteoglycans (CSPGs)^{87,89}. Although MAIs are secreted in the PNS by Schwann cells, or the peripheral counterparts of oligodendrocytes, they undergo rapid clearance by macrophages and Schwann cells and are not further secreted after clearance⁸⁷. Genetic programs in the PNS and CNS also vary^{89,90}, and overexpression of certain PNS-enriched proteins in the CNS allows the central nervous system neurons to grow in typically inhibitory conditions⁹⁰.

For appropriate regeneration of mammalian CNS axons following injury, both external and internal factors will need to be modulated. Research on animal models outside of mammals has already provided potential intrinsic therapeutic targets for CNS regeneration. Dlk-1 and LIN-41, implicated in *C. elegans* axonal regeneration, are exciting therapeutic targets for mammalian CNS regeneration. Similarly to *C. elegans*, cAMP promotes axon regeneration in vertebrates. For example, axon regeneration in the rat spinal cord is promoted with the administration of phosphodiesterase 4 inhibitor rolipram, which increases levels of cAMP⁹¹. Likewise, elevation of cAMP in the CNS of both rats and zebrafish has been shown to increase regeneration of spinal neurons^{92,93}.

Additionally, experiments in rodents strengthen the importance of microtubules observed in *C. elegans* neuronal regeneration. Moderate stabilization of MTs by Taxol has been shown to promote growth of CNS axons and reduce fibrotic scarring following spinal cord injury in the rodent Raphe-spinal tract⁹⁴. In vivo imaging of dorsal root ganglia neurons has provided evidence that MT stability leads to the formation of new growth cones in severed axons.

Disorganization of MT dynamics resulted in non-growing retraction bulbs⁹⁵. Injury to axons altered the organization of MTs to become highly dynamic and supported axonal regeneration by potentially generating free plus ends for MT remodeling⁹⁶.

miRNAs, which are emerging as a critical determinant of timing in neuronal plasticity, are likely to be conserved in their roles in mammalian neurons. Dysregulation of miRNA plays a role in neurodegenerative diseases⁹⁷. miR-21 overexpression has been shown to promote neurite growth in adult rat dorsal root ganglia⁹⁸ and to be elevated in the injured cortex of rats⁹⁹, while increases in miR-29b have been demonstrated in injured sciatic nerves and may be implicated in neuronal survival in adult mice¹⁰⁰. CNS injury experiments have shown changes in the levels of miR-211, miR-142-5P, and miR-124a⁹⁷. Additionally, miR-124 was shown to act as a developmental timer in retinal ganglion cells to control axon response to the guidance cue Semaphorin¹⁰¹. Since evidence for the ubiquity of miRNAs in the mammalian nervous system is now accumulating, it will be fascinating for future studies to uncover whether miRNAs are indeed the temporal regulators of neuronal plasticity in mammals similarly to their role in *C. elegans*.

Age-related decline in neuronal regenerative capacity

One of the hallmarks of aging is the decrease in the regenerative capacity of the neuron in response to injury. In many of the previously mentioned studies, it has been shown that overexpression of key proteins can prolong or enhance regenerative capacity in aged animals. However, there is much to be uncovered on the timing mechanisms that trigger the decline of regenerative capacity in neurons as they age^{1–5}.

The role of miRNA in the temporal regulation of regenerative ability was discovered when animals with mutations in *alg-1*, a protein required for miRNA biogenesis, displayed enhanced regeneration after injury in aged AVM neurons¹. As described previously, *let-7* miRNA was identified as the temporal regulator that signaled the decline of regeneration capacity by down regulating LIN-41 from the L3 stage onwards (Fig. 1A; Table 1). The *let-7* level remained high until day 2 into adult stage, after which the level fell as the animals aged (Fig. 1A). However, even at this reduced level, *let-7* retains its ability to regulate regeneration in aged animals as the inhibition of *let-7* or the overexpression of LIN-41 rejuvenated post-injury regeneration in animals as old as 12 days into adulthood^{1,102} (Fig. 2). It is also intriguing that although mature *let-7* miRNA levels become elevated only in the late L3 stage, the expression of the primary *let-7* transcript oscillates in the L1-L4 larval stages in a manner which coincides with larval transitions¹⁰³. This raises the possibility that *let-7* can potentially function as a molecular oscillator involved in early developmental transitions in addition to its role as a timing control molecule that signals the decline of regenerative capacity in aging animals.

Longevity mutants have also provided insight into neuronal aging and the decline of regenerative potential in aging animals. It is interesting to note that only some longevity mutants conferred altered regeneration in aged animals. For example, mutants of molecules found in the insulin/IGF pathway, *daf-2* and *daf-16*, showed, respectively, enhanced and reduced life span and regenerative capacity (Fig. 1B). *eat-2* mutants, which experience

calorie restriction and longevity, did not show enhanced axon regeneration after injury⁴ even though the beneficial effect of calorie restriction on attenuating age-induced neuronal changes is well documented¹⁰⁴. Thus, mitigation of organismal aging does not necessarily lead to increased neuronal regeneration. Using *daf-16* mosaic animals, it was further shown that *daf-16* (which mitigates *daf-2*'s role in regeneration) functions intrinsically in neurons to regulate regeneration independently of organismal aging. Thus, at least in the case of *daf-16*, mitigation of organismal aging may not be directly related to increased neuronal regeneration⁴.

Concluding remarks and future directions

Many neuronal connectivity mechanisms are reused in regeneration. Studies in *C. elegans*, *Drosophila*, and vertebrates have highlighted critical mechanisms regulating regenerative potential. *C. elegans* has provided a unique platform that has led to the discovery of intrinsic neuronal timers and their roles in regulating developmental and age-related decline in neuronal capacity. Since the initial discovery of heterochronic genes, the field has made immense progress in understanding their roles in temporal regulation of neuronal development and regeneration. However, one of the remaining questions that awaits to be answered is how different neuronal timers can govern a variety of neuron differentiation and post-differentiation events at distinct stages of an animal's life.

Much of the research on nerve regeneration has focused on the molecular mechanisms that regulate the specific events of regeneration, and several studies have investigated the role of miRNAs in organismal aging^{105,106}. However, very few have focused specifically on the role of miRNAs in neuronal aging and their link to neuronal plasticity and regeneration¹. Moving forward, it will be highly relevant to investigate the temporal organizers that regulate these processes. Furthermore, with the goal of therapeutic intervention to promote neuronal recovery after injury, it will become necessary to target not only the intrinsic and extrinsic regulators of regeneration but also the timing mechanisms in order to incorporate neuronal rejuvenation or delay neuronal aging for enhancing neuronal regeneration.

Acknowledgments

This work was supported by grants from the NSF (IOS-1455758), the Whitehall Foundation Research Award, and the NIH (5R01GM111320) to C.C.

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Figure 1. Pathways regulating age-related decline in neuronal regeneration in *C. elegans*
 (A) The neuronal timers, *let-7* and *lin-41*, signal developmental and age-related decline in neuronal regeneration as animals age. *lin-41* levels decline with advanced adult stage even though *let-7* levels also decline, suggesting that *lin-41* levels are likely regulated by both post-transcriptional (through the *let-7* and *lin-41* 3'UTR interaction) and transcriptional (through the *lin-41* 5'UTR) mechanisms in adult stage. (B) The aging pathway involving *daf-2*, *daf-16* and *fkh-9* that was known to regulate organism longevity also influenced axon regeneration decline in aging.

Figure 2. *let-7* mutations and *lin-41* overexpression restore regenerative capability in aged neurons

Neuronal regeneration capacity changes throughout the lifespan and diminishes with age. Inhibition of *let-7* miRNA or overexpression of its target, *lin-41*, in aged neurons restores youthful regenerative capacity. Dashed blue lines represent detached axons affected by degeneration. Solid blue lines indicate newly regenerated axons.

Table 1

Genes involved in neuronal plasticity and regeneration

Gene	Gene Ontology	Regulatory Mode	Neuronal Connectivity	Neuronal Plasticity	Neuronal Aging	Intrinsic Timer	Reference
<i>lin-4</i>	miRNA	Inhibits/Promotes	✓			✓	Lee et al, 1993; Wightman et al, 1993; Olsson-Carter and Slack 2010; Zou et al, 2012
<i>lin-14</i>	Transcription factor	Inhibits/Promotes	✓			✓	Lee et al, 1993; Wightman et al, 1993; Olsson-Carter and Slack 2010; Zou et al, 2012; Howell et al, 2015
<i>lin-28</i>	RNA binding protein	Inhibits	✓			✓	Moss et al, 1997; Olsson-Carter and Slack 2010
<i>let-7</i>	miRNA	Inhibits		✓	✓	✓	Pasquinelli et al 2000; Wynsberghe et al 2011; Zou et al 2013
<i>tin-41</i>	Tripartite motif protein	Promotes		✓	✓	✓	Slack et al, 2000; Zou et al, 2013
<i>tin-29</i>	Zinc finger transcription factor	Inhibits		✓		✓	Rougvie et al, 1995; Zou et al, 2013
<i>unc-30</i>	Homeodomain transcription factor	Inhibits	✓				Howell et al, 2015
<i>oig-1</i>	immunoglobulin domain protein	Inhibits	✓				Howell et al, 2015
<i>daf-2</i>	Insulin/IGF receptor	Inhibits		✓	✓		Byrne et al, 2014; Kaletsky et al, 2016
<i>daf-16</i>	Forkhead transcription factor	Promotes		✓	✓		Byrne et al, 2014; Kaletsky et al, 2016
<i>flk-9</i>	Forkhead transcription factor	Promotes		✓	✓		Kaletsky et al, 2016
<i>rpm-1</i>	E3 ubiquitin ligase	Inhibits	✓	✓			Nakata et al, 2005; Hammarlund et al, 2009; Yan et al, 2009
<i>dlk-1</i>	MAPKKK	Promotes	✓	✓			Nakata et al, 2005; Hammarlund et al, 2009; Yan et al, 2009
<i>pmk-3</i>	p38 MAP kinase	Promotes	✓	✓			Hammarlund et al, 2009; Yan et al, 2009
<i>ceb-1</i>	bZip domain-containing protein	Promotes		✓			Yan et al, 2009
<i>kin-2</i>	Regulatory subunit of:AMP-dependent protein kinase	Promotes	✓*	✓			Ghosh-Roy et al, 2010; Wu et al, 2010
<i>efa-6</i>	Exchange Factor for ARF-6	Inhibits		✓			Chen et al, 2015; Tang and Chisholm 2016
<i>unc-6</i>	Netrin	Promotes	✓	✓			Chang et al, 2004; Gabel et al, 2008

Gene	Gene Ontology	Regulatory Mode	Neuronal Connectivity	Neuronal Plasticity	Neuronal Aging	Intrinsic Timer	Reference
<i>sit-1</i>	Slit	Promotes	✓	✓			Hao et al, 2001; Gabel et al, 2008
<i>ced-10</i>	Rac GTPase	Promotes	✓	✓			Gitai et al, 2003; Gabel et al, 2008; Pastuhov et al 2016
<i>unc-34</i>	ENA/VASP protein	Promotes	✓	✓			Hao et al, 2001; Gabel et al, 2008

* The role of cAMP and its effector cAMP-dependent protein kinase in neuronal development has not been demonstrated in *C. elegans*, but has been implicated in other systems^{107,108}.