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Review article

Axonal fusion: An alternative and efficient mechanism of nerve repair

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ABSTRACT

Injuries to the nervous system can cause lifelong morbidity due to the disconnect that occurs between nerve cells and their cellular targets. Re-establishing these lost connections is the ultimate goal of endogenous regenerative mechanisms, as well as those induced by exogenous manipulations in a laboratory or clinical setting. Reconnection between severed neuronal fibers occurs spontaneously in some invertebrate species and can be induced in mammalian systems. This process, known as axonal fusion, represents a highly efficient means of repair after injury. Recent progress has greatly enhanced our understanding of the molecular control of axonal fusion, demonstrating that the machinery required for the engulfment of apoptotic cells is repurposed to mediate the reconnection between severed axon fragments, which are subsequently merged by fusogen proteins. Here, we review our current understanding of naturally occurring axonal fusion events, as well as those being ectopically produced with the aim of achieving better clinical outcomes.

1. Main text

1.1. Mechanisms of axonal regeneration

Whilst axonal injuries to neurons in the peripheral nervous system can lead to robust axonal regeneration, the same is not true for injured axons within the central nervous system. This is largely due to an inhibitory microenvironment. Much knowledge has been gained on the role of myelin-associated inhibitors, such as myelin-associated glycoprotein, Nogo, oligodendrocyte myelin glycoprotein, and Ephrin B3, which promote a preclusive environment through interactions with axonal receptors that include the Nogo-66 Receptor (NgR), NgR2, and EphA4 (Harel and Strittmatter, 2006; Hilliard, 2009; Yiu and He, 2006). Similarly, we have gained important insights into the intrinsic regulators of axonal regrowth, including the key role of calcium and cAMP levels, and the role of signaling pathways involving mitogen-activated protein kinases, mammalian target of rapamycin (mTOR), Janus kinase/signal transducers and activators of transcription (JAK/STAT), and the Krüppel-like transcription factors (Liu et al., 2011; Sun et al., 2011). However, despite these major advances in our understanding of nervous system injury and repair, we still have limited knowledge of how a damaged axon can re-establish connection with its target tissue.

Following injury, an axon can use one of several different strategies to re-join with its target tissue (Fig. 1). It first needs to initiate regrowth (Bradke et al., 2012), which can originate from the tip of the severed axonal end still attached to the cell body, from a branch extending out from this fragment, or from a new axon derived from the soma (Hilliard, 2009). To then reach its target, the axon can navigate along the entire length of its original pathway, or use a different, ectopic route (Harel and Strittmatter, 2006; Gribble et al., 2018; Nguyen et al., 2002; Rosenberg et al., 2014). Due to the extensive length of regrowth that may be required, regrowing axons can instead make functional connections with other neurons in the vicinity taking a similar route or with newly born neurons after cellular migrations into the damaged zone, thereby shortening the growth requirements (Harel and Strittmatter, 2006; Tuszynski and Steward, 2012). Alternatively, if the separated axon segment remains intact for a sufficient amount of time, the regrowing axon can reconnect and fuse with it to restore the original axonal structure. This final scenario, known as axonal fusion, is the focus of this review.

Axonal fusion occurs through the reconnection and merging of separated axon segments. In order for this to occur through spontaneous mechanisms, several essential steps are required (Box 1). Although not unique to regeneration through axonal fusion, the damaged membranes

Abbreviation: ABC, ATP-binding cassette; AFF-1, anchor cell fusion failure-1; DRG, dorsal root ganglion; EFF-1, epithelial fusion failure-1; NMNAT, nicotinamide mononucleotide adenylyltransferases; PEG, polyethylene glycol; PS, phosphatidylserine; RGC, retinal ganglion cells; UV, ultraviolet; WLD^S, Wallerian degeneration slow

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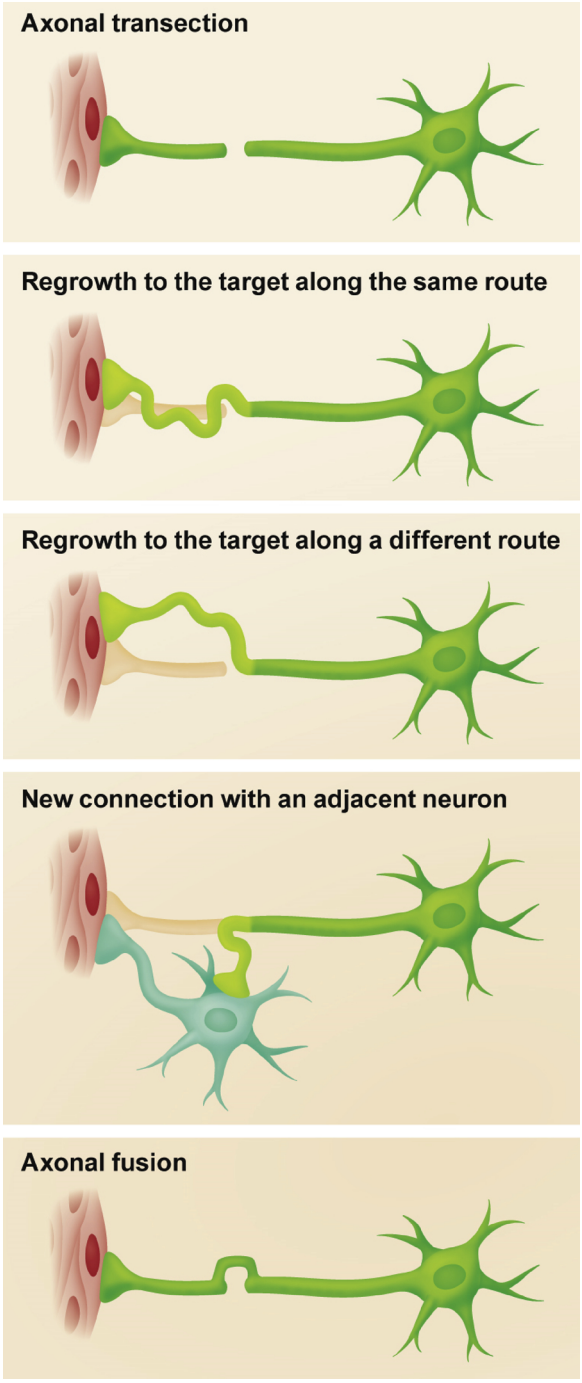

Fig. 1. The different modalities of axonal repair that can be used to achieve target re-innervation. A transected axon (top image) can use one of several different strategies to regrow and reconnect with its target tissue. These include regrowing the entire length beyond the injury site along the original route (second panel), or along a different route (third panel), or establishing connection with an adjacent neuron (fourth panel), or by re-joining with its separated segment (axonal fusion – bottom panel).

must be rapidly resealed to prevent the death of both the cell body and separated axon segment. This resealing process is largely driven by the influx of Ca^{2+}, which drives vesicle mediated repair of the plasmalemmal membrane (Bittner et al., 2016a; Cheng et al., 2015; Jimenez and Perez, 2017). Next, an axon must rapidly initiate regrowth to bridge the site of damage. Concurrently, degeneration in the separated axon segment must be sufficiently delayed to permit recovery of that segment upon fusion with the regrowing axon. The regrowing axon must then find and make physical contact (reconnect) with its separated segment. Subsequently, the reconnected membranes need to fuse to re-establish continuity between the segments. Finally, the internal structure of the axon must be repristinated to restore the transport networks required for neuronal function. Remarkably, this series of events occurs naturally following axonal transection in a variety of invertebrate species (Table 1). Taking cues from these phenomena, pre-clinical studies are harnessing similar paradigms to repair severed nerves in mammals.

1.2. Spontaneous axonal fusion

Axonal fusion was first reported in the peripheral nervous system of the crayfish (*Procambarus clarkii*) in 1967 (Hoy et al., 1967). In this publication, the authors provide evidence that severance of the single motor axon controlling the opener muscle of the claw can be repaired through a fusion mechanism. Similar mechanisms have subsequently been observed in central nervous system neurons of earthworms (*Lumbricus terrestris*) (Birse and Bittner, 1976), snails (*Helisoma trivolvis*) (Murphy and Kater, 1978), leeches (*Hirudo medicinalis*) (Deriemer et al., 1983; Frank et al., 1975), and sea slugs (*Aplysia californica*) (Bedi and Glanzman, 2001), as well as peripheral-like neurons of nematodes (*Caenorhabditis elegans*) (Ghosh-Roy et al., 2010; Neumann et al., 2011). As eloquently described more than 50 years ago by Hoy et al. “repair by fusion clearly offers two advantages: it is less expensive metabolically, since so much less resynthesis is required; and it demands only a single correct reconnection rather than the reassembly of an entire set of peripheral terminals” (Hoy et al., 1967).

In 2004, advances in laser technology applied to microscopy opened the door for single neuron axotomy to be performed in living *C. elegans* (Yanik et al., 2004). This system has since been successfully utilized to identify many conserved pathways regulating regeneration following axon transection (Hammarlund and Jin, 2014). Severing the axons of the *C. elegans* peripheral-like mechanosensory neurons (ALM, AVM or PLM), or the oxygen-sensing ALN and PLN neurons, can lead to spontaneous repair through axonal fusion (Ghosh-Roy et al., 2010; Neumann et al., 2011; Wu et al., 2007; Yanik et al., 2006; Basu et al., 2017). Electron microscopy, together with the movement of cytosolic fluorophores and active transport observed across the fusion site, have been used to confirm that these events represent true fusion between separated fragments, as opposed to mere gap junctions or synaptic connections (Ghosh-Roy et al., 2010; Neumann et al., 2011; Basu et al., 2017; Abay et al., 2017a). It is not yet established whether axonal fusion also occurs in other classes of *C. elegans* neurons, as a number of factors make some neurons less amenable to studying fusion. In the case of the ALM/AVM/PLM and ALN/PLN neurons, their location along the lateral surface of the animal, and the availability of specific fluorescent markers, permits clear visualization of these neurons and clear evaluation of fusion events. In contrast, the GABAergic motor neurons (6 DD class and 13 VD class), which have been used extensively for axonal regeneration studies, project their axons circumferentially from the ventral to dorsal nerve cords, where their neurites bundle together with those of other motor neurons. This characteristic, together with the lack of specific markers to highlight a single or only a few neurons, limits the visualization of individual axons and the evaluation of fusion in the dorsal cord (Yanik et al., 2006). As such, it is not currently known if axonal fusion is a widespread repair mechanism across the 302 neurons of the nematode nervous system.

1.2.1. Dendrite fusion

Regenerative neurite fusion has also been observed in dendrites. Maintaining the integrity of dendrites is crucial for correct sensory input to a neuron. Studies in the pair of *C. elegans* peripheral-like nociceptive neurons PVDs have revealed that its dendrites can undergo regenerative fusion similar to that observed in axons. These highly arborized neurons develop complex dendritic branches in periodic

Box 1
General requirements for axonal fusion

1 Sufficient delay in the onset of degeneration in the separated axon segment / initiation of regeneration in the segment still attached to the cell body.
2 Reconnection between the regrowing axon and its separated counterpart through the establishment of molecular bridging between the two segments.
3 Fusion of the two membranes to reestablish membrane and cytoplasmic continuity.
4 Repristination of internal axonal structure.

stereotypical patterns that resemble the Jewish candelabra, and are thus called menorahs. Development of menorahs involves dynamic processes of growth, pruning, retraction and mechanisms of self-avoidance that prevent overlap of branches (Oren-Suissa et al., 2010; Smith et al., 2010, 2012). Intriguingly, this developmental remodelling also involves fusion between dendritic branches (Oren-Suissa et al., 2010). Electron microscopy of wild-type PVD neurons revealed that the terminal PVD branches can fuse to form loop-like structures, which may function to restrict further growth of the dendrites and sculpt their final morphology. More recently, the PVD dendrites were also shown to undergo fusion as a means of repair (Oren-Suissa et al., 2017). Following UV laser transection, the PVD dendrites exhibited regrowth and formed reconnections between branches to bypass the injury site. Crucially, this was shown to restore cytoplasmic continuity and hence morphological recovery. Thus, fusion as a mechanism of neuronal repair is not restricted to the axonal compartment, but can also occur in dendrites.

1.2.2. Axonal fusion is a functional means of neuronal repair

The ultimate goal of any regenerative mechanism is to restore tissue structure and regain lost function. Axonal fusion appears to restore neuronal function in every species in which it has been observed. In the crayfish and snail, both behavioural function and the propagation of electrical activity across the fusion site are restored (Hoy et al., 1967; Murphy and Kater, 1978; Kennedy and Bittner, 1974). Similarly, in earthworms and leeches, axonal fusion restores axonal morphology and re-establishes action potentials across the site of transection (Birse and Bittner, 1976; Frank et al., 1975). In the earthworm, full function is not completely achieved (at least at a four-week post-injury time point), with a 25% reduction in conduction velocities in fused versus uninjured giant axons (Birse and Bittner, 1976). Cytoplasmic continuity is restored following axonal fusion in leeches and nematodes (Deriemer et al., 1983; Neumann et al., 2011). In cultured *Aplysia* neurons, axonal fusion not only restores cytoplasmic continuity, but also suppresses the hyperexcitability and excessive branching normally associated with axotomy in this species (Bedi and Glanzman, 2001; Walters et al., 1991). More recently, two independent studies established that axonal fusion in *C. elegans* restores full function to severed mechanosensory neurons (Basu et al., 2017; Abay et al., 2017a). In this case, the lost sensitivity to gentle mechanical stimulation returned to control levels, and active axonal transport was reinstated. Thus, axonal fusion represents a rapid means of spontaneous functional repair after neuronal injury.

1.2.3. Specificity of axonal fusion

Can a regrowing axon recognise its own separated segment and specifically reconnect with it? This question has partially been addressed in both earthworms and nematodes, with evidence strongly supporting specificity of recognition (Birse and Bittner, 1976; Neumann et al., 2011; Birse and Bittner, 1981). The ventral nerve cord (VNC) of the earthworm is composed of three morphologically and physiologically distinct large diameter axons (Bullock, 1945). Severance of the VNC therefore presents the regrowing axons with three alternative separated segments with which to reconnect and fuse. Birse and Bittner severed the VNC and tested the specificity of fusion using electron microscopy, electrophysiology and behavioural assays (Birse and Bittner, 1976). This battery of tests provided no evidence of aspecific fusion, leading the authors to conclude that the specificity of fusion is very high. Similar conclusions have been made in the snail (Murphy and Kater, 1978), whereas in the leech specificity of reconnection is maintained with distal segments of the same modality, but not necessarily with their own separated segments (Deriemer et al., 1983). In the nematode, a regrowing axon was challenged with two different severed axon segments: its own separated segment, and one from a neighbouring, largely fasciculating axon. Two pairs of neurons were tested in this fashion (ALM-ALN and PLM-PLN), with each pair demonstrating extremely high rates of specific fusion with their own separated axon segments (> 90% of the time). However, a low percentage (~10%) of axons also displayed concurrent aspecific events in which the regrowing axon also fused with the severed segment of the adjacent axon (Neumann et al., 2011). Thus, from the current evidence it is clear that a regrowing axon can display a high level of specific reconnection with its own separated segment. However, how this specificity is generated has not yet been resolved in any system, and it remains a fascinating area of investigation. It is possible that secreted ligands or cell-adhesion molecules might contribute to this specificity, or that despite the proximity of fasciculating axons, such as the *C. elegans* PLM and PLN neurons, they are still too distant for the non-specific fusion to take place at a higher rate.

Further supporting the notion of recognition between severed axon segments is the fact that regrowing axons do not appear to fuse with intact axons (Bedi and Glanzman, 2001; Neumann et al., 2011). Although fusion between neurons is now a recognized component of certain developmental and post-developmental programs (Giordano-Santini et al., 2016), fusion of a regrowing axon with an intact neighboring cell has not yet been reported. Thus, it is likely that specific molecular signals are displayed following injury that are required for axonal fusion. As described later in this review, one such 'save-me' signal has been identified as the phospholipid phosphatidylserine.

1.3. Molecular control of axonal fusion

The discovery of axonal fusion in *C. elegans* not only expanded the number of species in which this form of repair has been observed, but provided a highly genetically amenable system in which to interrogate the molecular mediators of this event. To achieve repair via axonal fusion, an axon must undergo sequential processes of regrowth, reconnection and fusion. That is, the proximal axon must first regrow towards its distal fragment (regrowth), then it must make contact with the distal fragment (reconnection), and finally it must merge the apposing membranes and create continuity between the two axonal fragments (fusion). These three processes are distinct and are mediated by different molecular mechanisms. The molecular pathways allowing for regrowth of *C. elegans* axons following injury have been studied extensively and are becoming well established (Hammarlund and Jin, 2014; Byrne and Hammarlund, 2017; El Bejjani and Hammarlund, 2012). In contrast, relatively few studies have focused on the mechanisms of reconnection and fusion. The ability of the proximal axon

Table 1
Axonal fusion in different species and neuronal classes.

Species	Neuronal class	Means of transection	Method of confirmation	Refs.
Aplysia californica (sea slug)	central nervous system - dissociated mechanosensory neurons isolated from pleural ganglia grown *in vitro*	glass microneedle	morphological assessment; dye filling with Lucifer yellow; suppression of phenotypes associated with axotomy	Bedi and Glanzman, 2001
Caenorhabditis elegans (nematode)	peripheral-like - mechanosensory neurons (PLM, ALM, AVM)	UV laser	morphological assessment; electron microscopy; photoconvertible fluorophores; axonal transport; super-resolution imaging	Ghosh-Roy et al., 2010; Neumann et al., 2011; Basu et al., 2017; Neumann et al., 2015
	peripheral-like - PLN/ALN oxygen-sensing neurons	UV laser	morphological assessment	Neumann et al., 2011
	peripheral-like – PVD nociceptive neurons	UV laser	morphological assessment; photoconvertible fluorophores	Kravtsov et al., 2017
Helisoma trivolvis (snail)	central nervous system - neurons 4R and 4L of the buccal ganglia	crush	morphological assessment; dye filling with Lucifer yellow; electrical recordings	Murphy and Kater, 1978
Hirudo medicinalis (leech)	central nervous system - mechanosensory neurons	forcep crush or scissor cut	morphological assessment; dye filling with horseradish peroxidase	Deriemer et al., 1983
Lumbricus terrestris (earthworm)	central nervous system - ventral nerve cord, which includes lateral and medial giant axons, and non-giant axons	mechanical	histological staining; electrical stimulation. Dye filling with Lucifer yellow	Birse and Bittner, 1976 Birse and Bittner, 1981
Procambarus clarkia (crayfish)	peripheral nervous system - motor axon of the opener muscle	forcep crush or scissor cut	methylene blue staining; electrical recordings across the crush/cut site	Hoy et al., 1967

to reconnect with its distal fragment is influenced by neuronal class, genetic background, age, and method of axotomy (Abay et al., 2017a; Bourgeois and Ben-Yakar, 2008; Guo et al., 2008). Additionally, loss of the executioner caspase CED-3 has been shown to delay reconnection in some mechanosensory neurons (Pinan-Lucarre et al., 2012). The process of fusion is promoted by elevated calcium and cAMP levels, which are also required for axonal regrowth (Ghosh-Roy et al., 2010).

1.3.1. The role of fusogen proteins

The first molecule implicated in axonal fusion was the membrane fusogen Epithelial Fusion Failure-1 (EFF-1) (Ghosh-Roy et al., 2010). Together with the functionally similar Anchor cell Fusion Failure-1 (AFF-1), EFF-1 mediates the majority of membrane fusion events in *C. elegans*, including those involving neuronal and non-neuronal cells (Giordano-Santini et al., 2016; Mohler et al., 2002). EFF-1 is a nematode-specific type I transmembrane glycoprotein with structural and functional similarity to class viral II fusion proteins (Perez-Vargas et al., 2014). EFF-1 must be inserted into both membranes to mediate fusion, as its mechanism of action involves the formation of trimers *in trans* across apposing membranes (Perez-Vargas et al., 2014; Zeev-Ben-Mordehai et al., 2014). Its activity is precisely regulated during development to ensure that it mediates fusion at the correct place and time (del Campo et al., 2005; Kontani et al., 2005; Shemer and Podbilewicz, 2002; Smurova and Podbilewicz, 2016; Smurova and Podbilewicz, 2017; Weinstein and Mendoza, 2013). EFF-1 and AFF-1 are both expressed in a subset of *C. elegans* neurons, and in cells closely associated with neurons (Mohler et al., 2002; Zeev-Ben-Mordehai et al., 2014). These two molecules have now been shown to mediate important roles in the fusion of *C. elegans* axons, as well as dendrites. Mutations in *eff-1* cause strong defects in axonal fusion in the *C. elegans* PLM neurons (Ghosh-Roy et al., 2010; Neumann et al., 2015). Cell-specific recue experiments have revealed that within this context, EFF-1 functions cell-autonomously in the PLM neurons, and displays a change in localization in response to axotomy (Neumann et al., 2015). In uninjured neurons, EFF-1 displays a vesicular punctate pattern, likely reflecting its localization in early endosomes (Smurova and Podbilewicz, 2016; Linton et al., 2018). However, following axotomy, EFF-1 displays a rapid shift in localization, accumulating at the tips of the severed axon and along the membrane of growth cones (Neumann et al., 2015). This localization pattern is consistent with EFF-1 actively shifting to the leading edge of the regrowing axon, allowing it to mediate membrane fusion following reconnection with the separated segment (Fig. 2). The membrane localization of EFF-1 in neurons is controlled by the small GTPase RAB-5, which functions to restrict EFF-1 activity at the membrane through endocytosis (Linton et al., 2018). As such, perturbation of RAB-5 function promotes EFF-1 membrane localization, which leads to enhanced levels of axonal fusion. This phenomenon is accompanied by the release of extracellular EFF-1-containing vesicles from the cell body (Linton et al., 2018).

In the *C. elegans* PVD neuron, the sculpting of dendrites, both during development and repair following dendrotomy, is also mediated by fusogens. In the context of development, EFF-1 was found to be required for dendritic branch remodeling (Oren-Suissa et al., 2010; Zhu et al., 2017). Specifically, it mediates the simplification of the dendritic arbor through controlled branch retraction and auto-fusion. The absence of EFF-1 leads to a hyperbranching phenotype, whereas cell-autonomous overexpression of EFF-1 causes a reduction in branching (Oren-Suissa et al., 2010). However, a recent study demonstrated that EFF-1 has a non-cell-autonomous function in this context (Zhu et al., 2017); selective expression of EFF-1 in the epidermis (the tissue surrounding the PVD branches) fully rescues the PVD dendrite defect of *eff-1* mutant animals. In the context of regeneration, dendritic repair is achieved through the combined activities of EFF-1 and AFF-1 (Oren-Suissa et al., 2017). Following dendrotomy, AFF-1 mediates the fusion of dendritic branches to bypass the injury site. In this case, AFF-1 is derived non-cellautonomously and is produced as extracellular vesicles by the neighboring epidermal cells. Dendrotomy was also associated with growth of ectopic branches, which were simplified through the cell-autonomous activity of EFF-1. The authors of this study proposed a stepwise process in which vesicle-derived AFF-1 acts in the earlier steps of regenerative dendrite fusion, and EFF-1 acts later for pruning of excessive regrowth (Oren-Suissa et al., 2017). Additionally, EFF-1 and AFF-1 can promote dendritic repair in ageing neurons (Kravtsov et al., 2017). The level of fusion in PVD post-dendrotomy declines with age, whereas ectopic branching increases. These changes can be suppressed with ectopic expression of either AFF-1 or EFF-1, respectively. Such concurrent activity of AFF-1 and EFF-1 has been described in other *C. elegans* tissues (Rasmussen et al., 2008), and potentially represents an elegant way of controlling fusogen activity to achieve different forms of membrane remodeling. These studies in the PVD neuron raise interesting questions regarding how fusogens are regulated in different contexts of neurite fusion. In the case of EFF-1, it appears that this molecule is programmed to function during development, but can be repurposed later in life to optimize dendritic repair. How EFF-1 and AFF-1 are regulated within these contexts of development and repair is still unclear. Just as the growth of an axon has different molecular

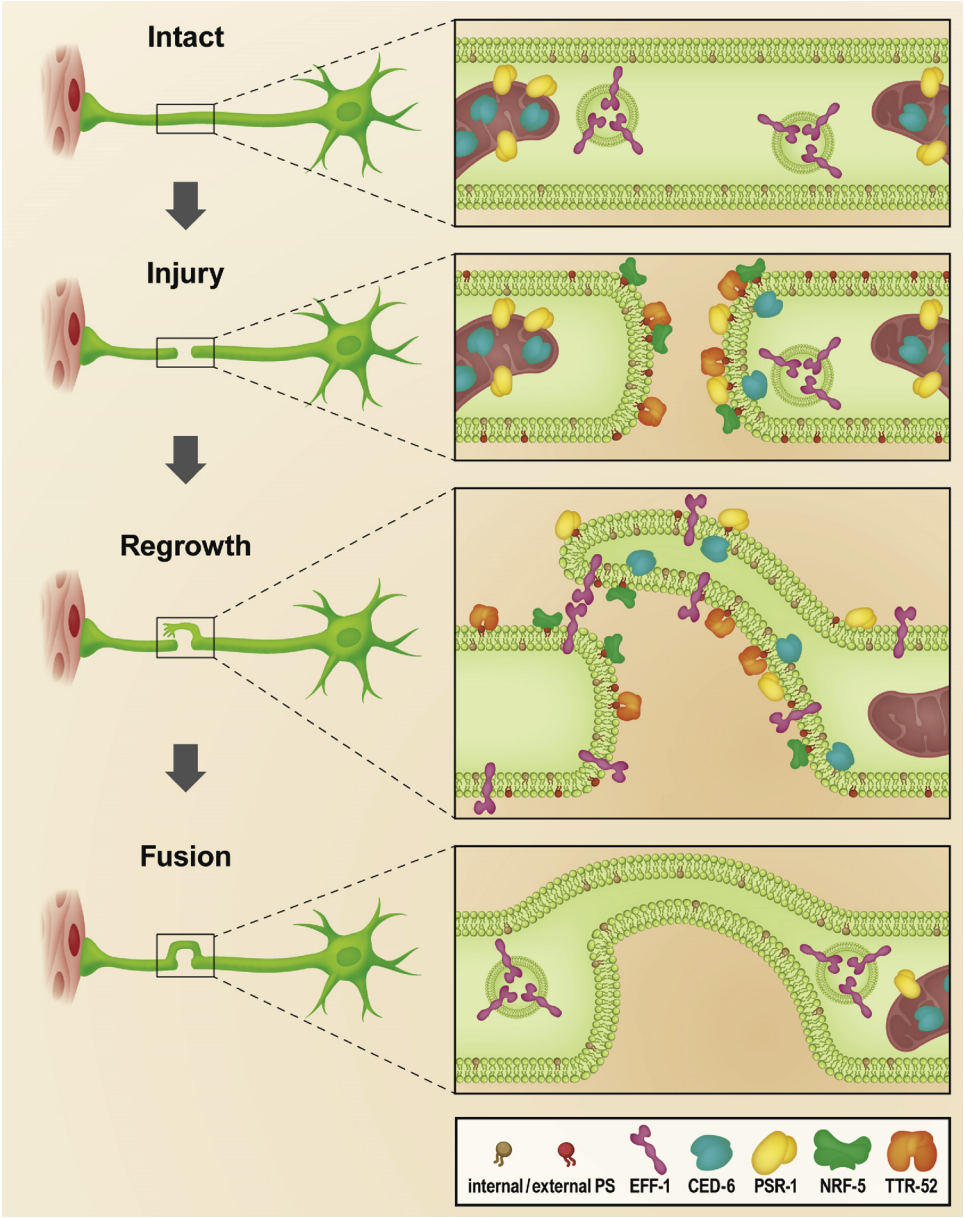

Fig. 2. Molecular changes associated with axonal fusion. Three major molecular changes have been associated with axonal fusion. 1. Rapidly after injury, phosphatidylserine (PS) is flipped from the inner leaflet of the plasma membrane to the external leaflet, to serve as a recognition or 'save-me' signal. Exposed PS is bound by the lipid-binding proteins TTR-52 and NRF-5, which likely then interact with molecules on the surface of the regrowing axon to mediate reconnection between the segments. 2. Molecules involved in reconnection accumulate at the tip of the regrowing axon, shifting from specific mitochondrial (CED-6) or mitochondrial and nuclear (PSR-1) localizations. 3. EFF-1 displays a change in localization from intracellular vesicles to the membrane, priming it to fuse the two membranes following reconnection.

regulators during development compared with regeneration (Wu et al., 2007; Gabel et al., 2008; Zou et al., 2013; Hammarlund et al., 2009), there may also be distinct mechanisms for regulating fusogens in these two contexts. In contrast, AFF-1 has not been reported to function during neuronal development, and may instead be activated by cellular responses unique to regeneration.

1.3.2. *The apoptotic recognition pathway*

In 2015, Neumann et al. used a candidate gene screening approach to identify the molecules involved in recognition between the regrowing axon and its separated segment (Neumann et al., 2015). This led to the surprising finding that the process of axonal fusion is remarkably similar to that of apoptotic cell recognition and engulfment. An early event during apoptosis is the presentation of the phospholipid phosphatidylserine (PS) on the external surface of the plasmalemmal bilayer as an 'eat-me' signal for recognition by phagocytes (Fadok et al., 1992) (Fig. 2). PS normally localizes asymmetrically to the inner leaflet of the membrane, where its highly anionic charge recruits proteins to the membrane and promotes membrane curvature (Leventis and Grinstein, 2010; Hirama et al., 2017). Disruption of this asymmetry, or 'flipping' of PS, occurs through the inactivation of aminophospholipid translocases that actively transport PS from the outer to the inner leaflet of the bilayer, and the concomitant activation of phospholipid scramblases that bidirectionally move phospholipids in the plasma membrane (Bratton et al., 1997; Nagata et al., 2016). Similarly, PS is rapidly exposed following axonal injury (Neumann et al., 2015; Wakatsuki et al., 2017; Almasieh et al., 2017), and has been proposed to serve as an essential 'save-me' signal on the separated axon segment for recognition by the regrowing axon (Abay et al., 2017a; Neumann et al., 2015; Teoh et al., 2018).

Two partly redundant, parallel apoptotic engulfment pathways recognize the PS 'eat-me' signal and mediate the clearance of dying cells by phagocytes (Kloditz et al., 2017) (Fig. 3). In the first, the phosphatidylserine receptor PSR-1/JMJD6 on the phagocytic membrane binds the exposed PS (Fadok et al., 2000; Yang et al., 2015) and activates an intracellular signaling pathway consisting of CED-2/CrkII, CED-5/DOCK180, CED-12/ELMO, and finally CED-10/Rac1 to orchestrate the actin cytoskeletal rearrangements necessary for engulfing the dying cell (Gumienny et al., 2001; Reddien and Horvitz, 2000; Wang et al., 2003; Wu and Horvitz, 1998a). The second pathway includes the secreted PS-

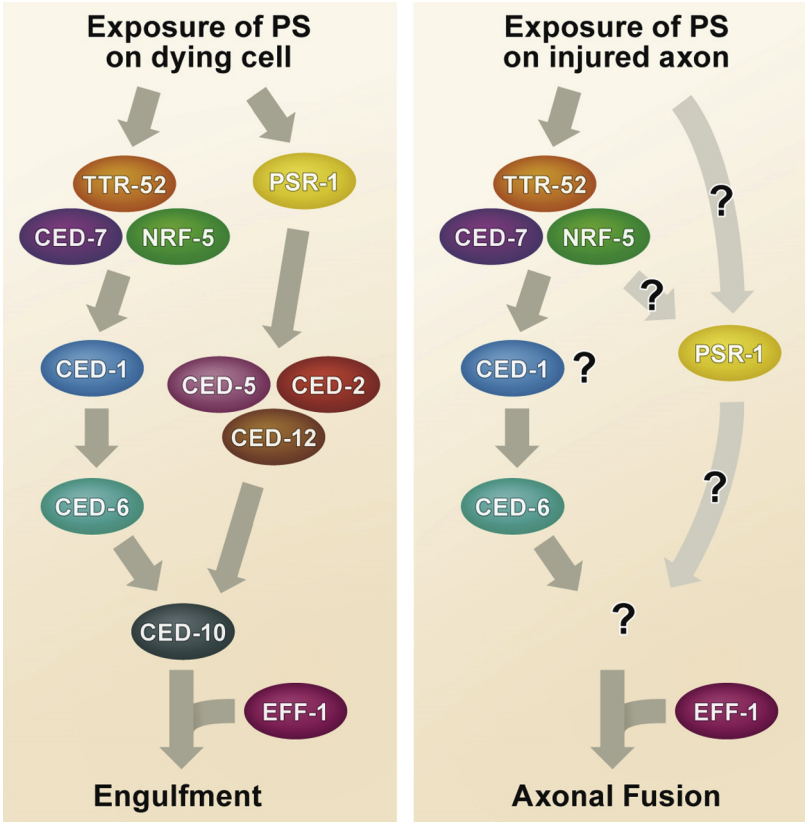

Fig. 3. Commonality and divergence of the molecular pathways controlling recognition processes necessary for apoptotic cell engulfment and axonal fusion.

binding proteins TTR-52/transthyretin (Wang et al., 2010) and lipid transfer/LPS-binding family protein NRF-5 (Zhang et al., 2012), the membrane-bound ATP-binding cassette (ABC) transporter CED-7/ABCA1 (Wu and Horvitz, 1998b), the transmembrane receptor CED-1/LRP1 (Zhou et al., 2001), the intracellular adaptor CED-6/GULP (Liu and Hengartner, 1998), and DYN-1/Dynamin (Yu et al., 2006). This pathway also converges upon CED-10 for cell engulfment (Kinchen et al., 2005). In a remarkable example of biological repurposing, many of these proteins also mediate the proximal-distal recognition required for axonal fusion (Neumann et al., 2015) (Fig. 3). In this context, the apoptotic recognition machinery functions upstream of EFF-1, as overexpression of this fusogen can compensate for axonal fusion deficits associated with loss of components of this pathway (Neumann et al., 2015).

Following axonal injury, TTR-52 and NRF-5 bind to the severed axon with similar dynamics to Annexin V, a widely-used marker of exposed PS (Neumann et al., 2015). Thus, these proteins likely bind specifically to PS on the damaged axon to mediate the recognition process. Mutations in TTR-52 or NRF-5 reduce the level of axonal fusion two-fold, as does loss of CED-7 function (Neumann et al., 2015). However, the role of this ABC transporter remains to be determined. Although CED-7/ABCA1 has been shown to mediate the presentation of PS on the outer surface of dying cells in both *C. elegans* (Li et al., 2015; Venegas and Zhou, 2007) and rodents (Hamon et al., 2000; Rigot et al., 2002), other studies have not confirmed this effect (Zullig et al., 2007) and it does not appear to be required for PS exposure after axonal injury (Abay et al., 2017a). One possibility is that CED-7 regulates PS localization following its initial exposure on the transected axon. Mapes et al. found that CED-7 and TTR-52 are important for the presentation of PS not only on dying cells, but also on the phagocytes (which is essential for engulfment) (Mapes et al., 2012). They proposed a mechanism whereby these proteins promote the efflux of PS from a dying cell to a phagocytic cell through the generation of extracellular vesicles containing PS on their outer surface. If this were the case following axonal injury, CED-7 and TTR-52 would promote the release of PS from the distal segment to the regrowing axon segment after it had been exposed. However, is not yet clear how such a model would fit with the observed diffuse localization pattern of CED-7 during axonal fusion (Neumann et al., 2015).

The phagocytic receptor CED-1 appears to have a minor role at best in axonal fusion, whilst CED-6 likely acts in similar fashion to its role in apoptotic engulfment: an adapter in a signal transduction pathway to mediate the recognition and fusion of the separated axon segments (Neumann et al., 2015). However, as CED-6 physically interacts with the cytoplasmic tail of CED-1 to transmit the apoptotic engulfment signal intracellularly (Su et al., 2002), CED-6 may interact with an alternative transmembrane protein during axonal fusion. Intriguingly, CED-6 was localized exclusively to the mitochondria prior to axotomy, before accumulating at the end of the regrowing axon following transection (Neumann et al., 2015) (Fig. 2). The role of CED-6 in mitochondrial function is currently unknown. It also remains to be determined whether CED-6 is secreted from these organelles following injury, and if so which mechanisms might promote this process.

Curiously, of the molecules involved in the parallel apoptotic engulfment pathway, only PSR-1 appears to be important for axonal fusion (Neumann et al., 2015). In this context, *psr-1* functions in the same genetic pathway as *ttr-52*, *nrf-5*, *ced-7* and *ced-6* (Neumann et al., 2015). Therefore, although the molecules involved in apoptotic engulfment are shared with axonal fusion, the specific interplay between them varies between these biological events. Similarly to CED-6, PSR-1 localizes to the mitochondria in intact axons, and accumulates at the regrowing tip after injury (Neumann et al., 2015) (Fig. 2). Whether CED-6 and PSR-1 physically interact in this context, and the link between PSR-1 and mitochondria, remain to be determined. In addition to its mitochondrial localization, PSR-1 was also found in the nucleus (Neumann et al., 2015). PSR-1 contains two conserved functional domains, a PS-binding

lysine-rich motif in the extracellular domain (Yang et al., 2015), and an intracellular Jumonji C (JmjC) domain (Chang et al., 2007). The JmjC domain has been linked to several different functions, including histone arginine demethylation (Chang et al., 2007), lysyl hydroxylation (Unoki et al., 2013; Webby et al., 2009), and RNA splicing (Hong et al., 2010; Heim et al., 2014). Both the PS-binding and JmjC domains are required for axonal fusion (Neumann et al., 2015). Thus, consistent with its localization pattern, PSR-1 may therefore have multiple functions during axonal fusion, both within the nucleus and at the tip of the regrowing axon.

The lack of function for CED-10 in axonal fusion (Neumann et al., 2015) poses another major outstanding question. CED-10 is a Rac GTPase that functions at the convergence point for the two apoptotic recognition pathways to induce the actin cytoskeleton rearrangements necessary for engulfment. In light of this notion, how do the molecules implicated in axonal fusion induce the appropriate cytoskeleton changes without CED-10? The *C. elegans* genome encodes three Rac GTPases, CED-10, MIG-2 and RAC-2, which possess overlapping functions in the regulation of intracellular actin dynamics for cell migration, and axon outgrowth and guidance (Lundquist et al., 2001). Thus, although MIG-2 and RAC-2 possess only subtle roles in apoptotic engulfment (Lundquist et al., 2001), it is possible that they may have more prominent roles in axonal fusion. Functions in axonal fusion for these Rac GTPases, or for DYN-1, which functions downstream of CED-6 to promote membrane extensions for phagocytosis (Yu et al., 2006), are yet to be reported.

In contrast to the age-dependent decline in regrowth capacity of axons observed across species (including both nematodes and mammals), the level of axonal fusion actually increases with advancing age (Abay et al., 2017a). However, the functional recovery associated with axonal fusion is significantly impaired in older animals (Basu et al., 2017). Although the molecular mechanisms mediating the increased frequency of axonal fusion over age are not known (Abay et al., 2017b,c,d), Basu et al. have demonstrated that the conserved microRNA *let-7* impairs functional recovery in older animals, in part by decreasing the level of *ced-7* mRNA (Basu et al., 2017). Initially described as a heterochronic gene in *C. elegans* (Reinhart et al., 2000), *let-7* family members have now been widely implicated in promoting differentiation during development in a number of species, and function as tumor suppressors in various types of cancer (Lee et al., 2016). In the context of axonal fusion, *let-7* likely binds directly to the 3`UTR of *ced-7* to inhibit CED-7-mediated reconnection between the regrowing axon and its separated axon segment (Basu et al., 2017). Loss of *let-7* function enhanced the efficiency of axonal transport, thereby promoting functional recovery in older animals (Basu et al., 2017). This increased axonal transport has been proposed to enhance the trafficking of EFF-1 to the membrane of the regrowing axon in order to further promote axonal fusion (Basu et al., 2017). Although precisely how *let-7* affects axonal transport remains to be determined, this microRNA represents an intriguing target for promoting functional recovery after neuronal injury.

1.3.3. Phosphatidylserine: the 'save-me' signal for axonal fusion

How the same pathways can be used to mediate two seemingly opposing cellular mechanisms of death and survival remains a fascinating open question. In particular, a phagocytic engulfment and regenerative axonal fusion appear to utilize the same initiating event – the presentation of PS to the external environment. Following axonal transection in *C. elegans*, PS is rapidly exposed on the axonal membrane (within 15 min) (Neumann et al., 2015), and the level of exposure strongly correlates with the level of reconnection achieved (Abay et al., 2017a). PS exposure is not affected by mutation of the genes required for regrowth, reconnection or fusion (Abay et al., 2017a), suggesting that it is an early, initiating event for axonal fusion. Importantly, PS is also exposed after transection of mammalian neurons. However, PS exposure has thus far been specifically associated with the degeneration of mammalian axons rather than their regrowth. Axonal transection of cultured retinal ganglion cells (RGC) from Sprague Dawley rats induced robust PS exposure within seconds on either side of the cut site (Almasieh et al., 2017). Externalized PS continued to bidirectionally spread along the axon over the proceeding thirty minutes after injury. Inhibition of intrinsic axonal degeneration pathways delayed the onset of PS exposure and slowed its spread (Almasieh et al., 2017), illustrating a close association between exposed PS and degeneration in this context. Transection of axons in dorsal root ganglion (DRG) explants from C56BL/6 J mice also resulted in the exposure of PS, but was not detected until 3 h post-injury (Wakatsuki et al., 2017). The differences in time scales is likely due to intrinsic differences in the specific neuronal classes. Non-transection insults can also induce PS exposure. Application of various apoptotic insults (including Alzheimer's associated ß-amyloid peptides) specifically to distal neurites of primary rat hippocampal neurons induced PS exposure (Ivins et al., 1998). Thus, PS exposure is a conserved mechanism following axonal injury; however, how it can serve either as a signal for degeneration or as a 'save-me' signal for axonal fusion remains to be determined.

1.3.4. Non-apoptotic exposure of PS

In addition to axonal fusion, non-apoptotic exposure of PS serves as an important facet of a number of diverse cellular events. The best characterized of these is haemostasis, in which activated platelets externalize PS to recruit and subsequently activate several clotting factors essential for coagulation (Leventis and Grinstein, 2010; Bevers et al., 1982). PS localization to the exoplasmic leaflet has also been shown to be important for fertilization of eggs (Gadella and Harrison, 2000), development of the placenta (Adler et al., 1995), exocytosis (Perez-Lara et al., 2016), and for both macrophage (Helming et al., 2009) and osteoclast (Verma et al., 2018) fusion. Non-apoptotic exposure of PS is also required for efficient fusion of viruses with their host cells during infection. PS from the host cell is incorporated into the viral membrane, allowing it to be recognized by PS receptors and thereby facilitating entry into the host (Amara and Mercer, 2015). This has been demonstrated for a number of viruses, including vaccinia (Mercer and Helenius, 2008), dengue (Meertens et al., 2012; Zaitseva et al., 2010), Ebola (Moller-Tank et al., 2013; Adu-Gyamfi et al., 2015), and HIV (Callahan et al., 2003; Zaitseva et al., 2017) and has been proposed as a common mechanism adopted by enveloped viruses to promote infection (Amara and Mercer, 2015). Fascinatingly, the dengue virus E glycoprotein and EFF-1 are both class II fusion proteins and possess related structures and functions (Perez-Vargas et al., 2014). Given the similarities in the mechanisms behind how these proteins mediate fusion events, especially in terms of the requirements for externalized PS, it appears plausible that structurally similar fusion proteins in other species may have the capacity for carrying out axonal fusion-like regenerative paradigms.

Transient exposure of PS to the external cell surface also occurs as part of the normal development of skeletal muscles. Precursor myoblasts fuse following exposure of PS at contacted points, thus forming myotubes in mature muscles (Jeong and Conboy, 2011; van den Eijnde et al., 2001). With parallels to axonal fusion, the molecular machinery driving apoptotic cell corpse engulfment appears to have been repurposed for myoblast fusion. In this context, exposed PS is recognized by at least two PS receptors previously implicated in the recognition of apoptotic cells: Brain-specific Angiogenesis Inhibitor 1 (BAI1) and stabilin-2 (Park et al., 2016; Hochreiter-Hufford et al., 2013). The signal is relayed intracellularly, predominantly through the first apoptotic engulfment pathway described above (Fig. 3), with CrkII/CED-2, DOCK180/CED-5, ELMO/CED-12, Rac/CED-10, and Dynamin/DYN-1 all implicated (Leikina et al., 2013; Hakeda-Suzuki et al., 2002; Nolan et al., 1998; Moore et al., 2007; Geisbrecht et al., 2008; Laurin et al., 2008; Vasyutina et al., 2009). Fusion of the cell membranes is successively achieved by the transmembrane protein Myomaker (Millay et al., 2016, 2013), which likely functions together with the Myomerger/

Myomixer/Minion micropeptide (Bi et al., 2017; Quinn et al., 2017; Zhang et al., 2017). This linear arrangement, consisting of PS exposure activating common components of the apoptotic recognition signaling pathways to bring two membranes within sufficiently close apposition for merging by fusogens, may therefore represent a common biological mechanism.

1.3.5. Molecular control of PS exposure

Whilst the molecular control of PS flipping during axonal fusion has not been elucidated, a number of proteins have been found to control PS localization under other cellular contexts. PS localization is mediated by three different families of lipid transporters: flippases, floppases and scramblases. Flippases are conserved P4-ATPase transmembrane proteins that transport PS in an ATP-dependent manner from the external surface to the inner surface of the membrane; floppases are less well-characterized members of the ABC transporter family that transport PS in the opposite direction, from the inner to outer leaflets; and scramblases are ATP-independent and instead of transporting PS in a unilateral direction, function to reduce membrane asymmetry by bi-directionally transporting lipids across the membrane (Leventis and Grinstein, 2010; Nagata et al., 2016; Pomorski and Menon, 2016). Identification of the proteins controlling PS exposure after axonal injury will be an important next step in our mechanistic understanding of axonal fusion. Whether injury itself can modulate the local membrane composition, which would therefore imply that longer-lived micro-injuries would correlate with more efficient PS exposure, remains to be determined.

Although all of the currently recognized molecular effectors of axonal fusion were identified in *C. elegans*, it is likely that similar molecular machinery is present in other species in which axonal fusion occurs. In particular, merging of the membranes, and the opening and expansion of the fusion pore between the two axonal segments, certainly require yet-to-be-discovered species-specific fusogens.

1.4. Mechanisms of axonal degeneration

For axonal fusion to be feasible, it is imperative that a separated axon segment undergoes a sufficiently slow program of degeneration and clearance to provide an adequate time-frame for the regrowing axon to reach it and achieve reconnection (Box 1). Severed axon segments undergo a process of stereotypical breakdown known as Wallerian degeneration (Waller, 1850), which is driven by intrinsic self-destruct pathways that are largely distinct from those involved in cell death (Conforti et al., 2014). This process results from a shift in the relative activity of pro-survival and pro-death signals following a period of latency once an axon is transected (Neukomm and Freeman, 2014). The NAD^+ biosynthetic enzymes nicotinamide mononucleotide adenylyltransferases (NMNATs) are major pro-survival factors (Conforti et al., 2014). In particular, NMNAT2 is continually trafficked along the axon, but due to its short half-life is rapidly depleted after axonal transection, which promotes degeneration (Gilley and Coleman, 2010; Milde et al., 2013). Ectopic expression of either of the three mammalian NMNAT proteins or the chimeric Wallerian Degeneration Slow (WLD^S) protein, which contains the full length NMNAT1 sequence, can robustly delay the onset of degeneration in a number of species (Mack et al., 2001; Adalbert et al., 2005; Babetto et al., 2010; Griffin et al., 2013; Hoopfer et al., 2006; MacDonald et al., 2006; Martin et al., 2010; Yahata et al., 2009), including in cultured human neurons (Kitay et al., 2013). Suppressing the turnover of NMNAT2 can also delay degeneration (Babetto et al., 2013; Xiong et al., 2012). Although the function of NMNAT2 in this context remains contentious, it appears likely that it functions to maintain NAD^+ levels (Gerdts et al., 2016). Once NAD^+ levels fall under a certain threshold, the pro-death Toll-like receptor (TLR) adaptor SARM1 is activated to promote degeneration, at least in part by further driving the breakdown of NAD^+ (Osterloh et al., 2012; Essuman et al., 2017; Gerdts et al., 2015). Downstream from these components is the pro-degenerative BTB and BACK domain protein Axundead. Axonal degeneration is completely suppressed over the lifetime of *Drosophila* lacking Axundead (Neukomm et al., 2017), although how this protein achieves this function is yet to be resolved. Thus, the loss of pro-survival (NMNAT, NAD^+) and activation of pro-death (SARM1, Axundead) factors drives the dismantling of severed axons. The final execution phase of axonal degeneration is driven by elevated levels of calcium activating the calpain family of cysteine proteases that dismantle the axonal cytoskeleton (Conforti et al., 2014). Given that axonal fusion is known to correlate inversely with degeneration in the severed axon segment (Abay et al., 2017a), manipulating the above pathways to completely suppress axonal degeneration may prove to be an effective approach for promoting axonal fusion.

Despite this wealth of information, it is clear that other molecules and mechanisms involved during axonal degeneration are yet to be discovered. Axonal degeneration after transection of *C. elegans* axons displays morphological similarities to Wallerian degeneration (Nichols et al., 2016), but is mediated by alternative pathways from those described above. The temporal dynamics of degeneration are unaffected by overexpression of endogenous NMNAT proteins, knockout of the SARM1 ortholog, or ectopic expression of mammalian NMNAT proteins or WLD^S (Nichols et al., 2016). However, some mechanisms, such as the role of mitochondria in axonal degeneration, are conserved across species. In *C. elegans*, blocking the transport of mitochondria into axons causes spontaneous axonal degeneration in some neurons, and greatly enhances axonal degeneration after transection (Nichols et al., 2016; Ding and Hammarlund, 2018; Rawson et al., 2014). This central role for mitochondria in axonal degeneration appears to be conserved in mammals (Court and Coleman, 2012; Iijima-Ando et al., 2012; Kuo et al., 2017), although whether these organelles prevent or promote degeneration in these species remains an open question (Conforti et al., 2014). Moreover, the cellular control over the clearance of degenerated axon segments is evolutionarily conserved, with components of the apoptotic recognition machinery also employed for clearing axonal debris (Hilliard, 2009; MacDonald et al., 2006; Nichols et al., 2016; Ziegenfuss et al., 2012). Intriguingly, some of the molecules implicated in axonal clearance in *C. elegans* are the same that promote axonal fusion in this species. Although clearance largely proceeds through the alternative CED-2/CED-5/CED-12/CED-10 pathway (Fig. 3), the lipid transfer proteins CED-7 and NRF-5, along with the intracellular adapter CED-6, all participate in both clearance and axonal fusion (Neumann et al., 2015; Nichols et al., 2016). Fascinatingly, a recent study in *C. elegans* demonstrated that the fusogen EFF-1 functions in phagosome sealing for the clearance of the distal region of epithelial cells (Ghose et al., 2018), further revealing the commonality between these processes. However, how these molecules can carry out apparently opposing functions remains an open question, the answer to which will help to define how the balance between axonal degeneration and axonal fusion is achieved.

1.5. Axonal fusion in mammals

1.5.1. Evidence for spontaneous repair in vertebrates

Despite accumulating evidence that intact mammalian neurons can fuse under certain conditions (Giordano-Santini et al., 2016; Sretavan et al., 2005), data demonstrating spontaneous fusion after injury in this animal class is limited. Spontaneous nerve repair commonly occurs in some vertebrate species, but this is driven largely by cell migration to form a bridge across the damaged zone to guide repair (Zochodne, 2008; Parrinello et al., 2010; Becker and Becker, 2014; Tazaki et al., 2017; Rasmussen and Sagasti, 2017; Rehermann et al., 2009). Axonal fusion has been observed in cultured rat neuroblastoma cells following UV-laser axotomy, but only in cases where very thin neuroplasmic bridges remain (Rieske and Kreutzberg, 1978). A study from close to a century ago reported that the severed segments of embryonic chick neurites could reunite, and suggested that this *restitutio ad integrum*

(restoration of the original condition) is the normal mechanism of repair for these cells (Levi, 1926). Conclusive proof that vertebrate neurons are capable (or incapable) of spontaneous axonal fusion repair is lacking. In contrast, more extensive evidence exists showing that axonal fusion-like mechanisms can be induced to aid neuronal repair in mammals, including humans.

1.5.2. Inducing axonal fusion-like mechanisms of repair in mammals

Developments in microtechnology now permit cellular-scale surgical approaches to be utilized for repairing transected mammalian axons *in vitro* (Chang et al., 2010). The use of microelectrofusion has proved successful in fusing separated axon segments and reinitiating cytoplasmic flow (Sretavan et al., 2005). More developed approaches conducted on a nerve, rather than the level of an individual axon, involve the addition of membrane fusing substances. Building on the findings of axonal fusion in invertebrates and aiming to overcome the slow rate of regeneration in mammalian neurons (~1 mm/day), Bittner and collaborators explored the use of the chemical fusogen polyethylene glycol (PEG) to repair the severed ends of transected axons by fusion (Bittner et al., 1986). PEG has long been used as a membrane fusogen, and it acts through a volume exclusion mechanism whereby it removes water from the area and forces membranes into close contact, thus promoting fusion (Lentz, 2007). Remarkably, PEG fusion has proved to be highly effective in restoring continuity and function in transected nerves. Over the three decades since this formative discovery, George Bittner and colleagues have made major steps forward with this approach, such that current strategies routinely report rapid and robust recovery of function in pre-clinical trials (Bittner et al., 2016b). The use of PEG fusion has been shown to be effective in both peripheral nerve and spinal cord injury paradigms in rodents, rabbits, and dogs (Kim et al., 2016a; Borgens et al., 2002; Donaldson et al., 2002; Lore et al., 1999; Shi and Borgens, 1999; Shi et al., 1999; Kim et al., 2016b; Liu et al., 2018; Mikesh et al., 2018; Riley et al., 2017). In its most effective form, PEG fusion is now a defined five-step process (Bittner et al., 2016b, 2012): 1) axon ends are trimmed to provide flat, uniform ends, 2) plasmalemmal sealing is prevented by the addition of Ca^{2+}-free hypotonic saline containing an antioxidant (typically methylene blue), 3) cut ends are re-joined with microsutures, 4) Ca^{2+}-free hypotonic PEG (500 mM) solution is applied to the sutured nerve to induce membrane fusion, 5) PEG is removed and vesicle-mediated repair of residual plasmalemmal disruptions is promoted by rinsing with Ca^{2+}-containing isotonic saline (Fig. 4). This approach has been shown to generate rapid functional recovery after sciatic nerve transection in rats, and to also deliver significantly faster recovery from crush injuries (Bittner et al., 2012).

Despite its impressive results in regaining lost function to damaged nerves, the mechanisms behind PEG fusion remain to be resolved. Experiments aimed at defining what occurs on an individual neuron level, whether pre-existing synaptic connections are re-engaged or remodelled, whether new connections are made, and precisely how function is restored, are all yet to be reported. Given the large number of fibers found within a nerve, specific reconnection between individual severed axon ends is not a plausible outcome using this type of repair (Bittner et al., 2016b). It is conceivable, however, that similar to what occurs spontaneously in the leech (Deriemer et al., 1983), specificity of reconnection may be sufficiently preserved between axons of the same modality (e.g. motor-motor, sensory-sensory). Indeed, a characteristic feature of axon tracts in both the CNS and PNS is topographic organization, such that axons innervating similar areas or carrying similar information, tend to be found next to one another. Thus, precise reconnection might not be required to allow adequate functional recovery. Furthermore, neuronal plasticity, which has been well described in permitting the reorganization of neuronal circuitry after injury (Chen and Zheng, 2014; Fink and Cafferty, 2016), may subsequently optimize neuronal function after reconnection has been induced.

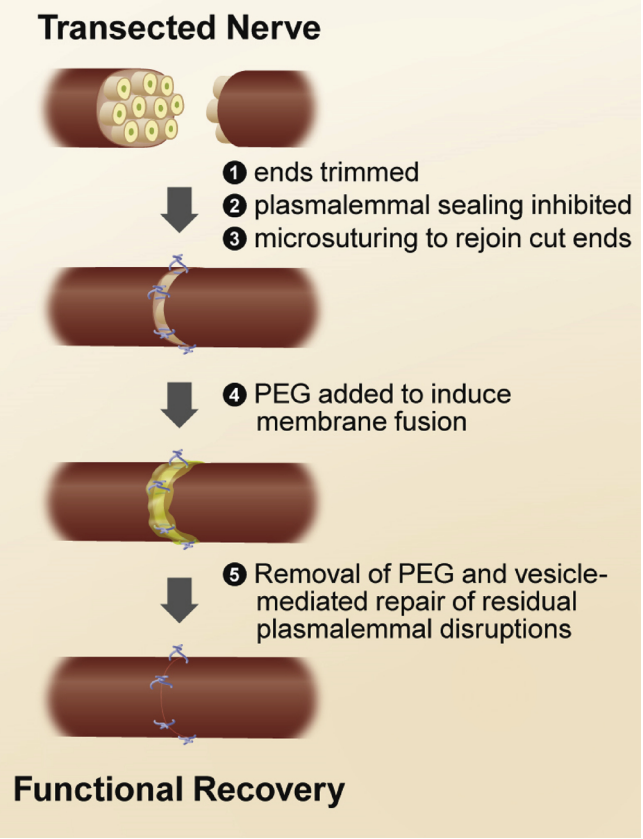

Fig. 4. Repairing mammalian nerve with PEG-driven fusion. Axonal repair using PEG-fusion has proven to be effective in restoring neuronal function to severed nerves in pre-clinical and clinical settings. The optimized methodology involves a five-step process (Bittner et al., 2016b, 2012): trimming of the severed ends, prevention of plasmalemmal sealing, rejoining segments with microsutures, PEG-induced membrane fusion, and finally vesicle-mediated repair of residual membrane disruptions.

Promisingly, PEG fusion has recently been adopted into the clinic. In 2016, Bamba et al. reported the first use of PEG fusion in humans, with the technique used to repair four fingers of two teenage patients following complete nerve transection injuries (Bamba et al., 2016). Within 12 h of injury, nerves were fused with PEG using the most effective five-step approach described in rodents (Bittner et al., 2016b, 2012). Compared to a control group of six patients, those who underwent PEG fusion displayed significantly faster recovery and improved functional outcomes over an eight-week trial period (Bamba et al., 2016). Despite its limitations, this trial highlights the potential of PEG fusion as an effective treatment for human nerve injuries. Nerve injuries frequently result in significant gaps between nerve segments that require more complex repair paradigms. Various types of biomaterials and conduits are currently being used to facilitate repair over larger gaps (Siemionow et al., 2010; Carballo-Molina and Velasco, 2015). PEG fusion has been combined with nerve grafts to treat these types of injuries in rodents, and has again been shown to be an effective means of restoring lost function (Riley et al., 2015).

Peripheral nerve injuries causing major gaps between segments are routinely repaired with autologous nerve grafts (Griffin et al., 2013). An alternative approach, especially for severe injuries, is end-to-side neurorrhaphy, in which a transected distal nerve stump is joined to the trunk of an adjacent intact, donor nerve (Tos et al., 2014). This technique has been used for a variety of peripheral nerve injuries, but mixed results and a lack of randomized clinical trials have limited its use (Tos et al., 2014). Based on the evidence described above in animal models and a human study, combining this approach with PEG fusion may aid

functional recovery and expand the clinical usefulness of neurorrhaphy approaches.

1.6. Major outstanding questions

1.6.1. Are fusogens expressed in mammalian neurons?

The fusogens responsible for the majority of cell fusion events remain undiscovered. In humans, many tissues are sculptured via developmental cell fusion, including myoblasts, osteoclasts, macrophages, and epithelial cells of the placenta (Aguilar et al., 2013). Contrary to classical thinking about the nervous system as being composed solely of individual units (the basis for the *neuron theory* developed by Ramón y Cajal (Lopez-Munoz et al., 2006)), fusion of neurons has been observed in specific circumstances such as aging, viral infections, and following the transplantation of stem and progenitor cells (Giordano-Santini et al., 2016). Intriguingly, although the two fusogens identified in humans to date (Syncytin-1 and Syncytin-2) are almost exclusively expressed in the placenta (Blaise et al., 2003; Mi et al., 2000), Syncytin-1 was found to be expressed in brain tissue of patients with multiple sclerosis (Antony et al., 2004; Mameli et al., 2015). Although many questions remain regarding the mechanisms behind neuronal fusion events, they strongly support the notion of fusogens being present and likely tightly regulated in the nervous system. Confirming the presence of such fusogens, and characterizing their function, could provide crucial insight into the potential of axonal fusion in repairing the mammalian nervous system.

1.6.2. Can axonal fusion occur spontaneously in mammalian neurons?

Do populations of neurons exist within the mammalian nervous system that can spontaneously regenerate through axonal fusion? Until recently, answering this question *in vivo* was not possible. Technological advances over the past decade have allowed injury responses to be visualized on a single cell level in the rodent brain and spinal cord *in vivo* (Akassoglou et al., 2017). Overall, these studies have revealed that axon segments severed from their cell bodies undergo a rapid program of degeneration (Canty et al., 2013; Kerschensteiner et al., 2005; Ylera et al., 2009; Bareyre et al., 2011; Erturk et al., 2007; Evans et al., 2014; Lorenzana et al., 2015) that would preclude any possibility of axonal fusion. However, under certain conditions with enhanced endogenous regeneration, regrowing axons can come in contact with their distal segments before these are cleared (Kerschensteiner et al., 2005; Bareyre et al., 2011; Di Maio et al., 2011), and in some instances, contact and extend along these segments (Di Maio et al., 2011). Although no indication of spontaneous axonal fusion has thus far been observed using *in vivo* imaging techniques in mammals, more extensive research is needed to confirm if this applies universally across the nervous system, or if there are certain neurons that possess fusion-competence. Further advances in technology now allow for imaging to be conducted in deeper regions of the brain and in freely behaving mice (Ouzounov et al., 2017; Sekiguchi et al., 2016), opening up further avenues for investigation.

1.6.3. If axonal fusion doesn't spontaneously occur in mammals, can it be induced?

For fusion-competence to be imparted there are several inhibitory factors that need to be mitigated. Firstly, as described above, the balance between degeneration and regeneration appears to be shifted towards degeneration in the mammalian nervous system. Consequently, a detached axon segment is cleared too quickly for axonal fusion to occur. Shifting this balance towards regeneration is therefore a crucial first step in inducing fusion-competence. Substantial progress has been made over recent years in our understanding of the molecular mechanisms behind axonal regeneration (Mahar and Cavalli, 2018), as well as those that function in the promotion or prevention of axonal degeneration (Neukomm and Freeman, 2014). Thus, by targeting these pathways, the speed and distance of regrowth could be enhanced and the onset of degeneration suppressed to provide an appropriate state for axonal fusion. Our growing understanding of the molecular mechanisms driving the reconnection and fusion of transected axon fragments in invertebrates will enable investigation of similar processes in mammals. Indeed, the level of reconnection is strongly correlated with PS exposure, regenerative branching, slower rates of axonal degeneration (Abay et al., 2017a), and can be enhanced by boosting intrinsic regeneration (Ghosh-Roy et al., 2010; Abay et al., 2017a). Furthermore, overexpression of fusogens can facilitate axonal fusion independently from reconnection pathways (Neumann et al., 2015). These studies provide insights into the cellular mechanisms that can be manipulated for the purpose of inducing fusion-competence. In addition, the PEG fusion approaches have demonstrated that the significant increase in complexity associated with mammalian nervous systems is not necessarily a barrier for regeneration though axonal fusion-like mechanisms.

1.6.4. How can the same mechanisms drive the clearance and fusion of injured axons?

In addition to the externalization of PS as a recognition signal, apoptotic cells display various other signals to attract or repel engulfing cells. These include additional 'eat-me' signals, as well as 'find-me', and 'stay-away' signals that are all distinct from those present on viable cells, which also possess 'don't-eat me' signals (Hochreiter-Hufford and Ravichandran, 2013). These studies highlight the complexity of signalling under different contexts and raise the possibility that additional signals may be exposed following axonal injury. Thus, it is probable that in addition to the PS 'save-me' signals, axonal injury induces a variety of changes in the composition of the external membrane. It may therefore be specific combinations of signals that are important for recruiting the machinery required for the apparently opposing biological signals. Changes in the dynamics of how these signals are exposed may also influence the outcome. Indeed, PS exposure displays greatly different dynamics depending on whether it is exposed in apoptotic or certain non-apoptotic contexts (Rysavy et al., 2014).

Perhaps a simpler explanation involves the sheer size of the severed axon segment, which may preclude clearance by phagocytic cells. Many invertebrate species display extremely slow axonal degeneration following transection injuries, and this appears to be a common trait amongst the species in which axonal fusion has been observed. Axons in these invertebrate species have been shown to survive for many days after injury (Birse and Bittner, 1976; Nichols et al., 2016; Benbassat and Spira, 1993; Bittner and Brown, 1981; Murphy and Kater, 1980), with severed crayfish motor axons remaining intact for up to an incredible 250 days (Bittner and Johnson, 1974). This delay in intrinsic axonal degeneration mechanisms is highly favorable for regeneration via axonal fusion. At the same time, the large axon segments remaining for large stretches of time may simply be too large for engulfment by phagocytic cells. Phagocytosis is defined as the engulfment of particles larger than 0.5 μm (Flannagan et al., 2012), and human macrophage and microglia cells are typically 20–50 μm in diameter (Kongsui et al., 2014; Krombach et al., 1997). Given that severed axon segments in the smallest of the organisms known to possess axonal fusion repair (*C. elegans*) are typically 300–400 μm in length, engulfment of large, intact axon segments appears impossible.

2. Conclusions

In 1976, Birse and Bittner stated "it would now appear that this axonal repair mechanism is not an oddity restricted to a small number of invertebrate species but, rather, may be utilized by a wide variety of invertebrate organisms" (Birse and Bittner, 1976). The relative simplicity and efficiency of spontaneous axonal fusion in these species makes it a highly attractive mechanism for repairing nervous system injuries in higher species. Although four decades have passed since this statement, recent discoveries and progress are continuing to heighten the promise

of axonal fusion for neuronal repair in vertebrates, and have finally revealed its clinical potential.

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