Extrinsic Repair of Injured Dendrites as a Paradigm for Regeneration by Fusion in Caenorhabditis elegans

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ABSTRACT
Injury triggers regeneration of axons and dendrites. Research has identified factors required for axonal regeneration outside the CNS, but little is known about regeneration triggered by dendrotomy. Here, we study neuronal plasticity triggered by dendrotomy and determine the fate of complex PVD arbors following laser surgery of dendrites. We find that severed primary dendrites grow toward each other and reconnect via branch fusion. Simultaneously, terminal branches lose self-avoidance and grow toward each other, meeting and fusing at the tips via an AFF-1-mediated process. Ectopic branch growth is identified as a step in the regeneration process required for bypassing the lesion site. Failure of reconnection to the severed dendrites results in degeneration of the distal end of the neuron. We discover pruning of excess branches via EFF-1 that acts to recover the original wild-type arborization pattern in a late stage of the process. In contrast, AFF-1 activity during dendritic auto-fusion is derived from the lateral seam cells and not autonomously from the PVD neuron. We propose a model in which AFF-1-vesicles derived from the epidermal seam cells fuse neuronal dendrites. Thus, EFF-1 and AFF-1 fusion proteins emerge as new players in neuronal arborization and maintenance of arbor connectivity following injury in Caenorhabditis elegans. Our results demonstrate that there is a genetically determined multi-step pathway to repair broken dendrites in which EFF-1 and AFF-1 act on different steps of the pathway. EFF-1 is essential for dendritic pruning after injury and extrinsic AFF-1 mediates dendrite fusion to bypass injuries.

KEYWORDS
EFF-1; AFF-1; dendrite auto-fusion; degeneration following dendrotomy; regeneration of dendritic trees

SENSORY perception relies on networks of neurons that monitor and modify behavior to assure that animals are able to locate food, sense their environment, and avoid predators or other threats (Goodman 2003). This perception depends on the integrity and spatial coverage of the receptive field (Hall and Treinin 2011). Axonal and dendritic trees play an essential role in processing and transducing information to ultimately evoke the appropriate response of the organism. In the central nervous system (CNS) of adult mammals, axon regeneration following injury is limited (Ruschel et al. 2015). Therefore, the regenerative process following axon severing has been the focus of numerous studies (Taylor et al. 2005; Park et al. 2008; Ruschel et al. 2015). It is believed that the main reasons why axons fail to regenerate are a reduction in neuronal growth capacity and inhibitory extrinsic factors. However, the molecular mechanisms of regeneration are not well-understood. Recent studies have suggested that modulation of intrinsic neuronal activity by mammalian target of rapamycin (mTOR) and G-protein-coupled receptor (GPCR) signaling promote axon regeneration (Park et al. 2008; Li et al. 2016). In parallel, there is evidence for a molecular pathway for axonal degeneration that affects regeneration (Coleman and Freeman 2010). The molecular mechanisms required for regeneration by regrowth following axonal injury are actively studied and numerous pathways have been identified (Taylor et al. 2005; Wu et al. 2007; Park et al. 2008; Hammarlund et al. 2009; Yan et al. 2009; Edwards and Hammarlund 2014; Hammarlund and Jin 2014; Ruschel et al. 2015). In contrast to regeneration by regrowth, a different strategy for axonal regeneration that has been observed in diverse invertebrates is reconnection by fusion of severed axons (Hoy et al. 1967; Bedi and Glanzman 2001; Yanik et al. 2004; Ghosh-Roy and Chisholm 2010; Neumann et al. 2011, 2015).
The nematode Caenorhabditis elegans is a powerful model to study neuronal regeneration after injury (Chisholm et al. 2016; Giordano-Santini et al. 2016). It has been recently found that injured axons of motor and mechanosensory neurons regrow and, in some cases, fuse after in vivo severing using laser surgery (Yanik et al. 2004; Bourgeois and Ben-Yakar 2007; Ghosh-Roy and Chisholm 2010; Ghosh-Roy et al. 2010; Neumann et al. 2011; Giordano-Santini et al. 2016). Moreover, screens for genes with roles in axon regrowth have identified many genes required for axon regeneration (Gabel et al. 2008; Ghosh-Roy and Chisholm 2010; Nix et al. 2014; Chisholm et al. 2016).

Compared to axonal regeneration and degeneration pathways, much less is known about dendritic regeneration following injury (Standler and Bernstein 1982; Hall and Cohen 1988; Stone et al. 2010, 2014; Song et al. 2012). Recent studies have identified the PVD and FLP neurons as highly branched bilateral neurons in C. elegans, which display a stereotypic dendritic arborization pattern composed of repetitive structural units known as menorhas (Figure 1, A and B) (White et al. 1986; Yassin et al. 2001; Halevi et al. 2002; Tsalik et al. 2003; Oren-Suissa et al. 2010; Pujadas et al. 2010; Smith et al. 2010; Albeg et al. 2011; Maniar et al. 2012). The PVD is highly polarized, with a single axon ventral to the cell body and complex but stereotyped dendritic arbors (Oren-Suissa et al. 2010; Maniar et al. 2012), making it an ideal system to study different aspects of the generation, maintenance, regeneration, and degeneration of dendritic trees. The PVD neurons are two polymodal nociceptors, responsible for an avoidance response generated after harsh mechanical stimuli to the main body or exposure to cold temperatures (Way and Chalfie 1989; Goodman 2003; Chatzigeorgiou et al. 2010). Animals in which PVD neurons are laser-ablated fail to respond to harsh touch (Way and Chalfie 1989). Recent studies uncovered the degenerin ion channels DEG/ENaC, MEC-10, and DEGT-1 that sense harsh touch, and the TRPA-1 channels that respond to cold temperatures (Albeg et al. 2011; Chatzigeorgiou and Schafer 2011). Moreover, researchers have identified numerous genetic pathways involved in dendritic arborization and maintenance of the PVD structure (Oren-Suissa et al. 2010; Aguirre-Chen et al. 2011; Smith et al. 2012; Salzberg et al. 2013; Liang et al. 2015; Taylor et al. 2015; Dong et al. 2016).

The dynamic pathway of PVD arborization revealed a function of EFF-1 fusogenic protein in sculpting neuronal trees (Oren-Suissa et al. 2010). EFF-1 mediates epithelial and muscle cell-to-cell fusion (Mohler et al. 2002; Shemer et al. 2004; Gattegno et al. 2007; Shinn-Thomas and Mohler 2011; Podbilewicz 2014; Shinn-Thomas et al. 2016; Smurova and Podbilewicz 2016a,b), auto cell fusion in the digestive tract (Rasmussen et al. 2008), and axonal fusion following injury (Ghosh-Roy and Chisholm 2010; Ghosh-Roy et al. 2010; Neumann et al. 2011, 2015). EFF-1 cell-autonomous expression in the PVD is sufficient to reduce the number of branches and to rescue disorganized menorhas (Oren-Suissa et al. 2010). EFF-1 controls dendritic plasticity via retraction of excess branches, by fusing branches, and by forming loops that restrict further growth (Oren-Suissa et al. 2010). AFF-1, a paralog of EFF-1, mediates fusion of the anchor cell to form the utse/hymen, fuses the lateral epidermal seam cells, merges some embryonic epithelial cells (Sapir et al. 2007; Avinoam et al. 2011), and fuses cells to form the tail spike (Chiorazzi et al. 2013). AFF-1 also fuses glial cells (Procko et al. 2011), is induced by Notch to auto-fuse a myoepithelial toroid (Rasmussen et al. 2008), and fuses the excretory duct cell to form a single-cell tube (Stone et al. 2009; Soulavie and Sundaram 2016). Here, we determine a cellular pathway for dendritic remodeling following injury. We uncover the functions of two fusion proteins, EFF-1 and AFF-1, in different stages of the regeneration of dendritic arbors of the PVD polymodal neuron in C. elegans.

Materials and Methods

Strains and transgenic animals

All nematode strains were maintained according to standard protocols (Brenner 1974; Sulston and Hodgkin 1988). In addition to the wild-type strain N2, the following mutations, transgenes, and strains were used: BP601 aff-1(tm2214)/mln1[dpy-10(e128) mls14] II (Sapir et al. 2007), MF190 hmls4[DES-2::GFP, pRF4], BP328 eff-1(ok1021) II, hmls4, BP450 hyEx30[myo-2::gfp, DES-2::GFP, KS], BP431 eff-1[ly(21) II, hmls4 (Oren-Suissa et al. 2010), NC1841 (wdIs52, F49H12.4::gfp; rwIs1, pme-7::RFP) (Smith et al. 2010), and CHB392 [hmnEx133(ser-2prom3::kaede)], kindly provided by Yip and Heiman (2016). Germline transformation was performed using standard protocols (Mello and Fire 1995). The KS bluescript plasmid was used as carrier DNA. Transgenic lines include: BP709 [hmls133 (ser-2prom3::kaede)]; BP1014 aff-1/mln1; dzIs53[pF49H12.4::mCherry]1s was created by crossing aff-1/mln1 with FdH12.4::mCherry (kindly provided by Y. Salzberg); BP1015 aff-1/mln1; hmls133[ser-2prom3::kaede]; hyEx66 [KS, pCFJ90 (myo-2::mcherry), pME4(DES-2::AFF-1)]; BP1017 aff-1/mln1; hmls133[ser-2prom3::kaede]; hyEx350 [KS, pCFJ90 myo-2::mcherry, pTG5 dpy-7::aff-1]; BP1052 aff-1/mln1; hmls133[ser-2prom3::kaede]; hyEx355 [KS, pCFJ90 myo-2::mcherry, pTG4 grd-10::aff-1]; BP1055 dzIs53[F49H12.4p:mCherry]; hyEx66[pRF4, AFF-1osmid::GFP, KS]; and BP1056 dzIs53[F49H12.4p:mCherry]; hyEx68[pRF4, AFF-1 osmid::GFP, KS].

Molecular biology

We used restriction-free cloning to insert the grd-10 promoter upstream to the aff-1 gene (Bond and Naus 2012), and Gateway cloning (Petersen and Stowers 2011) to clone aff-1 into a plasmid containing the dpy-7 promoter fragment (pDest Dpy7 and pDONR221). Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Scientific, Waltham, MA) was used to facilitate the cloning process.

Confocal microscopy and live imaging of C. elegans

Nematodes were mounted on 3% agar pads mixed with 10 mM NaN3 in M9 buffer. For time-lapse analysis, worms
were anesthetized with 0.1% tricaine and 0.01% tetramisol in M9 solution (Kirby et al. 1990; McCarter et al. 1997, 1999). Animals were analyzed by Nomarski optics and fluorescence microscopy, using a Zeiss laser scanning microscope (LSM) 510 META confocal (Zeiss [Carl Zeiss], Thornwood, NY), the Zeiss LSM 700 confocal or Nikon eclipse Ti inverted microscope (Nikon, Carden City, NY) equipped with Yokogawa CSU-X1 spinning disk (Yokogawa, Tokyo, Japan) and a sCMOS (Andor, Belfast, UK) camera. Z-stacks were taken with PlanApochromat 60 × oil NA = 1.4 objective using the spinning disk confocal (SDC) or 63 × NA = 1.4 objective using the LSM. When using the sCMOS (Andor) camera z-stacks were taken with ~0.35 μm z-step. When the LSM 510 meta was used, z-step was ~0.8 μm. Image acquisition was done using Andor iQ or Metamorph software (Molecular Devices, Sunnyvale, CA) when using the SDC, and Zen software when using the LSM 510 meta microscope or Zeiss LSM 700. Multidimensional data were reconstructed as projections using the ImageJ and Metamorph softwares. Figures were prepared using ImageJ, Adobe Photoshop CS5, and Adobe Illustrator CS6.

Quantifying PVD branching phenotypes and statistics

We defined primary branch fusion as reconnection of the distal and proximal primary branches via fusion, following injury. We verified continuity by analyzing GFP-signal continuity using confocal microscopy and live imaging. In addition, we used a photoconvertible Kaede cytoplasmic reporter expressed in the PVD to validate fusion. We define menorah–menorah fusion as connections via fusion between high order branches in injured animals. Menorah–menorah fusion was never observed in noninjured animals.

Quantification was done as previously described (Oren-Suissa et al. 2010). Using confocal microscopy, at least five sequential z-series pictures were taken from each worm. Each z-section was analyzed separately. The results from each worm were normalized to a longitudinal length of 100 μm in all relevant experiments. Significant differences between

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**Figure 1** Dendrite regeneration of multibranched PVD neurons following laser microsurgery in C. elegans. (A) A wild-type animal expressing DES-2::GFP, illustrating the PVD neuron elaborate branching pattern (inverted image). Branches of one menorah are numbered primary to quaternary (1ry to 4ry) and color-coded: blue, purple, red, and green, respectively (Oren-Suissa et al. 2010); c, cell body. Bar, 20 μm. (B) Schematic model of hypodermal cells and PVD menorahs in a young adult, left view. The wild-type PVDs grow between the hypodermis (outer cylinder, light blue) and the basement membrane of the hypodermis (data not shown), extending processes that branch out to form the menorah structures. In light red is the left hypodermal seam syncytium. Modified from Oren-Suissa et al. (2010); se, seam cells; c, cell body. (C) Cartoon summarizing the different stages in PVD regeneration following injury. Two-photon dendrotomy (see Materials and Methods) of the primary process (red arrowhead) leads to dynamic changes in the PVD arbor; loss of branch self-avoidance and growth is followed by primary branch fusion (blue arrowhead), menorah–menorah fusion (magenta bracket), or both. There is an additional phase of dynamic growth (asterisk) and pruning (black arrow), leading to arbor refinement. When the branches fail to fuse, the distal end undergoes degeneration. FF?, fusion family unidentified fusogen.
were imaged and a primary dendrite was injured anterior to cell body. Animals also were observed for each experiment. For all worms, the between processes, and ectopic sprouting. At least 10 individualized neurites using a femtosecond laser (Yanik et al. 2012; Rao et al. 2016). To study the process of regeneration of the PVD dendrites following injury, we performed dendrotomy of arborized neurites using a femtosecond laser (Yanik et al. 2004; Bourgeois and Ben-Yakar 2007; Wu et al. 2007; Ghosh-Roy and Chisholm 2010). We found that the fate of the dendritic tree relies upon the ability of its branches to reconnect via fusion following injury. Failure to rejoin the two parts of the severed primary dendrite results in degeneration of the distal part and, in some cases, a complete degeneration without regrowth of the dendritic tree. Thus, we defined a successful regeneration event as a process in which the severed branch was able to reconnect with its target (Hilliard 2009). Temporal analysis of PVD dendrite dynamics following injury revealed several overlapping steps in arbor regeneration (Figure 1C).

Dendrotomy brings loss of self-avoidance

The dendritic architecture of the PVDs is maintained by a contact-dependent self-avoidance mechanism. The tertiary branches withdraw upon contact of a neighboring branch, maintaining the menorah architecture (Smith et al. 2010, 2012; Yip and Heiman 2016). To test whether self-avoidance is maintained after injury, we explored the spatial dynamics of regenerating dendrites. Two hours after injury, we observed tertiary branches from neighboring menorahs that contacted each other and extended far from their initial location, resulting in a structure of overlapping menorahs (Figure 2). Some of these overlaps extended and occurred between menorahs originating from both sides of the lesion (Figure 2B, brackets and Supplemental Material, File S1 and File S2). These overlapping structures persisted even 46 hr after the injury (Figure S1 in File S12).

Dendrotomy at earlier stages, such as the L3 stage, showed similar results; animals exhibited loss of avoidance mechanisms and branch overlap (data not shown). These results suggest that, upon injury, the avoidance mechanisms are lost, making it more likely that a new connection will form to compensate for the injury.

Following injury dendrites regenerate and reconnect via primary branch fusion

PVD dendrites showed robust regeneration when severed at the L4 stage. We found that severed primary dendrites grew

Results

Dissection of dendritic regeneration in C. elegans mechanosensory neurons

The regenerative ability of axons following injury has been previously described in vertebrates and invertebrates ( Cajal 1899; Hoy et al. 1967; Devor 1976; Bradbury et al. 2002; Fernandez-Gonzalez et al. 2002; Giordano-Santini et al. 2016). The morphological and molecular changes that occur following dendritic severing remain mostly unexplored (Standler and Bernstein 1982; Oren-Sussia and Podbilewicz 2010; Oren-Sussia et al. 2010; Stone et al. 2010, 2014; Nawabi et al. 2012; Song et al. 2012; Rao et al. 2016). To study the process of regeneration of the PVD dendrites following injury, we performed dendrotomy of arborized neurites using a femtosecond laser (Yanik et al. 2004; Bourgeois and Ben-Yakar 2007; Wu et al. 2007; Ghosh-Roy and Chisholm 2010). We found that the fate of the dendritic tree relies upon the ability of its branches to reconnect via fusion following injury. Failure to rejoin the two parts of the severed primary dendrite results in degeneration of the distal part and, in some cases, a complete degeneration without regrowth of the dendritic tree. Thus, we defined a successful regeneration event as a process in which the severed branch was able to reconnect with its target (Hilliard 2009). Temporal analysis of PVD dendrite dynamics following injury revealed several overlapping steps in arbor regeneration (Figure 1C).

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Data availability

Strains are available upon request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.
toward each other (File S1 and File S2), and in 40% of the animals we observed reconnection of the distal end to the soma via fusion of the primary branches (Figure 3, B and C, “primary branch fusion,” blue arrowhead). To directly measure reconnection by fusion, we used the photoconvertible reporter Kaede expressed in the PVD (Yip and Heiman 2016). The primary branch of ser-2prom3::Kaede-expressing animals was dendrotomized, and the animals were recovered for 23 hr before dendrite reconnection was assessed by Kaede photoconversion (Figure 3, D–H). Red Kaede was observed spreading from the cell body beyond the reconnected site of injury to the distal part of the primary and higher ordered dendritic branches. Thus, Kaede photoconversion and diffusion beyond the injury site demonstrate fusion between the severed primary dendrites. In animals where reconnection failed, photoconverted Kaede did not spread beyond the injury site (data not shown).

It was shown that, following axotomy of the PLM mechanosensory neuron, reconnection of the axon to the distal branch is dependent upon EFF-1, but not AFF-1 activity (Ghosh-Roy et al. 2010; Neumann et al. 2011, 2015). In contrast, we found that primary dendrotomies in eff-1-null mutants were repaired and regeneration via primary branch fusion occurred (Figure 3C). Thus, following primary branch microsurgery, the two ends grow toward each other, the tips meet, connect, and the integrity of the distal arbors is maintained.

**Menorah–menorah fusion bypasses the severed primary dendrites**

We have previously shown that during wild-type development, PVD and FLP terminal quaternary branches can auto-fuse with one another to maintain menorah structure and to limit further growth (Oren-Suissa et al. 2010). To determine whether fusion of terminal dendrites is part of the regeneration process, we analyzed the overlapping branches after injury and found that most of the reconnections bypassed the injury site through menorah–menorah fusion, resulting in giant menorahs (Figure 4, magenta brackets). To judge the connectivity of the tertiary branches, we verified that the distal processes do not degenerate, and analyzed GFP-signal continuity using confocal microscopy and live imaging (Figure 4, File S1, File S2, and File S4). In addition, we used a photoconvertible Kaede cytoplasmic reporter expressed in the PVD (Yip and Heiman 2016) to demonstrate that the menorahs have fused to bypass the lesion site (Figure 4, E–G). We found that, in animals where menorah–menorah fusion took place, the distal fragment did not degenerate, regardless of primary–primary branch reconnection (Figure 4H; n = 20). Thus, terminal branch auto-fusion acts as a mechanism to bridge the gap between the PVD soma and the distal end to maintain connectivity and avoid degeneration.

**Ectopic branching, pruning, and arbor simplification complete regeneration**

In all animals assayed, failure to reconnect via fusion, following dendrotomy, resulted in degeneration of the distal end and took place within 12 hr in ~20% of the operated animals (Figure 5A, arrow, and File S3). Thus, the PVD fusion is essential for the survival of the elaborate tree structure following injury. There are several possible fusion outcomes following injury. The two severed primary branches could reconnect directly to one another via primary branch fusion. Alternatively, terminal branches from menorahs on both sides of the injury can reconnect via fusion (“menorah–menorah” fusion bypasses the severed primary dendrites).
**Figure 3** Primary branch fusion occurs following PVD dendrite injury. (A and B) Primary branch fusion following dendrotomy. L4 animal just after injury (red arrowhead in A) and 16-hr postsurgery (B). In (B), the severed distal and proximal ends of the primary branch reconnected (blue arrowhead). Bar, 10 μm. (C) Percentage of primary branch fusion during regeneration in wild-type \((n = 14)\) and \(\text{eff-1}(ok1021)\) \((n = 13)\) dendrotomized animals. Differences are not statistically significant (Fischer’s exact test). (D-H) PVD dendrite reconnection confirmed by Kaede photoconversion. A PVD primary dendrite of \(\text{ser-2prom3-Kaede}\)-expressing animals was dendrotomized, the animal was recovered for 23 hr, and the dendrite reconnection to its stump was assessed by Kaede photoconversion. The green Kaede form in the PVD cell body was irreversibly photoconverted to the red Kaede form using a 405 nm laser with the Mosaic system, and its spreading throughout the dendritic branches was followed 1 and 60 min post-photoconversion. Panels left to right are confocal reconstructions of a wild-type dendrotomized animal in the 488 green channel, 561 red channel, two channels merged view, and a schematic representation of the merged view. (D) Confocal reconstructions of the animal before dendrotomy, (E) immediately post dendrotomy, (F) 23-hr post-dendrotomy, (G) 1-min post-Kaede photoconversion, and (H) 60-min post-Kaede photoconversion. Red Kaede form (cyan in merged and schematic representations), though diluted when spreading through the dendritic tree, can be observed beyond the reconnected site of injury in the distal part of the primary and higher ordered dendritic branches. Red arrowhead, site of injury; blue arrowhead, site of primary branch fusion. In the merged and schematic columns: magenta, green Kaede and cyan, photoconverted red Kaede. *c*, PVD cell body; NS, not significant.
We also found that a third scenario existed, where primary branch fusion and menorah–menorah fusion both occurred. Analysis of these outcomes reveals that menorah fusion is the main mechanism used by PVD dendrites to reconnect following injury and remodeling (Figure 5B).

We analyzed the tree architecture following fusion and observed that the PVD dendrites appeared highly dynamic after injury, showing ectopic growth of terminal branches. These results suggested that growth is not restricted to a subset of branches and can occur throughout the neuron, allowing for massive regeneration (Figure 1C and Figure 2B, asterisks). To verify that growth is stimulated specifically due to the dendrite injury and not because of laser damage, we examined mock-injured animals for PVD morphology changes. We injured animals near the PVDs, at the same focal plane, but without hitting any dendrites. PVD mock-operated animals showed normal growth with no excess sprouting or changes in the PVD morphology (data not shown). These results demonstrate that dendrite severing specifically induced terminal branch reconnection by fusion, ectopic branching, and regeneration (Figure 1C and File S1 and File S2).
Interestingly, some of the distal secondary stems were eliminated following the reconnection, leaving just one or two secondary stems per giant menorah (Figure 4C and Figure 5C; black arrows). These dendritic rearrangements persisted even 48 hr after surgery, leading to simplification of the dendritic trees and leaving mainly giant menorahs. Thus, active elimination of excess branches occurs to recreate a pattern resembling wild-type PVD architecture. Analysis of time-lapse movies showed that excess branches were eliminated, and pruning occurred concomitantly with growth around the injury site (Figure 4D and File S4). Taken together, the PVD dendrites are able to successfully regenerate following dendrotomy, inducing dynamic remodeling by branch growth and elimination (Figure 1C).

**EFF-1 is essential for pruning excess branches after dendrotomy**

The central function of **EFF-1** in PVD developmental arborization is in quality control trimming of excess and abnormal branches (Oren-Suissa et al. 2010). To determine whether **EFF-1** acts in simplification following injury, we amputated primary dendrites in **eff-1** mutants and followed the repair process. We found that **eff-1** mutants maintained hyperbranched and disorganized menorahs and failed to simplify the dendritic tree following injury (Figure 6A). These phenotypes suggest that **eff-1** acts in branch retraction and simplification induced by severing of the primary branch. We were not able to determine whether **eff-1** participates in menorah–menorah fusion because the hyperbranched and severely disorganized arbors prevented us from identifying menorah fusion and additional ectopic sprouting (Figure 6, B and C). Since the injured **eff-1**(ok1021) mutant animals analyzed degenerated to the same extent as wild-type (Figure 6E), we conclude that **eff-1** is neither required for dendrite reconnection nor for degeneration. We propose that in injured **eff-1** mutants where primary branch fusion was not observed (Figure 3C), it is possible that reconnection occurred via menorah–menorah fusion. In contrast, both uncut and dendrotomized **eff-1** mutants showed no pruning, demonstrating that **eff-1** is required for branch simplification following dendrotomy (Figure 6D). In addition, cell-autonomous expression of **EFF-1** in the PVD resulted in excess pruning (Oren-Suissa et al. 2010; Kravtsov et al. 2016). Thus, **EFF-1** may act cell-autonomously to simplify excess sprouting following dendrotomy and is sufficient to trim branches and simplify arbors.
**AFF-1 is required to bypass cut dendrites via menorah–menorah fusion**

Since the *C. elegans* known fusogens, EFF-1 and AFF-1, are essential and sufficient to fuse cells in *C. elegans* and heterologous cells in culture (Mohler et al. 2002; Shemer et al. 2004; Podbilewicz et al. 2006; Sapir et al. 2007; Avinoam et al. 2011), we hypothesized that they may be required to regenerate broken neurites by homotypic fusion. Because EFF-1 prunes dendrites by branch retraction (Oren-Suissa et al. 2010; Kravtsov et al. 2016), we decided to determine a possible role for aff-1 following dendrotomy by asking whether menorah–menorah fusion occurs in aff-1 injured-mutants. We found that while dendrite development was normal in aff-1 mutants (Figure 7A), most of the reconnections were between the regrowing primary dendrite and its distal fragment following dendrotomy, rather than through menorah fusion as in wild-type animals (Figure 7E). This observation suggests a fusogenic function for AFF-1 in terminal branch fusion. In aff-1 mutant animals, we found some exceptions in which some menorahs overlapped, but we did not observe fusion between menorahs followed by secondary stem degeneration (Figure 5C). In dendrotomized aff-1 animals, failure to rejoin the dendritic trees resulted in degeneration of the distal part of the arbor (Figure 7, A and D). Thus, while aff-1 has no apparent role in normal PVD arborization, it is required for terminal branch fusion following dendrotomy. Due to the subviability of eff-1 aff-1 double mutants (Sapir et al. 2007), we were unable to test whether there is redundancy between these genes in primary branch fusion. It is conceivable that there is redundancy in the fusion machinery that fixes broken neurites or that an unidentified fusogen is required for postembryonic primary dendrite auto-fusion after microsurgery.

AFF-1-mediated membrane fusion of terminal branches emerges as the main mechanism by which dendritic repair occurs in *C. elegans*. Using an aff-1promoter::GFP fusion and a fosmid-based AFF-1::GFP translational fusion, we did not detect aff-1 expression in the PVD either before or after dendrotomy. Furthermore, PVD-expressing des-2p::AFF-1 was not able to rescue the fusion failure phenotype (Figure 7, D and E and Table 1). To determine whether AFF-1 acts extrinsically to the PVD to reconnect dendrites, we attempted to rescue fusion in aff-1 mutants using expression of grd-10p::AFF-1 in the epithelial seam cells. This reduced degeneration to wild-type levels (Figure 7, B–D) and increased primary branch fusion (Figure 7E), but had little or no effect on menorah–menorah fusion (Figure 7E). These results suggest that AFF-1 functions cell nonautonomously for dendrite...
repair, but AFF-1 rescues primary dendrite fusion rather than menorah–menorah fusion. A possible explanation for this result is that the source of AFF-1 in the seam cells is only <1000 nm away from the primary dendrite, and this proximity may facilitate primary dendrite reconnection. Moreover, if EFF-1 is required in the PVD to interact heterotypically with AFF-1, there may be a higher concentration of EFF-1 at the injury site. Last, the machinery of engulfment has been reported to act together with EFF-1 in the repair of injured PLM mechanosensory axons (Neumann et al. 2011, 2015). In summary, it is conceivable that reconnection of dendrites functions more efficiently at the injury site.

Expression of dpy-7p::aff-1 from the hypodermis was toxic in aff-1 mutant animals, suggesting that only nonautonomous expression from epithelial seam cells is sufficient to improve the ability of the PVD to rejoin the branches.

Table 1 PVD postinjury outcomes

| Genotype                                  | Menorah–Menorah Fusion, % | Primary Branch Fusion, % | Menorah–Menorah and Primary Fusion, % | Degeneration, % | n  |
|-------------------------------------------|---------------------------|--------------------------|--------------------------------------|----------------|----|
| Wild-type                                 | 50                        | 18.2                     | 13.6                                 | 18.2           | 22 |
| aff-1(tm2214)                             | 15.6, *P = 0.01           | 34.4                     | 3.1                                  | 46.9, *P = 0.04 | 32 |
| aff-1(tm2214); des-2p::aff-1              | 20                        | 26.7                     | 13.3                                 | 40             | 15 |
| aff-1(tm2214); grd-10p::aff-1             | 13.6, *P = 0.02           | 50, *P = 0.05            | 18.2                                 | 18.2, *P = 0.04 | 22 |

*Statistics calculated using Fischer’s exact test.*
by fusion (Figure S2 in File S12). The cell nonautonomous activity of AFF-1 in PVD regeneration by auto-fusion was unexpected since for cell–cell fusion, AFF-1 is required in both fusing plasma membranes (Avinoam et al. 2011).

**AFF-1-containing extracellular vesicles (EVs) may repair the PVD by fusing with it**

To determine AFF-1 expression and localization before and after dendrotomy, we imaged AFF-1 in worms expressing mCherry in the PVD, using a 30-kb fosmid-based GFP reporter (Sarov et al. 2006). We could not detect AFF-1 in the PVD at any stage during development or following dendrotomy. Instead, AFF-1 is strongly expressed on the plasma membrane, filopodia, and internal puncta in the epidermal lateral seam cells. This was expected since the seam cells fuse homotypically between the L4 and adult molt via AFF-1-mediated fusion (Sapir et al. 2007). Using structured illumination microscopy, we found extracellular puncta containing AFF-1::GFP that were apparently derived from the seam cells (Figure 8A, arrowheads). Using live SDC microscopy, we found that the vesicles containing AFF-1::GFP were observed outside the seam cells in control animals that were not dendrotomized (Figure 8B and File S5, File S6, and File S9). Following dendrotomy, the AFF-1::GFP signal in the seam cells was brighter (Figure 8C) and there was a fivefold increase in the mobility and number of EVs (Figure 8, D–F, File S7, File S8, File S10, and File S11). Thus, taken together, our results show that AFF-1 regenerates severed PVD dendrites in a surprisingly cell nonautonomous way from the seam cells.
Figure 9 Model of AFF-1-mediated repair via extracellular vesicle-cell fusion. PVD (red) is in close proximity to the epithelial seam cells (blue). AFF-1 (black pins) is expressed in seam cells and additional tissues, but not in the PVD. Upon injury, AFF-1-containing extracellular vesicles (EVs) are highly released from the seam cells. Some of these EVs reach the PVD and promote fusion of severed dendrites. EFF-1 (green pins) is expressed in the PVD but it does not act to fuse severed dendrites on its own. Instead, it may collaborate with AFF-1-EVs. We propose that menorah–menorah fusion is mediated by AFF-1-EVs that merge with the structurally compatible EFF-1 expressed in the PVD. EFF-1-coated pseudotyped viruses can fuse with cells expressing AFF-1 on their surface and vice versa (Avinoam et al. 2011).

Discussion

Hypothesis: AFF-1-EVs merge injured neurons from without

Cell–cell fusion from within occurs when a fusogenic protein (e.g., a viral fusion protein following infection) or an endogenous cellular fusion protein (e.g., EFF-1) is expressed intrinsically in cellular compartments, including the plasma membrane (Podbilewicz and Chernomordik 2005; Oren-Suissa and Podbilewicz 2010). Fusion from without occurs when a viral particle fuses target cells without infecting them (Bratt and Gallaher 1969; White et al. 1981; Clavel and Charneau 1994; Duelli and Lazebnik 2007). Here, we discovered an example of fusion from without during neuronal regeneration. We propose that epidermal seam cells shed EVs that travel 1000 nm or less to reach the PVD severed dendrites and the menorahs. These vesicles contain AFF-1 and can fuse dendrites from without (Figure 9).

AFF-1-containing vesicles derived from the lateral epidermal seam cells mediate fusion of severed dendrites and menorah auto-fusion to bypass the injury and to maintain dendritic tree structure and function. EVs (microvesicles, exosomes, and ectosomes) from different subcellular and tissue origins have been proposed as vehicles for cell–cell communication during normal physiology, participate in the immune response, control coagulation, and promote metastatic cancer (Tkach and Thery 2016). These EVs have been shown to exist in bacteria, archea, protists, plants, fungi, and animals (Beveridge 1999; Liegeois et al. 2006; Miyado et al. 2008; Kwon et al. 2014; Hyenne et al. 2015). In C. elegans, EVs derived from ciliated neurons affect mating behavior and communication between animals (Wang et al. 2014). Signaling EVs derived from the sperm activate oogenesis and ovarian muscle contraction (Kosinski et al. 2005). EVs also participate in engulfment of dead cells (Mapes et al. 2012) and morphogenesis of the embryo (Wehman et al. 2011).

EVs are probably universal but diverge in size, shape, and place of origin. They contain lipid bilayers, transmembrane proteins, and nucleic acids. One of the characteristics of these EVs is their ability to fuse to target cells and deliver RNAs, plasmids, toxins, and signaling molecules. However, the fusion proteins necessary to deliver and merge the diverse EVs have not been identified and characterized in any system. Mammalian cells transfected with C. elegans AFF-1 produce EVs that have been biochemically and ultrastructurally characterized (Avinoam et al. 2011; Fridman 2012). Moreover, AFF-1-containing vesicles and pseudotyped particles are able to fuse to mammalian cells expressing EFF-1 or AFF-1. Thus, AFF-1 can mediate fusion of EVs to cells expressing EFF-1 on the plasma membrane in a tissue culture system (Avinoam et al. 2011; Fridman 2012). Recently, pseudotyped particles and vesicles containing a sperm protein from plants that is structurally similar to EFF-1 were shown to mediate fusion to cells expressing EFF-1 (Valansi et al. 2017). Here, we provide the initial evidence for a proposed mechanism that can fuse EVs to target neuronal cells in vivo. Surprisingly, these EVs can cause auto-fusion from without mediated by AFF-1 transmembrane fusion protein on their surface. Moreover, in our working model, these AFF-1-EVs derived from the C. elegans lateral epithelia can fuse neurons in vivo, thus directly promoting regeneration (Figure 9).

Neurodevelopmental genetic stages in dendrite repair after injury

The mechanism of PVD dendritic regeneration can be divided into five stages: (1) reattachment at site of injury, (2) loss of self-avoidance between adjacent menorahs, (3) menorah–menorah fusion to bypass lesions, (4) sprouting of compensatory branches, and (5) pruning of excess branches (Figure 1C).

The interplay between two effector fusogens revealed a genetic pathway that links membrane remodeling during development and following neuronal injury. Here, we focused on two cellular stages: extrinsic stage (3) AFF-1-mediated menorah–menorah fusion from without and intrinsic stage (5) EFF-1-mediated trimming of excess branches from within (Figure 1C and Figure 9).

AFF-1 merges terminal branches to bypass broken dendrites

Axonal fusion after injury is crucial for reestablishing synaptic contacts, to prevent degeneration, and for regaining neurological functionality (Hilliard 2009; Giordano-Santini et al. 2016). Although eff-1 mutants failed to fuse broken axons (Ghosh-Roy et al. 2010), eff-1 mutants succeed to merge injured dendrites. We tested the two known C. elegans fusogens, EFF-1 and AFF-1, as well as the EFF-1 paralog C26D10.7 (Mohler et al. 2002; Avinoam and Podbilewicz 2011) (M. Oren-Suissa and B. Podbilewicz, unpublished results). We found that terminal branch fusion following dendrotomy was significantly reduced in aff-1 mutants compared to wild-type. However, none of these genes was independently required for primary dendrite fusion.
following an injury, suggesting either redundancy or that yet another C. elegans fusogen awaits identification. Based on these observations, we conclude that eff-1 is required to heal dendritic wounds, specifically via menorah–menorah fusion.

Our data support a model in which eff-1 and eff-1 expression is highly regulated in the PVD. In this working model, dendrotomy may initially repress EFF-1 surface expression allowing ectopic sprouting of terminal branches and loss of self-avoidance, culminating with AFF-1-mediated menorah–menorah fusion via a surprising cell nonautonomous mechanism of fusion from without (Figure 9).

Is auto-fusion an alternative pathway to repair severed neurons?

Fusion of severed axons occurs in invertebrates, for example in Aplysia (Bedi and Glanzman 2001), crayfish (Hoy et al. 1967), and C. elegans (Yanik et al. 2004; Ghosh-Roy and Chisholm 2010; Neumann et al. 2011, 2015), but rarely in vertebrates (Palsyn et al. 2013; Li et al. 2016). Why is this the case and how can our study help us understand this? Invertebrates and vertebrates do have conserved pathways to regenerate injured branches via regrowth (Park et al. 2008; Yan et al. 2009; Ghosh-Roy and Chisholm 2010; Brakde et al. 2012; Yaniv et al. 2012; Mar et al. 2014; Stone et al. 2014; Ruschel et al. 2015; Rao et al. 2016; Tao et al. 2016). However, it appears that fusion of broken neurites or bypassing the injured site using fusion instead of rebuilding complex trees is a more energetically economical process. The use of EVs could be a useful strategy to stimulate repair of injured branches in the CNS of vertebrates that usually cannot regenerate.

Severed neurites can reconnect by suspending self-repulsion mechanisms

We have found that in C. elegans PVD, following dendrotomy, there is a transient loss of self-avoidance between tertiary branches that allows the reconnection by merging the menorahs and bypassing the site of injury, thus maintaining the dendritic trees. This is consistent with studies in leech embryos showing that laser microbeam severing of neurites of mechanosensory neurons result in that the detached branch stop being avoided by the rest of the cell. This is consistent with a mechanism that controls self-avoidance and that requires physical continuity between the neurites (Wang and Macagno 1998). In C. elegans, this mechanism appears to involve netrins (Smith et al. 2012). In contrast, in zebrafish, detached fragments continue to repel the parent arbor (Martin et al. 2010). Thus, in zebrafish and probably in other vertebrates, it is required to have a WD-like mechanism to remove fragments of sensory neurites before the process of regrowth can occur. It would be useful to find ways to induce merging of the severed neurites as occurs in some invertebrates.

Spinal cord injuries, experimental axotomies, surgical accidents, stroke, and diverse forms of neurodegeneration are all conditions that currently cannot be generally repaired (Devor 1976; Fernandez-Gonzalez et al. 2002; Moritz et al. 2008; Ruschel et al. 2015; Li et al. 2016). Unveiling the mechanism of intrinsic eff-1-mediated dendritic simplification and extrinsic eff-1-mediated neuronal auto-fusion from without may pave the way for overcoming neurodegenerative diseases or brain injuries. In C. elegans, AFF-1-containing vesicles derived from epithelia appear to fuse dendrites, emerging as a potential effector that could repair broken neurons in heterologous systems.

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