Let-7 miRNA controls CED-7 homotypic adhesion and EFF-1-mediated axonal self-fusion to restore touch sensation following injury

Atrayee Basu, Shrishendu Dey, Dharmendra Puri, Nilanjana Das Saha, Vidur Sabharwal, Pankajam Thyagarajan, Prerna Srivastava, Sandhya Padmanabhan Koushika, and Anindya Ghosh-Roy

National Brain Research Centre, Manesar, Nainwal Mode, Gurgaon, Haryana 122051, India; Bruker India Scientific Private Ltd, New Delhi 110019, India; Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai 400 005, India; and Wellcome Trust–Department of Biotechnology India Alliance, Banjara Hills, Hyderabad, Telangana 500034, India

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Neuronal injury often leads to devastating consequences such as loss of senses or locomotion. Restoration of function after injury relies on whether the injured axons can find their target cells. Although fusion between injured proximal axon and distal fragment has been observed in many organisms, its functional significance is not clear. Here, using Caenorhabditis elegans mechanosensory neurons, we address this question. Using two femtosecond lasers simultaneously, we could scan and sever posterior lateral microtubule neurons (posterior lateral microtubules [PLMs]) on both sides of the worm. We showed that axotomy of both PLMs leads to a dramatic loss of posterior touch sensation. During the regenerative phase, only axons that fuse to their distal counterparts contribute to functional recovery. Loss of let-7 miRNA promotes functional restoration in both larval and adult stages. In the L4 stage, loss of let-7 increases fusion events by increasing the mRNA level of one of the cell-recognition molecules, CED-7. The ability to establish cytoplasmic continuity between the proximal and distal ends declines with age. Loss of let-7 overcomes this barrier by promoting axonal transport and engagement of the EFF-1 fusogen at the growing tip of cut processes. Our data reveal the functional property of a regenerating neuron.

femtosecond laser | axotomy | gentle touch | fusion | let-7

Accidental damage of neuronal processes often causes functional losses that affect locomotion, sensation, and many other higher brain functions (1, 2). Functional recovery in the adult nervous system is limited, since the injured nerves fail to find their postsynaptic partners while regrowing (1). After axotomy, the distal axon undergoes a degenerative process named “Wallerian degeneration” (3). In many organisms, such as Aplysia, crayfish, leeches, and worms, injured processes can reconnect with their severed distal processes (4–9). This phenomenon, “axonal fusion” (4–9), prevents the degeneration of the severed distal axon. Evidence suggests that axonal fusion suppresses the electrophysiological changes caused by injury (8). Based on these observations, it is logical to postulate that axonal fusion would lead to functional repair. However, quantitative behavioral evidence toward this is lacking.

Caenorhabditis elegans is a useful model for studying axon regeneration after injury (10). Genetic screens have identified a large number of pathways specifically required for adult axon regeneration (11, 12). The mechanosensory neurons responsible for gentle touch sensation are often used for studying axonal response to laser injury (10, 13). After laser axotomy of a posterior touch neuron (posterior lateral microtubule, PLM), a considerable number of axons become fused to their distal counterparts (9, 14). This phenomenon is dependent on the fusogen molecule EFF-1 and cell-recognition pathways (9, 15). Although, functional regeneration in a D-type GABAergic motor neuron was reported earlier (16), no study to date has addressed the functional aspect of regeneration in touch neurons. One constraint in studying the behavioral consequences of axotomy is that the severing of multiple neurons is often required to observe the effect of injury on behavior. Touch neurons are located laterally on both the sides of the worm body (17). Using conventional illumination methods, it is challenging to simultaneously visualize and cut the PLM axon that is located on the bottom side (16, 18).

We overcame this limitation by using the 3D sectioning capability of a two-photon microscope (19). By employing two independent laser beams, one femtosecond laser to localize axons and the other to sever them, we could systematically analyze the effect on gentle touch sensation when one PLM is axotomized versus when two PLMs are axotomized. Cutting both PLMs strongly reduces the posterior touch response. During the regenerative growth phase, we find that only the axons that successfully fuse with their distal counterparts contribute to functional recovery. Axonal fusion is also correlated with the restoration of axonal transport at the distal fragment. We further showed that let-7 miRNA inhibits the establishment of cytoplasmic continuity in a cell-autonomous manner during late larval and adult stages. Loss of let-7 decreases the level of ced-7 mRNA, and the probability of a fusion event is increased in late larval stage. As the worm ages, the axonal transport is perturbed, and a key fusogen molecule, EFF-1, becomes limiting at the growing tips of cut axon. Loss of let-7 overcomes these barriers by delaying neuronal aging and stimulating functional recovery.

Significance

In many organisms axonal fragments can rejoin by self-fusion after neuronal injury. It is hypothesized that cell fusion would be an efficient way to repair functional loss after injury. In this study, we tested this hypothesis using the Caenorhabditis elegans sensory neurons that are responsible for gentle touch sensation. We found that fusion between the proximal and distal fragments of an injured posterior touch neuron (the posterior lateral microtubule) promotes functional recovery in an age-dependent manner. We also discovered that let-7 miRNA inhibits functional restoration via EFF-1-mediated axonal self-fusion by reducing ced-7 expression. Our work established that the axon fusion process has functional significance in the maintenance of neuronal integrity throughout the life span of an organism.

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To whom correspondence should be addressed. Email: anindya@nbrc.ac.in.

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Results

Axotomy of PLM Neurons on both Sides Perturbs Posterior Touch Sensation. Gentle touch sensation on either the anterior or posterior end of a worm involves three neurons, two of which are located at the cuticle (Fig. 1A) (17). Using imaging techniques such as widefield or confocal microscopy and a single femtosecond laser, researchers to date have axotomized only one PLM neuron located on either the left or the right side (18, 20–23). Due to this limitation, researchers also had noted the side of axotomy and subsequently track the worms during further analysis (9, 11, 20). The neuron on the other side can be illuminated using two-photon microscopy (19). Here, we simultaneously used the two femtosecond lasers of a two-photon microscope, one at 920 nm for imaging GFP and the other at 720 nm for cutting, controlled independently by two galvanometer-based laser scanners (Fig. L4 and Fig. S1A), and cut PLM neurons on both sides (Fig. 1B, Fig. S1B, and Movie S1).

A worm that is moving forward responds to a gentle touch in the anterior side by reversing, and a worm that is moving backwards likewise responds to a posterior touch by reversing (Movie S2). Using this assay, one can measure touch sensation with a posterior touch response index (PTRI) (24). Since the worms were subjected to axotomy under anesthetized conditions (9, 11, 20), the earliest behavioral test was conducted 10 h after axotomy. Cutting the PLM neuron on one side 50 μm away from the cell body (Fig. 1A and B) reduces the PTRI at the experimental side significantly, from 0.95 ± 0.01 (mean ± SEM) to 0.62 ± 0.009 (***P < 0.0001; Newman–Keuls multiple comparisons test) (Fig. 1C). A mild but significant reduction was also noticed in the control side compared with laser-treated control worms, which were subjected to axotomy or ablation at the L4 stage. The position of the axotomy or ablation is indicated by an orange arrow. White arrows indicate the distal ends disconnected from the cell body.Insets show an enlarged view of the synaptic branch, with white arrowheads pointing to the branches (magnification: 2×). In case of ventral branch cutting plus axotomy, the yellow arrowhead points to the absence of the branch. When the PLM was ablated in the L2 stage, the distal axon became degenerated (magenta dotted arrow) at the young-adult stage. (C) The bar chart shows the PTRIs and ATRIs measured in worms before and 10 h after axotomy of the PLM at 50 μm from the cell body. Cutting both PLMs has a stronger effect on the PTRI than cutting one PLM, whereas the ATRI remains unaffected. N = 2–6 independent replicates. (D) PTRI after axotomy on one side at the L4 stage in worms in which the PLM on the other side and the PVM were ablated at the L2 stage. N = 2. (E) PTRI after axotomy and branch cutting in various combinations. Branch cutting along with axotomy further reduces the PTRI. N = 2 or 3. For C–E, ***P < 0.0001; ANOVA with Newman–Keuls multiple comparisons test. The numbers in each bar indicate the number of sides tested. Error bars represent SEM. ns, not significant.

Fig. 1. Effect of axotomy of PLMs on gentle touch sensation (A) Schematic of a worm with six touch neurons highlighted in green. The laser paths for imaging (LC 1, green) and severing (LC 2, red) the bottom PLM neuron are shown. (B) Confocal images of a PLM neuron labeled with Pmec-4-GFP (muIs32) after axotomy or ablation at the L4 stage. The position of the axotomy or ablation is indicated by an orange arrow. White arrows indicate the distal ends disconnected from the cell body. Insets show an enlarged view of the synaptic branch, with white arrowheads pointing to the branches (magnification: 2×). In case of ventral branch cutting plus axotomy, the yellow arrowhead points to the absence of the branch. When the PLM was ablated in the L2 stage, the distal axon became degenerated (magenta dotted arrows) at the young-adult stage. (C) The bar chart shows the PTRIs and ATRIs measured in worms before and 10 h after axotomy of the PLM at 50 μm from the cell body. Cutting both PLMs has a stronger effect on the PTRI than cutting one PLM, whereas the ATRI remains unaffected. N = 2–6 independent replicates. (D) PTRI after axotomy on one side at the L4 stage in worms in which the PLM on the other side and the PVM were ablated at the L2 stage. N = 2. (E) PTRI after axotomy and branch cutting in various combinations. Branch cutting along with axotomy further reduces the PTRI. N = 2 or 3. For C–E, ***P < 0.0001; ANOVA with Newman–Keuls multiple comparisons test. The numbers in each bar indicate the number of sides tested. Error bars represent SEM. ns, not significant.
30% of the response (Fig. 1E). Then we found that cutting the branch (yellow arrowhead in Fig. 1B) along with axotomy reduced the PTRI to 0.21 ± 0.01, which is comparable to the PTRI in animals lacking the mechanosensory channel MEC-4 (Fig. 1E). When we ablated PLM cell bodies at the L2 stage, we found that in the young-adult stage the distal axon is completely degenerated (broken purple arrows in Fig. 1B), and the PTRI value dropped to 0.18 ± 0.02 (Fig. 1E). Hence, these experiments suggested that, after axotomy, the distal axon partly contributes to the residual response. The rest of the response is contributed by the gap junction near the cell body (24). Nevertheless, these results show that the axotomy-induced drop in PTRI is due to the failure in communication mediated by the PLM gap junction and synapse.

**Axonal Fusion Between the Proximal and Distal Ends Drives Functional Recovery.** The PTRI value following axotomy serves as a baseline to investigate functional recovery over time. We found that the PTRI at 24 h is 0.65 ± 0.01 (mean ± SEM), significantly higher than the PTRI at 10 h postaxotomy (0.52 ± 0.008) (***P < 0.0001; Newman–Keuls multiple comparisons test) (Fig. 2B). We found that about 42% of the regrowing axons reconnect with their corresponding distal fragments by self-fusion of proximal and distal fragments (Fig. 2A, b’ and c’), as reported previously (9, 14, 15). The rest of the axons regrow toward the distal ends but fail to connect to their respective distal fragments (Fig. 2A, a’). We asked whether there would be a difference in PTRI between these two classes, fusion and non-fusion. We separated the fusion class into two subclasses, one that joins end-to-end, called “type 1” (T1), and the other that joins end-to-side, called “type 2” (T2) (Fig. 2A, b’ and c’). The PTRIs measured from the sides showing T1 and T2 fusion were 0.74 ± 0.012 and 0.73 ± 0.016, respectively (Fig. 2C). These values were significantly higher (***P < 0.0001; Newman–Keuls multiple comparisons test) than the PTRI of 0.54 ± 0.010 found in the nonfusion class (Fig. 2C). The average index of the non-fusion class was same as that found at 10 h postaxotomy (Fig. 2C). This indicated that the touch response is significantly recovered in animals in which severed axons could fuse to their distal counterparts. A similar observation was made in a different transgenic reporter background, tibh222 (Pneu-4;mCherry) (Fig. S2 A and B). To check whether the behavioral recovery in the animals in the fusion category is truly due to fusion or to some other compensatory mechanism, we axotomized PLM neurons twice (Fig. 2D). The first axotomy was done at the L4 stage, and 24 h later, we picked the worms showing fusion events to perform the second axotomy (Fig. 2D). We found that the PTRI at 24 h increased from 0.52 ± 0.008 to 0.76 ± 0.015 in worms showing T2 fusion (Fig. 2E). Then, upon the second axotomy, the PTRI dropped significantly, down to 0.45 ± 0.014 (***P < 0.0001; Newman–Keuls multiple comparisons test).

As fusion leads to functional recovery, we expected that the mutants impaired in fusion would display poor recovery. We axotomized the mutants lacking the fusogen molecule EFF-1 (9, 28), the phosphatidylinerse receptor PSR-1 (15), and the ABC transporter CED-7, which is involved in cell engulfment (9, 15).

Fig. 2. Fusion between the proximal and distal fragments promotes recovery. (A) The confocal images merged with the brightfield differential interference contrast showing three different types of regrowth patterns at 24 h after axotomy: A, a’ shows regrowth without fusion; A, b’ shows T1 fusion; and A, c’ shows T2 fusion. The white dashed box indicates the fused region, and the green arrowhead indicates the point of fusion. (B) The double-axis bar plot represents PTRI on the left y axis and the percent fusion on the right y axis at 10 and 24 h after axotomy. At 24 h after axotomy, both the percentage of fusion events and PTRI increased, N = 3–6 independent replicates. (C) PTRI measured in the worms showing nonfusion, T1 fusion, and T2 fusion revealed that the PTRI of fusion events was significantly higher. N = 6. (D) Schematic representation of the experiment in which axotomy was performed twice on the same PLM. (E) Bar graph presenting the PTRI values obtained at different time points in the double-axotomy experiment. N = 2. (F) The double-axis bar chart presents the PTRI and percent fusion values measured in wildtype, ced-7 (fl), psr-1 (fl), and eff-1 (fl) strains at 3 and 24 h postaxotomy. These mutants show poor functional recovery. N = 2–7 independent replicates. (G) Fusion events in the ALM lead to recovery of the ATRI. N = 2. In B–E and G, ***P < 0.0001; ANOVA with Newman–Keuls multiple comparisons test; In F, ***P < 0.0001; **P < 0.001; paired t test. The number within each bar indicates the number of sides tested. Error bars represent SEM. ns, not significant.
Each of these genes is important for the axon fusion process (9, 15). We found that these mutants showed a significantly lower percentage of fusion (Fig. 2F and Table S1), and their respective PTRIs did not increase significantly at 24 h postaxotomy (paired t test) (Fig. 2F). In the psr-1(lf) strain there was a further drop in PTRI at 24 h postaxotomy (Fig. 2F), most likely caused by the enhanced degeneration of the distal ends (white arrows in Fig. S2F), which was reported previously (15). The mutants blocked in regeneration, such as dkl-1, pmk-1, and ehp-1, also showed very little rise in their respective PTRIs at 24 h postaxotomy (Fig. S2 C–E). Similarly, after the ablation of ALMs, we found that the PTRI value corresponding to the fusion class is significantly higher than the value belonging to the nonfusion class (**P < 0.0001; Newman–Keuls multiple comparisons test) (Fig. 2G).

These experiments indicated that axonal fusion is the main reason for the improvement in the functional index during the course of regeneration.

Loss of let-7 Promotes Axonal Fusion and Functional Restoration.

Previous studies have identified molecular pathways that inhibit axon regrowth (11, 12, 29). Mutations in these genes accelerate the regrowth process (11, 12, 29). We asked whether any of these mutants would promote functional recovery. To compare recovery across various mutants, we expressed functional recovery as fraction of recovery (FR). FR was obtained by normalizing the gain in PTRI at 24 h due to regeneration over the loss in PTRI after axotomy (Supporting Information). The wild-type control showed an FR value of 0.12 ± 0.02 (mean ± SEM). Among the mutants we tested, a significant enhancement in recovery value, 0.28 ± 0.02 (**P < 0.0001; Newman–Keuls multiple comparisons test), is seen in the let-7(lf) mutant (Fig. 3A). We also tested this in a temperature-sensitive allele, n2853ts, of let-7. We found that at the restrictive temperature of 20 °C the fusion events increased significantly (Fig. 3B), and the FR value increased significantly to 0.40 ± 0.03 from 0.11 ± 0.03, as observed at 15 °C (**P < 0.0001; Newman–Keuls multiple comparisons test) (Fig. 3C). The enhanced recovery in the let-7(lf) mutant was also reproduced in a different marker background, ihb-222 (Fig. S3A). At every time point we tested, the let-7(lf) mutant showed significantly higher PTRI values than the wild-type control (Fig. 3D). The percentage of fusion events was also significantly enhanced at similar time points in the let-7(lf) mutant (**P < 0.0001; Fisher’s exact test). For example, the wild-type strain and the let-7(lf) mutant showed 4% vs. 32%, 16% vs. 46%, and 42% vs. 67% fusion at the 3-, 10-, and 24-h time points, respectively (Fig. 3E).

We further asked whether enhanced fusion events in the let-7(lf) mutant are due to the cell-autonomous function of let-7 miRNA. We expressed the sequence for let-7 miRNA under the control of either the touch neuron-speciﬁc promoter Pnmc-4 or the epidermal promoter Pdp-7 using extrachromosomal arrays. We found that only the transgenic lines shrEx11 and shrEx12 that are expressed in touch neurons rescue the enhanced fusion and functional restoration phenotype in let-7(lf) (Fig. 3E and F). However, epidermal expression of let-7 rescued neither the axonal fusion phenotype (Fig. 3E) nor the FR phenotype (Fig. 3F). This suggested that the enhanced fusion and functional recovery seen in the let-7(lf) mutant is due to the cell-autonomous effect of let-7 miRNA. Overexpressing let-7 in a touch neuron in the wild-type background reduced the fusion events and the FR strongly (Fig. 3 E and F).

As both axon fusion and regrowth are the outcomes of axotomy, one can argue that the enhanced fusion events in the let-7(lf) mutant are the outcome of its increased ability to regrow. To verify this, we tested other mutants that showed enhanced total regrowth in our previous study (11). One such mutant is efa-6(lf), which showed enhanced regrowth due to increased microtubule growth (11, 30). The total regrowth of nonfusion events at 24 h postaxotomy in the let-7(lf) mutant is 94.4 ± 5.2 μm (mean ± SEM), which is significantly higher than the 75.2 ± 3.84 μm obtained from the wild-type control (**P < 0.01; Newman–Keuls multiple comparisons test) (Fig. S3 B and C). Even at 6 h postaxotomy, the elongation in let-7(lf) mutants is significantly higher (Fig. S3C), and there are more filopodia-like structures at the axon tip (white arrowheads in Fig. S3 B and D). Nonfused axons in the efa-6(lf) mutant showed a total regrowth of 103.0 ± 11.5 μm at 24 h (***P < 0.0001; Newman–Keuls multiple comparisons test). However, the fusion frequency in efa-6(lf) was 17%, significantly lower than in the control (42%) (**P < 0.0001; Fisher’s exact test) (Fig. S3C).

Other mutants that were reported to show enhanced total regrowth, e.g., atfs-1(lf), tab-19(lf), and elt-1(lf), displayed 12%, 25%, and 46% fusion events, respectively. These rates are either lower than or comparable to the fusion events in the wild-type strain (Table S1). This suggests that fusion and total axon outgrowth are not necessarily linked phenomena.

Our observation that let-7(lf) enhances the total regrowth of nonfused PLM axons complements the previous report that let-7(lf) promotes the regrowth of the anterior ventral microtubule (AVM) (29). To verify whether AVM neurons also showed fusion-like phenomena, we cut the AVM neuron at 60 μm from the cell body at the L4 stage (orange arrow in Fig. S3E) and observed that 45% of the neurons showed fusion (green arrowhead in Fig. S3F). This percentage is enhanced to 65% in let-7(lf) mutants (**P < 0.01; Fisher’s exact test). Therefore, the activity of let-7 in regulating regeneration responses is similar in PLM and AVM neurons.

let-7 Regulates Axon Fusion Through ced-7.

Previous studies indicated that axon fusion is mediated by the fusogen molecule EFF-1 (9, 15) and is promoted by cell-recognizing mechanisms involving CED-7 and PSR-1 (15). Therefore, we asked whether the enhanced fusion events in the let-7(lf) mutant can opt any parallel pathway or rely on the above-mentioned factors. The frequency of fusion events in ced-7(lf); let-7(lf) mutants is 22%, similar to the 24% observed in ced-7(lf) mutants (Fig. 4A). Similarly, the fusion frequency in eff-1(lf); let-7(lf) mutants was same as in eff-1(lf) mutants (Fig. 4A). The enhanced FR in let-7(lf) is completely dependent on either eff-1 or ced-7 (Fig. 4B). This genetic epistasis indicates that let-7 miRNA, either directly or through its targets, regulates the molecules required for axonal fusion during regeneration.

let-7 miRNA binds and degrades the mRNA of lin-41 at the L4 stage and helps the developmental transition from larval to adult stage (31, 32). This antagonistic relationship was applied to the regrowth of AVM axons after laser injury (29). FRs in lin-41(lf) and lin-41(lf); let-7(lf) mutants were not significantly different: −0.029 ± 0.043 (mean ± SEM) and −0.03 ± 0.049, respectively (Fig. 4B). FR values in each of these backgrounds are significantly lower than the values in let-7(lf) mutants (**P < 0.0001; Newman–Keuls multiple comparisons test). lin-41 was epistatic to let-7 for the axonal fusion phenotype as well (Fig. 4A).

To test whether let-7 directly or through the lin-41 pathway regulates the mRNA level of any of the molecules required for axonal fusion, we used the qRT-PCR approach. We tested the mRNA level of fusogen genes in let-7(n2853ts) mutants, which show an elevated level of lin-41 transcript at the restrictive temperature of 25 °C (33). To determine the fold change in the mRNA level of the genes tested, we normalized the level changes in the wild-type strain as 1 (33, 34) (see Materials and Methods for details).

We found a 2.23 ± 0.28-fold (mean ± SEM) (2−ΔΔCT) increase in lin-41 level (**P = 0.0005; unpaired t test with Welch’s correction) in the let-7(n2853ts) background (Fig. 4C) when L1 worms were kept at a nonpermissive temperature (25 °C) for 29 h before RNA preparation. Among the genes tested, we found that the level of ced-7 mRNA is higher by 1.53 ± 0.11-fold in the let-7(n2853ts) strain compared with the wild-type background (**P = 0.0003; unpaired t test with Welch’s correction) (Fig. 4C and Table S2). However, we found that the ced-7 level
remained unaffected in the total loss-of-function mutant lin-41 (Fig. 4D). This indicated that let-7 regulates the expression of ced-7 independently of the lin-41 pathway. While searching for a potential binding site for let-7 miRNA in genes controlling axon fusion during regeneration, we found that the 3’ UTR of ced-7 contains a sequence resembling the let-7-dependent complementary site (LCS) in the 3’ UTR of lin-41 (Fig. S44) (31, 33, 35). Independently, by silico prediction using miRNA-mirSVR (www.microrna.org/microrna/home.do) and Target (www.targetscan.org) scans, we found that the UTR of ced-7 contains a putative LCS, 5’-aacatgataatactcg-3’ (Fig. 4E). To evaluate the role of let-7 miRNA in regulating the ced-7 UTR region, we designed a sensor expressing GFP in touch neurons with the control of the UTR of the ced-7 gene (Fig. 4E). We found that the GFP level in the touch neuron is relatively low (arrow in Fig. 4F) in all transgenes, each made using a concentration ranging from 1–10 ng/µL. To compare the GFP intensity in the PLM cell body in different strains, we examined the GFP/mCherry ratio expressed in same neuron under the control of the 3’ UTR of unc-54. In let-7(n2853ts) and let-7(n2853ts) mutants, the GFP/mCherry ratios were 0.47 ± 0.04 and 0.64 ± 0.08, respectively. These values are significantly higher than the ratios of 0.28 ± 0.02 and 0.26 ± 0.03 obtained from their respective controls (***P < 0.0001; Newman–Keuls multiple comparisons test and **P = 0.003; unpaired t test with Welch’s correction) (Fig. 4F and G). Conversely, overexpression of the let-7 miRNA sequence in...
touch neurons reduces the GFP/mCherry ratio from 0.28 ± 0.023 to 0.12 ± 0.007 (**P < 0.001; Newman–Keuls multiple comparisons test) (Fig. 4 F and G). The mCherry intensity in the cell body mostly remained constant in these backgrounds having different doses of let-7 (Fig. S4C). These results suggest that the loss of let-7 promotes axonal fusion by increasing the ced-7-level, most likely independently of the lin-41 pathway, and that the ced-7 mRNA level could be regulated by let-7 through its UTR sequence. However, they counter the evidence that lin-41(II) reduces axonal fusion. Loss of lin-41 reduced the initiation of axon regrowth in AVM neurons (29). We also found that in the lin-41(II) strain the regrowth of the PLM is strongly reduced (P < 0.0001, Newman–Keuls multiple comparisons test) (Fig. S4 D and E). Frequently, axons fail to initiate regrowth even at 24 h after axotomy (Fig. S4D). If regrowth is strongly perturbed, axon fusion will also be perturbed, as seen in the ehp-1(II) and dkl-1(II) mutants.

Loss of let-7 Helps Overcome the Aging-Related Decline in Regeneration by Promoting Effective Fusion Events. It is known that the axon regeneration potential declines with age in worms (13, 20, 36, 37) and other organisms (38, 39). We tested how functional restoration would be affected by aging and whether let-7 miRNA would influence any of the parameters of axon fusion in an age-dependent manner. We measured the PTRI at different ages: L4 (late larva), A2–A5 (days 2–5), A7 (day 7), A8 (day 8), A11 (day 11), and A16 (day 16), and found a significant reduction in PTRI from A5 onwards (Fig. S5). We chose worms at the A3 and A4 stages for our experiment, as the basal PTRIs at these stages were not altered (Fig. S5). We found that the FR in animals operated at the A3 and A4 stages are −0.02 ± 0.02 and −0.04 ± 0.023 (mean ± SEM), respectively, which are significantly lower (**P < 0.0001; Newman–Keuls multiple comparisons test) than the FRs of 0.14 ± 0.031 found in animals operated at the L4 stage (Fig. 5A). However, the fusion events were not reduced at A3 or A4 (Fig. 5B). This raised a question whether the fusion events in the old animal are functional or not. To answer this, we compared the FR values between fusion and nonfusion groups (Fig. 5C).

As noticed earlier, in the L4 stage the FR in the fusion events is 0.42 ± 0.029, significantly higher (**P < 0.0001; Newman–Keuls multiple comparisons test) than the value of −0.013 ± 0.03 in the nonfusion events (Fig. 5C). However, at the A3 and A4 stages the FR values in the fusion category are lower than those in the nonfusion class (Fig. 5C). This indicated that for some reason the fusion events in old animals are not effective in repairing the lost function. Similar experiments in let-7(II) mutants revealed that there is no drop in recovery values in the A3 and A4 stages (Fig. 5A). The FRs at the A4, A3, and A4 stages were 0.25 ± 0.042, 0.26 ± 0.045, and 0.23 ± 0.036, respectively. These values were significantly higher than the values in the wild-type strain (**P < 0.0001; Newman–Keuls multiple comparisons test) (Fig. 5A). Also, in the let-7(II) mutant, the FR values for fusion class at the A3 and A4 stages remained significantly than those of nonfusion class (Fig. 5C). This indicated that the loss of let-7 overcomes the age-related decline in functional restoration through effective axon fusion. Conversely, when we overexpressed let-7 in touch neurons, the FR at L4 becomes −0.098 ± 0.059, significantly lower (**P < 0.0001; Newman–Keuls multiple comparisons test) than the value in the wild-type strain at same stage and comparable to the FR value in the worms axotomized at the A3 stage. Therefore, let-7 is sufficient to induce aging-like symptoms, such as the lack of functional restoration, in the late larval stage. On the other hand, the daf-2(II) mutant, which has increased life span due to the down-regulation of insulin signaling, showed no enhancement in fusion events or in functional recovery (Fig. 5 A and B).

Loss of let-7 Restores Age-Dependent Vesicular Transport and Helps Enrichment of EFF-1 in the Growing Tip. We speculated that in aged worms, although injured axons showed fusion-like phenomena at the anatomical level, the cytoplasmic continuity is not established. To test this, we used in vivo imaging of Rab-3 protein tagged with GFP (GFP::RAB-3) to study axonal transport (40, 41). We imaged bidirectional movement of RAB-3 particles (Fig. 6 A, a′ and b′ and Movie S4) in the proximal and distal ends in fused axons (Movie S4A). We present the GFP::RAB-3 dynamics in given regions of interest (ROIs) (Fig. 6 A, a′ and b′) as a kymograph (Fig. 6 C and D). In a kymograph (Fig. 6C), diagonal tracks represent events of anterograde or retrograde movement. We present the movement as particle flux, i.e., the number of moving particles per unit length of axon per unit of time, as described before (41). We reasoned that a successful fusion event would lead to cytoplasmic continuity between the proximal and distal ends, and therefore, the particle flux in the distal and proximal ends will be similar. The values of anterograde and retrograde particle flux in the proximal fragment were 0.016 ± 0.0015 particles·μm−1·s−1 and 0.0078 ± 0.0013 particles·μm−1·s−1 (mean ± SEM), respectively, in T2 fusion events (Fig. 6 A, a′). In the distal pares, these values were 0.013 ± 0.001 particles·μm−1·s−1 and 0.007 ± 0.0009 particles·μm−1·s−1, respectively (Fig. 6E), similar in range to those in the proximal part. After the region of fusion was photobleached, the movement of particles through the point of fusion became much clearer (Movie S4B). In nonfusion events (Fig. 6 A, b′), the values of anterograde and retrograde flux in distal end axons were 0.0012 ± 0.0002 particles·μm−1·s−1 and 0.0011 ± 0.0004 particles·μm−1·s−1, respectively, indicating that the Rab-3 particles are relatively static in the distal end (Fig. 6D and E and Movie S6). Flux was significantly higher in the proximal part (**P < 0.0001; Newman–Keuls multiple comparisons test) (Fig. 6 D and E and Movie S5). The PTRI values corresponding to the fused and nonfused events in this experiment were 0.75 ± 0.01 and 0.48 ± 0.04, respectively, and they differed significantly (**P < 0.0001; Newman–Keuls multiple comparisons test).

We found a correlation among fusion events, restoration of particle flux in the distal end, and functional recovery. We noticed that in A3 worms the steady-state value of anterograde flux is significantly reduced (**P < 0.001; Newman–Keuls multiple comparisons test) (Fig. S6B), and this value is significantly higher in let-7(II) mutants (**P < 0.0001; Newman–Keuls multiple comparisons test) (Fig. S6 D and E and Movie S5). The PTRI values corresponding to the fused and nonfused events in this experiment were 0.75 ± 0.01 and 0.48 ± 0.04, respectively, and they differed significantly (**P < 0.0001; Newman–Keuls multiple comparisons test). Therefore we found a correlation among fusion events, restoration of particle flux in the distal end, and functional recovery. In older worms, incomplete cytoplasmic continuity might have occurred due to the lack of EFF-1 during the axon fusion process. To test this hypothesis, we developed an EFF-1::mCherry reporter (Fig. 6G). This construct is functional, as the axonal fusion in eff-1(II) worms carrying the transgene became 73%, compared with the 10% fusion observed in eff-1(II) worms without transgene (**P = 0.0001; Fisher’s exact test). We found that at 6 h postaxotomy, the EFF-1::mCherry signal is up-regulated near the growing end of axons (dashed box, R-I in Fig. 6G). Imaging the region near the growth cone reveal that EFF-1 is present throughout the growth cone but is also enriched at the tip of the filopodia-like protrusions (arrows in Fig. S7A). The GFP::RAB-3 puncta are also enriched at the growth cone.
If eff-1 is limiting at the growing tip of axons for functional repair, increasing the level of EFF-1 might be sufficient to obtain functional fusion events in aged worms. We performed axotomy on aged worms expressing a functional EFF-1::mCherry transgene (shrEx59) and found that at A3 and A4 the FR index of fusion events in these worms (0.35 ± 0.043 and 0.24 ± 0.035 respectively) is higher than the respective indices in wild-type worms (0.02 ± 0.05 and 0.029 ± 0.02, respectively) (Fig. 6I). Also the FR values corresponding to the fusion category in the A3 and A4 stages remained significantly higher (**P < 0.01; ***P < 0.0001; Newman–Keuls multiple comparisons test) than the values of nonfusion classes in the eff-1(+) background (Fig. 6I). This evidence suggested that the EFF-1 concentration is limiting for the functional restoration in old worms. The loss of let-7 overcomes this barrier by enhancing the axonal transport and enriching EFF-1 protein in the growing tip of the axon.

Discussion

C. elegans mechanosensory neurons have long been used to understand the molecular pathways controlling their regrowth ability. In this study, we directly address the functional consequences of the regeneration patterns. Our data reveal that the axon fusion is the major phenomenon that drives functional regeneration in touch neurons.

Use of Multiple Femtosecond Lasers in Neurosurgery. Our analysis with this multilaser system reveals that cutting both sides has a severe effect on gentle touch sensation as opposed to cutting one side. This further opens up the possibility of functional analysis of the touch circuit. The functional connectivity of the other neurons, such as PVD, AWC, and ASER, that are located bilaterally in C. elegans (23) can be dissected out using this multilaser two-photon system.

We found that, after axotomy, the intact distal end has the ability to give some response. This is consistent with the previous report that severing the axon or ablating the cell body alone in a microfluidic device has less effect on gentle touch sensation (27). However, the effect is more significant after the removal of the synaptic branch (27). After axotomy, one would imagine that the contribution of the gap junction present near the PLM cell body would be absent. Therefore, gap junction-mediated communication to the PVC, though not being present, would be abolished. The synaptic branch in the distal end makes a synapse to the AVA interneuron (42). It is predicated that AVA might communicate with PVC (24, 43). The synaptic branch within the intact distal end might communicate with the PVC through the AVA. Thus, a worm can respond to a posterior touch without a contribution from the gap junction after axotomy.

Axonal Fusion in Functional Recovery. For functional recovery to occur after neuronal injury, the regenerating axon must establish connectivity with its original target, either by the formation of a chemical synapse to its postsynaptic neuron or by reconnecting with its own distal fragments. In many systems, such as earthworm, Aplysia, C. elegans, crustaceans, and crayfish, the second phenomenon, known as “fusion” occurs (4–6, 9). In C. elegans, fusion during regenerative growth is dependent on the cell-recognition molecules CED-7, the phosphatidylserine receptor PSR-1, and the fusogen molecule EFF-1 (9, 15). In this study, we have correlated the anatomical regrowth patterns with the behavioral recovery in the same animal using microscopy and behavioral tests. We have shown that an efficient fusion restores the axonal transport in the distal end. Therefore, we have provided evidence for cytoplasmic continuity in the case of axon fusion. This undoubtedly proves that fusion could help recover function significantly and provides promising evidence that harnessing the fusion pathways after neuronal injury could be beneficial. Fusion might also protect long axonal tracts from degeneration after small breakages caused by
Our data indicated that the enhanced functional recovery seen in *let-7* mutants is dependent on the fusogen molecule *EFF-1* (28) and on cell-recognition molecules such as *PSR-1* and *CED-7*. *let-7* miRNA inhibits the heterochronic gene *lin-41* and in turn releases the inhibition of the transcription factor LIN-29 to help the developmental transition from L4 to the adult stages (32, 47). Normally *let-7* expression becomes prominent in the late L4 stage, and its level remains high in adult stages. We found that in *let-7(lf)* mutants the mRNA level of *ced-7* is up-regulated in the L4 stage, and the frequency of axon fusion is increased. We further found that the 3’ UTR of *ced-7* has a putative *let-7* miRNA-binding site and that this UTR is sensitive to the dose of *let-7*. This indicated that the *let-7* miRNA pathway negatively regulates functional restoration.

**Loss of *let-7* miRNA Promotes Functional Restoration in Adult Animals.** Manipulation of intracellular pathways has some promise in giving rise to improved axon regeneration. For example, activation of cAMP messenger signaling, inhibition of the PTEN subunit of the mTOR pathway, and stabilization of the microtubule cytoskeleton have individually helped overcome regeneration blocks in the neurons after spinal cord injury (44–46). By screening mutants of axon regrowth-related genes for functional recovery, we found that *let-7* miRNA negatively regulates functional restoration.

Fig. 6. Loss of *let-7* improves axonal transport during axonal fusion. (A, a’ and b’) Still images from time-lapse movies of the PLM with *tbs222 (Pmec-4-mCherry)* and *jsls21 (Pmec-7-GFP::RAB-3)* using spinning-disk confocal at 24 h postaxotomy. *mCherry* is shown in magenta, and *GFP::RAB-3* is shown in green. White arrows indicate GFP::RAB-3 particles in the axon. (A, a’) T2 fusion. (A, b’) Nonfusion. (B) Schematic representation of images shown in A. (C) Kymographs from the movies of 180-s duration were generated from the 30-μm ROIs (yellow dotted box in A, a’) placed on the distal and proximal ends of fused neuron. Diagonal tracks represent events of anterograde (dashed yellow arrow) or retrograde (dashed red arrow) movements. (D) A kymograph from the distal and proximal ends of a nonfused axon as shown in A, b’. The perpendicular tracks (arrowheads) represent static particles. (E) The bar chart represents particle flux in the proximal and distal ends measured in fusion and nonfusion events. *A*, anterograde events; *R*, retrograde events. Particle flux = number of particles or tracks per micrometer per second. *N* = 5–8 independent replicates. (F) The bar chart shows flux values in the proximal and distal ends in L3 worms measured in T2 fusion events. *N* = 4–7 independent replicates. (G) Spinning-disk confocal images of worms coexpressing *muls32 (Pmec-7-GFP)* and *shrExS9 (Pmec-4-EFF-1::mCherry)* before and after axotomy. Orange arrows indicate the site of axotomy. Two ROIs are shown: R-I at the cut tip and R-II placed 10 μm away from ROI-I to quantify the average intensity of *EFF-1::mCherry*. *EFF-1* intensity is increased at R-I at 6 h after axotomy. This enrichment is reduced at the A3 stage. (H) The average intensity of *EFF-1::mCherry* in two ROIs obtained 30 min before and 6 h after axotomy is plotted for L4- and A3-stage experiments. *N* = 2–4 independent replicates. (I) In the *eff-1(+)* strain *shrExS9 (Pmec-4-EFF-1::mCherry)*, FR values in the fusion category are increased significantly compared with the values in wild-type strain at the A3 and A4 stages. *N* = 2. In E, F, H, and I, *P < 0.01; ***P < 0.001; ANOVA with Newman–Keuls multiple comparisons test. Error bars represent SEM. (J) The working model depicting how the *let-7* miRNA pathway negatively regulates effective axon fusion in L4 and aged worms. ns, not significant.
that let-7 might target ced-7 directly by binding to its UTR. Overall, our data suggest that let-7 negatively regulates the axon fusion process by reducing the mRNA level of ced-7.

One intriguing finding is that the correlation between fusion-like phenomena and functional recovery is lost in old worms. Previous work suggested that EFF-1 is enriched near the contact point between the proximal and distal ends during the fusion process (15). We showed that EFF-1 fusogen is limiting at the growing tip of axon in the older worms and is sufficient to establish functional fusion events. We found that axonal transport is affected in older worms, and the enrichment of EFF-1 is also perturbed. So EFF-1 could be transported to the axon tip following neuronal injury.

We propose that let-7 regulates axon fusion in two ways (Fig. 6d). Loss of let-7 increases the probability of contact between the proximal and distal ends by increasing the level of ced-7 mRNA. However, ced-7 is not sufficient to establish cytoplasmic continuity in older animals. In older animals, let-7(′f) maintains the healthy bidirectional axonal transport by delaying neuronal aging, and therefore EFF-1 is not limiting at the axon tip. Similarly, the fusogen molecule AFF-1 is limiting in older animals for proper branching of the PVD dendrite during regeneration (48).

Materials and Methods

C. elegans Strains. C. elegans strains were grown on nematode growth medium (NGM) agar plates at 20 °C using standard methods (49). For growing the temperature-sensitive mutant let-7(n2853ts), we used a 1°C incubator as a permissive condition and 20°C as a nonpermissive condition (31). All the loss-of-function alleles except let-7(n2853ts) are denoted as ‘′f′′. For example, the dtk-1 mutant is presented as dtk-1(′f). All the strains used for this study are identified in Supporting Information.

Femtosecond Lasers, Axotomy, and Imaging. Most of the axotomy experiments other than the experiments involving age-related changes in functional recovery were done at the L4 stage. During axotomy and imaging, the worms were immobilized by using 10 mM levamisole hydrochloride (Sigma L0380000) anesthesia in a 5% agarose pad or with a suspension of 0.1-M diameter polystyrene beads (00876-15; Polysciences) in a 12.5% agarose pad (L0380000) anesthesia in a 5% agarose pad, when PCR reactions were run (33, 34). For every experiment described in this report, the gentle touch assay was performed on both sides of the worm. Before or at any time point after axotomy, the worms were placed in individual plates. A worm generally lies on one of its sides. The side facing upward was assayed first, and whether it was the right or the left side was noted. In this assay, the worms were subjected to anterior and posterior touch alternatively, 10 times each, with the help of an eyelash and the time it took for the worm to respond. We used a 1× ultraviolet microscope to observe the motility of the worms. The assay was repeated 3 times. The worms were immobilized by using 10 mM levamisole hydrochloride (Sigma L0380000) anesthesia in a 5% agarose pad, when PCR reactions were run (33, 34). For quantitative imaging of P

Molecular Biology and Transgenes. For touch neuron-specific expression of let-7, a gateway (invitrogen) entry clone let-7 (pCR: let-7 (pNBRGWY21)) was converted to pCR: R and recombined with pCR: pZHY23 (pNBRGWY19) and pNBRGWY44 (pPdy7-destination vector) to generate Pmec-4-let-7 (pNBRGWY16) and Pp70-let-7 (pNBRGWY36), respectively. The primers used for generation of pCR:let-7 were 5′-CTACACTGGATCCGCCG-3′ and 3′-GCTTCGAAGAGTTCTGTCTC-5′. To make Pmec-4-EFF-1::mCherry and Pmec-4-let-7::E10214, an entry clone pNBRGWY39 (PCR:mCherry-1-CDNA) was constructed by PCR using primers 5′-GGCTTCACTGGATCCGCCG-3′ and 3′-GATTGCACCTGG- TACGCATAG-3′. Then the PCRI plasmid was recombined with pZHY26 (Pmec-4-Gateway-mCherry) and pZGY55 (Pmec-4-Gateway-GFP) to generate Pmec-4-EFF-1::mCherry (pNBRGWY41) and Pmec-4-let-7::E10214 (pNBRGWY40), respectively. To generate Pmec-4-GFP-3′ UTR ced-7, first the boundary of the UTR was found from the annotated gene sequence of ced-7 in the Caenorhabditis Genetics Center (CGC) database (https://cbi.umn.edu/cgco/home). Then the UTR was amplified with the forward primer (ced-7-3′ UTR f) 5′-CCGCAATCCTGGCCGCTCAGGAC-3′ and the reverse primer (ced-7-3′ UTR r) 5′-GGGACTAGTCCGCGATCCCGACAGTATTCTTCC-3′ and was cloned into a pCR: vector to obtain pNBRGWY37 (PCR: 3′ UTR ced-7). Then the pCR: clone was recombined with pZHY1867 (Pmec-4-GFP-Gateway destination vector) to generate pNBRGWY38 (Pmec-4-GFP-3′ UTR ced-7).

RNA Extraction and qRT-PCR. Standard RT-PCR analyses were performed following previous methods (52). We used RNA samples from L4-staged N2, let-7(n2853ts) and lin-41(na104) worms. To prepare RNA samples from let-7 (n2853ts) strain, −50–80 gravid adult worms grown at 15 °C were allowed to lay eggs for 3–4 h in a 60-mm NGM plate at 15 °C. After hatching, one batch of worms at the L1 stage was transferred and kept at 25 °C for 29 h as described previously (54), and the other batch was kept at 15 °C. lin-41(na104) are sterile, and respective wild-type controls were kept at 20 °C. After 4 days, the L4 worms were collected and washed three times in M9 buffer, pelleted down, frozen, and stored at −80°C. RNA was extracted from the thawed worms using the Qiagen RNeasy Mini Kit (no. 74104; Qiagen). The extracted RNA was then treated with DNase I to remove genomic DNA contamination using Ambion’s DNA-free kit (AM1906). cDNA was prepared from −2 μg RNA using SuperScript III Reverse Transcriptase (no. 18080093; Thermo Fisher Scientific). Twenty-five nanograms of RNA were used for qRT-PCR in 20 μL of Power SYBR Green PCR Master Mix (no. 3367659; Applied Biosystems Life Technologies). The reactions were performed for 40 cycles. Relative positions of the primers are indicated in Fig. S4A, and their sequences are given in Supporting Information. Some of the primers were designed for priming at the intron and exon boundary to avoid amplification from contaminating genomic DNA (Fig. S4A). The rest were designed so that the genomic DNA contamination would result in a PCR product larger than the reverse transcribed product. However, no contaminating bands were observed in 3% agarose gel, when PCR reactions were run (Fig. S4B). The relative mRNA amounts of target genes in the let-7(n2853ts) and wild-type strains were calculated using the ∆∆CT method and were normalized to tba-1 control for endogenous mRNAs (33). The value for wild type was set to one for calculations of fold modulation (33, 34).

Time-Lapse Imaging of GFP::RAB3 and EFF-1::mCherry. The worms were immobilized using 10 mM levamisole hydrochloride on 5% agarose pads. GFP::RAB3 and mCherry were imaged from worms coexpressing jnsb281 (Pmec-4-GFP::RAB-3 (40) and tbs222 (Pmec-4-mCherry). For live imaging of GFP::RAB3 fluorescence, we collected 535 frames at the rate of three frames/min using the 100× lens of a NA 1.46, Yokogawa 20× objective and a HAMAMATSU SMZ589, Japan EMCCD camera (512 × 512) controlled by Velocity software in a spinning disk system (Perkin-Elmer UltraView) assembled in an Olympus IX83 microscope. EFF-1::mCherry and internal control GFP were imaged from the worms coexpressing shvEs59 (Pmec-4-EFF-1::mCherry) and multis32 (Pmec-7-GFP). We imaged worms at the L4 and A3 stages, before and after axotomy. EFF-1::mCherry and GFP were imaged using 20× laser power of 561-nm and 488-nm lasers, respectively. We used exposure times of 350 μs and 50 μs for EFF-1::mCherry and GFP, respectively. Imaging was done using a CSU-X1 scan-head and a Photometric ECLIPSE EMCCD camera (512 × 512) controlled by Zen Blue software in a Zeiss Axio Observer.Z1 microscope. Details of the quantitative measurements of GFP::Rab-3 movement and the intensity of EFF-1::Cherry are provided in Supporting Information.

Point-Scanning Confocal Imaging. For quantitative imaging of Pmec-4-GFP-3′ UTR ced-7 (sensor) lines, the worms coexpressing Pmec-4-GFP-3′ UTR ced-7 (sensor) and Pmec-4-mCherry-3′ UTR unc-54 (tbs222) were imaged with 60.5% of 488-nm and 43.6% of 543-nm lasers, respectively, under a 100× oil objective.
Statistical analyses were performed using GraphPad Prism software. For two-way comparisons, an unpaired test was performed when comparing the PTRI values obtained at 3 h postaxotomy from the same worm. Fisher's exact test was used for proportions. Three or more samples were compared with ANOVA (non-parametric) with a Newman-Keuls multiple comparisons test. In all the bar graphs, we denote “n” as the number of samples and “N” as the number of independent replicates. The error bars represent the SEM.

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