Novel DLK-independent neuronal regeneration in *Caenorhabditis elegans* shares links with activity-dependent ectopic outgrowth

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During development, a neuron transitions from a state of rapid growth to a stable morphology, and neurons within the adult mammalian CNS lose their ability to effectively regenerate in response to injury. Here, we identify a novel form of neuronal regeneration, which is remarkably independent of DLK-1/DLK, KGB-1/JNK, and other MAPK signaling factors known to mediate regeneration in *Caenorhabditis elegans*, *Drosophila*, and mammals. This DLK-independent regeneration in *C. elegans* has direct genetic and molecular links to a well-studied form of endogenous activity-dependent ectopic axon outgrowth in the same neuron type. Both neuron outgrowth types are triggered by physical lesion of the sensory dendrite or mutations disrupting sensory activity, calcium signaling, or genes that restrict outgrowth during neuronal maturation, such as SAX-1/NDR kinase or UNC-43/CaMKII. These connections suggest that ectopic outgrowth represents a powerful platform for gene discovery in neuronal regeneration. Moreover, we note numerous similarities between *C. elegans* DLK-independent regeneration and lesion conditioning, a phenomenon producing robust regeneration in the mammalian CNS. Both regeneration types are triggered by lesion of a sensory neurite via reduction of neuronal activity and enhanced by disrupting L-type calcium channels or elevating cAMP. Taken as a whole, our study unites disparate forms of neuronal outgrowth to uncover fresh molecular insights into activity-dependent control of the adult nervous system's intrinsic regenerative capacity.

lesion conditioning | axon regeneration | femtosecond laser ablation | DLK-1 | activity-dependent ectopic axon outgrowth

One of the principal goals of modern neuroscience is the comprehensive understanding and therapeutic application of neuronal regeneration (1). This goal is particularly relevant in the case of the mammalian central nervous system (CNS), which regenerates poorly. Efforts to enhance axon regeneration generally fall into two broad categories: eliminating or blocking nonpermissive extrinsic inhibitors or promoting a neuron's intrinsic regenerative capacity (2). Although much research in previous decades focused on the extrinsic angle, recent encouraging developments, particularly in invertebrate models, increasingly examine the cell intrinsic control of regeneration. Studies demonstrate that axon regeneration recruits or recapitulates mechanisms involved in a diverse range of biological processes, including synapse formation, stress response, apoptosis, and development.

During development, neuronal electrical activity acts as a common intracellular feedback mechanism to establish appropriate connections and modulate outgrowth (3). Subsequently, a neuron transitions from a state of rapid growth to a stable morphology, and neurons within the adult mammalian CNS lose their ability to effectively regenerate in response to injury. A striking exception to this paradigm is lesion conditioning, a phenomenon exemplified by the dorsal root ganglion (DRG), where peripheral axon damage enables robust central axon regeneration (4). The peripheral lesion is thought to revert the neuron to a growth-permissive state in an activity-dependent manner (5). These observations suggest that activity-dependent inhibitors of neurite outgrowth may represent a potent therapeutic target for enhancing neuronal regeneration in the face of injury or disease.

The nematode *Caenorhabditis elegans* is a well-established and powerful model system (6) for the genetic and molecular study of both activity-dependent neuronal development and regeneration. Specifically, several studies (7–9) have observed spontaneous ectopic axon outgrowths from four bipolar sensory neurons of mutant animals raised under stressed conditions (high temperature). An extensive genetic study indicated that the mutations disrupt sensory activity, which triggers cell-specific ectopic outgrowth after the neurons establish their initial pattern of innervations (10). More recently, the application of advanced subcellular laser ablation techniques (11) to *C. elegans* has permitted quantitative in vivo study of single-neuron regeneration in this genetically tractable system. Several studies have revealed the role of conserved mitogen-activated protein kinase (MAPK) pathways, including the dual leucine zipper kinase (DLK) and the parallel but coordinated c-Jun N-terminal kinase (JNK) pathways (12, 13, 14). Other studies have clarified the beneficial role of caspase activity (15) and the role of calcium signaling (16, 17). Thus, *C. elegans*, by virtue of its facile genetics and amenability to precise laser surgery, is an excellent system for studying axon outgrowth and regeneration.

In a previous study, we sought to dissect the subcellular components of the *C. elegans* ASJ sensory neurons by femtosecond

Significance

By laser surgery, genetics, and pharmacology, we demonstrate that neurons of the nematode *Caenorhabditis elegans* undergo a novel form of regeneration that is largely independent of defined regeneration pathways, including DLK, which underlies axon regeneration from *C. elegans* to mammals. Our results indicate genetic and molecular connections between DLK-independent regeneration and a previously studied activity-dependent ectopic axon outgrowth in *C. elegans*. We also note numerous similarities with lesion-conditioned regeneration, in which reduction of sensory activity triggers robust axon regeneration in the mammalian CNS. Our study unites disparate forms of neurite outgrowth to uncover the molecular mechanisms that modulate regeneration in the adult CNS and suggests that ectopic outgrowth might represent a powerful gene discovery platform for regeneration.

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See Commentary on page 5465.

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laser surgery to establish their role in development and behavior (18). ASJ axons rapidly regenerate to the vicinity of their original synaptic targets, but regeneration is prevented by mutation of *dlk-1*, which plays a crucial role in neuronal regeneration in *C. elegans* (12), *Drosophila* (19), and mammals (20, 21). Remarkably, although *dlk-1(ju476)* completely prevents regeneration after axotomy, we discovered that strong DLK-independent regeneration returns under two conditions, which we explore in this study. First, regeneration occurs after severing both the axon and sensory dendrite, indicating that sensory dendrite cuts trigger robust DLK-independent regeneration. Second, even without a dendrite cut, regeneration occurs under mutations that also trigger ectopic axon outgrowth. Here, we demonstrate that this novel type of regeneration is largely independent of both the DLK-1/DLK and the KGB-1/JNK pathways. We find numerous direct links between this DLK-independent regeneration and ectopic outgrowth in the same neurons. Finally, we note similarities between DLK-independent regeneration in *C. elegans* and regeneration after lesion conditioning in mammals, including shared phenotypes, role of L-type voltage-gated calcium channel (L-VGCC), and mediation by cyclic adenosine monophosphate (cAMP). As such, our study establishes a novel regeneration pathway in *C. elegans*, where physical, genetic, or pharmacological lesion of a sensory neurite or sensory signaling removes activity-dependent developmental inhibitors to permit robust regeneration.

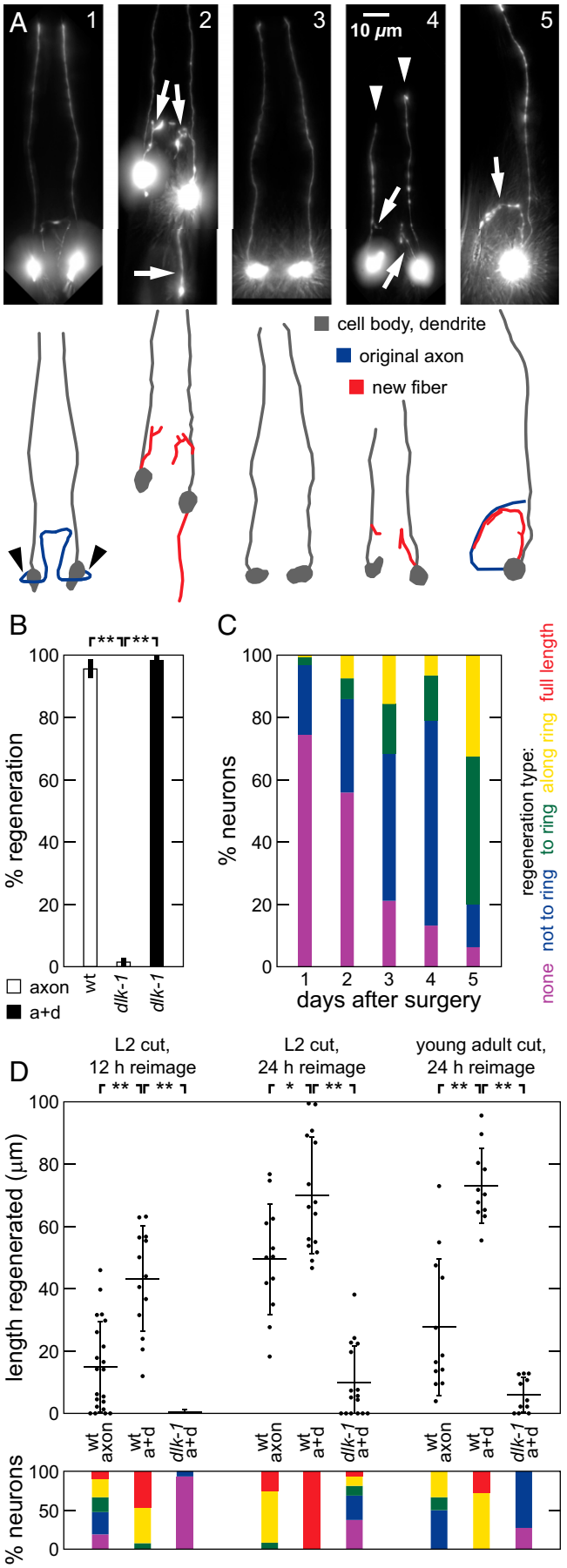
Results

Transection of the Axon and Sensory Dendrite Triggers DLK-Independent Regeneration in the ASJ Neuron. The ASJ neuron in *C. elegans* is one of the amphids, a set of 12 stereotyped bilateral bipolar sensory neurons, each composed of a cell body, a dendrite terminating in sensory cilia in the nose, and an axon mediating the synaptic connections in a central neuropil called the nerve ring (Fig. 1 *A1* and *A5*) (22). In wild-type (WT) *C. elegans*, in vivo laser severing of ASJ axons results in robust regeneration, with >95% of neurons displaying regenerative outgrowth within several days (Fig. 1 *A2* and *B*), whereas dendrite transection results in little or no growth, even after 4 days (18, 23). Consistent with previous studies of several neuron types in *C. elegans* (12, 13, 18), the *dlk-1(ju476)* mutation eliminates conventional DLK-1–mediated axon regeneration after axotomy alone, with <5% of postsurgery neurons regenerating (Fig. 1 *A3* and *B*). Remarkably, we find that concomitantly ($\Delta t < 1$ min) severing both the ASJ axon and the sensory dendrite (axon+dendrite, a+d) restores regeneration rates in *dlk-1* animals (Fig. 1 *A4* and *B*), suggesting that severing the sensory dendrite triggers a nonconventional (i.e., DLK-independent) regeneration pathway that is not activated by axotomy alone. To confirm results in a different neuron, we performed the same surgeries on the ASH amphid neuron (Fig. S1*B*). Results are consistent with findings in the ASJ neuron, showing a reduction in regenerated length from *dlk-1* and an increase in regenerated length from an additional dendrite cut.

Both conventional regeneration after axotomy alone in WT animals and DLK-independent regeneration generate similar outgrowths of three basic morphologies, although the dynamics of regeneration are distinct (see below). The ASJ axon normally projects ventrally from the cell body and then extends along the nerve ring, where it makes numerous synaptic connections with other neurons (blue lines in Fig. 1 *A1* and *A5*). As shown in Fig. 1*A* and Fig. S1*A*, regeneration projects from the cell body anteriorly to the ring and follows the ring posteroventrally (Fig. 1 *A2*, *A4*, and *A5*), projects posteriorly from the cell body (Fig. 1*A2*), or occasionally projects off the dendrite and follows the ring posteroventrally (Fig. 1 *A2* and *A4*). The outgrowth morphologies also match ectopic axon outgrowths from the same neurons triggered by genetic defects in sensory activity (see below) (10). As shown in Fig. 1*C*, we quantified the regeneration extent and found that >80% of DLK-independent regeneration successfully grows into the nerve ring by 5 d postsurgery (e.g., Fig. 1 *A2*, *A4*, and *A5*). These data include regeneration from *dlk-1* axon+dendrite cuts as well as axon cuts in double mutants, with *dlk-1* and mutations triggering regeneration listed in Fig. 2*B* (see below). *SI Results* (Fig. S1 *C* and *D*) details additional observations of this regeneration, illustrating and quantitatively comparing regeneration in single backgrounds. Thus, DLK-independent regeneration is morphologically similar to conventional regeneration, and both are clearly directed toward the original axon targets in the nerve ring.

Given that a dendrite cut enhances regeneration in *dlk-1*, we next sought to determine if this enhancement exists in the WT or arises by disruption of *dlk-1*. We, therefore, examined the impact of dendrite cuts on regeneration in WT animals. After surgery, most ASJ neurons in WT animals substantially regenerate; therefore, to quantify regeneration after different surgeries, we measured regenerated outgrowth length and penetrance into the nerve ring (*Materials and Methods* and Fig. S2). As shown in Fig. 1*D*, in WT ASJ neurons the addition of a dendrite cut generates significantly longer regeneration with greater success penetrating into the nerve ring compared to axon lesion alone. Enhancement occurs both at relatively short regeneration times of 12 and 24 h after surgery during the L2 larval stage and after surgery during the later young adult stage. In contrast, DLK-independent regeneration resulting from surgery in L1 or L2 typically begins during the L4 larval stage: reimaging before L4 (about 24 h after L2) reveals little regeneration in *dlk-1* mutants (Fig. 1*D* and Fig. S1*E*). Although DLK-mediated regeneration appears to initiate within about 12 h after surgery or less if enhanced by dendrite cut, DLK-independent regeneration initiates from 24 h to several days after surgery (Fig. 1*C*). Axon+dendrite surgeries have a similar effect in young adult animals, resulting in relatively modest regeneration in *dlk-1* compared to WT after 24 h. Despite the limited DLK-independent regeneration at earlier reimaging times shown in Fig. 1*D*, reimaging at later times confirms continued regeneration that, although slower than DLK-mediated regeneration, eventually reaches and extends along the nerve ring (Figs. 1 *C* and *D* and 3*D* for length measurement). These observations suggest that, in WT animals, both DLK-mediated regeneration and a novel DLK-independent regeneration are active in a single neuron and enhanced by dendrite surgery. In this context, mutation of *dlk-1* eliminates DLK-mediated regeneration, revealing the weaker but distinct DLK-independent regeneration.

DLK-Independent Regeneration after ASJ Axon+Dendrite Lesion Is Independent of Defined MAPK Signaling Pathways. To further define the mechanisms underlying ASJ regeneration, we tested additional known genetic modulators by noting the frequency of regeneration after the axon or axon+dendrite surgery types (Fig. 2*A*). First, we tested *dlk-1(km12)*, which produces regeneration rates not statistically different from *dlk-1(ju476)* after either type of surgery and confirms that the *dlk-1* regeneration phenotypes are not limited to one *dlk-1* allele. Second, we tested MAPK genes that mediate conventional motor neuron regeneration in *C. elegans* (24) for a role in conventional ASJ regeneration. As shown in Fig. 2*A*, *Inset*, conventional axon regeneration in motor neurons requires a coordinated activation of the DLK-1/PMK-3/CEBP-1 p38 and the parallel but coregulated MLK-1/KGB-1/FOS-1 JNK MAPK pathways (14, 25). Although mutation of *cebp-1* and *fos-1*, which encode C/EBP and Fos basic region-leucine zipper transcription factors, significantly reduces conventional regeneration, mutation of *mlk-1* and *kgb-1* does not (Fig. 2*A*, *Left*). Thus, unlike motor neuron regeneration, ASJ conventional regeneration (Fig. 2*A*, *Inset*) does not require *mlk-1* or *kgb-1*. Third, we tested MAPK genes for roles in DLK-independent regeneration. Double mutants of *dlk-1* and these genes (Fig. 2*A*) do not exhibit significantly different regeneration rates from *dlk-1* after either type of surgery (only *kgb-1* shows a mild 20% reduction after a+d). These results suggest that DLK-independent regeneration involves a novel mechanism largely independent of both the *dlk-1* and *kgb-1* MAPK pathways shown to mediate most *C. elegans* regeneration.



DLK-Independent Regeneration Shares Phenotypic and Genetic Links with Ectopic Axon Outgrowth. Although DLK-independent regeneration is distinct from previously defined axon regeneration, we observe striking similarities to another type of amphid growth called activity-dependent ectopic axon outgrowth, which occurs primarily at 25 °C cultivation. Both mechanisms produce similar morphologies, with projections running either posteriorly (7) or into the nerve ring (Fig. 1*A*). In addition, both ectopic outgrowth (10) and ASJ DLK-independent regeneration appear in the later stages of the animal's lifecycle, well after the amphid sensory neurons have developed their final morphology: regeneration in animals with *dlk-1* usually does not emerge until the L4 larval stage or later (Fig. 1*D* and Fig. S1*E*). *SI Results* lists more details on ectopic outgrowth characteristics.

Based on these phenotypic similarities, we asked if the same physical or genetic lesions might trigger both ectopic outgrowth and DLK-independent regeneration. First, dendrite cuts trigger DLK-independent axon regeneration, and we find that dendrite lesions alone can trigger ectopic axon outgrowth (Fig. 2*B*, left gray bars) under conditions required for ectopic outgrowth (i.e., 25 °C cultivation and surgery on two successive generations—see *SI Results* for discussion of maternal effect). Second, we tested an array of mutations that reduce sensory activity and trigger ectopic outgrowth to assess their role in triggering DLK-independent regeneration. These "outgrowth" mutations disrupt genes encoding the L-VGCC α_1 subunit $Ca_V1.2$ (*egl-19*/*CACNA1C*) and α_2/δ-1 subunit (*unc-36*/*CACNA2D1*), cyclic nucleotide-gated channel subunits α and β (*tax-4*/*CNGA1* and *tax-2*/*CNGB1*, respectively), a transmembrane guanylate cyclase involved in signal transduction (*daf-11*), and intraflagellar transport protein 52 (*osm-6*, which fails to develop functional sensory dendrites) (10). As shown in Fig. 2*B*, gray bars, most outgrowth mutations also successfully trigger DLK-independent regeneration after axotomy without a dendrite lesion at lower temperatures that generate less ectopic outgrowth (additional details are in *SI Results*). The rates of ectopic outgrowth and regeneration are strongly correlated (Fig. S3*B*). The results above strongly suggest that the same activity-dependent mechanism that triggers ectopic outgrowth also triggers DLK-independent regeneration after axon damage.

Dendrite cuts enhance regeneration under DLK-mediated and DLK-independent processes (Fig. 1*D*), and, therefore, we next asked if ectopic outgrowth can occur independent of *dlk-1*. Comparing ectopic outgrowth in WT *dlk-1(+)* and *dlk-1(ju476)* backgrounds (Figs. 2*B*, black and clear bars, and 3*C*, *Right*), we find a significant but incomplete reduction of ectopic outgrowth under *dlk-1*. These

Fig. 1. Sensory dendrite cuts enhance ASJ regeneration in *C. elegans* WT and *dlk-1*. (*A*) Postsurgery GFP fluorescence images and line drawings of ASJ neuron with soma and dendrite (dark gray), normal axon path (blue), and regenerated fiber (red) in (*A1*–*A4*) dorsal-ventral and (*A5*) lateral views. White arrows indicate regenerative growth, white arrowheads indicate cut dendrites, and black arrowheads indicate typical axotomy location. (*A1*) Mock surgery. (*A2*) WT regeneration. (*A3*) Lack of regeneration in *dlk-1* after axon surgery. (*A4*) Regeneration in *dlk-1* after axon+dendrite surgery. (*A5*) Regeneration directed along ring in *dlk-1; tax-4* after axon surgery. (*B*) *dlk-1* eliminates and dendrite cut restores regeneration. Percentage of neurons regenerating after surgery ($45 \leq n \leq 68$). (*C*) Regenerated fibers aimed at the nerve ring, where original synaptic targets are located. Percentage of neurons with indicated regeneration extent defined by fiber with farthest outgrowth. Full-length regeneration included in regeneration "growth along ring" category. Data are aggregated across multiple genetic backgrounds, all containing *dlk-1* ($80 \leq n \leq 337$). Fig. S1*C* shows single-background data. (*D*) Dendrite cuts enhance axon regeneration in the WT. (*Upper*) Total regeneration lengths for various surgeries and backgrounds. Each point indicates a single animal measurement. Only 1 of 15 *dlk-1* neurons regenerated for L2 surgery (12-h reimaging; at L3); points are not shown for simplicity in this case. (*Lower*) Percentage of neurons with indicated regeneration extent defined by farthest outgrowth as in *C*. Data are represented as average ± SD. a+d, axon+dendrite. $^*P < 0.05$; $^{**}P < 0.001$.

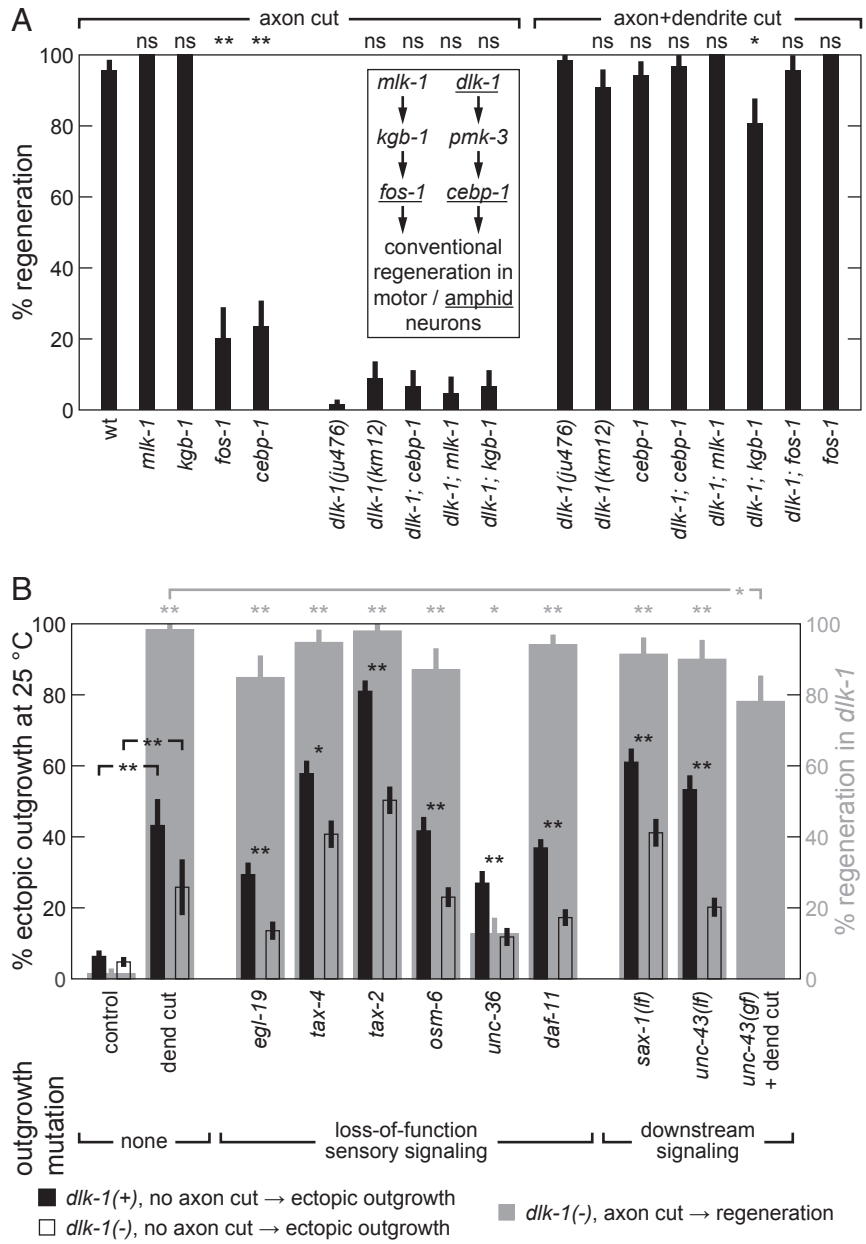


Fig. 2. (*A*) MAPK genes are not critical for DLK-independent regeneration. Percentage of neurons regenerating after indicated surgery ($30 \leq n \leq 68$, except for $n \geq 20$ for strains with *fos-1* or *mlk-1*). WT and *dlk-1* data are replicated from Fig. 1*B*. *P* values are calculated comparing data point with control value (leftmost data point in each group). (*B*) Comparison of DLK-independent regeneration and ectopic outgrowth. Gray-shaded results: Outgrowth-inducing mutations and dendrite cuts trigger DLK-independent regeneration. Percentages of neurons regenerating after axon lesion are shown ($30 \leq n \leq 68$). *P* values (indicated above plot) are calculated comparing data point with control (i.e., *dlk-1* with axon cut), except for *unc-43(gf)*. Leftmost two columns are replicated from Fig. 1*B*. Black and clear bars: Dendrite cuts trigger and *dlk-1* partly mediates ectopic outgrowth. Percentages of neurons with ectopic outgrowth ($n \geq 150$, except $n \geq 31$ for dendrite cut data). *P* values (indicated directly above data) are calculated comparing measurements from different *dlk-1* alleles or as indicated. Data are represented as average ± SD. ns, Not significant. $^{*}P < 0.05$; $^{**}P < 0.001$.

data suggest that both DLK-mediated and DLK-independent processes underlie ectopic outgrowth, similar to regeneration.

Finally, we asked if ectopic outgrowth and DLK-independent regeneration might share the same underlying regulation. Ectopic outgrowth is regulated by proteins involved in the control of neurite outgrowth in development and cell maintenance (9). For example, SAX-1 is a conserved nuclear dbf2-related (NDR) family kinase that stabilizes neurite morphology cell autonomously (26), and UNC-43 is a homolog of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) that modulates neurite outgrowth during development and mediates activity-dependent neural plasticity (27–29). These two proteins also restrict ectopic outgrowth in *C. elegans* (9). We found that the *sax-1(ky211)* and *unc-43(n1186)* loss-of-function (lf) mutations generate DLK-independent regeneration without a dendrite cut (Fig. 2*B*, gray bars). In a complementary manner, the gain-of-function (gf) mutation *unc-43(n498)* successfully inhibits regeneration after axon+dendrite surgery, decreasing it by ∼20%. To confirm these findings in another neuron, we performed surgery on the ALM axon in *dlk-1* and *unc-43* mutant backgrounds. As further described in *SI Results*, results are consistent with ASJ findings indicating two parallel regenerative pathways that are at

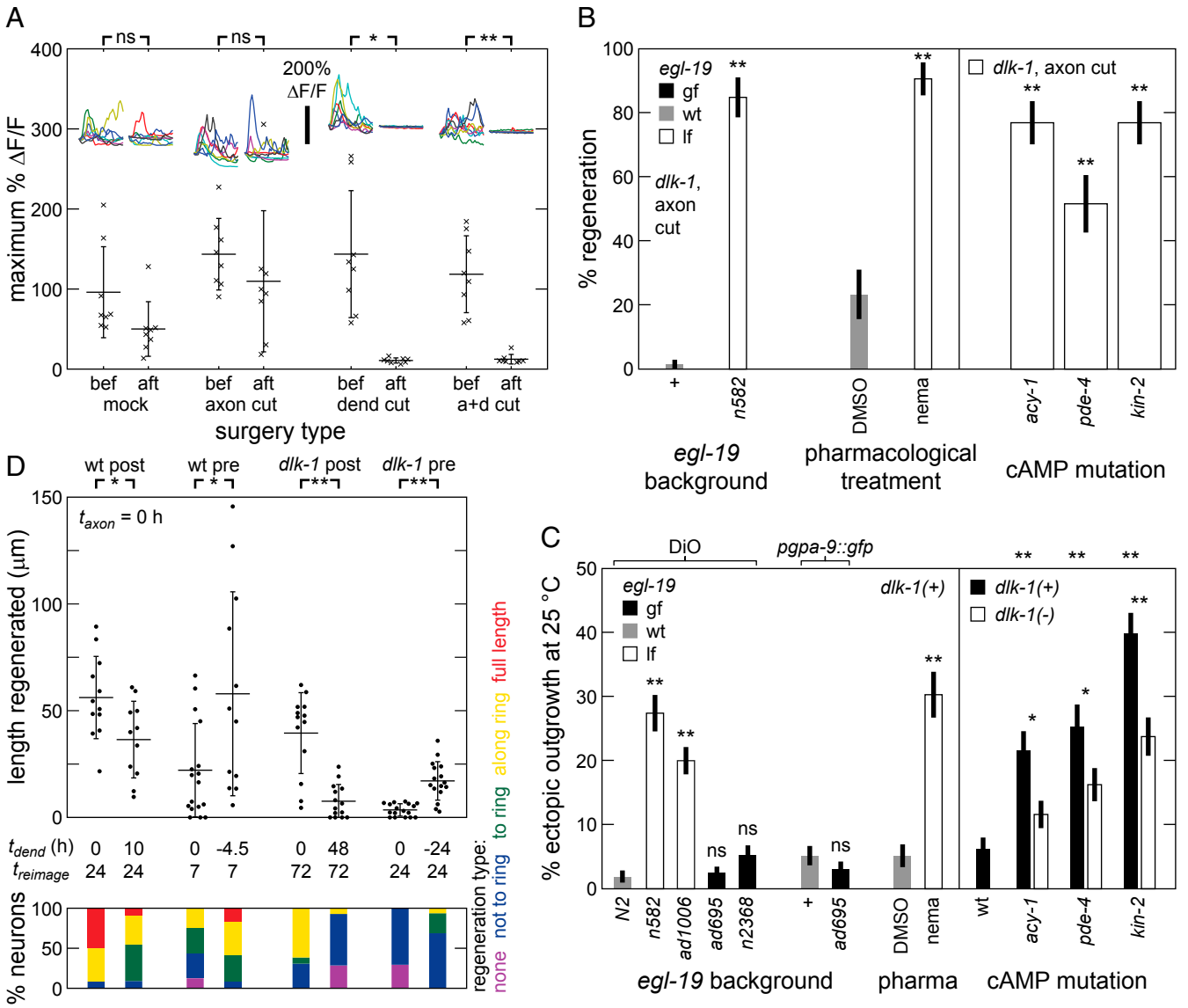


Fig. 3. (*A*) Dendrite lesion abolishes sensory-evoked activity in ASJ neuron. Intracellular calcium dynamics before (bef) and after (aft) ASJ surgery. Each x indicates a single animal measurement. *Insets* show 30-s calcium traces. (*B* and *C*) Reduction of EGL-19 function and elevation of cAMP signaling trigger DLK-independent regeneration and ectopic outgrowth. Modulation of function by genetic lesion of *egl-19* and pharmacology. (*B*) Percentage of neurons regenerating ($n \geq 30$). *P* values are calculated comparing data point with *dlk-1*, except for that nemadipine-treated was compared with DMSO. Leftmost two columns are replicated from Fig. 2*B*. (*C*) Percentage of neurons with ectopic outgrowth ($n \geq 150$). ASJ visualized by DiO or fluorescent protein. Significance indicators positioned directly above data are calculated comparing data point with control value (leftmost data point in each group). Significance indicators positioned between data points compare data from different *dlk-1* alleles. WT data in *Right* are replicated from Fig. 2*B*. (*D*) Dendrite cuts enhance axon regeneration in a time-dependent manner. (*Upper*) Total regeneration lengths for various surgeries and backgrounds. Each sequential surgery set is processed concurrently with a concomitant surgery set. All axons were cut at $t_{axon} = 0$ h. Dendrite cut (t_{dend}) and reimaging ($t_{reimage}$) were at indicated times. Each point indicates a single animal measurement. (*Lower*) Percentage of neurons with indicated regeneration extent defined by farthest outgrowth. Data are represented as average ± SD. a+d, axon+dendrite; ns, not significant. $*P < 0.05$; $**P < 0.001$.

least partly redundant: one that is DLK-mediated, and a second one that is DLK-independent and negatively regulated by *unc-43*. In summary, the results above demonstrate many connections between DLK-independent regeneration and ectopic outgrowth, suggesting that they share a common activity-dependent mechanism for triggering and control of neuronal growth.

Reduction of Sensory and Calcium Activity and Elevation of cAMP Signaling Trigger DLK-Independent Regeneration and Ectopic Outgrowth. Recent publications have directly demonstrated in multiple amphid neurons that ectopic outgrowth mutations reduce neuronal activity (summarized in *SI Results*). Given its role in triggering activity-dependent ectopic outgrowth, we directly tested if sensory dendrite lesion might reduce ASJ activity. Bright light stimulates ASJ neuron activity (30), which we measured by monitoring cytoplasmic calcium levels using the genetically encoded calcium-sensitive fluorophore GCaMP3 (31). Before surgery, the ASJ neurons generate a robust response to 30 s of 10 mW/mm^2 blue light ("bef" conditions in Fig. 3*A*). After mock ablations or axotomy, the light response ("aft") to a subsequent 30 s of 10 mW/mm^2 blue light is not statistically different from the initial response. However, severing sensory dendrites (either alone or with axotomy) completely eliminates the light response. Thus, sensory dendrite lesion but not axon lesion abolishes ASJ sensory-evoked activity. Interestingly, the DRG

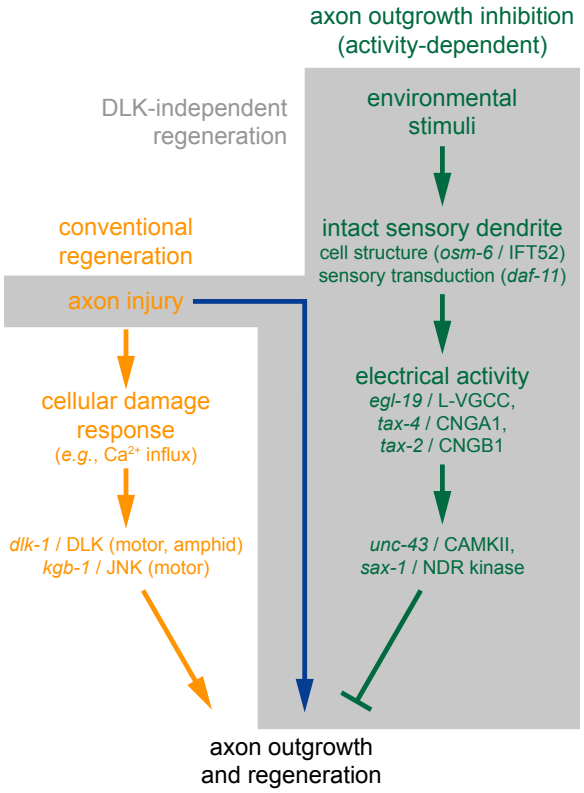


Fig. 4. Neurite growth mechanisms. Simplified working model of neurite growth mechanisms in *C. elegans*. The orange and green pathways represent conventional regeneration and ectopic outgrowth, respectively. The shaded pathways comprise a DLK-independent regeneration, which shares characteristics with mammalian lesion conditioning.

neurons of the mammalian CNS display the same phenotype (5), where lesion of the peripheral sensory but not central axon abolishes DRG sensory-evoked activity. Although endogenous (i.e., resting) activity of mammalian DRG neurons is not significantly changed by neurite lesions, the loss of sensory-evoked activity appears to be an important signal to trigger central axon regeneration by lesion conditioning.

The L-VGCC and, specifically, its pore-forming subunit $Ca_v1.2$ are important components in neuronal calcium signaling and play a central role in lesion conditioning (5). We performed experiments to further define the channel's role in DLK-independent regeneration and ectopic outgrowth. As noted above, genetic disruption of the *CACNA1C* homolog *egl-19* triggers DLK-independent regeneration without dendrite cut (Fig. 3*B*). Likewise, the EGL-19 antagonist nemadipine-A (32) also triggers robust DLK-independent regeneration without dendrite cut (Fig. 3*B*): we find significantly more neurons regenerated under nemadipine-A than its solvent vehicle dimethyl sulfoxide (DMSO). By contrast, the original ectopic outgrowth studies indicate that gain-of-function mutation *egl-19(ad695)* triggers ectopic outgrowth (10). To more fully understand this possible discrepancy, we examined the impact of both *egl-19(lf)* and *egl-19(gf)* mutations on ectopic outgrowth. Contrary to the original study, our results, including the sequencing of strains from the original study (*SI Results*), demonstrate that *egl-19(lf)* but not *egl-19(gf)* triggers ectopic outgrowth (Fig. 3*C*). Moreover, blocking EGL-19 with nemadipine-A also triggers ectopic outgrowth (Fig. 3*C*). These results demonstrate that reductions of EGL-19 function trigger ectopic outgrowth and DLK-independent regeneration. Because calcium influx through voltage-gated calcium channels plays a critical role in neuronal activation and sensory signaling, these findings are consistent with an activity-inhibited regenerative pathway. In mammals, reduced L-VGCC calcium current contributes to axon regeneration of DRG neurons (5), suggesting a possible mechanistic link to the outgrowth mechanisms that we observe here.

Increased cAMP signaling enhances regeneration in *C. elegans* as well as the mammalian CNS (16, 33–36). Therefore, we tested if elevated cAMP signaling can induce DLK-independent regeneration in the ASJ neuron. Adenylate cyclase catalyzes the production of cAMP, which is degraded by cAMP phosphodiesterase. Gain-of-function adenylate cyclase *acy-1(md1756gf)* (37) and loss-of-function phosphodiesterase PDE4 *pde-4(ce268)* (38) both increase neuronal cAMP signaling and enable robust regeneration of the ASJ after axotomy alone in a *dlk-1* mutant background (Fig. 3*B*). Likewise, constitutive elevation of the cAMP signaling effector protein kinase A by mutation of its regulatory subunit *kin-2* (37) also enables DLK-independent regeneration after axotomy alone. Consistent with our other findings, these mutations also trigger ectopic outgrowth (Fig. 3*C*). These results indicate that, similar to lesion conditioning, elevated cAMP signaling enhances DLK-independent regeneration and ectopic outgrowth.

In a classic lesion conditioning experiment, peripheral sensory axotomy before central axotomy, known as preconditioning, more strongly improves central axon regeneration than concomitant axotomies and vice versa, suggesting a cellular intrinsic growth capacity that is enhanced by peripheral axotomy (39). To assess if the ASJ exhibits a similar phenotype, we separated sensory dendrite and axon surgeries by 5–48 h. Azide anesthesia likely reduces regeneration, and therefore, we processed each sequential surgery set concurrently with a concomitant surgery set, treating these animals alike except for laser surgery. Comparing the regenerated axon length in both WT and *dlk-1* animals, we find that dendrite surgery after axon surgery ($t_{dend} > 0$ h; "post" comparison) produces less regeneration than concomitant surgeries ($t_{dend} = 0$ h), whereas dendrite surgery before axon surgery ($t_{dend} < 0$ h; "pre" comparison) produces more (Fig. 3*D*). These results underscore the dendrite lesion's crucial role in enhancing axon regeneration and provide another phenotypic similarity between DLK-independent regeneration and mammalian lesion-conditioned regeneration.

Discussion

Our work describes regeneration in *C. elegans* amphid neurons that is triggered by lesion of both the axon and sensory dendrite. This novel regeneration is largely independent of DLK-1/DLK, KGB-1/JNK, and other known MAPK signaling factors underlying neuronal regeneration in *C. elegans* and other organisms. Although a recent study showed that dendrite regeneration in *Drosophila* dendritic arborization neurons is also independent of both DLK and JNK (40), an analogous mechanism initiated by axon surgery has not been defined.

Our results indicate a direct link between DLK-independent regeneration and activity-dependent ectopic axon outgrowth, with comparable morphologies, late time of outgrowth, overlapping set of inducing mutations and surgeries, and at least partial DLK independence. Our work suggests that examining nematode ectopic outgrowth could provide a fruitful source of insight into other vertebrate and invertebrate regeneration mechanisms and vice versa. Similar ectopic outgrowths occur in mammals and are often associated with disease states. For instance, defects in the *tax-2* homolog *CNGB1* and the L-VGCC gene *CACNA1F* trigger ectopic outgrowth in mouse models of retinitis pigmentosa (41) and congenital stationary night blindness (42). Information gained from these models may be combined with results in neuronal regeneration to inform multiple areas of study.

DLK-independent regeneration and ectopic outgrowth in *C. elegans* also share numerous similarities with lesion-conditioned regeneration observed in the mammalian CNS. They are all

triggered by genetic or pharmacological inhibition of L-VGCCs or by damage to sensory neurites causing a reduction of sensory activity. Furthermore, all are mediated by cAMP activity. Importantly, DLK-independent and lesion-conditioned regeneration also exhibit a similar regeneration phenotype dependent on the sequence and timing of neurite lesions, including a preconditioning effect. In mammals, DLK underlies preconditioning of peripheral axon regeneration (21), a conditioning effect on DRG neurons in vitro (43), and retinal ganglion cell regeneration after optic nerve crush (44). Nonetheless, the similarities with the *C. elegans* DLK-independent regeneration observed here suggest the possibility of modeling aspects of CNS lesion conditioning in genetically accessible systems.

Although regenerative processes vary with cell type and context (*SI Results*), our findings suggest a simple working model for regeneration and outgrowth in *C. elegans* (Fig. 4). Physical damage of the axon leads to conventional regeneration through specific damage-induced calcium signaling that initiates *dlk-1*– and *kgb-1*–mediated regeneration (orange pathway in Fig. 4). Independently, sensory activity of the intact neuron normally suppresses ectopic outgrowth (green pathway in Fig. 4) in *C. elegans*. Our results and previous studies (*SI Results*) indicate that the dendritic cilia transduce external stimuli to generate amphid sensory activity. Activity propagates by ion channels, resulting in calcium influx through EGL-19/L-VGCC and activation of SAX-1/NDR kinase and UNC-43/CaMKII, which inhibit outgrowth. Disruption of this pathway at any point by dendrite lesion, genetic mutation, or pharmacology removes the suppression, enabling neurite growth within two contexts. Ectopic outgrowth occurs without axotomy when animals are grown under stressed conditions. Alternatively, axon injury (blue line in Fig. 4) can activate DLK-independent regeneration. Thus, this novel DLK-independent regeneration (gray area in Fig. 4) requires both a central axon injury signal and the elimination of activity-dependent developmental blocks mediated by the peripheral sensory neurite.

Our study identifies key negative regulators of regeneration, *sax-1* and *unc-43*, which also restrict outgrowth during neuronal maturation. SAX-1 and its *Drosophila* homolog Tricornered negatively regulate dendritic growth during development to establish correct sensory neurite tiling in worms and flies (45, 46). In mammals, NDR kinases suppress formation of supernumerary axons during polarization of developing hippocampal neurons in vitro and are activated by calcium (47, 48), suggesting a possible regulatory link with neuronal activity and the underlying calcium signal (9). Likewise, increased CaMKII activity causes maturation of dendritic morphology, slowing outgrowth and stabilizing dendritic structures in developing neurons of the *Xenopus* optic tectum (29). A recent study revealed a specific developmental pathway, whereby calcium entry through L-VGCCs in response to sensory activity controls dendrite complexity in the *Xenopus* visual system as well as cultured rat cortical neurons. These results suggest that CaMKII activation in response to elevated calcium up-regulates transcription of the GTPase Rem2 to restrict dendrite outgrowth and branching (49). Taken together, our findings suggest that the same activity-dependent pathways restricting neurite outgrowth during development through activation of SAX-1/NDR kinase and UNC-43/CaMKII can also constitutively block regeneration after axonal injury in the adult.

Electrical activity is a key modulator of neurite outgrowth during development and after injury. Within this context, our work establishes the ASJ neuron in *C. elegans* as a powerful experimental model for studying DLK-independent regeneration and suggests ectopic outgrowth as a tractable gene discovery platform. Employing this model, we demonstrate that, after axonal injury, neuronal sensory activity restricts outgrowth by known developmental regulators of neurite outgrowth. Removal of this inhibition triggers a novel regeneration pathway largely independent of DLK and JNK signaling. The striking similarities between DLK-independent regeneration in *C. elegans* and lesion-conditioned regeneration in the mammalian CNS suggest a conserved mechanism. As such, our study establishes a framework for defining the mechanisms that modulate a neuron's intrinsic growth capacity and ultimately enable robust regeneration within the adult nervous system.

Materials and Methods

Cultivation and Strains. Following standard methods (6), we cultured strains on nematode growth medium (NGM) plates inoculated with OP50 bacteria (i.e., seeded plates), the primary food source for laboratory *C. elegans*, and maintained animals at 20 °C unless otherwise stated. We cultivated strains at 25 °C for ectopic outgrowth experiments, except for *daf-11*, which we temperature-shifted from 15 °C to 25 °C after the dauer entry decision (8). Experiments in liquid solution used NGM buffer, which includes the same inorganic salts as NGM agar. We confirmed the genotype of all mutant strains [except *egl-19(ad1006)*, which is not defined] by polymerase chain reaction and gel electrophoresis (larger deletions) or sequencing (small-scale mutations) as well as defined phenotypes. Primer sequences are available on request. *SI Materials and Methods* lists the fluorescent and mutant parent strains used to generate this study's strains. Unless otherwise stated, *dlk-1* refers to *dlk-1(ju476)*.

Imaging and Neurite Surgery. We followed established procedures for immobilization, imaging, surgery by femtosecond laser ablation, and post-surgery reimaging (23, 50). We immobilized L4 and adult animals with 5 mM sodium azide and younger animals with 2 mM sodium azide, except for in calcium imaging experiments (see below). We used cell-specific green fluorescent protein (GFP) (51), GCaMP (calcium-sensitive fluorophore) (31, 52), and $DiOC_{18}$(3), also known as DiO (53) to image neurons. We primarily visualized the ASJ by *ptrx-1::trx-1::gfp*, an extrachromosomal translational fusion GFP, because it was the clearest and brightest cell-specific GFP. Overexpression of this transgene has no effect on outgrowth rates (Fig. S4). We scored individual neurons for ectopic outgrowth of greater than 2 μm, similar to established procedures (10). In the course of this study, we found that ectopic outgrowth displays a strong maternal effect (i.e., treatment of one generation modulates ectopic outgrowth rates in the subsequent generation). These results are detailed in *SI Results* and Fig. S5. Thus, to avoid confounding effects, all strains were subject to the same conditions or surgery for multiple generations in the ectopic outgrowth experiments, except as noted.

Femtosecond laser surgery has submicrometer precision in the sample bulk (23) and permits cell-specific surgery of the ASJ dendrite within the amphid bundle and axon proximal to the amphid commissure (18). Unless otherwise noted, we performed laser surgery in L2 or L3 life stage with 3-nJ pulses at 10-kHz repetition rate, severing axons within several micrometers of the cell body and dendrites near their midpoint. Under these conditions, severed dendrites can persist for days, but severed axons typically decay within 1 d. Although a report noted AWB regeneration after dendrite surgery (54), we do not observe regeneration after ASJ dendrite surgery (23). Unless otherwise stated, we reimaged animals 3–5 d after surgery, and we only counted animals with successful surgery, noted by the absence of a neurite with normal morphology. For most experiments, we scored individual neurons for the presence of regeneration (greater than 2 μm) rather than measuring outgrowth length. The reasons underlying the above methodology are listed in *SI Materials and Methods*. We measured regenerated fiber lengths by using the ImageJ plugin 3D Distance Tool available at the NIH ImageJ website. The plugin measures the distance between successive selected points in a *z* stack. As depicted in Fig. S2*A*, we selected points unidirectionally along the entire length of the regenerated fiber (slices 4–6) and measured distinct branches separately (red and yellow points in Fig. S2*A*). Summing the distances, we obtained the total regenerated length of new fibers (maximum projection shown in Fig. S2*B*).

Calcium Imaging. We measured intracellular calcium levels as an indicator of neuronal activity using animals expressing ASJ-specific GCaMP3. Following standard procedures (15), we immobilized *C. elegans* using 2% (wt/wt) agar pads with 0.05% tetramisole. The ASJ neuron displays a robust response to higher intensities of the same 480-nm blue light (30) used for GCaMP3 imaging, and therefore, we located the ASJ cell body using low light illumination (<1 mW/mm^2) that does not elicit a response and stimulated neurons with higher intensities. In agreement with earlier studies, we first measured response to a range of illumination stimuli and found robust response to intensities of 10 mW/mm^2 and above but lower response at lower intensities. We used 10 mW/mm^2 for all subsequent experiments. Animals

were left in the dark for >30 s before exposure to 30 s of intense light while recording 1 frame per second. As described previously (55), we quantified GCaMP3 fluorescent intensities as the average intensity across the ASJ cell body normalized to the initial intensity (i.e., in the first frame). As shown in Fig. 3*A*, responses are temporally variable, and therefore, we quantified the response size from each trial as the maximum amplitude change in fluorescence across the 30-s recording. For experiments combining Ca^{2+} imaging with laser surgery, we measured the neurons response, performed the surgery as noted above, allowed animals a 30-min recovery, and measured the light response of the neuron again.

Nemadipine-A Treatment. We made a stock solution of 10 mg/mL nemadipine in dimethyl sulfoxide (DMSO) and diluted 4.2 μL stock solution (or DMSO) in 76 μL NGM buffer. We made nemadipine (or DMSO) plates by spreading this mixture on the surface of a 6-cm seeded plate. We allowed plates to dry overnight, introduced gravid animals on the plates the next day, and picked L2 or L3 animals for surgery after two more days.

Statistics and Interpretation of Results. Most of the ASJ regeneration and ectopic outgrowth data are binary: we score whether or not neurons regrow or outgrow. We calculated *P* values for these data by Fisher's exact test. For regenerated length measurements, we calculated *P* values by the unpaired, unequal variance, two-tailed *t* test. For calcium imaging data, we calculated *P* values by the paired, two-tailed *t* test. For ALM regeneration length data, we conducted a one-way analysis of variance (ANOVA) to compare the effect of genetic background on regenerated length in WT and mutant *dlk-1* and *unc-43* conditions. There is a highly significant effect of genetic background on regenerated length for the six conditions [$F(5,131) = 14.7$; $P < 0.0001$]. We performed posthoc comparisons using the Tukey test. Data are represented as average ± standard deviation (SD). We indicate values that differ at $P < 0.05$ (*) and $P < 0.001$ (**) levels.

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