Supplemental Information for

In vivo dendrite regeneration after injury is different from dendrite development

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Supplemental Materials and Methods
Figures S1 to S6

Supplemental Materials and Methods

Drosophila strains

We used the following Drosophila strains : w; ppk-CD4-tdGFP^{1b} (Han et al. 2011); ppk-GAL4^{1A} (Grueber et al. 2003a), w ;; ppk-CD4-tdGFP^{8}/TM6B, Tb (Han et al. 2011), w; UAS-DCR2 / CyO ; GAL4$^{2-21}$, UAS-tdGFP / TM6 (Cheng et al. 2010), w;; GAL4$^{19-12}$, UAS-CD4-tdGFP, repo-Gal80 / TM6B (Yan et al. 2013), w; ppk-CD4-tdGFP^{1b} ; repo-Gal4, UAS-CD4-tdTomato (Song et al. 2012), arm-GFP ;; ppk-Gal4, UAS-mCD8-RFP/TM3 (Parrish et al. 2009), w; UAS-mys UAS-αPS1 / TM6B, Tb (Han et al. 2012), ;;UAS-DSCAM-GFP [3.36.25.1+.19+.23] (Soba et al. 2007), w;; UAS-KCNJ2$^{[7]}$ (Bloomington #6595), UAS-ORKΔC (Nitabach et al. 2002), UAS-EKO (White et al. 2001), ppk-CD4-tdTomato ppk-GS, UAS-Kir2.1-GFP (Nicholson et al. 2008), w; UAS-cut.L / CyO, Weep (Grueber et al. 2003a), UAS-ss (M. D. Kim et al., 2006), UAS-knot (Jinushi-Nakao et al. 2007).

Two Photon Injury and Imaging

Eggs were collected on grape plates with yeast paste, to synchronize the age of the animals. After allowing for embryonic and early larval development, dendrites were injured as follows. Larvae were immobilized individually on agarose pads sandwiched between a slide and a coverslip. All dendrites were severed from da neurons by focusing a Chameleon 2-photon 930nm laser mounted on a custom-built Zeiss fluorescence microscope at every primary branch point proximal to the cell body, in a modified version of previous assay (Song et al. 2012; Stone et al. 2014). Mock injured neurons were adjacent uninjured neurons that went through the same

process of mounting on an agarose pad, but whose dendrites were uninjured by the 2-photon. For some experiments, the class IV da neuron to the anterior and posterior were ablated by focusing the 2-P laser on their cell body (**Figure 1**). Images of neurons before or just after balding are single Z-slices, so the dendrite branch sometimes goes above or below the focal plane (for example, **Figure S1A**). After injury, animals were transferred to solitary grape plates with yeast paste, and allowed to recover and develop there between imaging sessions. Neurons were imaged approximately 24hrs after injury (to confirm that all dendrite branches had been severed) and 72hrs after injury (to assess regeneration). These images of animals on agarose pads were collected on a Leica SP5 confocal microscope. Images of neurons 24hrs or 72hrs after injury are maximum intensity projections of Z-stacks through the entire dendrite arbor. Class I da neurons are ddaE. Class III da neurons are ddaA. Class IV *da* neurons are ddaC, unless otherwise noted (vdaB in **Figure 2C**, v'ada in **Figure S4**). For pruning experiments, ddaC neurons were imaged at specified times after pupal formation (APF), and the autofluorescence of the pupal case was subtracted from the final images. For class I da neuron RNAi knockdown and transcription factor overexpression experiments (**Figure S2**), a single dendrite branch was cut instead of balding the entire arbor. Unless otherwise noted, all scale bars are 50 µm; some **Figure 6** and **Figure S6** scale bars are 10 µm, as noted.

Quantification of Regeneration

We traced dendrite arbors using the Simple Neurite Tracer plug-in within ImageJ. Using this approach, we determined the number of dendrite branch tips, and the total length of all the dendrite branches. On these traced arbors, we performed Sholl analysis of dendrite branches crossing circles separated by 5µm. To determine percent territory coverage, we measured the

total area of the hemisegment by locating the segment boundaries and midline, then measured what fraction of that area had dendrite branches from the neuron of interest. Statistical tests were performed in Prism 6 (GraphPad) and are noted in the figure legends.

Immunostaining

Larvae were filleted and fixed for 30 minutes in 4% paraformaldehyde at room temperature after final imaging time point. Non permeabilized staining, using exclusively PBS, was used for *ppk1*, *ppk26* and NOMPC antibodies. PBS with 0.01% Triton-X was used for all other immunohistochemistry. Antibodies were used at: 1:10,000 α-ppk26 rabbit (Gorczyca et al. 2014), 1:300 α-abrupt mouse (DHSB-Stephen Crews), 1:1,000 α-NOMPC rabbit (Cheng et al. 2010), 1:20 α-*cut* (2B10) mouse (DHSB-Gerald Rubin); 1:100 α-Collier mouse (Dubois et al. 2007), 1:8,000 α-ppk1 rabbit (Gorczyca et al. 2014), 1:500 α-rabbit IgG, alexa 647 (Jackson labs 711-605-152), 1:500 α-mouse IgG, cy3 (Jackson labs 715-165-150).

Self-crossings

To determine self-crossings, animals were anesthetized with ethyl ether prior to mounting on agarose pads. Dendrites were imaged with a 63x objective with fine Z-slices. Contacting versus non-contacting crossings were determined based on whether dendrite branches were found in separate Z-planes, as before (Han et al. 2012). As the total dendrite length within the field of view is different for different genotypes and conditions (**Figure S3B**), the number of self-crossing events was normalized to the total dendrite length within the 63x objective's field of view.

For short term time lapse imaging of dendrite tip encounters, neurons were balded at 48hrs AEL, balding was confirmed 24hrs later, and time lapse images were acquired 48hrs after injury. Unanesthetized larvae were mounted for imaging, and coarse Z-stacks were captured every 2 minutes for 30 minutes.

Geneswitch

For drug-induced expression of Kir2.1, RU486 Mifepristone (Sigma M8046) was dissolved in ethanol, then mixed into the grape agar plates to a final concentration of 100 µM (Nicholson et al. 2008). Eggs were collected normally, and allowed to hatch on normal grape plates. Animals were moved onto plates with RU-486, or control plates without drug, 24hours before injury to allow Kir2.1 expression starting around the time of injury, and kept on plates with RU-486 for the rest of the experiment.

Electrophysiology

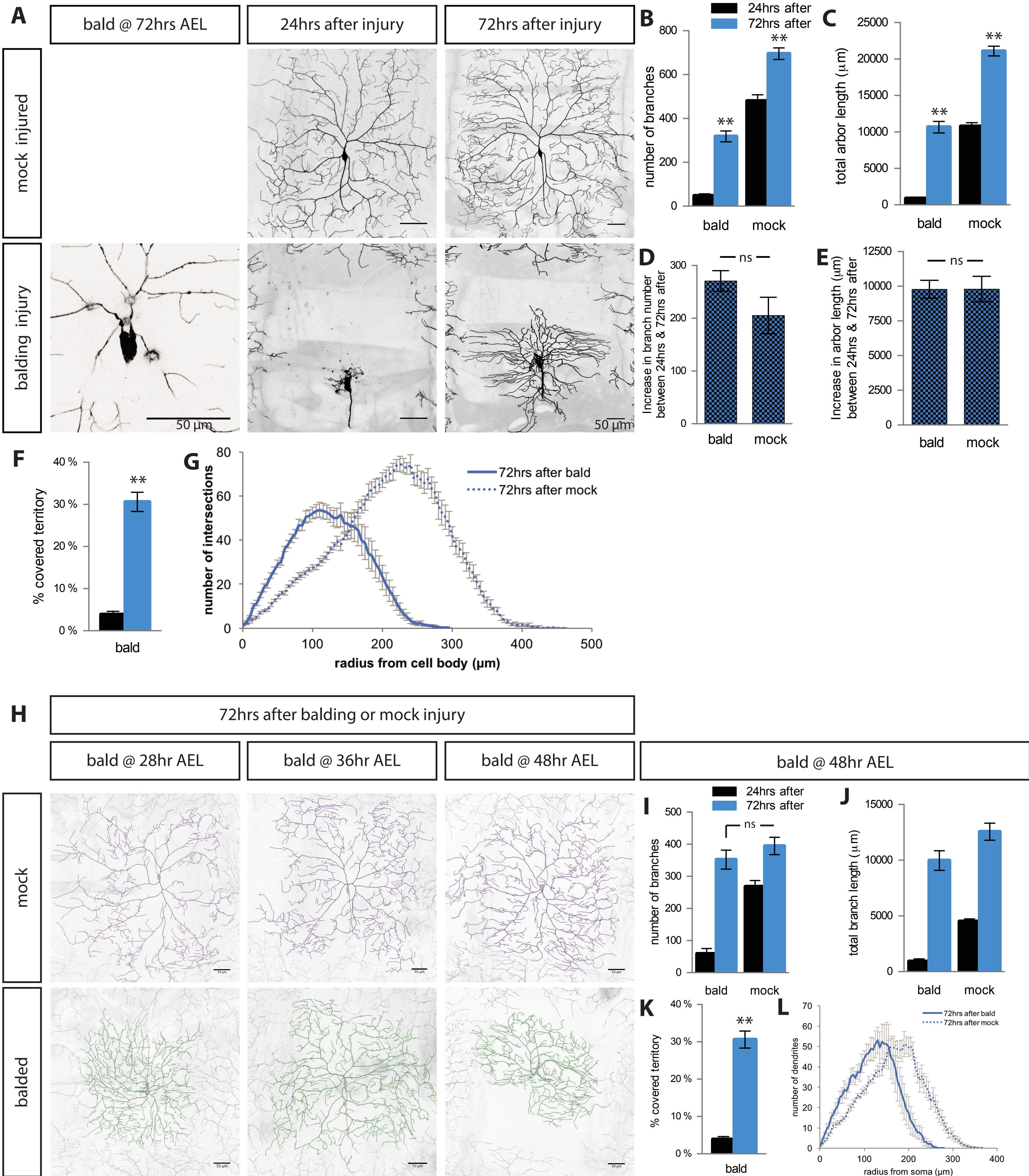
Extracellular field recordings of action potentials from class III ddaA neurons in response to gentle touch were performed in filleted larvae preparations as previously described (Yan et al. 2013) with the following modifications. Animals were 144hrs AEL at the time of fillet. Neurons were either uninjured control neurons; had been balded at 72hrs AEL and allowed 72hrs after injury to regenerate (with imaging at 24hrs after injury to confirm balding and 48hrs after injury to confirm regeneration); or had been balded approximately 5hrs before recording, allowing time for degeneration of the distal dendrites but not regeneration. Ablation of one specific ddaF neuron in another segment at the time of balding was used as a landmark to ensure that the correct ddaA neuron was identified and recorded from in the fillet. Gentle touch probe

stimulus was applied either adjacent to the cell body, or at the far hemisegment border. Number of action potentials 1 second after stimulus minus the number of action potentials 1 second before stimulus is reported. While control and regenerated neurons occasionally had endogenous unstimulated APs, recently balded neurons did not. Comparison of the response to various strength stimulus between conditions was analyzed using two-way ANOVA.

SUPPLEMENTAL FIGURES

Supplemental Fig. S1. Class IV da neurons exhibit robust growth after all dendrites are severed at any larval stage. **(A-G) At 72hrs after egg laying.** (**A**) Single Z-plane showing a class IV neuron, ddaC, just after severing all four primary dendrites. Maximum intensity projections show the same neuron 24hrs and 72 hours after injury, compared to a mock injured control neuron. (**B-C**) Number of branch tips (B) total dendrite length (C) in injured and control neurons, at 24 hrs (black) and 72hrs (blue) after dendrite severing or mock injury. (**D-E**) Increase in branch number (D) and total arbor length (E) between 24hrs and 72hrs after injury for injured and control neurons. (**F**) Increase in coverage of hemisegment territory between 24 hrs (black) and 72hrs (blue) after dendrite severing. (**G**) Sholl analysis of dendrite arbor complexity 72hrs after injury, for balded (solid) and control (dashed) neurons. All scale bars are 50 µm. ** $p<0.0001$ comparing 72hrs after injury (blue) to 24hrs after injury (black) by one-way ANOVA with Tukey's multiple comparisons test (B-C) or paired t-test (F). *ns,* comparing balded neurons to mock injured neurons, $p>0.05$ by paired t-test (D-E). n=14 balded neurons, 12 mock neurons. **(H-L) Younger class IV da neurons regenerate dendrite arbors**. (**H**) Neurons balded at 28hr AEL or 36hr AEL regenerate full dendrite arbors, but much of their territory is invaded by adjacent uninjured neurons, so their arbor occupies a smaller territory. (**H-L**) Neurons balded at 48hr AEL regenerate the number of branches of an injured neurons (I), almost regenerate the arbor length (J), and cover much of the body wall territory (K), but do not recover the architecture as determined by Sholl analysis (L) of an uninjured neuron. *ns*, $p>0.05$ by one-way ANOVA with Tukey's multiple comparisons test (I). ** $p<0.0001$ by paired t-test (K). n=8 bald neurons, 8 control neurons.

Thompson-Peer_Fig S1



Supplemental Fig. S2. Most manipulations equally affect dendrite branching, regardless of whether there is injury or not. (**A-B**) Class I neurons with a single branch severed (blue) regrow dendrite branches to match the number in control uninjured neurons (black outline) by 72hrs after injury or mock injury. RNAi knockdown (A) or overexpression of transcription factors (B) that cause branching defects in class I neurons effect similar changes in uninjured and injured neurons. *ns* $p>0.05$ by one-way ANOVA with Tukey's multiple comparisons. n=19 injured wt, 18 mock wt neurons, 6 injured Akt RNAi, 4 mock Akt RNAi neurons. n=18 injured wt, 37 mock wt, 14 injured UAS-cut, 12 mock UAS-cut, 13 injured UAS-ss, 9 mock UAS-ss, 12 injured UAS-knot, 21 mock UAS-knot neurons.

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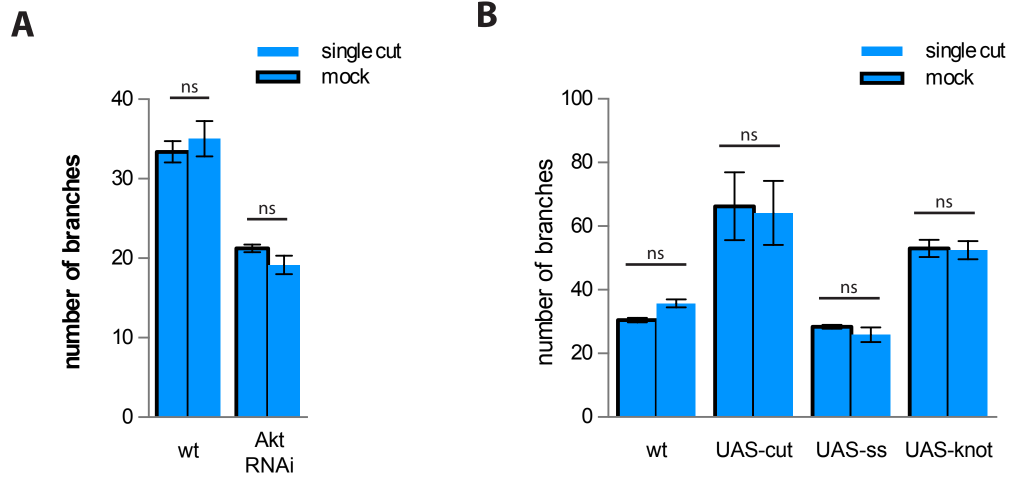
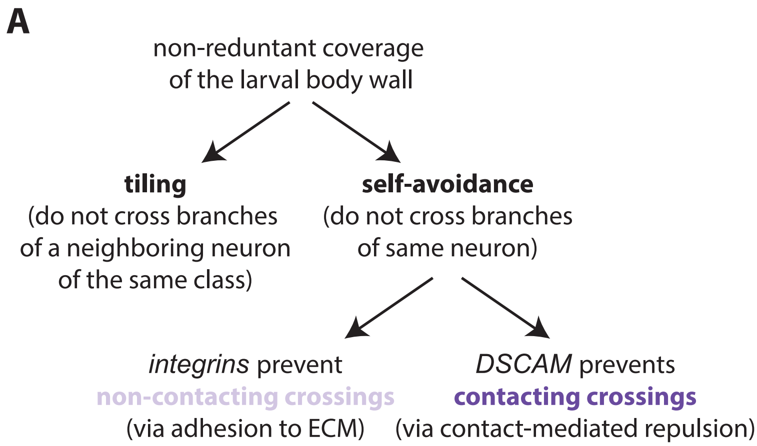


Supplemental Fig. S3. (**A**) Class IV da neurons use both tiling and self-avoidance to cover the body wall non-redundantly. Self-avoidance includes the absence of both contacting and non-contacting crossings. (**B**) Self-crossings are normalized to the total length of the dendrite arbor visible in the field of view, which varies greatly across genotypes. n=6 wt balded, 6 wt mock, 9 wt bald at 48hrsAEL, 10 wt mock at 48hrsAEL, 9 integrin OE bald, 9 integrin OE mock, 4 DSCAM bald, 4 DSCAM mock neurons. *ns* $p>0.05$, * $p<0.01$, ** $p<0.001$ comparing mock treated (no outline) to regenerated (black outline) dendrites by t-test. (**C**) Altering self-crossing can change territory coverage. *ns* $p>0.05$, * $p<0.05$, **$p<0.001$, compared to wild type balded at 72hrs AEL by t-test. n=14 wt, 9 wt balded at 48hrsAEL, 11 integrin OE, 4 DSCAM OE balded neurons. (**D**) A DSCAM transgene tagged with GFP forms puncta that are regularly spaced and localized in primary and terminal branches in uninjured neurons (top panels). In regenerated arbors, some branches have few DSCAM-GFP puncta (middle panels) while other branches have normal levels of DSCAM-GFP (bottom panels). Scale bars are 50 µm or 10 µm for insets. (**E**) Time lapse imaging of dendrite tip encounters shows that 48hrs after balding at 48hrs AEL, regenerated dendrite tips contact other branches of the same arbor more times over a 30 minute session, and that more of those events result in branch tips crossing over the other branch, rather than turning away or retracting. Average number of encounters per neuron reported, with branch tips crossing over (light red) or retracting and turning away (dark red). n=7 mock neurons, 8 regenerated neurons. * $p<0.05$ by one-way ANOVA.

A



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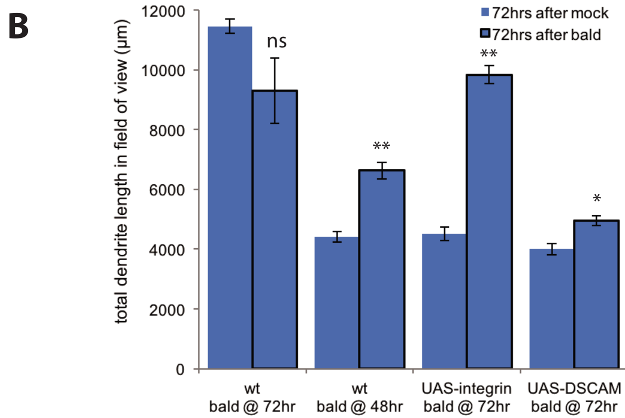

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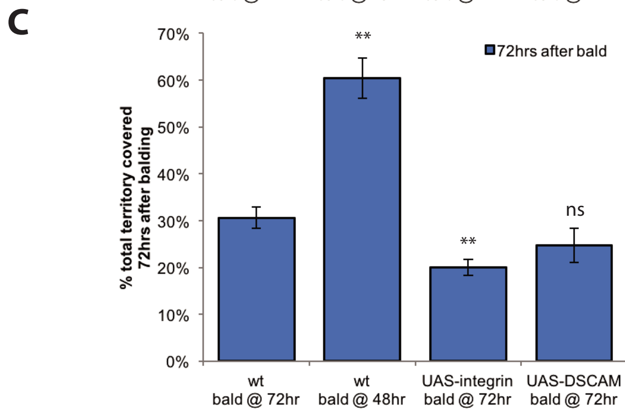

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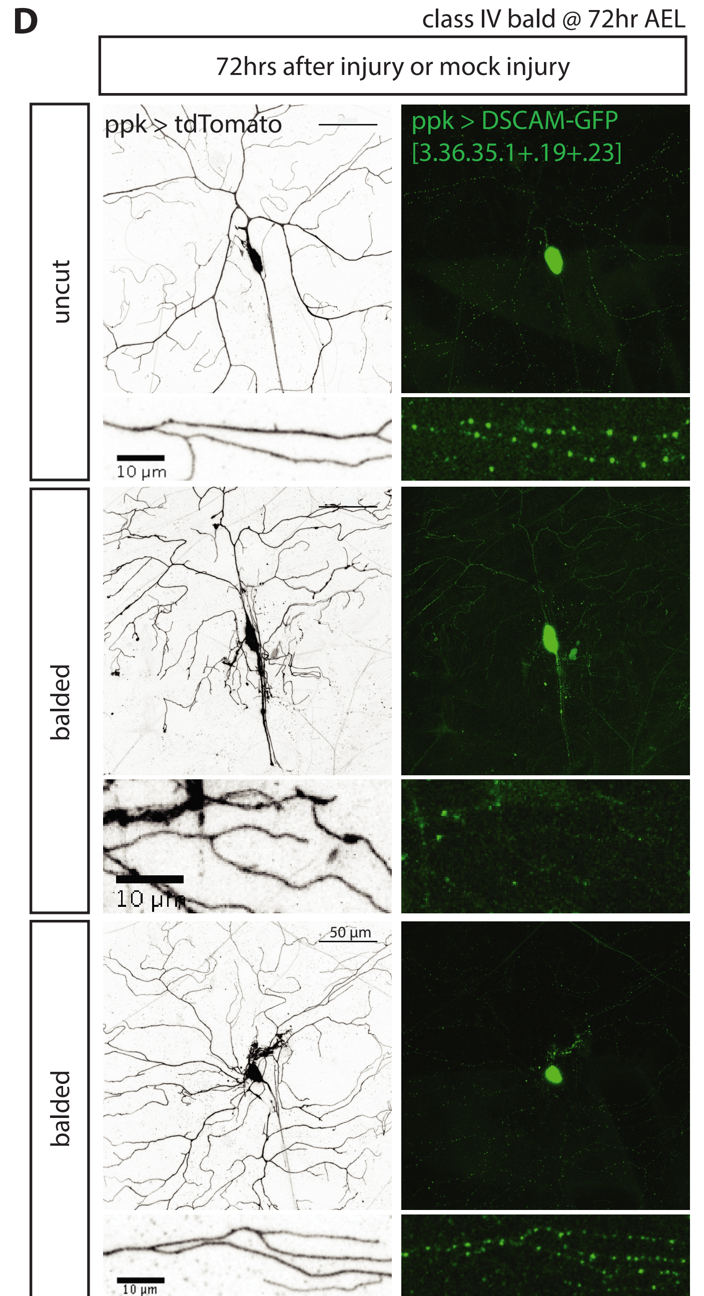

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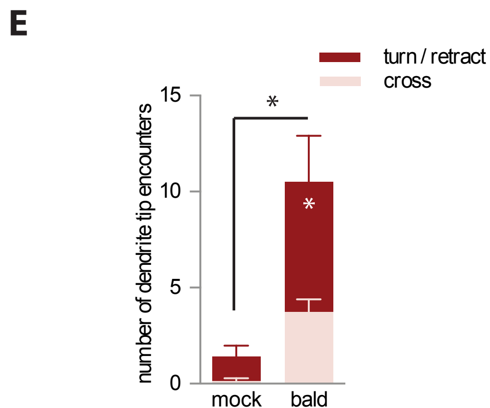

Supplemental Fig. S4. Neurons can go through multiple rounds of dendrite regrowth. Class IV v'ada neurons, which were balded and regenerated as larvae, then pruned during pupal metamorphosis, regrow adult dendrite arbors that look normal. n=6 adult neurons that had been balded during the larval phase, 5 control neurons.

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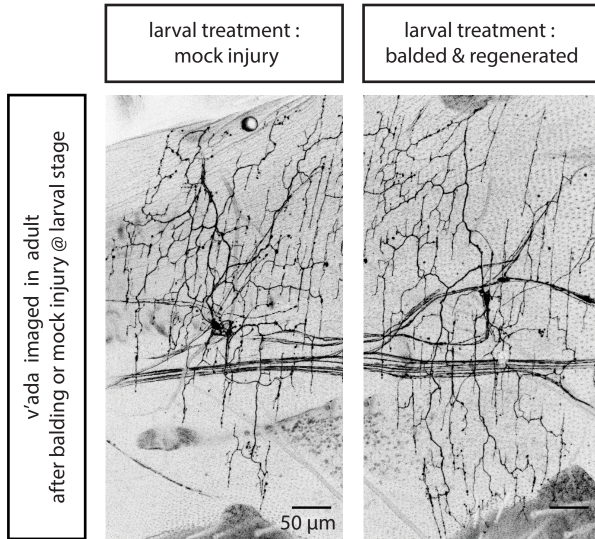

Supplemental Fig. S5. Hyperpolarizing neurons using K^+ channel transgenes blocks dendrite regeneration in both class I and class IV neurons. Compared to wild type neurons, overexpressing Kir2.1, ORK, or EKO is sufficient to significantly block dendrite regeneration in class IV *da* neurons (A-D) or class I *da* neurons (E-H), including formation of new branches (A, C, E, G) or lengthening of the dendrite arbor (B, D, F, H). Data are plotted at 24hrs after injury or mock injury (black, A-B, E-F) and 72hrs after injury or mock injury (blue, A-B, E-F), or the difference between those two time points (blue-black hatching, C-D, G-H). Comparison between 24hrs versus 72hrs of the same genotype (and the same mock or bald injury) indicated within the bars, by one-way ANOVA with Sidak's multiple comparisons test, ** p<0.001, *ns* p>0.05. Comparison of the increase in branch number or total length between wild type and other genotypes of the same condition indicated on top of the bars, * p<0.05, ** p<0.001, *ns* p>0.05 by one-way ANOVA with Sidak's multiple comparisons test. For class IV da neurons: n = 14 wt bald, 12 wt mock, 17 Kir bald, 15 Kir mock, 14 ORK bald, 6 ORK mock, 6 EKO bald, 7 EKO mock neurons. For class I da neurons: n = 24 wt balded, 36 wt mock, 20 Kir balded, 31 Kir mock neurons.

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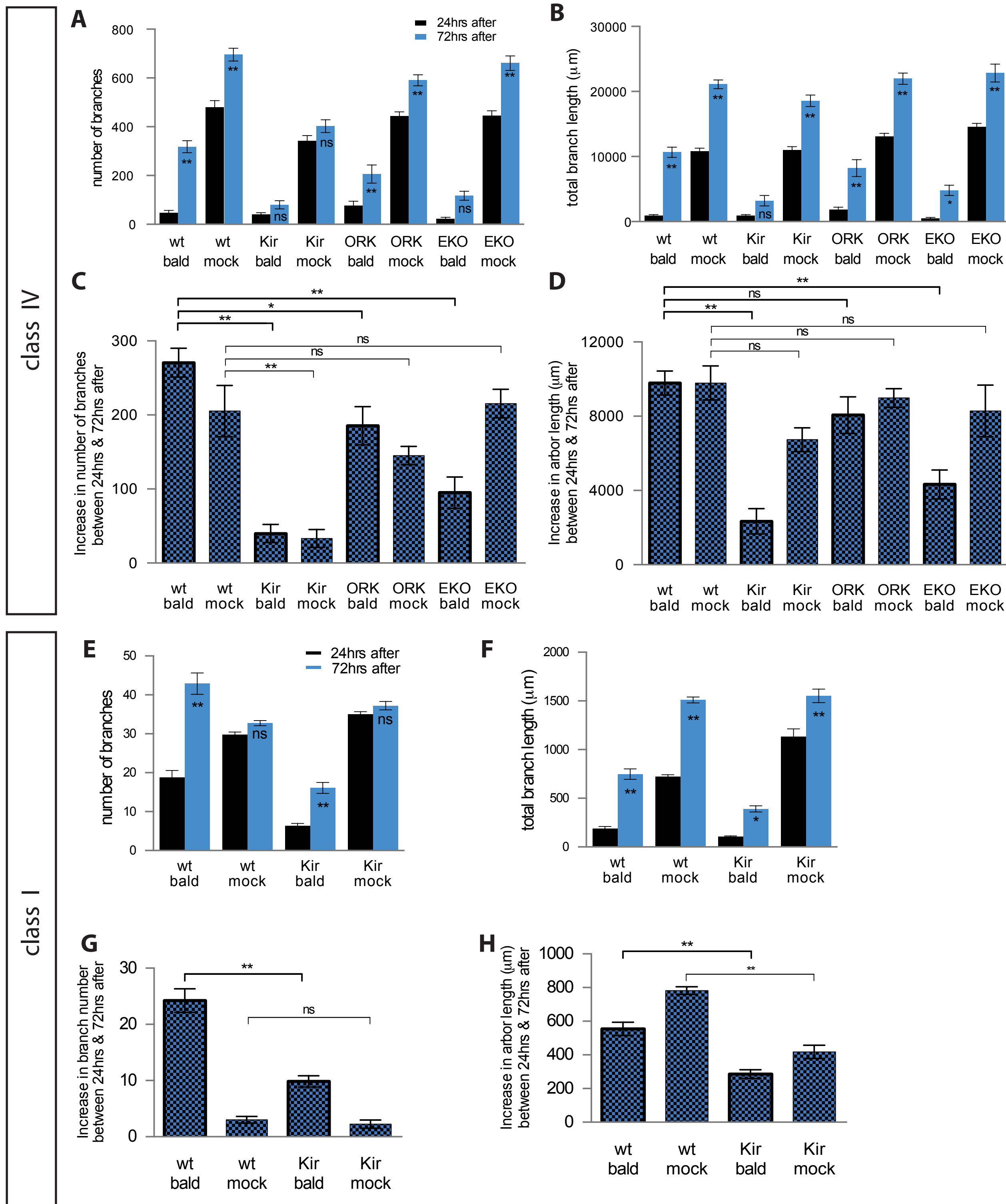
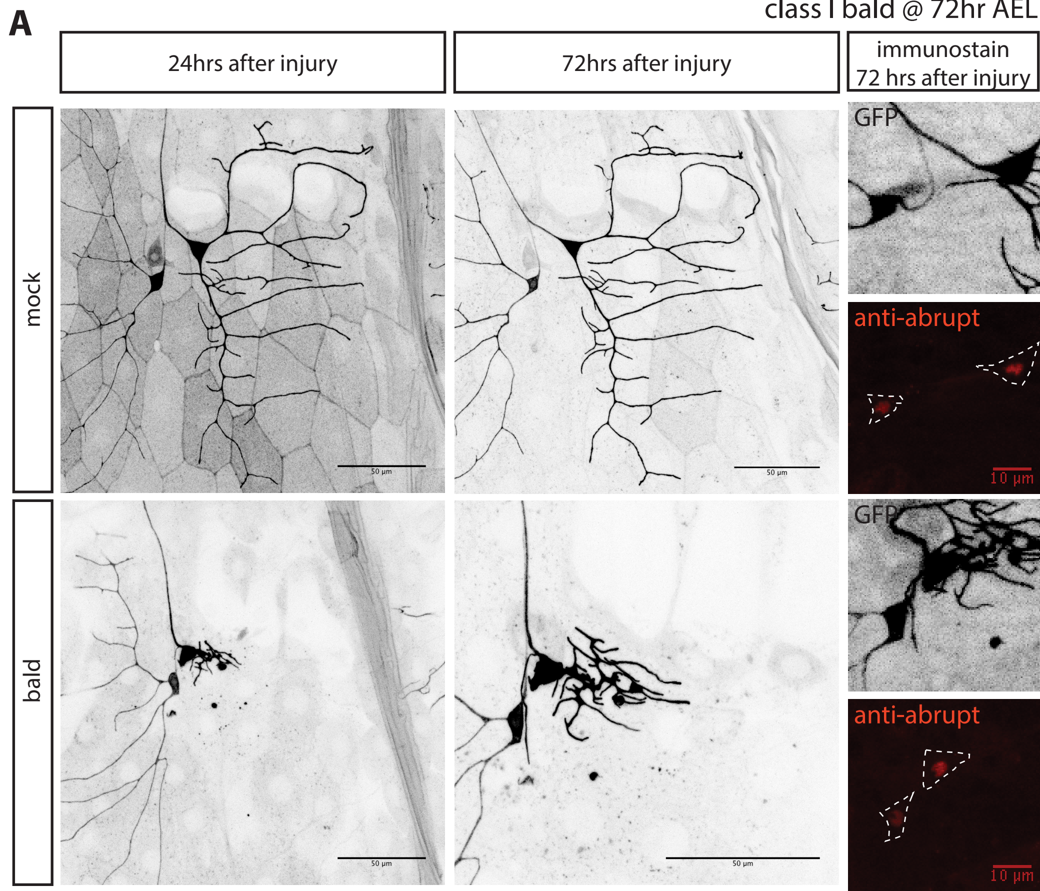
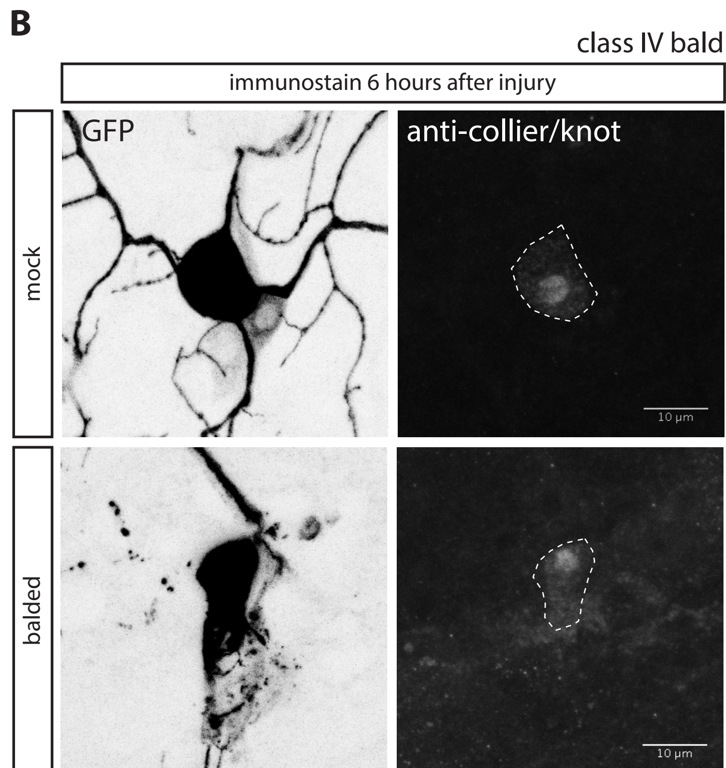
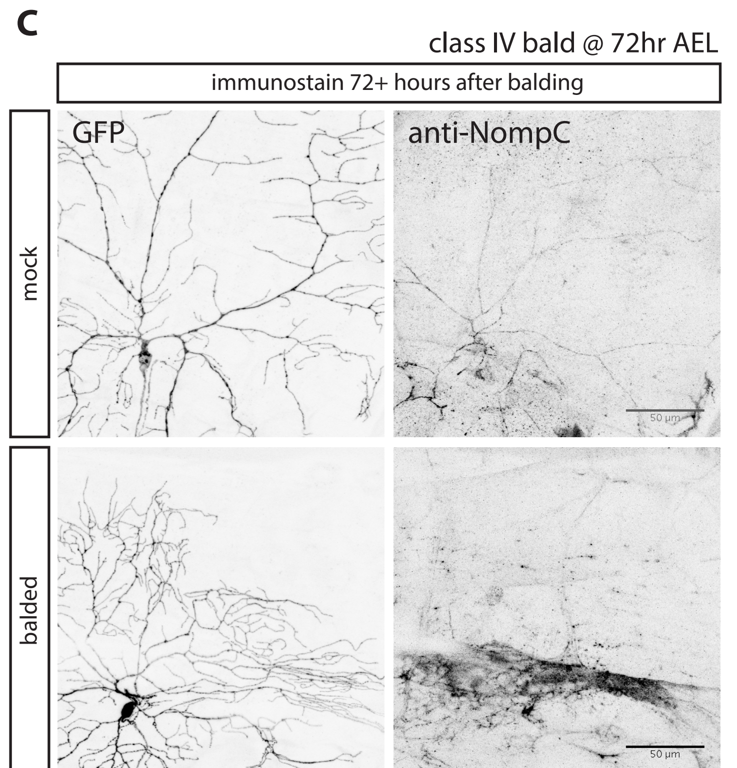


Supplemental Fig. S6. Dendrite balding and regeneration do not alter expression of the class-specific markers abrupt, knot/collier, or NOMPC. (**A**) Immunostaining for the class I specific transcription factor *abrupt* in regenerated class I neurons 72hrs after balding does not reveal large changes in *abrupt* expression. Class I ddaD (uninjured, left) and ddaE (right, balded or control) cell bodies are outlined by dotted lines. (**B**) Immunostaining for the transcription factor *knot/abrupt* in regenerated neurons 6 hours after balding does not reveal significant changes in expression level in class IV neurons. (**C**) Immunostaining for the class III specific gene NOMPC 72 hours after balding class IV neurons shows no expression, implying that class IV neurons do not start aberrantly expressing markers of other da neuron classes after injury. Scale bars are 50 μm (A large panels, C) or 10 μm (A cell bodies, B).


A
class I bald @ 72hr AEL
24hrs after injury
72hrs after injury
immunostain
72 hrs after injury
mock
bald
GFP
anti-abrupt
10 μm
GFP
anti-abrupt
10 μm


B
class IV bald
immunostain 6 hours after injury
mock
balded
GFP
anti-collier/knot
10 μm
10 μm


C
class IV bald @ 72hr AEL
immunostain 72+ hours after balding
mock
balded
GFP
anti-NompC
50 μm
50 μm