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Supplementary Information for

HSP90 is a chaperone for DLK and is required for axon injury signaling

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Table S1. Screen hits from the ICCB Known Bioactives Library

Compound	Description	Growth (Fold/DMSO)	Dose [μM]	Manual validation	Role in axon regeneration?
17-Allylamino-geldanamycin	HSP90 inhibitor	0.03, 0.07	8.5, 1.7	pass	
Lycorine	multifunction antiviral, anticancer, anti-inflammatory	0.12	3.1		
Nocodazole	tubulin inhibitor	0.06, 0.37	16.6, 3.3		
Geldanamycin	HSP90 inhibitor	0.06, 0.06	8.9, 1.8		
Camptothecin	Topoisomerase 1 inhibitor	0.07, 0.15	14.4, 2.9	pass	
10-Hydroxycamptothecin	Topoisomerase 1 inhibitor	0.07, 0.14	13.7, 2.7		
Vinblastine	tubulin inhibitor	0.07	5.5		
Thapsigargin	SERCA inhibitor	0.09, 0.08	7.7, 1.5		
Tosyl-Phe-CMK (TPCK)	Serine protease inhibitor	0.12, 0.34	14.2, 2.8		
Latrunculin B	Actin inhibitor	0.12	12.6		
5-Iodotubercidin	inhibits casein and adenosine kinases, ERK2	0.12	2.5	pass	ERK1/2 are pro-regen. injury signals (1)
Curcumin	multifunction anticancer, inhibits NF-κB, STAT3, AP-1	0.16	13.6		
TMB-8	Inhibits intracellular Ca2+, nAChR, PKC	0.17	11.6		
Trichostatin-A	histone deacetylase inhibitor	0.2, 0.13	16.5, 3.3		
Taxol = Paclitaxel	microtubule stabilizer	0.2	5.9		
Roscovitine	Cdc2, CDK2, CDK5 inhibitor	0.23	14.1	pass	Roscovitine blocks axon elongation (2)
Capsazepine	TRPV1 inhibitor	0.25	13.3	pass	
Ionomycin	Ca2+ ionophore	0.26, 0.2	7.1, 1.4		
SP-600125	JNK inhibitor	0.3	22.7	control	JNK is pro-regen. injury signal (3)
SB-415286	GSK3 inhibitor	0.34	13.9		GSK3 is pro-regen. injury signal (4)
Resveratrol	multifunction anticancer	0.34	2.2		
K252A	Kinase inhibitor (Broad spectrum)	0.37	1.1		
Quercetin	broad anti-inflammatory	0.38	14.8		
SB 203580	p38 inhibitor	0.41	13.2	pass	p38 required for *Ce* and *Dm* axon regen (5, 6)
Mitomycin C	cross-links DNA	0.42	15		
Ebselen	glutathione peroxidase mimetic	0.42	18.2		
Ala-Ala-Phe-CMK	Tripeptidyl peptidase II inhibitor	0.43	11		
Rapamycin	mTOR inhibitor	0.43	5.5		mTOR is required for axon regen (7)
AG1478	EGFR inhibitor	0.44	14.2	fail	EGFR promotes substrate inhibition (8)
Olomoucine	Cdc2, CDK2, CDK5, ERK1 inhibitor	0.45	16.8		
CITCO	Constitutive androstane receptor (CAR) agonist	0.46	11.4		
SB 202190	p38 inhibitor	0.48	15.1		p38 required for *Ce* and *Dm* axon regen (5, 6)
AG-490	JAK2 inhibitor	0.48	17		JAK2 is pro-regen injury signal (9)
Cypermethrin	multifunction ion channel inhibitor	0.49	12		
Chelerythrine	multifunction PKC inhib., anti-inflam., antibacterial	0.47	2.6		

Hits that were validated in the 96-well replating assay and did not cause overt toxicity (toxicity defined as neuron count < 50% of controls). Two numbers in Growth and Dose columns signify that compound was a hit at both doses tested. The 7 novel compounds tested in the manual assay are designated by entries in the "Manual Validation" column. Manual assay = low-throughput replating onto slides and quantification of longest neurite (see Methods). *Ce* = *C. elegans*, *Dm* = *D. melanogaster.*

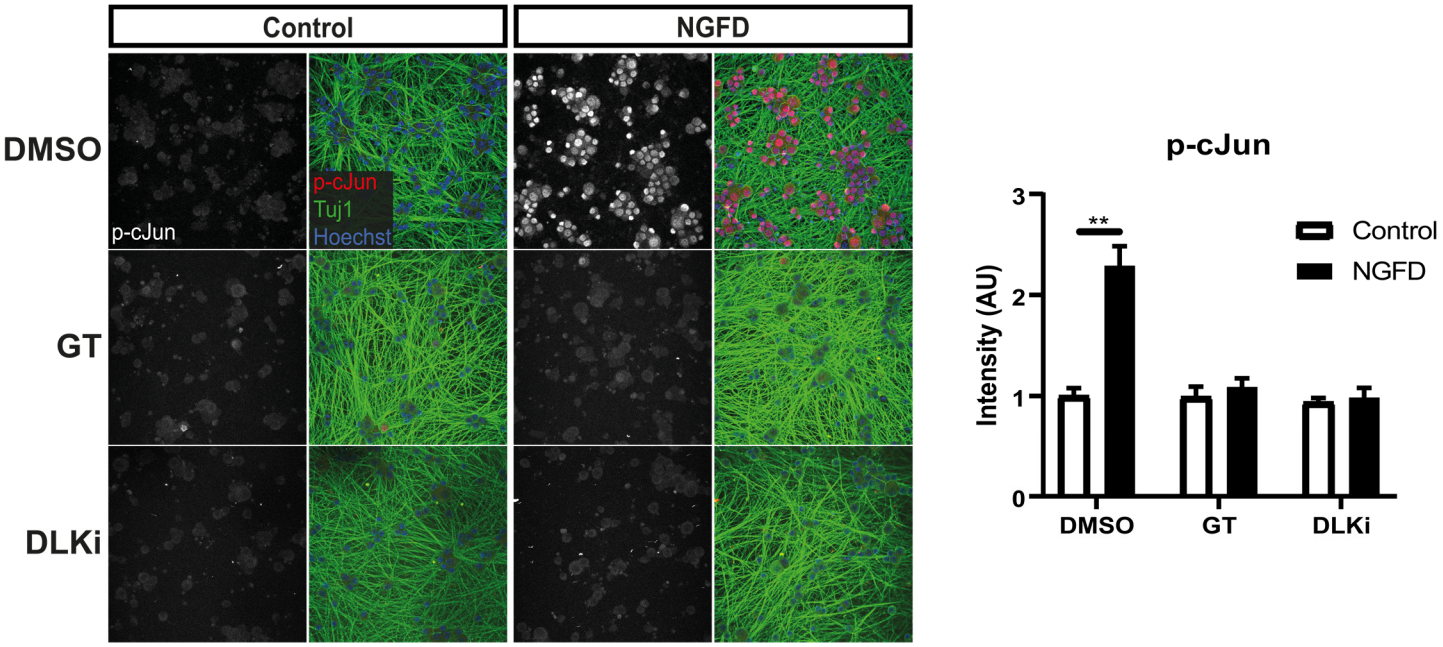

Fig. S1. HSP90 inhibition blocks cJun induction following NGF deprivation (NGFD). Embryonic DRG neurons were cultured for 5 days, then treated with either DMSO or 15 nM ganetespib (GT) for 8h before NGF was withdrawn from the culture medium. Cells were fixed 3h post-NGFD. 500 nM DLKi was used as a positive control. Within each experiment, two technical replicates (~300 cells each) were averaged to yield one biological replicate. n=2 independent experiments, mean ± SEM, 1-way ANOVA w/Tukey's multiple comparisons, DF=11, F=23.4, **DMSO Ctrl v. NGFD p=0.001, GT Ctrl v. NGFD p=0.98, DLKi Ctrl v. NGFD p=0.99.

Extended Materials and Methods

Primary DRG neuron culture

Adult dorsal root ganglia (DRG) were isolated and pooled from cervical, thoracic, and lumbar spinal regions (10). DRG were incubated for 15 min at 37C in a solution containing 0.35 mg/mL Liberase Blendzyme (Roche), 0.6 mg/mL DNase (Sigma), and 10 mg/mL bovine serum albumin (Sigma). DRG were then moved to 0.05% Trypsin-EDTA (Invitrogen) and incubated for 15 min at 37C. Trypsin was removed and replaced with culture media consisting of DMEM (Invitrogen), 10% fetal bovine serum (FBS, Invitrogen), and 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). DRG were dissociated via trituration with a 1 mL pipette tip and plated at a density of 2-3 DRG/mL. Neurons were plated onto glass chamber slides (Fisher Scientific) or polystyrene tissue culture plates (Corning). Prior to plating, plates or slides were incubated overnight with 10 mg/mL poly-D-lysine (Sigma) and then washed twice before being coated with 10 mg/mL laminin (Sigma) for at least 2h. Neurons were cultured at 37C with 5% CO_2. When neurons were cultured for more than 24h, media was changed on day *in vitro* (DIV) 1 and 10 µM AraC (antimitotic, Sigma) was added.

Embryonic DRG neurons were isolated from E13.5 CD1 embryos as previously described (11). All DRG were collected into cold DMEM and then placed into 0.05% trypsin-EDTA for 20 min at 37C. DRG were dissociated with a 1 mL pipette and all cells were resuspended in 60 µL. Neurons were plated as 2.5 µL spots on PDL/laminin-coated plates and cultured in Neurobasal medium (Invitrogen) with 50 ng/mL nerve growth factor (NGF, Harlan Laboratories), 2% B27 (Invitrogen), 1 µM 5-fluoro-2'-deoxyuridine, and 1 µM uridine (antimitotics, Sigma). Cells were cultured at 37C with 5% CO_2 and media was changed on DIV2 and DIV5. To achieve enough protein for western blot analysis, neurons from 9 spots (roughly 16 DRG) were pooled for each condition.

Replating assay and neurite length analysis

Replating was performed as previously described (10). Media (including any drug treatment) was removed from each well and cells were briefly washed with warmed DMEM. DMEM was removed and replaced with 0.025% trypsin-EDTA and cells were incubated for 5 min at 37C, 5% CO_2. Trypsin was replaced with fresh culture media and the plate was gently washed several times to release neurons before the entire cell suspension was replated onto PDL/laminin-coated glass chamber slides. After 18h of culture at 37C and 5% CO_2, neurons were fixed with 4% paraformaldehyde (Electron Microscopy Services) in PBS for 20 min at room temperature. Following a wash with PBS, cells were blocked with 0.1% Triton X-100 in PBS (PBST) with 10% goat serum. Blocking solution was removed and replaced with fresh blocking

solution containing mouse anti-Tuj (βIII Tubulin) primary antibody (BioLegend, 1:500). Following overnight incubation at 4C, primary antibody solution was removed, slides were washed 3x with PBS, and then secondary antibody solution with goat anti-mouse A488 (Life Technologies, 1:1000) and Hoechst 33342 (2 μg/mL, Life Technologies) was applied for 1-2h. Following antibody removal and 3 more PBS washes, slides were mounted with Vectashield (Vector Laboratories) and a glass coverslip, then sealed with clear nail polish. Tuj1-positive neurons were imaged under a 10x air objective on a Leica DMI4000B microscope with a DFC7000T fluorescent camera. The longest neurite of each neuron was traced using the ImageJ plugin, NeuronJ (12). Within each experiment, two technical replicates (~100 cells each) were averaged to yield one biological replicate. Data represents 3-8 independent experiments.

Immunocytochemistry

As described above, neurons were fixed with 4% paraformaldehyde in PBS and blocked with 10% goat serum in PBST. Cells were then incubated overnight at 4C with primary antibody solution consisting of mouse anti-Tuj1 and either rabbit anti-GAP43 (Millipore, 1:500), rabbit anti-phospho-cJun (Cell Signaling, 1:300), or rabbit anti-SCG10 ((13), 1:5000). SCG10 antibody was purified from anti-SCG10 rabbit antiserum (Novus Biologicals) using the SulfoLink Kit (Thermo). Following primary antibody removal, cells were washed with PBS and incubated with secondary antibody solution containing Hoechst 33342 (2 μg/mL), goat anti-mouse A488 (1:1000), and goat anti-rabbit A568 (1:1000) in PBST + 10% goat serum (all from Life Technologies). After 1-3h, secondary antibody solution was removed, slides were washed 3x with PBS and then mounted with Vectashield and glass coverslips as described above. Images were taken on a Leica SPE confocal microscope with a 40x oil immersion objective. 30-50 Tuj1-positive neurons were imaged per group for each experiment. Gain was set based on the DMSO 24h group so that intensity was not saturated. Nuclear (Hoechst-positive) p-cJun or somal (Tuj1-positive) GAP43 and SCG10 intensities were quantified in ImageJ. Within one experiment, all images were taken with the same gain and each group was normalized to the 1h baseline intensity. n=3-5 independent experiments were performed.

Quantitative real-time PCR

qRT-PCR for regeneration-associated genes (RAGs) was performed as previously described (10). Adult DRG neurons were cultured as previously described. At the time of plating, neurons were treated with either DMSO, 1 μM 17AAG, or 500 nM GNE-3511. At 24h, lysate was collected in Trizol Reagent (Invitrogen) and total RNA was isolated using chloroform extraction. RNA was treated with RQ1 RNase-free DNase (Promega) for 30 min at 37C and then terminated with RQ1 stop solution for 10 min at 65C. First strand cDNA synthesis was performed using the qScript cDNA

Synthesis Kit (Quanta Biosciences). For each group within one experiment, an equal amount of DNase-treated RNA was used to obtain cDNA preparations of equal concentration. This cDNA was diluted and loaded in triplicate into a reaction plate with PerfeCTa SYBR green FastMix Rox reagent (Quanta Biosciences). We confirmed the absence of genomic DNA contamination using controls of nuclease-free water and non-reverse transcribed DNase-treated RNA. Amplification values were obtained using a QuantStudio 3 Real-Time PCR System (Applied Biosystems) and analyzed using the ΔΔCT method with *Gapdh* as the loading control (14). Primers were validated previously using standard curves (10, 15). Five to eight independent experiments were performed. For each RAG, transcript levels were normalized to the 24h DMSO-treated group. In 3 runs (2 *Sprr1a* and 1 *Galanin*), RAG mRNA at 1h was undetectable and so was not used in the statistical analysis. For example, *Sprr1a* was probed for in 8 independent experiments, but in two of them, *Sprr1a* transcript from the 1h group was undetectable. The remaining 6 values were used for statistical analysis in Fig. 3E. Primers: *Galanin* (Forward: 5′-GCCCACATGCCATTGACAAC-3′, Reverse: 5′-CGGACAATGTTGCTCTCAGG-3′), *Sprr1a* (Forward: 5-AGAGAACCTGCTCTTCTCTGAGT-3′, Reverse: 5′- CTGGTGCAGCTGAGGAGGTA-3′), and *Gapdh* (Forward: 5′-TGTGAACGGATTTGGCCGTA-3′, Reverse: 5′- ACTGTGCCGTTGAATTTGCC-3′) (10, 15).

Western blot

To analyze protein levels in cultured neurons, embryonic DRG neurons were cultured for 6 days as described above. Neurons from 3 littermate embryos were pooled for each experiment. To assess 17AAG effect on DLK levels, groups were treated with either 5 μM 17AAG or an equivalent volume of DMSO for either 4 or 8h. To measure turnover of DLK and SCG10, cycloheximide was added for 4 or 8h. For all experiments, lysate was collected on ice with Laemmli buffer with mini-cOmplete protease inhibitor (1x, Roche) and phosphatase inhibitor cocktail 3 (1:100, Sigma). Lysate was boiled at 100C for 10 min and run on a 4-15% polyacrylamide gel (BioRad). Protein was transferred to a nitrocellulose membrane and then blocked with 5% milk solution in Tris-buffered saline with 0.2% Tween-20 (TBST). Membranes were incubated overnight at 4C in primary antibody solution consisting of 5% milk, TBST, and one of the following antibodies: mouse anti-TUJ1 (1:5000, BioLegend), rabbit anti-SCG10 (1:2500,(13)), rabbit anti-HSP90 C45G5 (1:500, Cell Signaling), rabbit anti-GAPDH D16H11xp (1:1000, Cell Signaling), mouse anti-MAP3K12 N377/20 (1:500, NeuroMab), rabbit anti-SEK1/MKK4 (1:1000, Cell Signaling), or rabbit anti-SAPK/JNK (1:1000, Cell Signaling). Primary antibody solution was removed and membranes were washed 3x with TBST before incubation with secondary antibody solution consisting of 5% milk in TBST and HRP conjugated to either anti-rabbit or anti-mouse antibody (Jackson, 1:10,000). After 1-2h of incubation, membranes were washed 3x and developed using Immobilon Western Chemiluminescent Substrate (EMD

Millipore). Membranes were imaged on a G:Box Chemi-XX6 (Syngene) and quantified using ImageJ. DLK band intensities of each lane were normalized to the intensity of their corresponding TUJ1 loading controls. Final values are expressed as fold change over time 0. Five independent experiments were performed. As necessary, membranes were stripped with Abcam stripping buffer (15 mg/mL glycine, 1 mg/mL SDS, 1% Tween-20, pH 2.2) following the manufacturer's instructions (Abcam).

To measure DLK levels of mice *in vivo*, sciatic nerves were isolated into ice-cold PBS where the epineurium was quickly removed. To minimize protein loss and degradation, nerves were quickly transferred to lysis buffer consisting of RIPA (0.52% w/v deoxycholic acid, 1% v/v NP40, 0.1% v/v SDS, 1X PBS), mini-cOmplete protease inhibitor (Roche), and phosphatase inhibitor cocktail 3 (1:100, Sigma). Tissue was digested with a pestle, sonicated, and centrifuged at 4C for 5 min at max speed. Supernatant was collected and quantified with a BCA assay kit (Thermofisher). Protein concentrations were equalized among groups in 1X Laemmli buffer, and then analyzed by western blot as described above. DLK levels are represented as fold change over DMSO controls.

To assess *Drosophila* protein levels, *elav3E-Gal4* flies were crossed to either *UAS-RFP* (control) or *UAS-Hsp83-RNAi*. Ventral nerve cords (VNCs) were isolated from third-instar larvae and homogenized on ice in lysis buffer (100 mM NaCl, 100 mM Tris ph 8.0, 1 mM EDTA pH 8.0, 2 M urea, 1% v/v SDS, 20 mM Na_3VO_4, 1x Roche protease inhibitor) with a pestle. VNCs from 10 genetically identical flies were pooled into one lysate to achieve sufficient protein concentration. Gel electrophoresis and transfer were performed as described above. Membranes were incubated overnight at 4C in primary antibody solution consisting of 5% milk, TBST, and one of the following antibodies: rabbit anti-Hsp90 #4874 (1:300, Cell Signaling), mouse anti-b-Tubulin E7 (1:20, Developmental Studies Hybridoma), rabbit anti-Wallenda (1:800,(16)). Secondary antibody incubation, development, and imaging were performed as described above. Wallenda or Hsp83 levels are represented as fold over control animals. This experiment was performed four times.

Co-immunoprecipitation

Human embryonic kidney (HEK293T) cells were cultured to 70-80% confluence and then transfected via polyethylenimine with either empty FUIV [FUGW-Ubiquitin promoter-Internal Ribosome Entry Site-Enhanced YFP (Venus)] vector (17) or FUIV containing flag-tagged DLK. Over 75% transfection efficiency 2 days after transfection was verified via the Venus reporter. Prior to lysate collection, M2 anti-flag affinity agarose beads (Sigma) were blocked in 1.5% BSA overnight at 4C. Two days after transfection, cells were gently washed with ice-cold PBS and then lysed on ice in co-immunoprecipitation (co-IP) buffer consisting of 1 mM EDTA pH 8.0, 10 mM HEPES pH 7.4, 0.5% Triton, 1x mini-

cOmplete protease inhibitor (Roche), 1mM phenylmethylsulfonyl fluoride, and phosphatase inhibitor cocktail (Sigma, 1:100) in PBS. Lysate was sonicated and cleared of cell debris by centrifuging at 13000 rpm for 5 min at 4C. A portion of lysate was saved as Input and the remainder was incubated with anti-flag beads overnight at 4C with gentle shaking. Beads were then washed 2x with coIP buffer, resuspended in 1x Laemmli buffer (containing SDS) and boiled for 10 min at 100C to release bound protein. Following a 5 min centrifugation at 15,000 rpm, lysates were analyzed by SDS-PAGE and western blotting as described above. To immunoprecipitate from embryonic DRG neurons, neurons were cultured in 6-well plates at a density of 3 embryos per condition. Lysate was collected as described for HEKs and precleared for 30 min with Protein-G Dynabeads (Invitrogen) at 4C. Lysate was incubated with mouse anti-DLK antibody (Neuromab clone N377/20 1:100) or an equivalent amount of mouse IgG antibody (Jackson ImmunoResearch) overnight at 4C. Next, each antibody was immunoprecipitated with Protein-G Dynabeads for 1h at 4C. Precipitates were washed, eluted into sample buffer, and analyzed via SDS-PAGE as described above. DLK pull-down was probed for with rabbit anti-MAP3K12 antibody (Novus Biologicals, 1:250). Membranes with immunoprecipitated DRG proteins were developed using a WesternBright Sirius detection kit (Advansta).

NGF Deprivation

Embryonic DRG neurons were cultured as previously described. Eight hours prior to NGF withdrawal, DMSO or 15 nM ganetespib were applied. One hour prior to NGF withdrawal, 500 nM GNE-3511 was added to the DLKi group. Cells underwent a complete media change, drugs were reapplied, and NGF was placed back into control wells while mouse NGF neutralizing antibody (Alomone Labs) was added to the NGF deprivation groups. Cells were fixed 3h after media change and immunostained and imaged as described above. Within each experiment, two technical replicates (~300 cells each) were averaged to yield one biological replicate. Data represents 2 independent experiments.

Fly stocks

Flies were maintained at 25C on standard fly food. The following stocks were used in this study: *BG380-Gal4* (18), *puc-LacZ* (19), *elav3E-Gal4* (20), *dvglut-Gal4* (21), and *Highwire*^{ND9} (22). Flies obtained from the Bloomington Stock Center included: *UAS-RFP* (#32218), *UAS-wallenda-RNAi* (TRiP Collection #25396), *UAS-hsp83-RNAi* (TRiP Collection #33947), and *UAS-white-RNAi* (TRiP Collection #33623).

Drosophila nerve crush assay

BG380-Gal4;puc-LacZ lines were crossed to flies expressing either *UAS-white-RNAi* (control), *UAS-Hsp83-RNAi*, or *UAS-wallenda-RNAi*. Third-instar larvae were positioned with their ventral surface up and segmental nerves were pinched

through the cuticle for 5s with Dumostar #5 forceps (23). Larvae were transferred to a grape plate with activated yeast paste added and kept alive for 24h at 25C. Larvae were then filleted open in PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. Larvae were washed with PBST and blocked with 5% goat serum in PBST for 30 min. Larvae were incubated in primary antibody solution consisting of rat anti-Elav 7E8A10 (1:50, Developmental Studies Hybridoma Bank), mouse anti-LacZ 40-1a (1:100, Developmental Studies Hybridoma Bank), and 5% goat serum in PBST overnight at 4C. After removing primary antibody, flies were washed 3x with PBST and incubated with secondary antibody solution containing goat anti-rabbit A488 (1:1000, Life Technologies) and goat anti-mouse Cy3 (1:1000, Jackson ImmunoResearch Laboratories) for 1h. After antibody solution was removed, larvae were washed 3x with PBST, equilibrated in 70% glycerol in PBS, and mounted on slides with VectaShield. Ventral nerve cords were imaged at 40x on a Leica TCS SPE confocal microscope. All images for each experiment were taken with identical gain, which was set using "control injured" flies to avoid oversaturating LacZ signal. The nerves crushed in this assay stem from motor neurons of the dorsal midline, a narrow strip of cells centered in the ventral nerve cord. The nuclei of these cells were identified via elav staining in the dorsal midline of the ventral nerve cord. Because *puc-LacZ* contains a nuclear localization sequence, LacZ intensity was quantified in these nuclei for at least 7 animals and normalized to uninjured neurons from control flies.

Drosophila synaptic overgrowth assay

To drive transgenic RNAi constructs in a *highwire*^{ND9} mutant background, *dvglut-Gal4,highwire*^{ND9} flies were crossed to *UAS-Hsp83-RNAi* or *UAS-wallenda-RNAi* lines. Third-instar larvae were filleted open and fixed in Bouin's fixative for 10 min at room temperature. Flies were stained as described above. To identify neuromuscular junctions (NMJs), larvae were probed with rabbit anti-DVGLUT (1:10,000, (21)). Secondary antibodies consisted of goat anti-rabbit A488 (1:1000, Life Technologies) and anti-HRP-Cy3 (1:1000, Jackson ImmunoResearch Laboratories) to label synaptic boutons and nerves, respectively. NMJs were imaged at 40x on a Leica TCS SPE confocal microscope. The number of DVGLUT-positive boutons were quantified from 18-23 NMJs at muscle 4 from at least 4 animals per genotype.

Experimental design and statistical analysis

Prior studies have determined that a minimum sample size of 3 independent experiments is required to reject the null hypothesis given $\alpha = 0.05$ (10, 11, 24). For cell culture experiments, one culture was considered one independent experiment (biological replicate). For adult DRG cultures, one mouse gave sufficient cells for one experiment. To achieve sufficient protein yield for western blot analysis, embryonic DRG neurons were pooled from several littermate embryos to give n=1 independent experiment. For *in vivo* mouse experiments, each mouse was considered n=1. Immunofluorescence

(**Figs. 3A-D, 6, SI Appendix Fig. S1**), CT values (**Fig. 3E**), and protein levels (**Figs. 5-6**) were all normalized to control or baseline values, which are detailed in the respective sections above.

For *Drosophila* protein level analysis, each independent experiment is representative of 10 pooled ventral nerve cords. For *puc*_LacZ experiments, each animal was considered an independent experiment. In the NMJ synapse overgrowth assay, each muscle 4 NMJ is confined to its own hemisegment and is thus considered one independent experiment (22). One animal provides approximately four muscle 4 NMJs to analyze.

Statistical tests were performed in Prism (GraphPad). All data are presented as mean ± SEM, except for **Fig. 1E**, which is shown as mean ± SD to depict the well-to-well variability of the screen. Experiments with two conditions (**Figs. 1E, 5F, 6B, 6C**) were analyzed for statistical significance with a Student's t-test. One-way ANOVA with a Tukey post-hoc test was used to determine significance and correct for multiple comparisons of experiments with 3 or more groups (**Figs. 2B, 2D, 3B-E, 6E, 7B, SI Appendix Fig. S1**). Two-way ANOVA with a Sidak post-hoc correction was performed on data in **Figs. 5B&D**, as time and treatment were two independent variables. "*", "**", and "***" indicate p-values less than 0.05, 0.01, and 0.001, respectively, while exact values are displayed in the figure legends.

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