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Supplemental Information

ALS-Linked SOD1 Mutants Enhance Neurite Outgrowth and Branching in Adult Motor Neurons

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Supplementary Figures

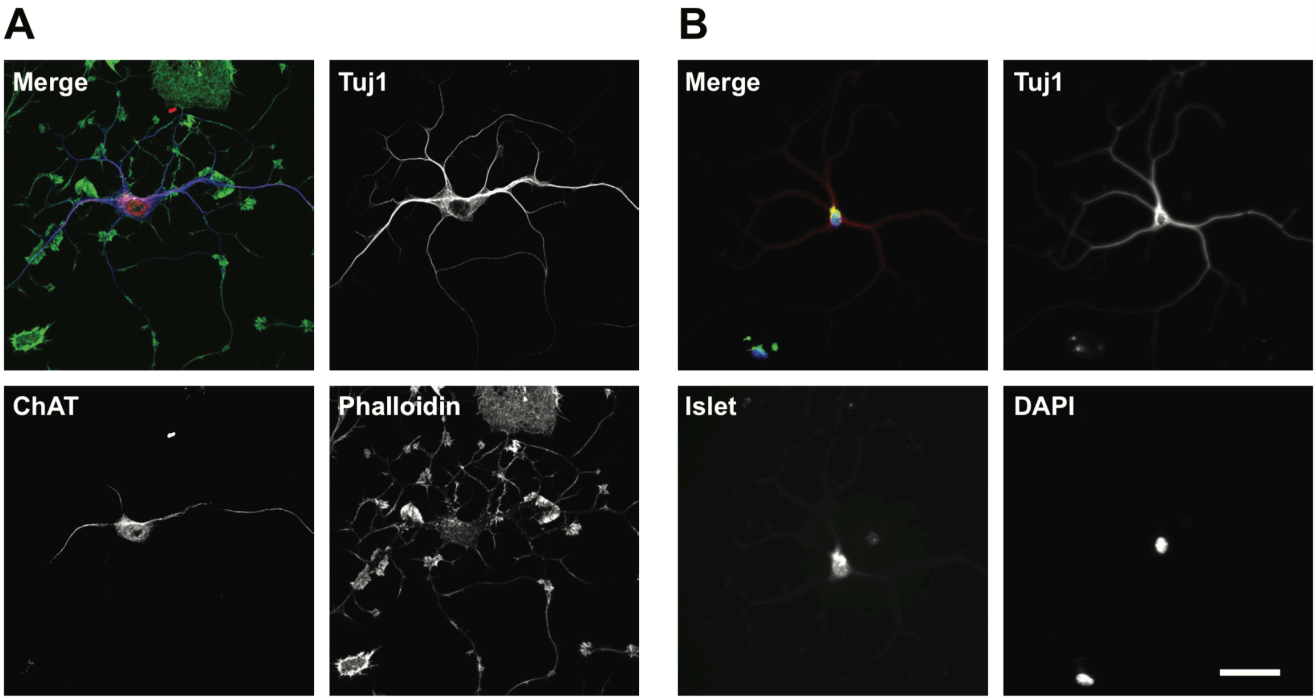

Figure S1. Expression of motor neuron specific markers in cultured adult motor neurons, related to Figure 1.

Images of cultured adult motor neurons stained for **(a)** choline acetyltransferase (Chat) and **(b)** islet. Tuj1 (neuron-specific class III β3 tubulin), phalloidin (F-actin), and DAPI. Scale bar is 50 μm

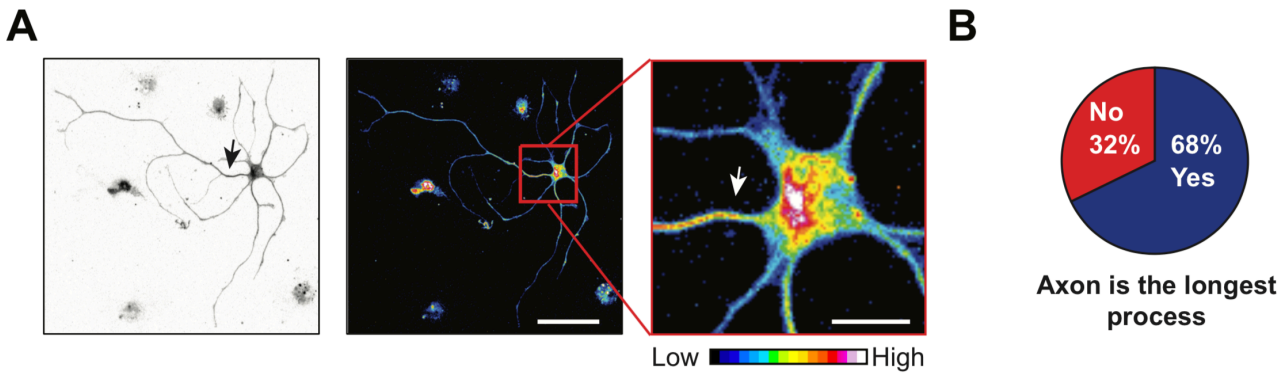

Figure S2. Neuron radius is a measurement of axonal outgrowth, related to Figure 1.

(a) Representative image of motor neuron immunostained for tau. The right images are pseudocolored to depict the tau fluorescence intensity. Scale bar is 10 μm.

(b) Quantification of the frequency of which the axon (measured as the process with the highest tau fluorescence) was the longest process of the cell. n=75 cells.

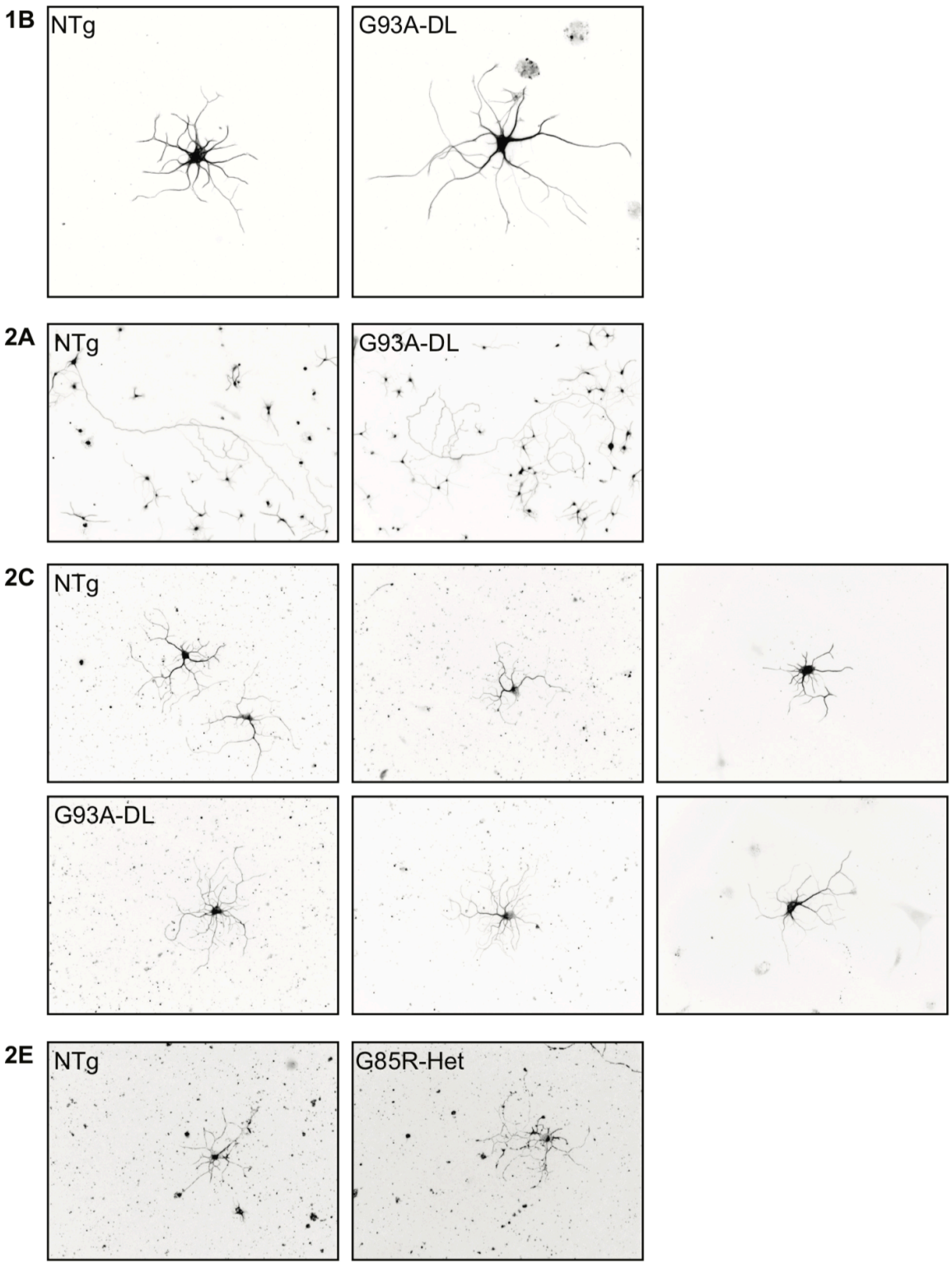

Figure S3. Original images used for neurite tracing, related to Figure 1, 2. The corresponding figure in the main text is listed with each image.

Transparent Methods

Mouse colony housing and breeding

All studies involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida in accordance with NIH guidelines. Adult mice were housed one to five per cage and maintained on *ad libitum* food and water, with a 12 hr light/dark cycle. Transgenic mouse strains SOD1-G93A and SOD1-G93A-DL (JAX stock #004435 and #002299 respectively (Gurney, 1994) were purchased from Jackson laboratory (Bar Harbor, Maine), and bred by the Rodent Breeding Services offered by the Animal Care Services at the University of Florida. SOD1-G93A and SOD1-G93-DL colonies were maintained by breeding hemizygous mice either to wild type siblings, or to C57BL/6J inbred mice (Jax Stock # 000664). Additional transgenic mice strains used in this study were G85R-SOD1:YFP (Wang *et al.*, 2009) and WT-SOD1 (JAX stock #002297), provided by Dr. Borchelt. The G85R-SOD1:YFP mice were maintained as heterozygotes on the FVB/NJ background. The WT-SOD1 were maintained on a C57Bl/6J and C3H/HeJ hybrid background. In addition to the adult mice, we also used timed-pregnant C57BL/6J and SOD1-G93A-DL mice at gestational day 14 generated by the Rodent Breeding Services at the University of Florida.

Colony maintenance genotyping for all strains was performed as previously described (Gurney, 1994; Wang *et al*., 2003). To control for possible transgene copy loss due to meiotic rearrangement, breeders were regularly screened by RT-PCR as previously described (Henriques *et al*., 2010) and replaced with fresh founder stocks from Jackson laboratory (Bar Harbor, Maine) every 5 generations. In our colony SOD1-G93A and SOD1-G93-DL mice reached late disease stage at 150-180 and 240-330 days old, respectively.

Assessment of ALS disease progression

Mice were considered symptomatic if they displayed a 15% loss of bodyweight or showed signs of leg paralysis, whichever was reached first. In our hands, the majority of mice (~70%) were euthanized because of leg paralysis, and the rest due to decreased body weight. All mice were euthanized by CO_2 inhalation following the guidelines provided by the University of Florida Animal Care Services (ACS) and approved by the Institutional Animal Care and Use Committee (IACUC). Late disease stage was defined by hind leg paralysis/weight loss.

Study design

To control for sex differences in disease progression and phenotype of SOD1-G93A mice, symptomatic adult G93A and G93A-DL mice were always paired with non-transgenic (NTg) mice of the same sex and of similar age for each experiment, in most cases (> 80%) using littermates. If a littermate was unavailable, an NTg mouse was selected that was within a 10-day age difference. Age and sex matching also allowed us to control for batch differences in the conditioned medium used to culture adult motor neurons as described below under “cell culture conditions”. Timepoints from pre-symptomatic mice reflect the exact age of the mouse ± 5 days (e.g. 6 months old = 180 ± 5 days). All experiments were performed in accordance with relevant guidelines and regulations of the IACUC and Animal Care Services at the University of Florida.

Adult and embryonic mouse spinal cord isolation

Embryo spinal cords were obtained from timed pregnant G93A-DL and C57BL/6J mice at embryonic day 14 as previously described in detail (Beaudet *et al*., 2015). Once embryos were removed from the

uterus, spinal cords were extracted under sterile conditions in a laminar flow hood with the aid of a dissecting microscope (Nikon SMZ800) and small forceps and placed into cold Leibovitz's L-15 medium (Life Technologies, Grand Island, NY) supplemented with 25 µg ml^{-1} penicillin-streptomycin (Life Technologies). The meninges and dorsal root ganglia (DRG) were peeled off and individual spinal cords were transferred into a 12 wells plate, identified and kept in cold L-15 medium on ice. Tails from each embryo were also harvested at this point for genotyping (G93A-DL mice).

Adult spinal cords were isolated by cutting the vertebrate column with scissors in front of the back legs and just below the medulla oblongata and flushed out of the spinal column using a syringe filled with cold supplemented DMEM/F12-medium with 18G needle (BD Biosciences). The DMEM/F12-medium used for this purpose consisted of DMEM/F12 in a 3:1 ratio supplemented with 36.54 mM NaHCO3 (Fisher Scientific), 0.18 mM L-adenine (Sigma), 312.5 µl L^{-1} 2N HCL (Fisher Scientific), 10% of fetal calf serum (Hyclone, GE Healthcare Life Sciences, South Logan, Utah) and 25 µg ml^{-1} of penicillin-streptomycin (Life Technologies). The adult spinal cords were transferred into cold DMEM/F12-medium.

Motor neuron cell extraction and separation

Both embryonic and adult motor neurons were extracted using the method and reagents described in detail by Beaudet *et al*. (Beaudet *et al.*, 2015) with a few modifications. Briefly, individual spinal cords were cut into small pieces and incubated for 30 min at 37°C in digestion buffer consisting of Dulbecco's PBS (DPBS, Life Technologies, Grand Island, NY) containing 10 U/ml^{-1} papain (Worthington, Lakewood, NJ, USA), 200 µg/ml^{-1} L-cysteine (Sigma St. Louis, MO) and 250 U/ml^{-1} DNase (Sigma, St. Louis, MO). The digestion buffer was then removed and replaced with DPBS containing 8 mg/ml Ovomucoid trypsin inhibitor (Sigma), 8 mg/ml bovine serum albumin (BSA, Sigma), and 250 U/ml DNase. The tissue was then triturated using glass pipettes to obtain a single-cell suspension. This step was repeated trice before all cells were collected and filtered through a 40 µm cell strainer (BD Falcon) and centrifuged at 280 g for 10 min at 4 °C for motor neuron. Adult mixed motor neuron cultures were ready to plate after this step. Embryonic motor neuron pellets were enriched by resuspending in 6 ml of cold Leibovitz's L-15 medium (Life Technologies) and laid over a 1.06 g ml−1 Nycoprep density solution (Axis-Shield, Dundee, Scotland) and spun at 900 g for 20 min at 4 °C without brake in a swinging bucket centrifuge (Eppendorf, Hauppauge, NY). Motor neurons were collected at the interface of the Nycoprep solution and poured in a new 50 ml collection tube which was then filled with cold L-15. Motor neuron cells were counted at this step. Motor neuron collecting tubes were centrifuged at 425 g for 10 min in a swinging bucket centrifuge at 4°C. Typical yield of 90% pure motor neuron cultures was $1x10^6$ cells per spinal cord (litters usually ranging from 7-10 pups).

Cell culture

Embryonic motor neuron pellets were gently resuspended at 200,000 cells/cm^2 in freshly prepared Motor Neuron Growth Medium (MNGM), which is described in detail by Graber DJ *et al* (Graber and Harris, 2013). Briefly, the MNGM consists of Neurobasal A medium (NB-medium, Life Technologies) supplemented with 1X B-27 Serum-Free Supplement (Gibco/Life Technologies), 1X SATO supplement, 5 µg mL^{-1} Insulin (Gibco/Life Technologies), 1 mM Sodium pyruvate (Gibco/Life Technologies), 2 mM L-Glutamine (Gibco/Life Technologies), 40 ng mL^{-1} of 3,3,5-triiodo-L-thyronine sodium salt (T3; Sigma-Aldrich), 1 µg mL^{-1} Mouse laminin (Gibco/Life Technologies), 417 ng mL^{-1} Forskolin (Sigma-Aldrich), 5 µg mL^{-1} N-acetyl-L-cysteine (NAC, Sigma-Aldrich) and 1x Penicillin-streptomycin (Gibco/Life Technologies). After filter-sterilization using a 22 µm syringe filter, 10 ng mL^{-1} of each of the following growth factors was added to the medium: brain-derived neurotrophic factor (BDNF; Sigma-Aldrich),

ciliary neurotrophic factor (CNTF; Peprotech, Rocky Hill, NJ) and glial-derived neurotrophic factor (GDNF; Peprotech). Embryonic motor neurons were either seeded on to Poly-D-lysine (PDL) coated 6cm tissue culture plates (10 μg mL^{-1} PDL, Sigma-Aldrich) to generate conditioned medium used for adult motor neuron cultures, or on to 1.5 cm glass coverslips pre-coated first with PDL (10 μg mL^{-1} for 1h at RT) then with Human Placental Laminin for 3 h at 37°C (1.67 μg mL^{-1} laminin in NB-Medium, Sigma-Aldrich). Embryonic motor neurons grown on coverslips were cultured for 3 days prior to fixation in 4% PFA and immunostaining for imaging and growth analysis.

Adult mixed motor neuron cultures were seeded onto PDL coated cover slips (10 μg $mL^{-1)}$ and cultured in MNGM which had been pre-conditioned for 4 days by embryonic motor neurons isolated from NTg C57BL/6J mice. Given that adult motor neuron pellets contain considerable amount of debris when first plated, cells were not counted prior to seeding. After the cells were allowed to attach to the coverslips in a humidified 37°C incubator for 1 h, they were washed twice with warm NB-medium to remove debris and cultured in 1 ml of conditioned MNGM mixed 1:1 with freshly prepared MNGM. Adult motor neurons were cultured for 2 days prior to fixation and immunostaining for imaging and growth analysis. To confirm that these cultures were indeed enriched with motor neurons, cells were cultured for two days *in vitro* and immunostained for the motor neuron specific markers choline acetyltransferase (ChAT) or LIM-homeobox gene islet-1 (Isl1) (Figure S1). Motor neurons where selected for analysis based on their expression of β3 tubulin, their large size, multi-polarity, and stellate cell shape. Additionally, all motor neurons that were selected were spatially isolated from any other cells in the prep to remove the possibility of contact inhibition altering outgrowth. The average total cell number isolated per G93A-DL mouse is approximately 92,000, with approximately 17% of these cells being β3 tubulin positive. Of the β3 tubulin positive cells 68% (13 of 19 that were selected at random) were identified as motor neurons through islet staining. Resulting in a total yield of approximately 11,000 motor neurons per mouse.

Cath.-a-differentiated (CAD) cells (purchased from Sigma-Aldrich) cells were cultured in DMEM/F12 medium (Gibco) supplemented with 8% fetal calf serum, 1% L-Glutamine, and 1% penicillin-streptomycin. CAD cells were differentiated in the same medium without serum. They were imaged in DMEM/F12 medium without phenol red (Gibco) supplemented with 15mM HEPES. Prior to imaging, CAD cells were plated on coverslips coated with 10 μg/mL Laminin (Sigma). CAD cells were transfected 12-24 hrs prior to imaging with the appropriate constructs using the Neon electroporation system (Invitrogen) using a single 1400 v 20 ms pulse. 1 μg of DNA was used for each 10 μL electroporation. This protocol routinely gave >99% transfection efficiency and <10% cell death.

Immunofluorescence

Cells were fixed with 4% electron microscopy grade paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA) for 10 min at RT, permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 3 min, and washed twice with 1X DPBS. Cells were stained overnight at 4°C with primary antibodies diluted in immunofluorescence staining buffer. They were then washed twice with DPBS for 5 min, incubated with secondary antibodies (diluted 1:1000) for 1 hr at room temperature in immunofluorescence staining buffer. F-actin was stained with Phalloidin-568 (diluted 1:100, Life Technologies) for 30 min at room temperature in immunofluorescence staining buffer. Finally, cells were washed three times with DPBS before mounting with Prolong Diamond W/ DAPI (Life Technologies). We used the following antibodies/stains: Mouse anti- β3 Tubulin (TUJ1 1:500 dilution, Covance, Princeton, NJ), Goat anti-ChAT (1:1000, AB144, ED Millipore), Rabbit anti-Islet1 (1:1000, NBP2-14999,

Novus Biologicals), Mouse anti-Tau (1:25000, gifted from the Giasson lab (Strang et al., 2017)) Alexa Fluor™ Phalloidin 568, anti-mouse IgG 488 and anti-rabbit IgG 488 (Life Technologies) used at 1:1000.

Adeno-associated virus (AAV) mediated overexpression of mutant SOD1

Motor neurons from adult (9-12 months) NTg mice were isolated and plated as described above. On the day they were plated, 20 µl of AAV2/8 (titer $1X10^9$) expressing wild type SOD1-YFP, $SOD1^{G93A}$ - YFP and GFP only was added to the wells and the cells were cultured for 10 days (growth medium was refreshed every 5 days). For these experiments we used a self-complementary virus (scAAV), driven by the chick beta actin promotor (CBA) as described in (Rosario et al., 2016). After 10 days in culture, cells were fixed and stained for β3 Tubulin then imaged/analyzed as described above.

Microscopy

High resolution images of motor neurons and CAD cells were acquired with a Nikon A1R+ laser scanning confocal microscope with GaAsP multi-detector unit using a Plan Flour 40X 1.3 NA objective or an Apo TIRF 60X 1.49 NA objective. Imaging of cells for outgrowth and branching pattern analysis was done using the EVOS XL digital inverted microscope using a Plan Neoflour 20X 0.5 N.A. objective (Life Technologies).

Image analysis

Neurite tracing and branching analysis: Images were taken on the EVOS XL microscope and imported into Fiji (ImageJ) software. All visible projections in these images were traced using the Simple Neurite Tracer plugin (Longair et al., 2011). An image stack was created from the tracing which was then analyzed using the Sholl analysis plugin (Ferreira et al., 2014). To compare the relative change in neuron radius between NTg and SOD1 mutants across different experiments each set was normalized to the average radius of the age matched NTg control group. The Sholl profile containing the number of branches per given distance from soma and overall neuron radius was then exported to Microsoft Excel or GraphPad Prism for analysis.

Axon identification and analysis: Staining of motor neuron cultures were performed as previously described with a mouse-anti tau antibody to specifically identify the axon. Images were taken on the EVOS XL microscope and imported into Fiji (ImageJ) software. The axon (neurite that was most intensely stained for tau) was then measured from the center of the soma to determine if it was the longest projection from the cell. We found that after two days in culture, the longest neurite correlated with highest tau expression 68% of the time (Figure S2). Thus, measuring the cell radius measurement quantifies axonal outgrowth.

Neurite growth cone analysis: Confocal z-stacks were converted into a single maximum intensity projection image. Terminal neurite growth cone size, filopodia length, and filopodia number were analyzed using Fiji (ImageJ) software. Filopodia length was defined as the distance between lamellipodium edge to the furthest end of the extending filopodia. Values were exported into Microsoft Excel and GraphPad Prism for statistical analysis.

Axonal filopodia analysis: Images were taken on the EVOS XL microscope and imported into Fiji (ImageJ) software. The longest projection for each motor neuron was measured using the Simple Neurite Tracer plugin. Filopodia were then counted on the identified longest projection using the cell counter function of ImageJ. Each of these measurements was then imported into Microsoft Excel for

analysis. Axonal filopodia density was calculated by dividing the total number of filopodia counted by the length of the axon.

Cath.-a-differentiated (CAD) cell filopodia analysis: YFP, wild type human SOD1-YFP, and human SOD1^{G93A}-YFP constructs were expressed from plasmids based on the pEF-BOS expression vector. After transfection, CAD cells were cultured in serum-free medium for 18 hours to induce the formation of neurite-like processes. Cells were then fixed and stained for phalloidin as described above. Neurite-like processes were imaged using deconvolution-based super-resolution confocal microscopy using the 60X Apo TIRF objective (Wilson, 2011) by using zoom settings that were higher than the Nyquist criteria, resulting in oversampled pixels (0.10 μm). 3D confocal z-stacks were created and then deconvolved with Nikon Elements software using the Landweber algorithm (15 iterations, with spherical aberration correction). The ImageJ plugin Filopodyan (Urbančič et al., 2017) was used to segment individual filopodia using the phalloidin channel on the deconvolved, maximum-intensity projected images and then measure each filopodia's YFP fluorescence.

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