Supporting Online Material

TGF-β Signaling Specifies Axons During Brain Development

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Antibodies and DNA Constructs

Primary antibodies used were rat anti-TβR1 and goat anti-TβR2 (both from R&D systems), rabbit anti-TβR1 (Cell Signaling), mouse anti-Tau-1 (Chemicon), mouse anti-Tuj1 (Covance), mouse anti-Nestin (BD Biosciences,), mouse anti-TAG1 (Developmental Studies Hybridoma Bank), chicken anti-GFP (Clontech) and goat anti-Par6 (Santa Cruz Biotechnology). pEGFP-C1 was from Clontech. HA-TβR2 and Myc-TβR2-KR were gifts from Dr. Gerald Blobe (Duke University). pCIG2 and pCIG2-Cre were previously described (1). pCIG2-Par6-S345A was generated by introducing a point mutation into pEGFP-C2 Par6 (gift from Dr. Yuh-Nung Jan and Dr. Lily Jan, University of California, San Francisco) using the sense primer 5'-cgaggtgatgttagcggattc**gca**ctctgaattaattctgcagtc-3' and the antisense primer 5'-gactgcagaattaattcagag**tgc**gaatccgctaacatcacctcg-3' in which the highlighted codon indicates the mutation. pCIG2-Par6-S345E was generated as above using the sense primer 5'- cgaggtgatgttagcggattc**gaa**ctctgaattaattctgcagtc-3' and the antisense primer 5'-gactgcagaattaattcagag**ttc**gaatccgctaacatcacctcg-3' in which the highlighted codon indicates the mutation. Inserts for both Par6-WT and Par6-S345A were PCR amplified and ligated into pCIG2 using SacI and EcoRI sites. All DNA constructs were verified by sequencing.

Dissociated Neuronal Cultures

Hippocampal neurons were plated onto glass coverslips coated with poly-D-lysine at a density of 200-300 neurons/mm^2. After neurons attached to the coverslip (typically 4-6 hr after plating), they were transfected using Lipofectamine 2000 (Invitrogen). After 48-72 hr, cells were fixed using 4% PFA/4% sucrose in PBS and permeabilized with 0.1% Triton-X100

prior to incubation with primary antibodies to label intracellular epitopes. Cultures were then incubated with dye-conjugated secondary antibodies and mounted using Vectashield mounting medium (Vector Laboratories). For surface labeling of TβR1 and TβR2, neurons were incubated with the appropriate antibody for 45 min at 37^{o}C in culture media. Afterwards, the cells were washed briefly in warm PBS, fixed, and processed for immunocytochemistry. For pharmacological inhibition of TGF-β receptors, 30 μm SB-431542 (Tocris) dissolved in DMSO was added directly to cultures 4-6 hours after plating and analyzed after 60-68 hours.

Fluorescence Microscopy and Immunohistochemistry

Fluorescent images were acquired on a Zeiss 510 confocal scanning microscope (Carl Zeiss, Inc.). Isolated brains from E14.5 mice were fixed overnight at 4^{o}C in a solution of 4% paraformaldehyde (PFA)/4% sucrose in phosphate buffered saline (PBS). Brains were sectioned at 150 μm on a Vibratome and blocked for 2 h at 4^{o}C in 7% BSA in PBS with gentle rocking. Slices were permeabilized using 0.3% Triton X-100 and incubated overnight with primary antibodies diluted in 7% BSA solution, at 4^{o}C with gentle agitation. The following day, sections were washed, incubated with appropriate Alexa-conjugated secondary antibodies, and mounted onto glass slides using Vectashield mounting medium.

For live imaging, electroporated organotypic slices were imaged after 60 h in culture. During the acquisition, slices and their intact inserts were placed in a 35 mm glass-bottom dish (MatTek) filled with slice culturing media consisting of Basal Medium Eagle (Sigma) supplemented with Hank's Buffered Salt Solution, 50 mM glucose, 2.5 mM HEPES (pH 7.4), 1 mM $CaCl_2$, 1 mM $MgSO_4$, 4 mM $NaHCO_3$, 1x Penicillin-Streptomycin (Invitrogen), and 5% horse serum (Invitrogen). Images were collected on a Nikon Eclipse TE300 microscope equipped with a mechanized and temperature-controlled stage. Confocal images were obtained using a Yokogawa spinning disk confocal (Solamere Technology Group), with excitation from a 50 mW Sapphire 488 LP laser (Coherent) selected and shuttered by an acousto-optical tuning filter (Neos Technologies), and the emission directed by a filter wheel

(Sutter Instruments) containing band pass filters (Chroma). Images were acquired using a 20x Nikon Plan-Fluor 0.45 NA extra-long working distance lens. All images in this study were analyzed using Metamorph software (Universal Imaging Corporation).

TβR2 immunofluorescence in immature SVZ neurons was measured as follows. First, linescans were performed from the soma to the end of the process. For quantification, pixel intensities corresponding to fractional distances of 0.25, 0.5, and 0.75 and 1.0 were measured, normalized to the original pixel value within the soma, and averaged. The collected averages of the sample population for the leading edge dendrite and trailing edge axon were then plotted. Fluorescence values at 0.25 and 0.5 fractional distances were designated as the proximal axon and values at 0.75 and 1.0 were designated as the distal axon.

Immobilized TGF-β Bead Assay

Purified recombinant TGF-β1,2,3 (R&D Systems) was biotinylated using the EZ-link Sulfo-NHS-LC-LC reagent (Pierce) according to the manufacturer's instructions. 2 μg of each protein was used per biotinylation reaction, and excess biotin was separated from the protein by spin filtration at 10,000 x g for 35 min at 4^{o}C on a 3 kDa Micron size exclusion column (Millipore). The unfiltered phase was removed and incubated with 1 x 10^{6} streptavidin-conjugated polystyrene beads (10 μm diameter, Pierce), with gentle shaking at 4^{o}C for 30 min according to the manufacturer's protocol. The beads were washed in PBS and stored at 4^{o}C until use. For control beads, an equal molar quantity of BSA was used in the preparation.

Experiments were performed on a Nikon Eclipse TE300 inverted microscope as described above. Neurons were imaged in a Ludin chamber (Life Imaging Services) filled with Neurobasal media lacking phenol-red and supplemented with B27, Glutamax, and buffered at pH 7.3 with 25 mM HEPES (Gibco Invitrogen). Approximately 300 beads were placed into the center of the imaging chamber and images were acquired at a frequency of one image per minute for the duration of the experiment (typically 90 min) using a 20x Nikon Plan-Fluor NA 0.45 NA extra-long working distance lens.

Co-Immunoprecipitation

Forebrains from E18 rat embryos were homogenized in phosphate-buffered saline containing 0.5% Triton X-100, 135 mM NaCl, complete Mini protease tablet (Roche), and PMSF (1 mM). The homogenate was cleared by centrifugation at 14,000 x g for 10 min at 4^{o}C. Samples were incubated with or without primary antibody overnight at 4^{o}C. The following day, GammaBind G Sepharose beads (GE Healthcare) were washed, blocked in a 5 mg/ml solution of BSA and resuspended in wash buffer (lysis buffer containing 250 mM NaCl). The beads were added to the immunoprecipitation reactions, incubated at 4^{o}C for 1 h, and collected by centrifugation at 5,000 x g for 5 min. The final complex was washed five times with wash buffer, resuspended in protein sample buffer, resolved by SDS-PAGE, and subjected to immunoblot analysis.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. TGF-β Ligand Expression During Embryonic Development of the Neocortex, Related to Figure 1.

(A) *In situ* hybridization showing the absence of TGF-β1 in the forebrain an E14.5 mouse embryo. A magnified view of the developing neocortex demarcated by the black box is shown below.

(B) *In situ* hybridization showing the expression of TGF-β2 within peri-ventricular zones of the neocortex in an E14.5 mouse embryo. The expression of TGF-β2 corresponds with anatomical areas where axon specification in pyramidal neurons occurs. A magnified view of the developing neocortex demarcated by the black box is shown below. Arrow indicates the peri-ventricular expression of TGF-β2.

(C) *In situ* hybridization showing the expression of TGF-β3 in the medial ganglionic eminence (MGE) and peri-ventricular zones in an E14.5 mouse embryo. The expression of

TGF-β3 in the MGE corresponds with anatomical areas of inhibitory neuron generation. A magnified view of the developing neocortex demarcated by the black box is shown below. Arrowhead demarcates the MGE. Arrow indicates the peri-ventricular expression of TGF-β3. All images were obtained from the Genepaint database (http://www.genepaint.org).

Figure S2. Loss of TGF-β Signaling Causes Axonal and Neuronal Migration Defects During Cortical Development, Related to Figure 2.

(A) TβR2 immunohistochemistry in neocortical slice cultures of E15 *Tgfbr2*$^{flox/flox}$ embryos electroporated with GFP (top) or GFP plus Cre (bottom). Arrows indicate GFP-positive neurons either exhibiting (top) or lacking (bottom) TβR2 immunoreactivity. Scale bar, 15 μm.

(B) TβR2-KO neurons that fail to migrate out of the SVZ (arrows) are negative for the radial glial marker nestin. Scale bar, 20 μm.

(C) Data represent means ± SEM of the percent cells with axons in the cortical plate (CP) and intermediate zone (IZ). *$p<0.05$ relative to WT, Student's t-test.

(D) Quantitative analysis of cortical migration. The fraction of cells in each cortical zone was pooled from at least 5 embryos. n = 5033, 2482 cells for WT and TβR2-KO, respectively. Note that fewer TβR2 KO cells reach the CP. *$p<0.05$ relative to WT, Student's t-test.

Figure S3. Validation of TβR1 and TβR2 Antibodies Using RNA Interference, Related to Figure 3.

(A) COS7 cells were co-transfected with two pan-Tgfbr1 shRNA constructs (2) and a pCMV-TβR1 plasmid. After 2 days of expression, a noticeable knockdown in TβR1 expression was observed.

(B) Quantification of shRNA mediated TβR1 knockdown. TβR1 expression was normalized to scrambled shRNA control. n = 3. *$p<0.05$, Student's t-test.

(C) DIV 5 rat hippocampal neuron transfected with the TβR1 shRNA constructs at DIV 1 and stained for surface TβR1. Cells expressing the TβR1 shRNA constructs (arrow) exhibited a significant decrease in immunoreactivity compared to neighboring untransfected cells. Scale bar, 20 μm.

(D) Cortical neurons from *Tgfbr2*$^{flox/flox}$ mouse were electroporated with GFP plus Cre at E14.5, dissociated, and cultured *in vitro* for three days prior to immunostaining for surface TβR2. GFP-positive cells expressing Cre lacked TβR2 immunoreactivity. Scale bar, 20 μm.

Figure S4. TGF-β Signaling is Both Necessary and Sufficient for Axon Specification, Related to Figure 4.

(A) Cortical progenitors from E14.5 *Tgfbr2*$^{flox/flox}$ mouse embryos were electroporated with GFP (top panels) or GFP plus Cre (bottom panels) and maintained in dissociated culture for 5 days *in vitro* (5 DIV). Whereas control neurons elaborate multiple short, MAP-2 positive dendrites (top arrowhead) and a long, single tau-1 positive axon (top, arrow), neurons lacking TβR2 did not form an axon (bottom panels).

(B) Quantification of cells containing axons in WT (green) and TβR2-KO (black) neurons Results were averages from at least 3 embryos. n = 61, 67 cells for WT and TβR2-KO, respectively. *$p<0.05$, Student's t-test.

(C) Dissociated hippocampal neurons from E18 rat embryos treated with DMSO as a vehicle control. Bottom panels show the morphology of a typical DIV 3 neuron consisting of a single long axon (arrow) and multiple short dendrites. Cells were fixed and visualized by staining with Tuj1 antibody. Scale bar: 50 μm top, 5 μm bottom panels.

(D) Images showing changes in morphology of DIV 3 neurons grown in the presence of the TβR1 inhibitor SB-431542 (30 μm). Bottom panels show individual neurons with multiple short neurites of roughly equivalent length. Scale bar: 50 μm top, 5 μm bottom panels.

(E) Data indicate means ± SEM of the fraction of neurons with a distinguishable axon after treatment with DMSO or 30 μM SB-431542. Data are pooled from 3 independent experiments, n = 85, 112 cells for DMSO, SB-431542. *$p<0.05$, Student's t-test.

(F) Hippocampal neurons were transfected with GFP plus constitutively active TβR2-WT at DIV5 and fixed 48 hours later. Arrows indicate tau-1-positive, MAP2-negative axons. Scale bar, 20 μm.

(G) Quantification of axon number in DIV5 cells expressing GFP alone or GFP plus TβR2-WT. GFP, n = 38; TβR2-WT, n = 43. *p<0.05, Student's t-test.

Figure S5. TGF-β but not Laminin Induces the Formation of Multiple Axons, Related to Figure 5.

(A-B) Hippocampal neurons were plated on laminin (A) or TGF-β (B) and cultured for 4 days prior to fixing and staining for tau-1 and MAP2. Arrows indicate tau-1-positive MAP2-negative axons. Scale bar, 20 μm.

(C) Quantification of axon number in DIV4 cells grown on laminin or TGF-β. Data are pooled from 3 independent experiments. Growth on TGF-β resulted in a five-fold increase in neurons with multiple axons (% with ≥2 axons: laminin, 4.5 ± 1.0; TGF-β 23.7 ± 4.8, n = 97). *p<0.05, Student's t-test.

Figure S6. Par6-S345A Suppresses the Gain-of-Function of TβR2-WT Overexpression, Related to Figure 6.

(A) Dissociated hippocampal neurons from E18 rat embryos were transfected 4-6 hours after plating with constructs encoding GFP , GFP + TβR2-WT, or TβR2-WT + Par6-S345A-IRES-GFP. Cells were fixed 65-72 hours later and stained for the axonal marker tau-1. Arrows indicate tau-1 positive axons. Note the emergence of multiple axons in cells expressing GFP + TβR2-WT. Scale bar, 20 μm.

(B) Quantification of axon number in cells expressing GFP, GFP + TβR2-WT, or TβR2-WT + Par6-S345A. Data were pooled from two independent experiments. GFP, n = 40; TβR2-WT, n = 30; TβR2-WT + Par6-S345A, n = 31. *p<0.05, Student's t-test.

SUPPLEMENTAL MOVIES

Movie S1, Related to Figure 2. Part1: Neocortical neurons in organotypic slice culture from an E15 *Tgfbr2*$^{flox/flox}$ embryo electroporated with GFP alone. Neurons can be seen migrating within the IZ toward the CP. Arrowheads indicate the soma of several GFP-positive neurons. Images were taken 60 h after electroporation and image stacks were acquired with a step size of 0.3 μm every 20 min for 20 h. Time is indicated in hours:min.

Part 2**:** Neocortical neurons in organotypic slice culture from an E15 *Tgfbr2*$^{flox/flox}$ embryo electroporated with a bicistronic plasmid expressing GFP and Cre recombinase. Neurons exhibit impaired migration within the IZ. Arrowheads indicated the soma of several GFP-positive neurons. Note the elaboration of leading edge processes, but the absence of trailing axons. Images were taken 60 h after electroporation and image stacks were acquired with a step size of 0.3 μm every 20 min for 20 h. Time is indicated in hours:min.

Part 3: Side-by-side magnified view of a neocortical neuron from an E15 *Tgfbr2*$^{flox/flox}$ embryo electroporated with GFP alone and a neocortical neuron from an E15 *Tgfbr2*$^{flox/flox}$ embryo electroporated with a bicistronic plasmid expressing GFP and Cre recombinase. For the wild-type neuron, the arrowhead indicates the soma and the arrow marks the trailing edge axon. For the TβR2-KO neuron, note the elaboration of a leading edge process, but the failure to extend a trailing axon. The arrowhead indicates the soma and the arrow marks the position where the trailing edge axon should emerge. Images were taken 60 h after electroporation and image stacks were acquired with a step size of 0.3 μm every 20 min for 20 h. Time is indicated in hours:min.

Movie S2, Related to Figure 5. Local Application of TGF-β Induces Rapid Neurite Outgrowth

A DIV 1 cell coming into contact with a streptavidin polystyrene bead conjugated with biotinylated TGF-β. The neurite which touches the TGF-β bead undergoes rapid elongation

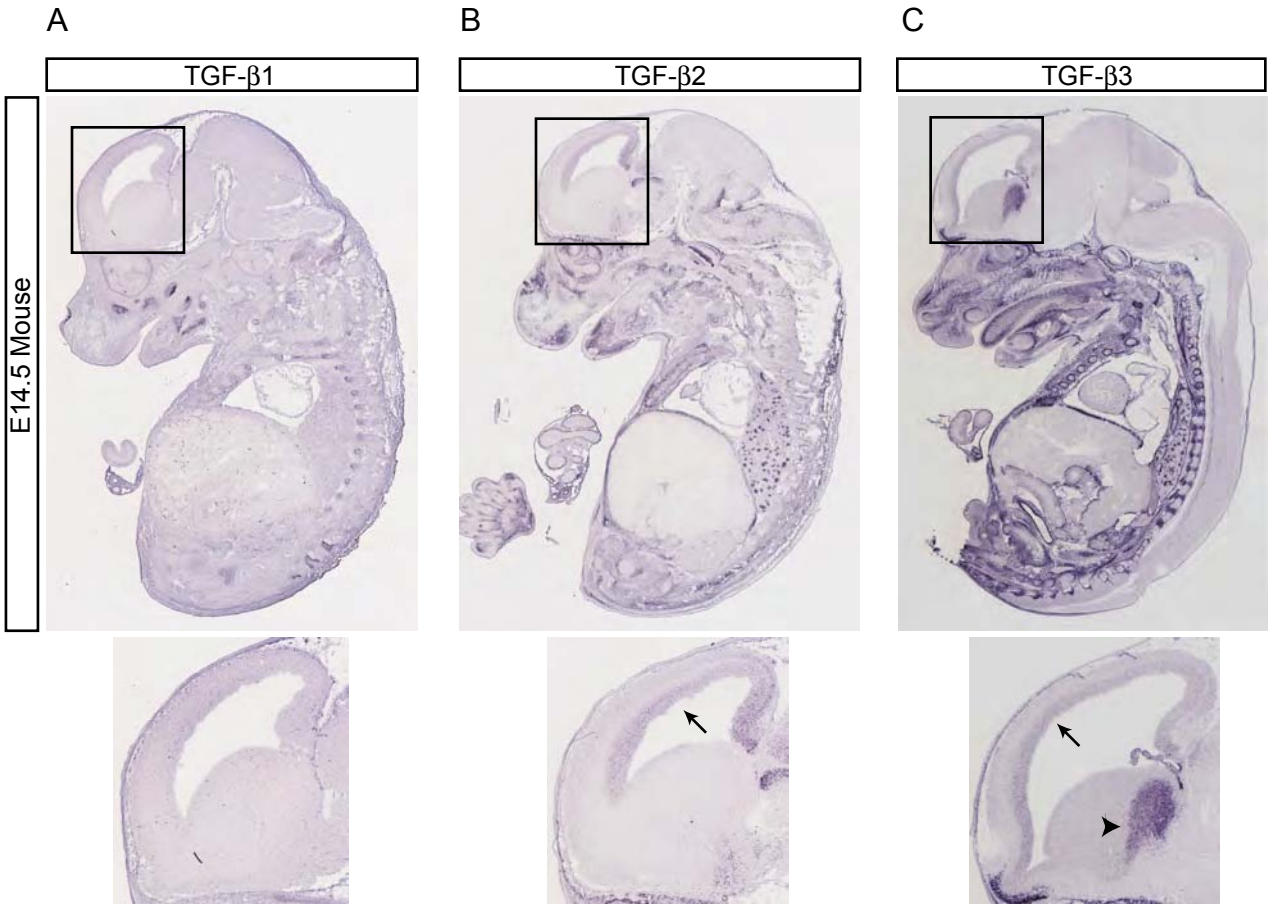
over the course of 1 h indicative of a nascent axon. Images were collected every 15 min.

The movie corresponds to cell shown in Figure 5A.

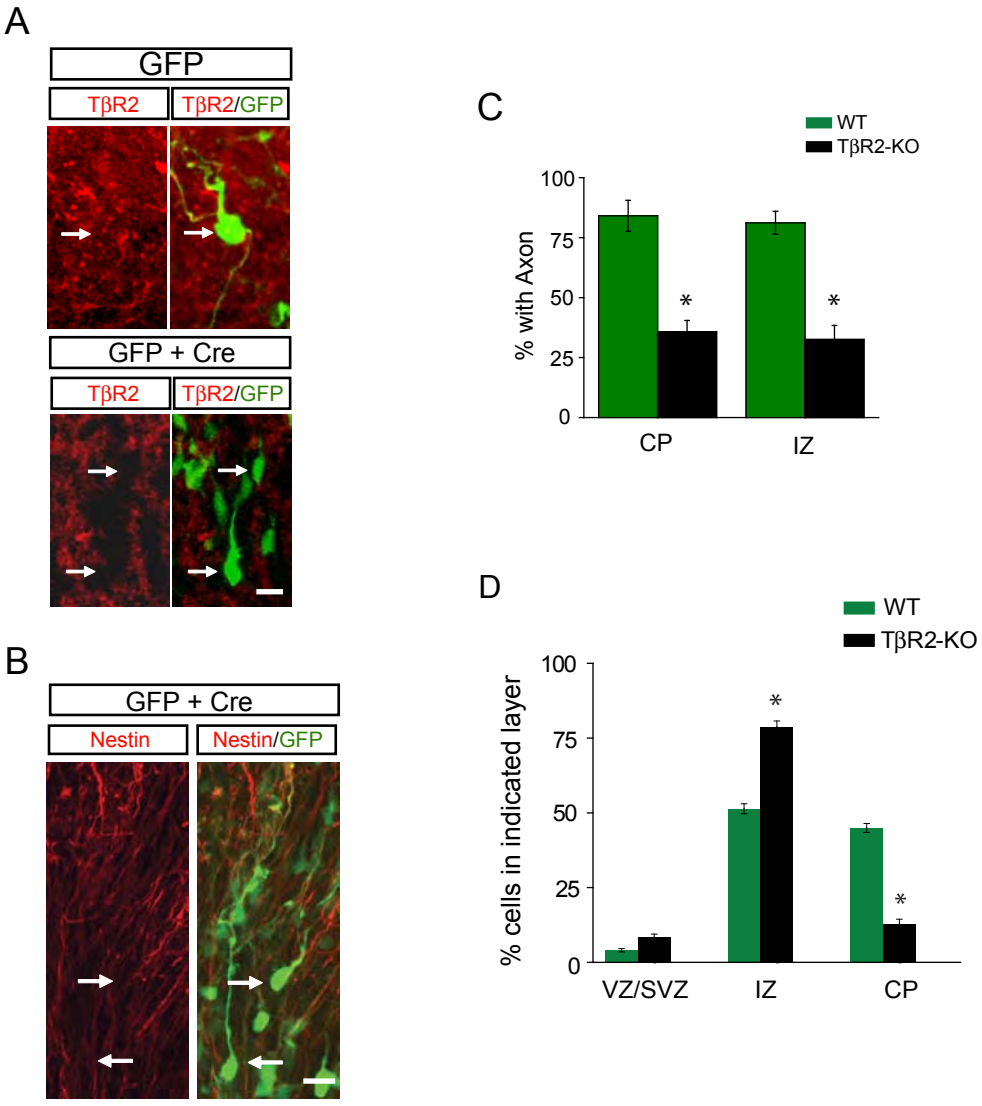
References

1. Hand R, Bortone D, Mattar P, Nguyen L, Heng JI, Guerrier S, Boutt E, Peters E, Barnes AP, Parras C, *et al.* (2005) *Neuron* **48,** 45-62.
2. Liu IM, Schilling SH, Knouse KA, Choy L, Derynck R, & Wang XF (2009) *EMBO J* **28,** 88-98.

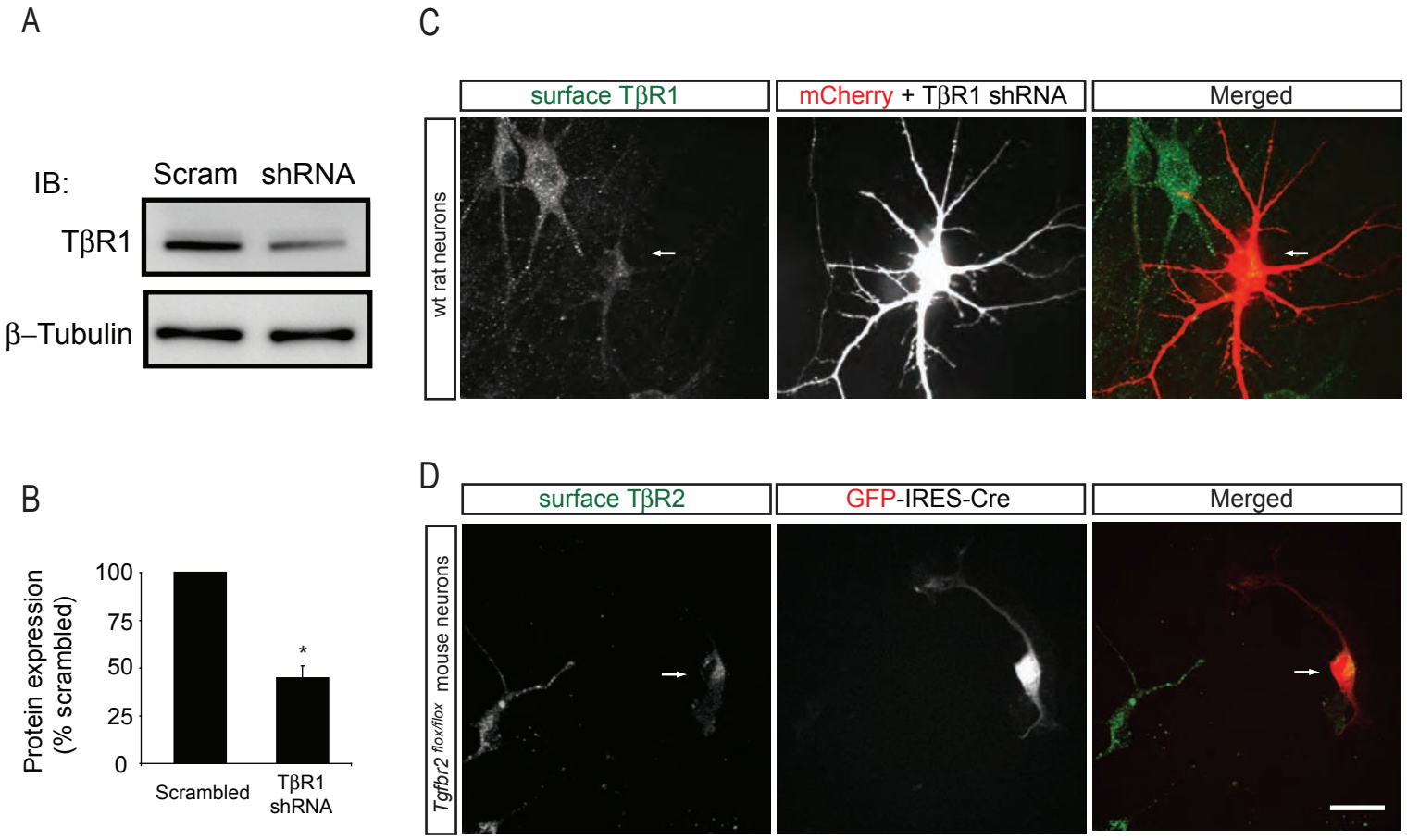
Yi et al., Figure S1

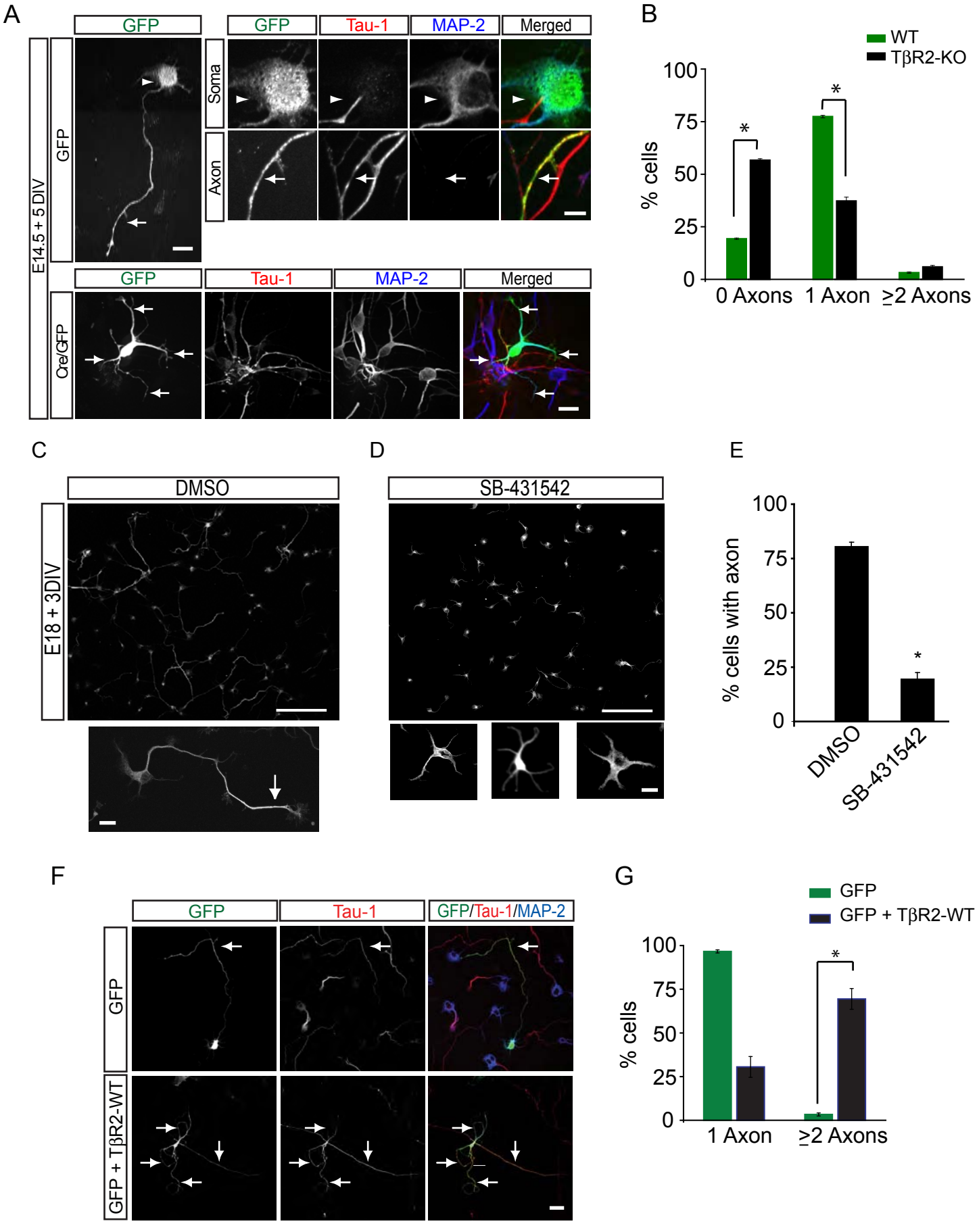


Yi et al., Figure S2



Yi et al., Figure S3

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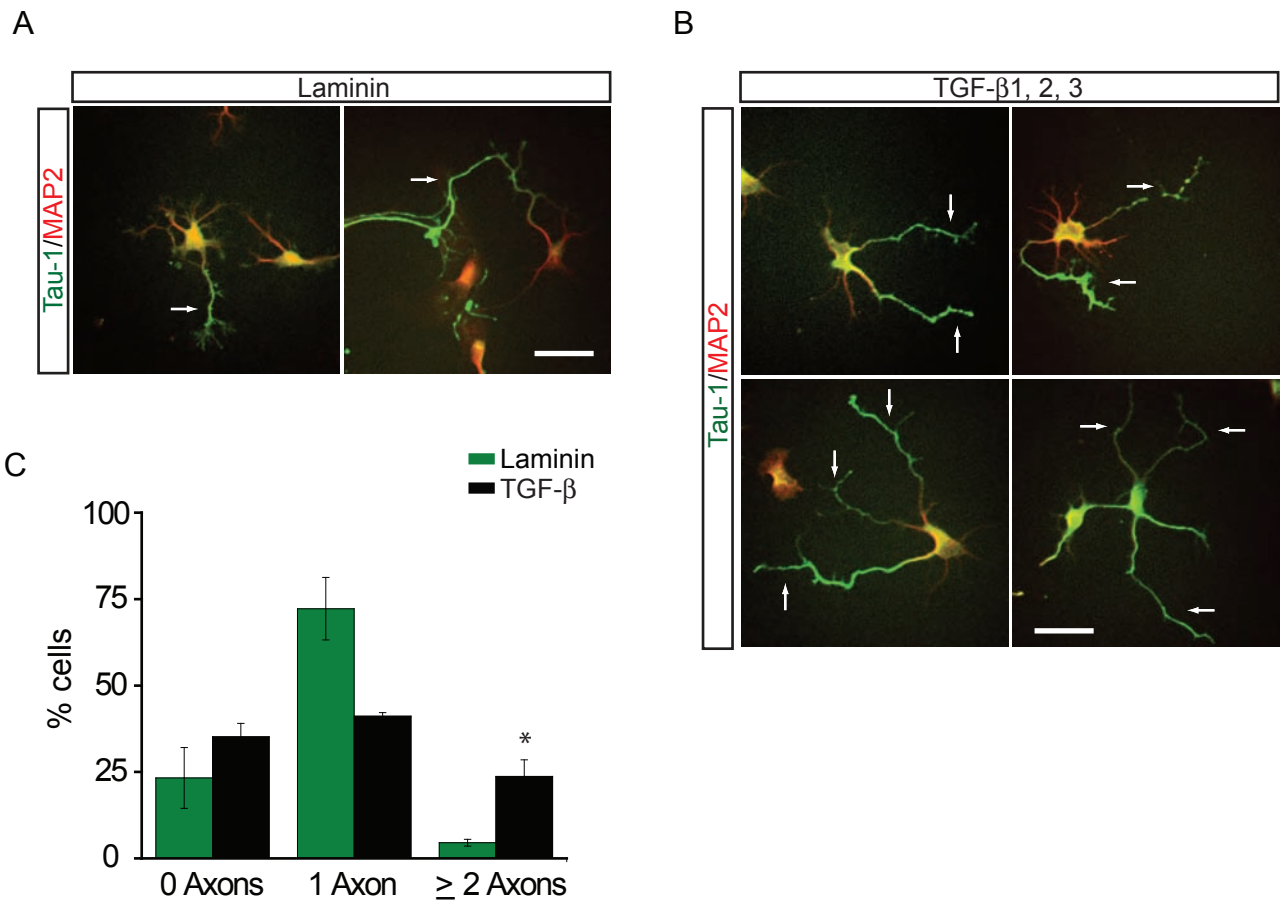
Yi et al., Figure S5



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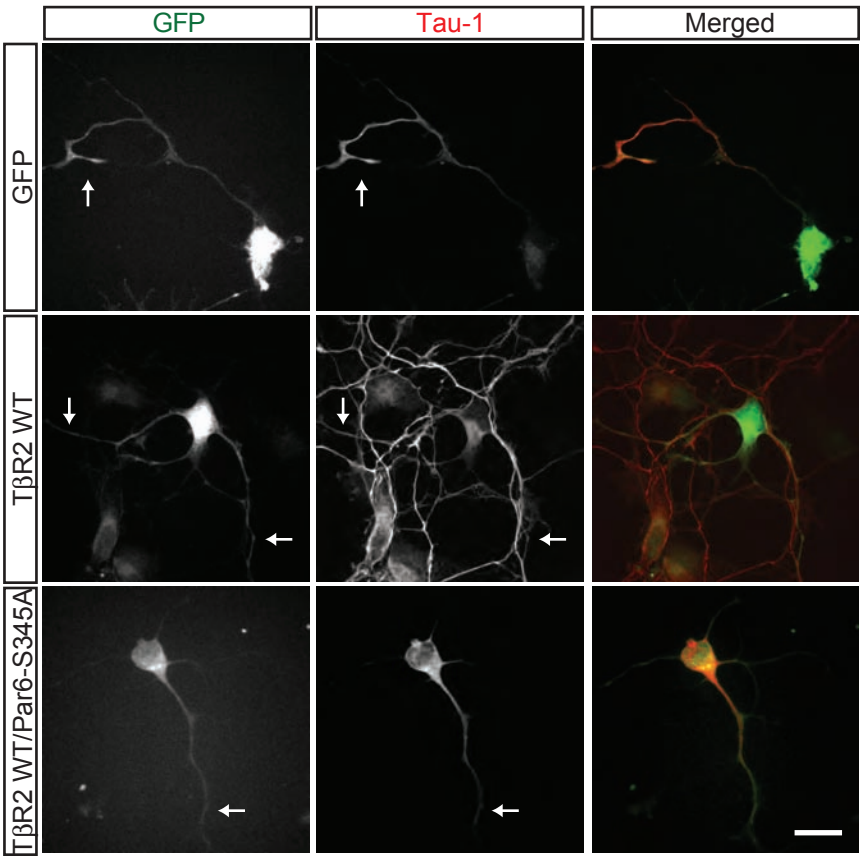
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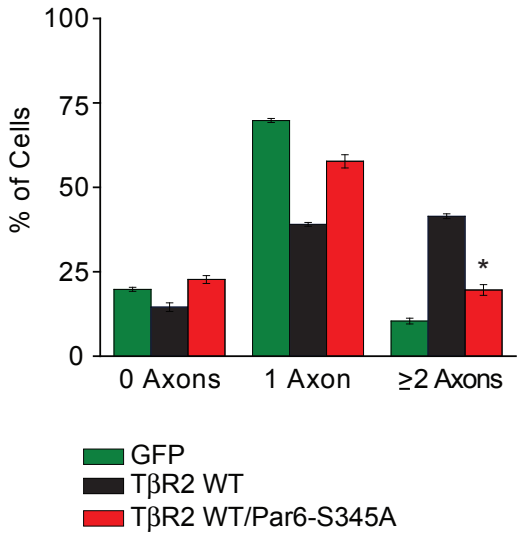
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