Nuclear pore complex remodeling by p75NTR cleavage controls TGF-β signaling and astrocyte functions

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Astrocytes modulate neuronal activity and inhibit regeneration.

We show that cleaved p75 neurotrophin receptor (p75NTR) is a component of the nuclear pore complex (NPC) required for glial scar formation and reduced gamma oscillations in mice via regulation of transforming growth factor (TGF)-β signaling. Cleaved p75NTR interacts with nucleoporins to promote Smad2 nucleocytoplasmic shuttling. Thus, NPC remodeling by regulated intramembrane cleavage of p75NTR controls astrocyte–neuronal communication in response to profibrotic factors.

TGF-β is a major growth factor promoting the transition of quiescent to reactive astrocytes, and it is implicated in neuronal functions and neurodegeneration1. However, the mechanisms that control astrocyte responses to TGF-β and their contribution to astrocyte-neuronal communication remain elusive. After brain injury, p75NTR is upregulated in astrocytes2–3. To investigate the role of p75NTR in astrocytes, we crossed NGFR+/− (named p75NTR+/−) mice with glial fibrillary acidic protein (GFAP)-TGF-β mice, which spontaneously develop astrotosis, hydrocephaly and neuronal dysfunction4. Remarkably, loss of p75NTR prevented astrocyte activation and rescued the hydrocephaly in GFAP-TGF-β mice (Fig. 1a,b and Supplementary Fig. 1a,b). p75NTR was expressed in astrocytes and its deletion did not affect TGF-β levels in GFAP-TGF-β mice (Supplementary Fig. 1c,d).

Astrocytes contribute to gamma oscillations5, which control learning, memory and attention6. TGF-β-induced astrocyte activation decreased gamma oscillations and altered locomotor activity; these were rescued upon genetic deletion of p75NTR (Fig. 1c,d). In accordance, after brain trauma, p75NTR−/− mice had reduced astrocyte activation and deposition of neurocan, a component of the glial scar that inhibits neural regeneration7,8 (Supplementary Fig. 2). TGF-β treatment of wild-type (WT) primary astrocytes stimulated protein secretion and gene expression of neurocan, whereas treatment of p75NTR−/− primary astrocytes failed to do so (Supplementary Fig. 3a). Indeed, treatment of cortical neurons with conditioned medium from TGF-β-treated p75NTR−/− astrocytes did not inhibit neurite outgrowth, unlike WT conditioned medium (Supplementary Fig. 3b). Neurocan expression in TGF-β-treated WT cells was unaffected by neutralization of neurotrophins or inhibition of tropomyosin-related receptor kinase (Trk) signaling (Supplementary Fig. 3c). Loss of p75NTR also reduced TGF-β-induced expression of GAT1 (Slc6a1) and Sl100b (Supplementary Fig. 3d), which regulate astrocyte–neuronal crosstalk9. These data reveal an unanticipated role for p75NTR in regulating astrocyte activation and neuronal activity in response to TGF-β.

TGF-β signals by nuclear accumulation of phosphorylated Smad (P-Smad) transcriptional regulators10. p75NTR−/− astrocytes had reduced nuclear, but not cytosolic, TGF-β-dependent accumulation of P-Smad2 compared to WT (Fig. 2a and Supplementary Fig. 4). P-Smad2 undergoes regulated intramembrane cleavage by γ-secretase resulting in the liberation of the p75 intracellular domain (p75ICD)11. Nuclear P-Smad2 and neuroen expression were reduced in WT astrocytes treated with γ-secretase inhibitors (Fig. 2b and Supplementary Fig. 5a). In p75NTR−/− astrocytes, transient transfection of p75-FasTM, a p75NTR mutant resistant to γ-secretase cleavage12, increased P-Smad2 nuclear accumulation and deposited neurocan, a component of the glial scar that inhibits neural regeneration7,8 (Supplementary Fig. 5b). Nuclear accumulation of Stat-1 was not affected (Supplementary Fig. 5c). These results suggest that γ-secretase cleavage of p75NTR and its proteolytic product p75ICD regulate P-Smad2 nuclear accumulation in astrocytes.

Sma4d nuclear translocation depends on direct binding to FG-repeat-containing nucleoporins (FG-Nups) that triggers the opening of the nuclear pore13. Since stabilization of the natively unfolded FG domains by binding of nuclear cofactors is necessary for opening of the pore13, we examined whether p75NTR regulates nuclear import by interacting with FG-Nups. In astrocytes, p75NTR showed perinuclear localization and colocalized with FG-Nups, whereas in neurons p75ICD localized primarily to the cytoplasm and plasma membrane, as expected (Supplementary Fig. 6a,b). The distinct ring-like perinuclear localization was specific for the p75ICD, and absorption of the p75NTR antibody with recombinant p75ICD abolished the staining (Supplementary Fig. 6c–e).

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Figure 1 p75NTR deficiency rescues TGF-β-induced hydrocephalus, astrocyte activation and neuronal dysfunction. (a) Hematoxylin and eosin stain and GFAP immunostaining (red) of representative brain sections of 4-week-old WT, p75NTR−/−, GFAP-TGF-β and GFAP-TGF-β, p75NTR−/− mice. Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Representative images from n = 4 mice. (b) Quantification of ventricle size (top) and GFAP intensity (bottom) (n = 4 mice per group). Mean ± s.e.m. Ventricle size: *P = 6.083 × 10−6, **P = 7.792 × 10−6, ***P = 5.849 × 10−5 (from left to right) and n.s., P = 0.467; GFAP intensity: *P = 5.136 × 10−6, **P = 1.337 × 10−6, ***P = 5.138 × 10−5 (from left to right) and n.s., P = 0.275; one-way ANOVA and Fisher’s least significant difference test. Scale bars, 750 µm (top) and 15 µm (bottom). (c) Locomotor activity in the open field during the 50 min of EEG recordings (n = 7 WT mice, 8 GFAP-TGF-β mice and 6 GFAP-TGF-β, p75NTR−/− mice per group). **P = 0.0025 by repeated-measures ANOVA and Bonferroni post hoc multiple comparisons test. (d) Gamma oscillatory power (30–80 Hz) during different locomotor activity intervals in an open field from c. GFAP-TGF-β mice, but not GFAP-TGF-β, p75NTR−/−, had impaired activity-dependent inductions of gamma oscillatory activity (n = 7 WT, 8 GFAP-TGF-β and 6 GFAP-TGF-β, p75NTR−/− mice), n.s., P = 0.4566, 0.3024, 0.1249, 0.0670, 0.9999 and 0.9999, left to right; *P < 0.05, **P < 0.01, ***P < 0.001: 1–5 movements/min, P = 0.0066; 6–25, P = 6.3841 × 10−5; 26–100, P = 4.0256 × 10−13 and P = 1.2077 × 10−12 (left to right); >100, P = 0.0002 and P = 0.0334; one-way ANOVA and Bonferroni post hoc multiple comparisons test.

Super-resolution imaging of p75NTR and FG-Nups in astrocytes by three-dimensional structured illumination microscopy (3D-SIM) identified p75NTR at the nuclear outer membrane adjacent to FG-Nups and inside the nucleus (Fig. 2c. Supplementary Fig. 7 and Supplementary Movie 1). In astrocytes, p75NTR interacted with FG-Nups located throughout the NPC14, including the cytoplasmic filament Nup358, the inner center Nup62, and the nuclear basket Nup153 (Fig. 2d and Supplementary Fig. 8a,b). In situ proximity ligation assay, which allows sensitive single-molecule detection of protein interactions, showed that TGF-β increased the interaction of both Smad2 and p75ICD with Nup153 and Nup358 (Supplementary Fig. 8c). Scanning peptide array analysis and deletion mutagenesis indicated that the Nup153 FG region and the death domain of p75NTR are required for the interaction (Supplementary Fig. 9a–c). Transfection of p75-Δ83 in p75NTR−/− astrocytes increased P-Smad2 nuclear accumulation upon TGF-β treatment by only 2.2-fold, compared to 4.7- and 7.7-fold increases induced by p75FL or p75ICD, respectively (Supplementary Fig. 5b), suggesting that the p75 death domain, which is implicated in TGF-β-induced nuclear entry, is necessary for TGF-β-induced nuclear accumulation of p75ICD. Scanning peptide array analysis and deletion mutagenesis indicated that the FG-Nup signal, suggesting that in unstimulated astrocytes the p75NTR reporter was translocated to the outer nuclear membrane to the inner center of the NPC (Fig. 2e). Quantification of the p75NTR reporter protein revealed that TGF-β increased nuclear accumulation of the p75NTR reporter by 2.2-fold in WT astrocytes, but not fibroblasts, transfected with Cherry-p75NTR-EGFP showed primarily green signal (Fig. 3a and Supplementary Fig. 12a). Inhibition of α-secretase, but not γ-secretase, increased the yellow signal, suggesting that in unstimulated astrocytes the p75NTR reporter was cleaved by α-secretase (Fig. 3b and Supplementary Fig. 12a). Time-lapse imaging in astrocytes transfected with the Cherry-p75NTR-EGFP reporter showed that TGF-β induced the release and translocation of cleaved p75NTR into the nucleus (Fig. 3b,c and Supplementary Movie 2). Similarly, TGF-β increased the release of endogenous p75ICD into the nucleus by ~2.75-fold (P = 0.016) (Fig. 3d). In accordance with the biochemical data for endogenous p75NTR (Fig. 2e), inhibition of γ-secretase abolished the TGF-β-induced accumulation of cleaved p75NTR in the nucleus, suggesting that TGF-β induces the release and nuclear accumulation of p75ICD (Fig. 3b,c and Supplementary Movie 2). Inhibition of p75NTR cleavage by long (24 h) treatment with α- or γ-secretase inhibitors prevented the nuclear localization of p75ICD (Supplementary Fig. 13).

To examine the redistribution of the p75ICD at the NPC upon TGF-β treatment at high resolution, we performed simultaneous imaging by 3D-SIM of endogenous p75NTR at ~800 individual nuclear pores. Upon TGF-β stimulation, the p75ICD traveled from the outer nuclear membrane to the inner center of the NPC (Fig. 3e). Quantification of the p75ICD, nucleoporin and DNA signals (Supplementary Fig. 14) showed that under basal conditions the p75ICD correlated with the DNA signal (Fig. 3e,f), suggesting that at baseline the p75ICD is excluded from the inner center of the NPC. In contrast, upon TGF-β stimulation the p75ICD correlated with the FG-Nup signal (Fig. 3e,f), suggesting redistribution of the p75ICD into the inner center of the NPC. As expected, the DNA

GFAP-TGF-β, p75NTR−/−
GFAP-TGF-β
hydrocephaly
GFAP
DAPI
Ventricle size (µm²)
Autosome activity (movements/min)
GFAP-TGF-β
GFAP-TGF-β, p75NTR−/−
GFAP-TGF-β
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signal was unaltered under basal conditions or upon TGF-β stimulation (Fig. 3e,f). Finally, three-dimensional electron tomography showed increased nuclear pore size in p75<sup>NTR</sup><sup>−/−</sup> astrocytes. Representative immunobLOTS from three independent experiments. Underlined numbers indicate the fold changes of nuclear p75<sup>NTR</sup>-Mediated TGF-β signaling, astrocyte cell-specific regulation of intramembrane proteolysis might contribute to the cell-specific composition of the NPC to create differences in growth factor signal transduction pathways between neurons and astrocytes. Further, we found that p75<sup>NTR</sup> mediated TGF-β signaling altered activity-dependent gamma oscillations, suggesting an unanticipated function for p75<sup>NTR</sup> in the regulation of neural information processing and cognition. Identification of γ-secretase-mediated cleavage of p75<sup>NTR</sup> as a molecular link between TGF-β signaling, astrocyte activation and neuronal functions could provide therapeutic targets for resolving the gliotic scar and promoting neuronal activity.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**Figure 3** TGF-β-induced p75NTR intramembrane cleavage regulates the NPC structure and function. (a) Cherry-p75NTR-EGFP fusion protein, p75NTR N terminal, Cherry (red); p75NTR C terminal, EGFP (green); uncleaved p75FL (yellow); cleaved p75ICD or p75 C-terminal fragment (CTF) (green). EGFP signal reduction diameter measurements by three-dimensional electron tomography in ~16 nuclear pores per condition. Arrowheads, individual nuclear pores; yellow line, TGF-β (relocalization of p75NTR into the NPC in TGF-β). (b) Representative merged Cherry/EGFP images from three independent experiments in the presence of the γ-secretase inhibitor compound E or the α-secretase inhibitor TAPI-2. (c) Quantification of nuclear NPC signal in ~180 NPCs obtained from three independent experiments. (d) TGF-β-treated WT astrocytes show increased nuclear p75ICD (right) compared to control (left). Scale bar, 5 μm. Representative images from five independent experiments. (e) Average signal for p75NTR in control (white) reveals realocalization of p75NTR into the NPC in TGF-β-treated astrocytes. p75NTR signal is absent (black) in the NPC in untreated astrocytes. Scale bar, 100 nm. (f) Quantification of p75NTR (green), FG-Nup (red) and DAPI (blue) signals in ~16 nuclear pores per condition. Arrowheads, individual nuclear pores; yellow line, nuclear pore diameter measurement, mean ± s.e.m. *P < 0.01, **P < 0.0001; n.s., not significant; by one-way ANOVA and Bonferroni multiple comparisons. WT untreated versus WT + TGF-β, P = 0.0003; WT untreated versus p75NTR −/− untreated, P = 3.79 × 10−7; WT + TGF-β versus p75NTR −/− + TGF-β, P = 8.77 × 10−10; TGF-β − untreated versus TGF-β + TGF-β, P = 0.054.

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**AUTHOR CONTRIBUTIONS**

C.S. performed the majority of the experiments. J.K.R. performed histology and surgery, K.M. performed STED microscopy and biochemical experiments. A.S.K. performed EEG recordings and behavioral measurements. P.M.C. performed 3D-SIM microscopy, A.P. performed electron tomography, F.C. performed peptide arrays, N.L.M. contributed to live cell imaging and histology, and E.V. maintained mouse colonies. B.B.-R., V.R. and J.J.P. contributed to animal colonies and histology, R.N. contributed to image analysis, M.D.H., M.H.E., T.W.-C. and J.J.P. contributed to the experimental design, data analysis and interpretation. C.S. and K.A. designed the study, analyzed data, coordinated the experimental work and wrote the manuscript with contributions from all authors.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Cortical stab wound injury. Cortical stab wound injury was performed as described previously in male mice. For the expression analysis of p75 NTR in astrocytes were treated with 2 ng/ml of TGF-β at 30–80% confluency on 24 × 24 mm cover glasses (Assistent), cultured in DMEM supplement with 10% heat-inactivated FBS and penicillin/streptomycin. Image stacks of astrocytes were taken using a 3-D SIM microscope. Briefly, super-resolution imaging was performed using a 3-D SIM microscope equipped with a 100×, 1.4 NA, oil-immersion objective (Olympus). For 3-D SIM recording, image stacks with a z distance of 0.125 µm were acquired. Exposure times were between 100 and 200 ms, yielding typically 3,000–10,000 counts in a raw image of 16-bit dynamic range. Raw images containing 3-D SIM illumination pattern were processed to reconstruct high-resolution information as described with Volocity 5 software (Perkin Elmer). Reconstructed images were registered to compensate for slightly differing camera positions and orientations by calibration with images of 100-nm fluorescent beads. For deconvolution of confocal data sets, a maximum-likelihood estimation algorithm was applied using theoretic point spread functions and a maximum number of ten iterations. To determine numbers and volumes of RF, a threshold-based segmentation was applied using the object separation option of Volocity. Despite the slightly bigger RF sizes within dense clusters possible owing to incomplete separation, the average volume of RF was found to correspond well to the twofold increase in resolution that 3-D SIM provides over confocal laser scanning microscopy.

Three-dimensional structured illumination microscopy (3D-SIM). Astrocytes were grown to 60–80% confluency on 24 × 22 mm cover glasses (Corning) coated with poly-D-lysine, cultured in DMEM supplemented with 10% heat-inactivated FBS and penicillin/streptomycin. Image stacks of astrocytes were taken using 3-D SIM microscope. Briefly, super-resolution imaging was performed using a 3-D SIM microscope equipped with a 100×, 1.4 NA, oil-immersion objective (Olympus). For 3-D SIM recording, image stacks with a z distance of 0.125 µm were acquired. Exposure times were between 100 and 200 ms, yielding typically 3,000–10,000 counts in a raw image of 16-bit dynamic range. Raw images containing 3-D SIM illumination pattern were processed to reconstruct high-resolution information as described with Volocity 5 software (Perkin Elmer). Reconstructed images were registered to compensate for slightly differing camera positions and orientations by calibration with images of 100-nm fluorescent beads. For deconvolution of confocal data sets, a maximum-likelihood estimation algorithm was applied using theoretic point spread functions and a maximum number of ten iterations. To determine numbers and volumes of RF, a threshold-based segmentation was applied using the object separation option of Volocity. Despite the slightly bigger RF sizes within dense clusters possible owing to incomplete separation, the average volume of RF was found to correspond well to the twofold increase in resolution that 3-D SIM provides over confocal laser scanning microscopy.

Stimulated emission depletion (STED) microscopy. Astrocytes were grown to 60–80% confluency on 24 × 24 mm cover glasses (Assistent), cultured in DMEM supplemented with 10% heat-inactivated FBS and penicillin/streptomycin. Astrocytes were treated with 2 ng/ml of TGF-β (R&D Systems) or were left without treatment. Cortical neurons were cultured with 80% ACM, conditioned medium (ACM) and neurite outgrowth assays were performed as described. For inhibitor studies, astrocytes were pretreated with 1 µM compound E (Calbiochem), an inhibitor of γ-secretase activity, or with 20 µM TAPI-2 (Calbiochem), an inhibitor of α-secretase, after transfection and 3 h before TGF-β treatment. TGF-β was added and the Cherry-p75NTR-EGFP intracellular dynamic was imaged in living cells under 5% CO₂ and 95% air through a 63× oil-immersion lens, NA 1.4, on a TCS SP5 confocal system (Leica) with a heated stage (37°C). EGFP and Cherry were excited with the 488-nm line of an argon laser and the 543-nm line of a helium-neon laser, respectively. The FITC/TRITC filter combination was used to detect the EGFP and Cherry signal. Fluorescence emissions were collected between 500 and 560 nm for the EGFP and, to prevent any bleedthrough, we selected a more efficient emission filter for TRITC corresponding to 600–640 nm. Images were acquired in the green and red channels at 11-s intervals over 15 min. The images were processed using LASAF software (Leica). The digitized images were analyzed using Image software (National Institutes of Health). The number of pixels per image with an intensity above a predetermined threshold level within the astrocyte nucleus was quantified by measurement of the EGFP signal of the Cherry-p75NTR-EGFP fusion protein representing the cleaved p75ICD. All quantitative analyses were performed in a blinded manner.

Astrocyte conditioned medium and neurite outgrowth assay. Astrocyte conditioned medium (ACM) and neurite outgrowth assays were performed as described with WT or p75NTR−/− primary astrocytes treated with 20 ng/ml TGF-β for 2 d. For inhibitor studies, astrocytes were pretreated with 10 µM TGF-β receptor type I inhibitor (SB431542, Calbiochem, catalog no. 616461) 1 h before TGF-β treatment. Cortical neurons were cultured with 80% ACM, allowed to extend processes for 24 h and stained with β-tubulin (Sigma). Neurite outgrowth was determined as the proportion of total cells bearing neurites longer than the diameter of the cell body, an indication of successful initiation of neurite outgrowth. The number of neurite-bearing cells was counted and percentage of the total number of cells was calculated. The number of neurite-bearing cells was measured from 400 to 500 neurons per condition. Ten representative images per well were taken. All experiments were repeated four times and were performed in triplicate.

C57BL/6J mice (Jackson Laboratory), C57BL/6-inbred p75NTR−/− mice and the transgenic mouse line GFAP-TGF-β (ref. 4) were used. Transgenic GFAP-TGF-β and p75NTR−/− mice were bred to obtain GFAP-TGF-β: p75NTR−/− mice. All mice were in C57Bl/6 background crossed for more than 10 generations and littermates were used in all experiments. Mice were housed under a 12-h light/dark cycle. Up to five animals per cage were house. They were fed standard chow and had access to food and water ad libitum. All animal procedures were performed under the guidelines set by the University of California, San Francisco, Institutional Animal Care and Use Committee and are in accord with those by the US National Institutes of Health.

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p75NTR cleavage. For TGF-β induced cleavage of p75NTR, primary mouse astrocytes were pretreated with 1 μM compound E (Calbiochem) 3 h before TGF-β treatment. Primary mouse astrocytes were treated with 20 ng/ml TGF-β (R&D Systems) for 1 h. Rat astrocytes were electroporated using Amaxa Astrocult Nucleofector kit (Lonza) with the p75NTR full-length construct. Cells were plated in DMEM with 10% heat-inactivated FBS and penicillin/streptomycin at a density of 500,000 cells per 75-cm² flask coated with poly-d-lysine. Primary p75NTR−/− MEFs were electroporated and cultured in the same conditions, but were plated at a density of 300,000 cells per well of a six-well plate. Fibroblasts were pretreated with 1 μM compound E (Calbiochem) or with 20 μM TAPI-2 (Calbiochem), 14 h and 1 h before TGF-β treatment. 10 μM MG132 (Calbiochem) was added to astrocytes and fibroblasts 1 h before TGF-β treatment. 24 h after electroporation, cells were treated with 20 ng/ml TGF-β (R&D Systems) for 12 h for astrocytes and for 3 h for embryonic fibroblasts, and cell lysates were harvested and processed for western blotting. The primary mouse astrocyte nuclear fraction was prepared using the Active Motif kit (Active Motif 54001). Cell lysates were probed with rabbit anti-p75ICD (1:300, 9992, kind gift of M.V. Chao, New York University), rabbit anti-p75NTR (1:1,000, 07-476, Millipore), rabbit anti-H3 (1:1,000, 9715, Cell Signaling) and rabbit anti–GAPDH (1:1,000, 2118, Cell Signaling).

Peptide array mapping. Peptide array mapping was performed as described. Peptide libraries were synthesized by automated SPOT synthesis. Synthetic overlapping peptides (25 amino acids in length) of Nup153 were spotted on Whatman 50 cellulose membranes according to standard protocols by using Fmoc chemistry with an AutoSpot Robot ASS 222 (Intavis Bioanalytical Instruments AG). Membranes were overlaid with 10 μg/ml recombinant GST-p75 NTR ICD. Bound recombinant GST-p75 NTR ICD was detected using goat anti-GST (1:2,000, 27-4557-01, GE Healthcare) followed by secondary rabbit anti–horseradish peroxidase antibody (1:2,500, RPN 4031, GE Healthcare).

Electron tomography. For electron microscopy, primary astrocytes were grown on poly-l-lysine-coated 75 cm² tissue culture flasks. Astrocytes were treated with 2 ng/ml of TGF-β (R&D Systems) for 1 h. Astrocytes were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde and 0.1 M sodium cacodylate pH 7.4 and pelleted, then postfixed in 1% osmium tetroxide, 0.8% potassium ferrocyanide and 3 mM calcium chloride in the same buffer, en bloc stained in 2% uranyl acetate, dehydrated in ethanol, and infiltrated and embedded in Durcupan. Sections were sectioned on a Reichert Ultracut S ultramicrotome at a thickness of 300 nm and sections picked up on 100-mesh clamsHELL grids. These sections were poststained for 15 min in a 1% uranyl acetate solution followed by 5 min in a Sato lead solution. To ensure stability in the beam, a thin coat of carbon was applied to each side of the sections. Collodion gold particles with a diameter of 15 nm were deposited on opposite sides of the section to serve as fiducial cues. For each tilt series, a single series of images was collected with a JEOL (Tokyo, Japan) 4000EX intermediate-voltage electron microscope operated at 400 kV. The specimens were irradiated before collecting data to limit anisotropic specimen thinning during tilt series collection. Using a CCD camera, tilt series were recorded at 20,000x magnification in angular increments of 2° from ~60° to ~60° about an axis perpendicular to the optical axis of the microscope. Precise angular increments were achieved using a computer-controlled goniometer. The pixel dimensions of the CCD camera were 3448 x 3448 and the pixel resolution was 0.865 nm. The IMOD software package was used for rough alignment of the tilt series and the Tbx8 software package was used for fine alignment and reconstruction. Nuclear pore diameter measurements were made using NIH Image. Measurements were taken in the x-y plane of multiple slices about the presumed center of the pore and the maximum value was taken to be the true center.

Proximity ligation assay (PLA) analysis. PLA analysis was performed following the manufacturer’s instructions (Olink Bioscience). Briefly, astrocytes were plated in 300 μl of DMEM with 10% heat-inactivated FBS and penicillin/streptomycin at a density of 30,000 cells per well in eight-well Nunc plates coated with poly-l-lysine in 300 μl of DMEM with 10% heat-inactivated FBS and penicillin/streptomycin at a density of 30,000 cells per well in eight-well Nunc plates coated with poly-l-lysine and cultured for 2 d at 37 °C in 5% CO₂. Cells were fixed with 4% PFA for 4 °C for 30 min, permeabilized using 0.1% Triton/PBS for 10 min at 4 °C, and blocked by using the blocking solution from the Olink PLA kit for 30 min at 37 °C. Cells were incubated with primary antibody diluted in 1% BSA/PBS overnight at 4 °C. The following primary antibodies were used: rabbit anti-p75ICD (1:300, 9992, kind gift of M.V. Chao, New York University), anti–mouse nuclear pore complex (mouse monoclonal (Mab414) to nuclear pore complex proteins) (1:2,000, ab24609, Abcam), anti–mouse Nup358 (1:100, sc-74518, Santa Cruz Biotechnology), anti–rat Nup153 (1:100, sc-101544, Santa Cruz Biotechnology), anti-rabbit P-Smad2 (1:500, sc-7316, Cell Signaling). Cells were incubated with the appropriate PLA probes (secondary anti-mouse, anti-rat and anti-rabbit antibodies, respectively) for 60 min at 37 °C. For ligation and circularization of the DNA oligonucleotides, cells were incubated with ligation-solution for 30 min at 37 °C. For rolling circle amplification, cells were incubated with amplification solution containing a fluorophore with the excitation wavelength of 594 nm for 100 min at 37 °C. Cells were mounted with a coverslip using a minimal volume of Duolink In situ Mounting Medium with DAPI and analyzed by confocal microscopy. Images were acquired on a Leica TCS SP8 laser confocal microscope with a 63× oil immersion objective, NA 1.4 and LAS AF image analysis software. For quantification, the PLA signal per nucleus was counted for 70 nuclei per each condition. All quantitative analyses were performed in a blinded manner.

Immunohistochemistry. P28 mice were transcardially perfused with ice-cold saline under avertin anesthesia and brain samples were removed, embedded in OCT (Tissue-Tek) and frozen on dry ice. Immunohistochemistry on sagittal brain cryostat sections was performed as described. The primary antibodies used were rat anti–glial fibrillary acidic protein (GFAP) (1:1,000; 13-0300, Invitrogen), rabbit anti-neurocan (1:2,000, sc-6189, Santa Cruz Biotechnologies), rabbit anti-p75ICD (1:300, 9992, kind gift of M.V. Chao, New York University), rabbit anti-p75NTR (1:300, AB1554, Millipore) and secondary antibodies were conjugated to Alexa Fluor 488 or 594 (1:200; Jackson Immunoresearch Laboratories). Quantitative image analysis for the immunostained mouse sagittal sections was performed on three separate tissue sections through the body of the lateral ventricle. For measurement of ventricle sizes in the brain, cresyl violet–stained sagittal sections an equivalent distance from the midline were chosen on the basis of common morphological landmarks. Ventricle size was calculated from the ventricle area divided by the total brain area in a blinded manner using ImageJ. For quantification of GFAP intensity at the lateral ventricle, five nonoverlapping rectangular boxes (100 × 100 μm) were placed along the subventricular zone of the lateral ventricle. The digitized images were analyzed using ImageJ software (US National Institutes of Health). The number of pixels per image with an intensity above a predetermined threshold level was quantified by measurement of the immunoreactive areas for GFAP. The measurement of total immunoreactivity is represented as percent area density defined as the number of pixels (positively stained areas) divided by the total number of pixels (sum of positively and negatively stained area) in the imaged field. All quantitative analyses were performed in a blinded manner.

Cell culture. HEK293T (ATCC), NIH3T3 (ATCC) and isolated MEF cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and penicillin/streptomycin. MEFs and primary cortical astrocytes were isolated from p75NTR−/− mice as we described. Astrocytes were used for experiments after they reached confluency.

Immunocytochemistry. Primary astrocytes were plated for 2 d at a density of 20,000 cells per well in eight-well Nunc plates coated with poly-l-lysine in 300 μl of DMEM with 10% heat-inactivated FBS and penicillin/streptomycin. Astrocytes were treated with 2 ng/ml of TGF-β (R&D Systems) for 1 h. Immunocytochemistry was performed as described using goat anti-p75ICD (1:100, sc-6189, Santa Cruz Biotechnologies), rabbit anti-p75NTR (1:300, 9992, kind gift of M.V. Chao, New York University), mouse anti–nuclear pore complex protein (1:2,000, ab24609, Abcam), rabbit anti–phospho-presenilin-1 (1:100, ab78914, Abcam), rabbit anti–phospho-Smad2 (1:500, ab3849, Millipore) in PBS with 1% BSA overnight.

RNA isolation and quantitative PCR. RNA was isolated from primary astrocyte cultures and quantitative real-time PCR was performed as described. The following primers were used:

- **Neurocan (Ncan)**: Forward: 5′-TGC AAC CAC GAC GGC TAA GCT C-3′
  Reverse: 5′-GGG GAT AAG CAG GCA ATG AC3′
- **Fibronectin (Fn1)**: Forward: 5′-GCA GTG ACC ACC ATT CCT G-3′
  Reverse: 5′-GTT AGC CAG TGA CCT GCT GAA C-3′
- **GAT1 (Slc6a1)**: Forward: 5′-GAAACGCTGCTTCTGTACGGT-3′
  Reverse: 5′-AGCAAACGATGAGTGGAGTCCC-3′

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S100b Fwd: 5′-TGTTGCCCTATTGATGTCT-3′
S100b Rev 5′-CCCATCCCCCATTGTGCT-3′
Gadph Fwd: 5′-CAAGGCGCGAAATGGGAAA-3′
Gadph Rev: 5′-GACCTACCCTTTGATGTTC-3′

Immunoblots. Immunoblots to detect neurocan in the supernatant of WT or p75NTR−/− astrocytes were performed as described. Astrocytes were treated with 20 ng/ml TGF-β (R&D Systems) for 2 d and pretreated with 10 µM SB431542 (Sigma), an inhibitor of TGF-β receptor type I, 1 h before TGF-β treatment as indicated. For rescue experiments, p75NTR−/− astrocytes were infected with lentivirus expressing p75NTR constructs, medium was changed 2 d after infection and cells were treated on the third day after infection. Astrocyte cytoplasmic and nuclear fractions were prepared using the Active Motif kit (Active Motif). For detection of P-Smad2, WT and p75NTR−/− astrocytes were treated with 2 ng/ml TGF-β (R&D Systems) for 1 h or, in inhibitor studies, WT astrocytes were pretreated with 1 µM compound E (Calbiochem), an inhibitor of γ-secretase activity, 1 h before TGF-β treatment. For detection of P-presenilin-1, WT primary astrocytes were treated with 2 ng/ml TGF-β. The following primary antibodies were used: rabbit anti-histone H3 (1:1,000, 9715, Cell Signaling), rabbit anti-P-Smad2 (1:1,000, 3101, Cell Signaling), mouse anti-Smad2 (1:1,000, 3103, Cell Signaling), rabbit anti-GAPDH (1:1,000, 2118, Cell Signaling), rabbit anti-neurocan (1:500), rabbit anti–P-presenilin-1 (1:1,000, 78914, Abcam), rabbit anti-presenilin-1 (1:1,000, ab71181, Abcam), mouse anti-α-tubulin (1:1,000, T6199, Sigma).

Coimmunoprecipitation. Coimmunoprecipitation was performed as described. For mapping experiments, immunoprecipitation was performed with anti-HA antibody (Cell Signaling) or anti-Nup153 antibody (Santa Cruz Biotechnologies). For endogenous coimmunoprecipitation, cell lysates were incubated with rabbit anti-mouse p75NTR antibody (1:100; 9992, kind gift of M.V. Chao, New York University) and rat anti-mouse Nup153 antibody (5 μg, Santa Cruz Biotechnology) bound to A-agarose beads for 4 h at 4°C. Cell lysates were probed with the following antibodies: mouse anti–nuclear pore complex protein antibody (1:1,000, ab24609, Abcam), rat anti-Nup153 antibody (1:500, sc-101544, Santa Cruz Biotechnology), mouse anti-Nup358 antibody (1:500, sc-74518, Santa Cruz Biotechnology), rabbit anti-HA (1:1,000, 3724, Cell Signaling), rabbit anti-p75NTR (1:1,000, AB1554, Millipore).

Statistics. Statistical significance was calculated using GraphPad Prism (GraphPad Software) by unpaired or paired two-sided Student’s t-test to analyze significance between two experimental groups or by one-way ANOVA, Fisher’s least significant difference test or Bonferroni’s post-test, or two-way ANOVA for multiple comparisons. Data are presented as mean ± s.e.m. Data distribution was assumed to be normal, but this was not formally tested. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications. No randomization was used to assign experimental groups or to collect data, but mice and cells were assigned to specific experimental groups without bias. All animals were present at the end of study. No data points were excluded. All histopathological analyses were performed in a blinded manner.

A Supplementary Methods Checklist is available.

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