Supporting Information

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SI Methods

Animals. All mice were fed a standard diet ad libitum and maintained on a 12-h light/dark cycle. For survival experiments, 6- to 9-wk-old mice were anesthetized intraperitoneally with a combination of ketamine (10 mg/kg) and xylazine (1 mg/kg; Phoenix Scientific), and then a drop of proparacaine hydrochloride ophthalmic solution (0.5%) was applied to the eye to deliver local anesthesia before corneal injury. The animal sample size was calculated using power analysis software; animals were randomly treated and evaluated, and all sample analysis was done in a double-masked manner.

TG Neuronal Growth Assay. TG neurons were treated with 100 ng/mL PlGF2 or HB-EGF (R&D Systems) and neurite imaged after 4 d in culture. For inhibition studies targeting the PI3K pathway, TG neurons were treated with 25 μM LY294,002 (Sigma L9908) or vehicle for 1 h, and then 50 μM of VEGF-A or -B was added. For inhibition studies targeting the Notch pathway, TG neurons were pretreated with DAPT (a γ secretase inhibitor that indirectly inhibits Notch, Sigma D5942) or vehicle for 1 h, and then 50 μM of VEGF-A or -B was added. Neurite growth was followed for 72 h, and the growth factor-induced neurite elongation was classified into four types: (*i*) nascent (neurite budding and less than 35 μm in length), (*ii*) short (neurites between 50 and 100 μm in length), (*iii*) medium (neurites between 150 and 300 μm in length), and (*iv*) long (neurites longer than 400 μm). All neurite growth was imaged with an AxioObserver Z1 fluorescence microscope in the YFP channel attached to an AxioCam HRm digital camera (Zeiss) operated by AxioVision 4.0 software. Images were analyzed using Neurolucida 9 (MBF Bioscience), a quantitative nerve tracing software.

Cell Viability Assay and Immunostaining. The effect of VEGF-B on the survival of TG neuronal cells was evaluated with the Live/Dead Viability/Cytotoxicity kit (Invitrogen) according to the manufacturer's protocol and as previously described (1). To determine the expression of VEGFR1 and NRP1 on TG neurons treated with VEGF-B, cells from thy1-YFP mice cultured for 3 d were incubated with a goat anti-mouse VEGFR1 (R&D Systems AF471) or rabbit anti-mouse NRP1 (Santa Cruz SC5541). Positive staining was visualized using Cy3 conjugated secondary antibodies.

RNA sequencing and gene expression analysis. TG neurons were isolated from 3- to 4-week-old thy1-YFP mice. Neurons were dissociated as previously described (1), pooled, and seeded on 35-mm tissue culture dishes with cover glass bottoms (World Precision Instruments). After 2 h incubation at 37 °C, neurons were treated with either 50 ng/mL VEGF-A or VEGF-B or left untreated. After 3 d in culture, medium was gently aspirated and RNA obtained using an RNeasy Plus micro kit (Qiagen). RNA quality was verified and RNA sequencing was performed on an Illumina HT DNA Sequencing–HiSeq2500/1500 by the Genomics Resources Core Facility from Weill Cornell Medical College. Gene expression was analyzed using ArrayStar 5 software (DNASTAR). These experiments were repeated four times and data analyzed for cluster differences and samples pooled for statistical comparisons.

Corneal Nerve Regeneration Analysis. Quantification of corneal nerves regeneration was obtained from corneal whole mount images traced and analyzed with Neurolucida 9 software. Five image fields were sampled in each corneal whole mount: one at the center cornea and the remainder at midway between the central and limbal cornea for each of four corneal quadrants. Central and peripheral cornea nerve densities were calculated and expressed as a percentage of control.

Corneal Nerve Sensation Testing. The degree of recovered nerve sensation in the injured cornea was evaluated using calibrated von Frey Hairs (North Coast Medical). In this method the center of the cornea of an unanesthetized mouse was touched with thin filaments of ascending stiffness (0.008–1.4 g) until the hairs buckled and a blink response was elicited. C57BL/6 wild-type and *Vegf-b*$^{-/-}$ mice subjected to corneal epithelial debridement and receiving subconjunctival injection of VEGF-B, as well as their respective controls, were evaluated to determine the minimal force (g) needed for a blink response. Eight mice were tested in each group in three individual experiments.

VEGF-B Protein Expression. VEGF-B protein expression was analyzed in corneal epithelia and stroma collected before surgery and at days 1, 3, and 7 after corneal debridement ($n = 6$ corneas per time point). The level of expression was determined with the mouse VEGF-B ELISA kit (USCN Life Science Inc. SEA144Mu) according to the manufacturer's protocol and as previously described (1). VEGF concentrations were normalized to total protein concentrations. To corroborate the ELISA data, proteins were also separated in SDS/PAGE gels by PAGE electrophoresis and then transferred onto polyvinylidene difluoride membranes for Western blot analysis. After blocking, membranes were incubated with mouse anti-VEGF-B (Santa Cruz Biotechnology sc-80442) and rabbit anti-actin (Abcam 1801) antibodies. Membranes were washed three times in PBS and 0.1% Tween-20 and incubated with IRDye 800CW donkey anti-rabbit IgG and IRDye 700CW goat anti-mouse IgG antibodies (1:10,000; Rockland Immunochemicals) for 1 h. Blots were imaged and analyzed with the Odyssey Infrared Imaging System (LI-COR).

In Vitro Scratch Wound Assay. Human corneal limbal epithelial (HCLE) cells were cultured in six-well plates until confluency as previously described (1). Cells were starved in keratinocyte serum-free medium (KSFM) medium (Gibco 10724-011) without supplements for 24 h. Then a straight-line scratch mark was made in each well with a 200-μL pipette tip. KSFM medium supplemented with VEGF-B^{186} (50 ng/mL) or EGF (10 ng/mL, Millipore 01-101) was added to the wells. Scratch areas were imaged at 0, 8, 24, and 48 h at the same locations using the mark and find module of the Axiovision software and the wound closure analyzed by Photoshop.

1. Pan Z, Fukuoka S, Karagianni N, Guaiquil VH, Rosenblatt MI (2013) Vascular endothelial growth factor promotes anatomical and functional recovery of injured peripheral nerves in the avascular cornea. *FASEB J* 27(7):2756–2767.

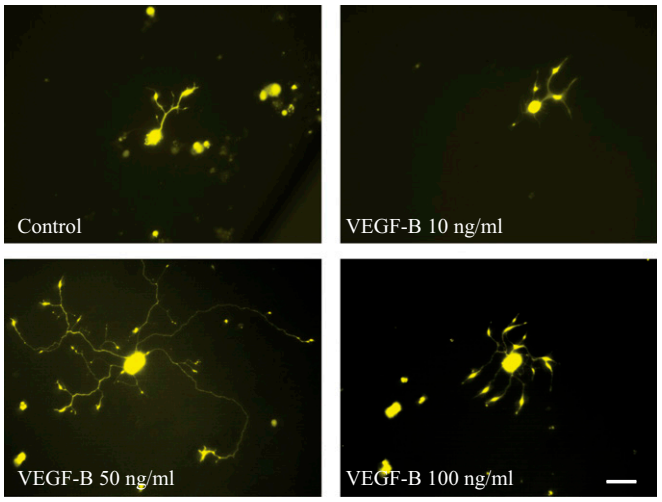


Fig. S1. VEGF-B induced dose-dependent neurite growth in cultured neurons. Isolated TG neurons from thy1-YFP mice were incubated in growth medium alone or with increasing concentrations of VEGF-B. After 3 d in culture the neurons were imaged and neurite growth analyzed using Neurolucida software. We found that 50 ng/mL was the most potent dose for inducing extensive neurite elongation and branching. Data are expressed as mean ± SEM of four independent experiments. $*P \leq 0.01$. (Scale bar, 50 μm.)

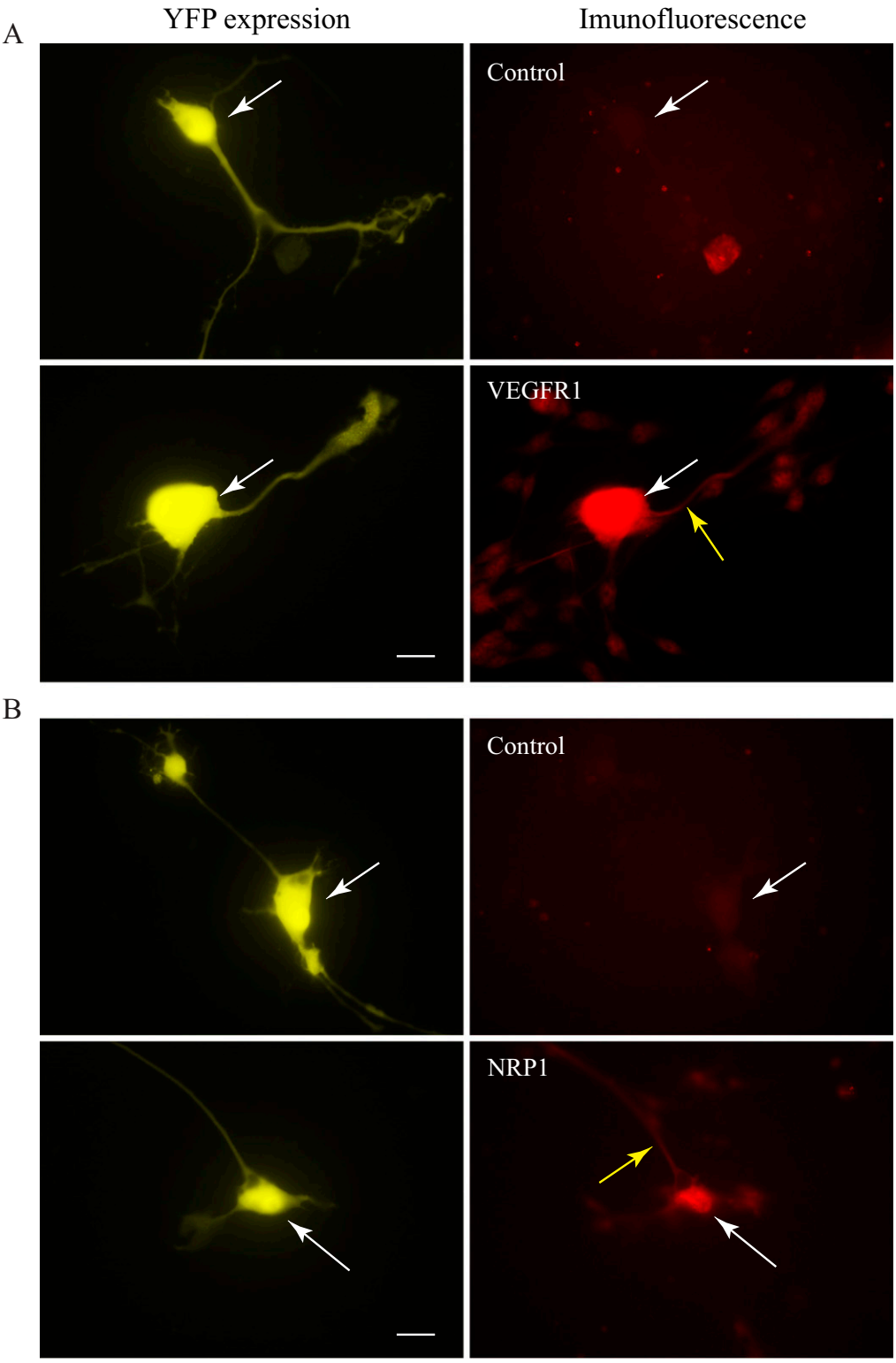


Fig. S2. VEGFR1 and NRP1 are expressed in TG neurons. TG neurons from thy1-YFP mice were treated with 50 ng/mL VEGF-B and incubated for 3 d. Cells were fixed in 2% PFA for 20 min, blocked with 1% BSA and 0.1% triton in PBS, and incubated with antibodies against VEGFR-1 and NRP1. Cy3-conjugated secondary antibodies were used to detect protein expression. (*Left*) Normal YFP neurofluorescence. (*Right*) Control cells were incubated without primary antibody. (*A*) VEGFR1 and (*B*) NRP1 expression is mostly localized in the cell body, although some positive staining is present in the neurites. White arrows indicate the localization of the cell body, yellow arrows indicate neurite staining. (Scale bars, 20 μm.)

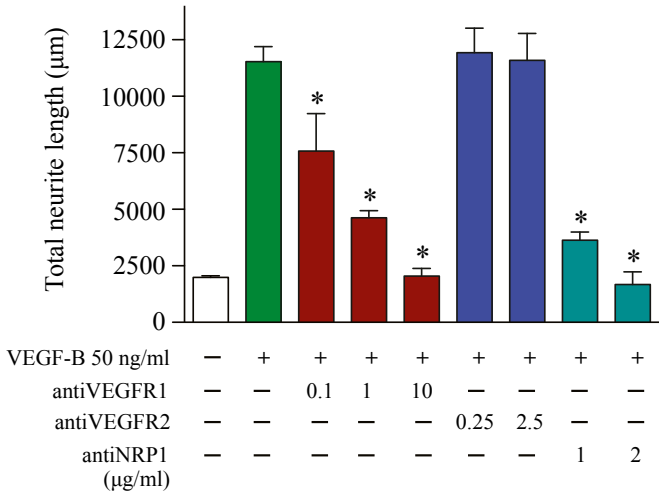


Fig. S3. VEGFR1 and NRP1 inhibition blocks VEGF-B–induced neurite growth. Isolated TG neurons were preincubated for 1 h with antibodies against VEGFR1, VEGFR2, and NRP1 in a dose-dependent manner. The cells were then treated with 50 ng/mL VEGF-B, and neurite growth was evaluated after 3 d. VEGF-B–induced neurite growth was inhibited dose dependently by antibodies against VEGFR1 and NRP1, but no effect was seen in cells pretreated with anti-VEGFR2 antibody. Data are expressed as mean ± SEM of three independent experiments. *$P \leq 0.01$.

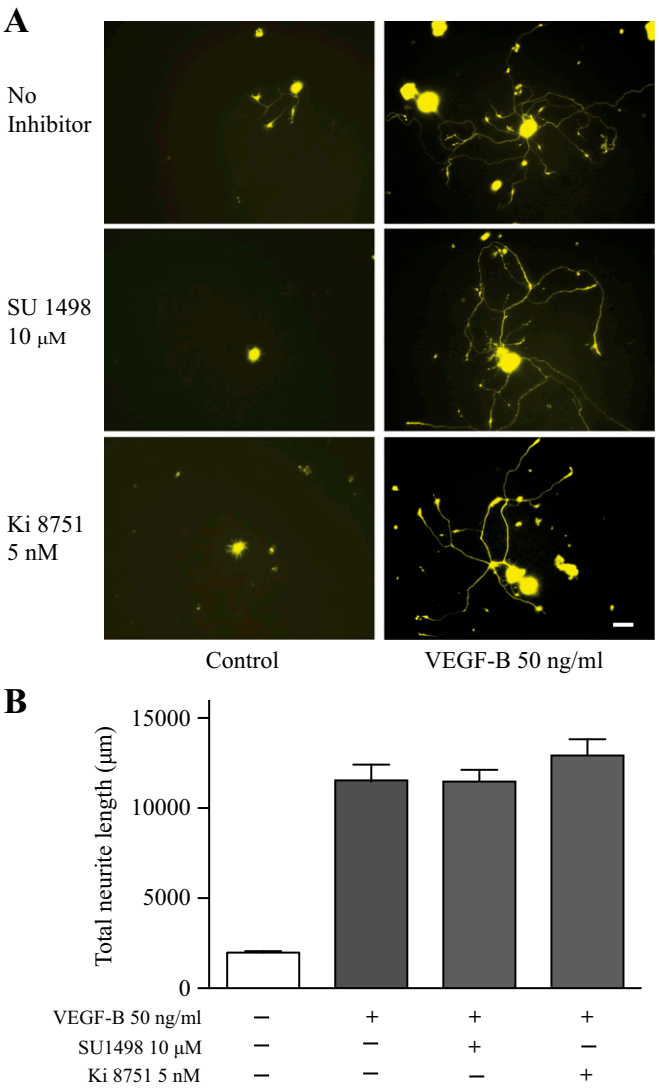
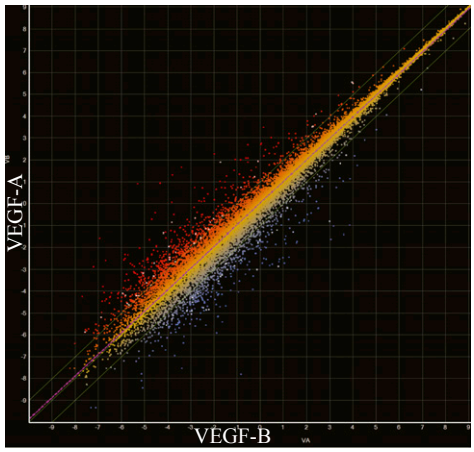


Fig. S4. VEGFR2 signaling is not involved in VEGF-B–induced neurite growth. To confirm that VEGFR2 signaling does not play a role in VEGF-B–induced neurite growth, TG neurons were preincubated for 1 h with 10 μM SU1498 or 5 nM Ki8751, two specific tyrosine kinase inhibitors that target the tyrosine kinase domains of VEGFR2. The cells were then treated with 50 ng/mL VEGF-B, and neurite growth was evaluated after 3 d. (*A*) There was no inhibition of neurite growth in any of the treatments and (*B*) the dendrite length was no different from control without inhibitors. Thus, VEGFR2 activation is not required for VEGF-B–induced neurite growth. Data are expressed as mean ± SEM of three independent experiments. *$P \leq 0.01$. (Scale bar, 50 μm.)

A Scatter plot of RNA seq gene expression in treated TG neurons



193 genes at 90 % confidence:
89 genes at 2 fold change
18 genes at 4 fold change
4 genes at 8 fold change

B Heat map indicating the change in gene expression in treated TG neurons

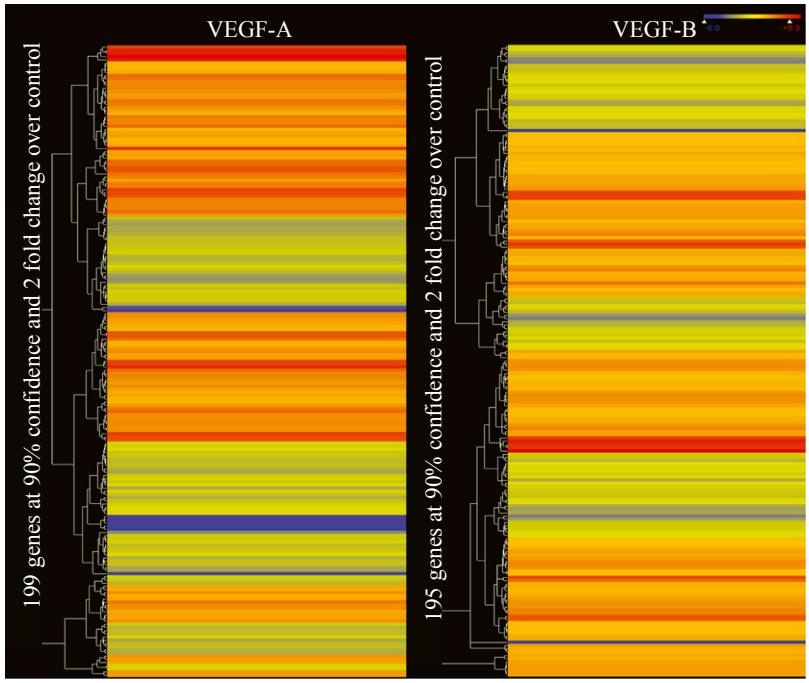


Fig. S5. VEGF-A and VEGF-B induced differential gene expression in TG neurons. Isolated TG neurons from 3 to 4 mice were pooled and treated with either VEGF-A or VEGF-B or left untreated. After 3 d in culture RNA was extracted, checked for quality, and RNA sequence analysis performed using an Illumina HT DNA Sequencing -HiSeq2500/1500. (*A*) Scatter plot showing that 193 genes presented between two- and eightfold change in gene expression when comparing neurons treated with VEGF-A and VEGF-B. (*B*) Heat map showing the distinct gene expression pattern found in VEGF-A or -B–treated neurons compared with untreated control.

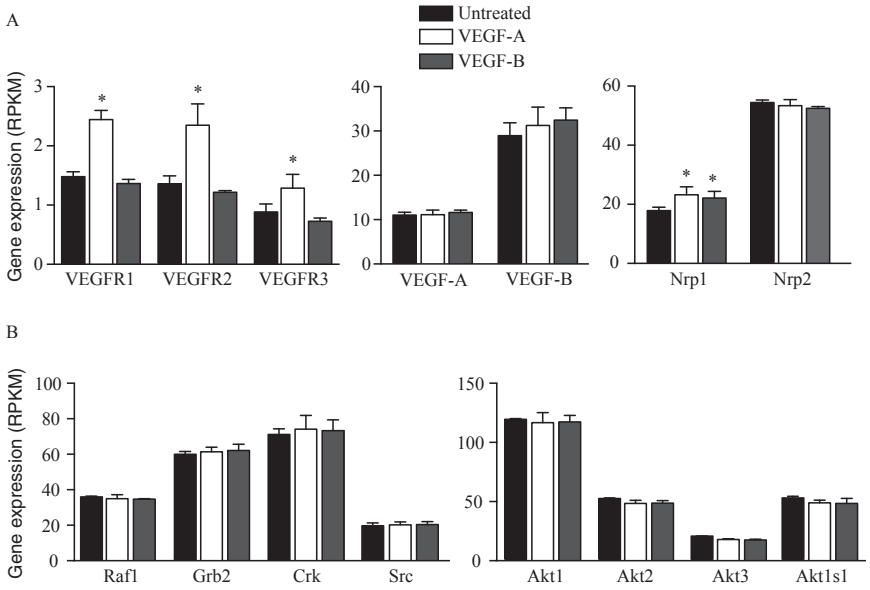


Fig. S6. VEGF-A treatment induces increased VEGF receptor expression. (*A*) RNA sequencing analysis demonstrated that higher levels of VEGF receptors occur in TG neurons treated with VEGF-A compared with those treated with VEGF-B or untreated control. The expression of NRP1 was higher upon VEGF-A or -B treatment, and both NRP1 and NRP2 showed 10- to 25-fold higher expression than the VEGF receptors. (*B*) We found no differences in the expression of common intracellular signaling candidates regularly involved in cellular growth and differentiation.

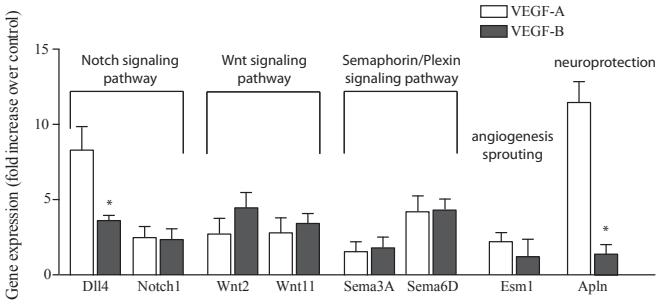


Fig. S7. Potential intracellular signaling pathways involved in TG neurite growth. RNA sequencing analysis shows that important elements of the Notch, Wnt, and Semaphorin/Plexin signaling pathways may play a role in neurite growth in TG neurons treated with either VEGF-A or VEGF-B. Additionally, genes that are involved in cellular sprouting and neuroprotection also showed differential gene expression when treated with either VEGF-A or -B.

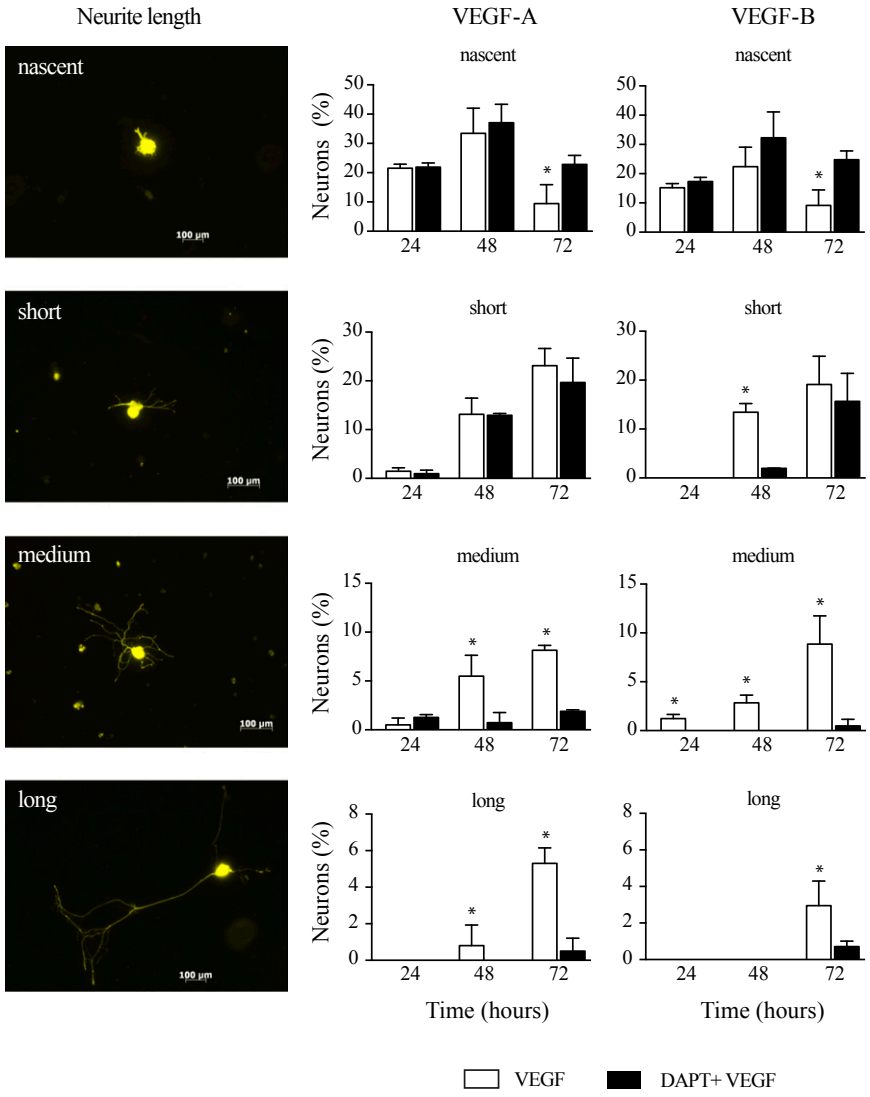


Fig. S8. Inhibition of Notch signaling impairs neurite elongation. TG neurons were pretreated with the γ secretase inhibitor DAPT and then incubated with either VEGF-A or VEGF-B. Neurite growth was followed, and the number of neurons showing distinct elongation patterns was analyzed (*SI Methods*). There was no impairment of initiation of neurite growth (budding) from the neuronal cell body in either treatment. However, cells treated with the inhibitor mainly form nascent or short neurites, whereas cells treated with either VEGF-A or -B alone induced significant neurite elongation in a time-dependent manner. Neurite elongation was inhibited at all points analyzed by the DAPT inhibitor, but there was no effect on cell viability, and a significant percentage of neurons still presented nascent or short neurites after 3 d in culture. These data indicate that signaling through the Notch pathway is required for VEGF-induced neurite growth. Data are expressed as percentage of total neurons in culture; bars represent the mean ± SEM. $*P \leq 0.01$.

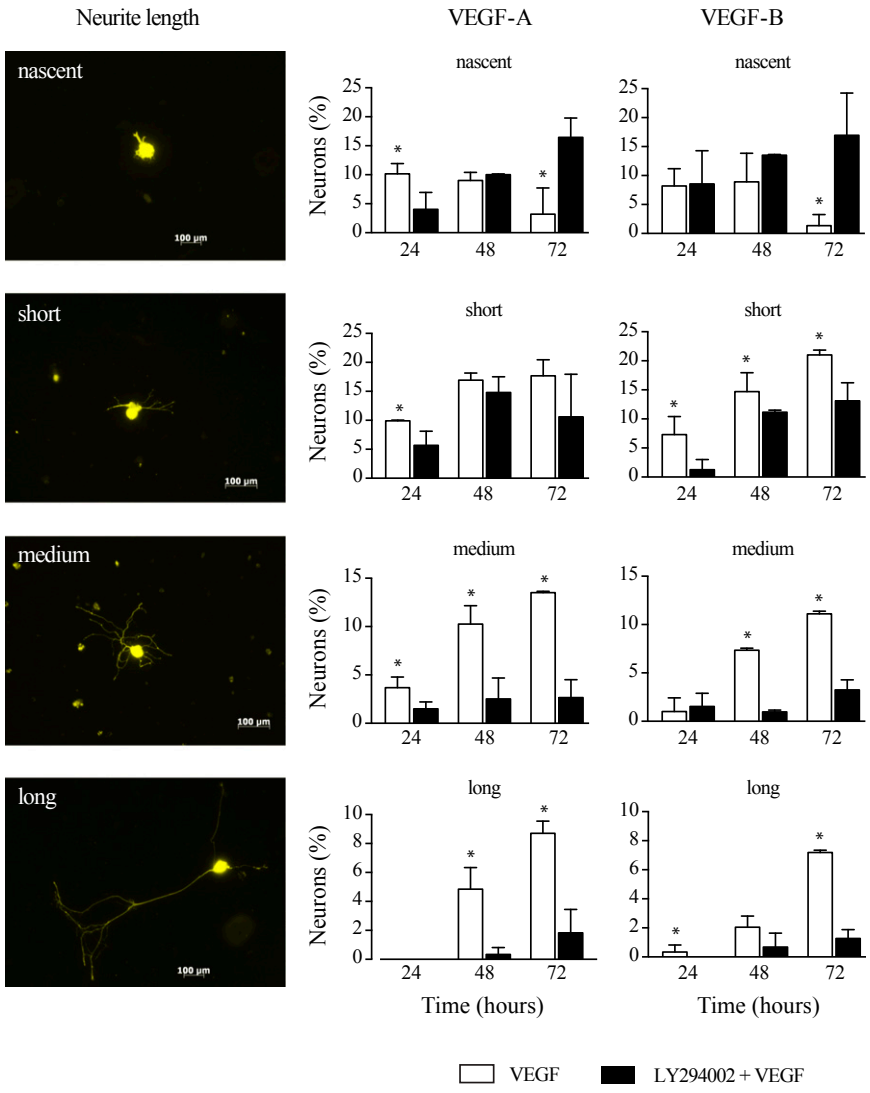


Fig. S9. Signaling through PI3K is required for neurite elongation. TG neurons were pretreated with the PI3K inhibitor LY294,002 and then incubated with either VEGF-A or VEGF-B. Neurite growth was followed, and the number of neurons showing distinct elongation patterns was analyzed (*SI Methods*). There was no impairment on initiation of neurite growth from the neuronal cell body in either treatment. However, cells treated with the inhibitor mainly form nascent or short neurites, whereas cells treated with either VEGF-A or -B alone quickly form all neurite sizes. Neurite elongation was inhibited at all points analyzed by the PI3K inhibitor, but there was not an effect on cell viability because a significant percentage of neurons still presented nascent or short neurites after 3 d in culture. These data prove that signaling through the PI3K pathway is required for VEGF-induced neurite growth. Data are expressed as percentage of total neurons in culture; bars represent the mean ± SEM. *$P \leq 0.01$.

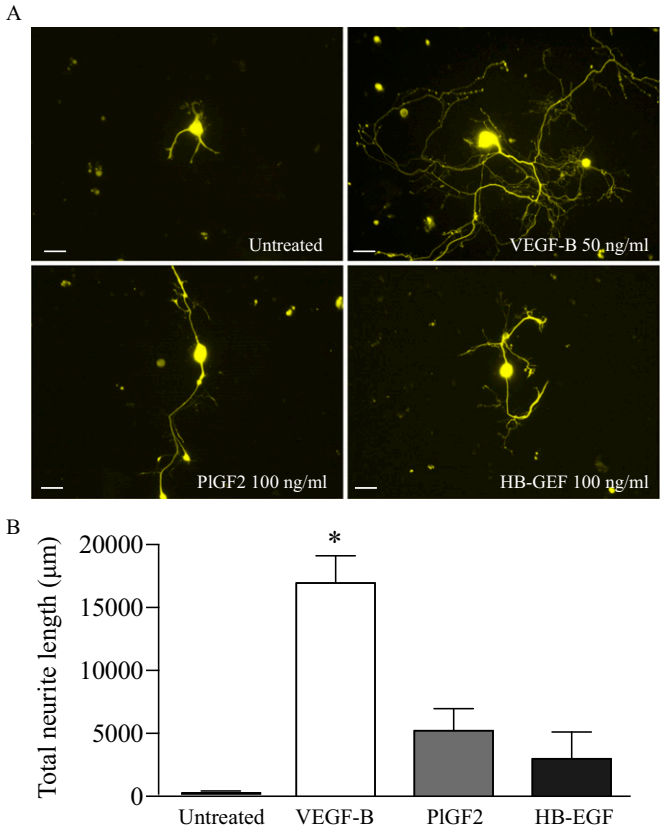


Fig. S10. PlGF2 and HB-EGF induced neurite growth. Isolated TG neurons were left untreated or incubated with 50 ng/mL VEGF-B or 100 ng/mL PlGF2 or 100 ng/mL HB-EGF. After 4 d in culture the neurons were imaged and compared for potency against the control. (*A*) Both PlGF2 and HB-EGF increased neurite growth compared with untreated control cells. (*B*) In general HB-EGF induced a stronger neurite growth than PlGF2, but when total neurite length was compared this difference was not significant, and both were less potent than VEGF-B. (Scale bars, 100 μm.)

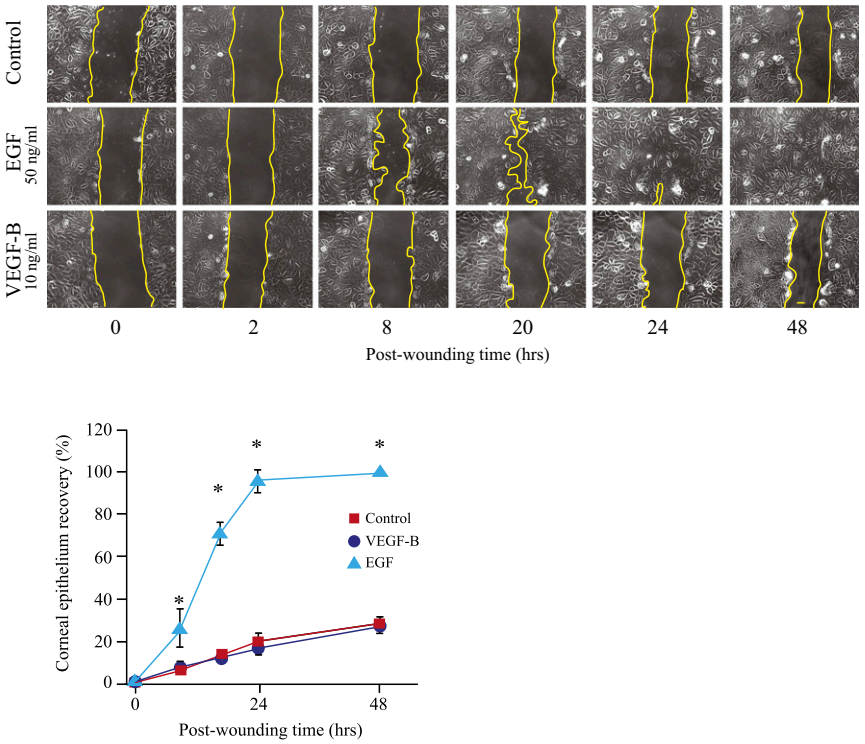


Fig. S11. VEGF-B does not directly induce epithelial wound healing. A scratch was made on HCLE cells grown to confluence in KSFM (*SI Methods*). Cells were cultured in the absence or presence of 50 ng/mL VEGF-B or 10 ng/mL EGF. The cell growth into the scratch areas was imaged at 0, 8, 24, and 48 h at the same locations in the culture using an automated phase-contrast microscope. VEGF-B had no effect on inducing HCLE cell growth compared with untreated control. Quantification demonstrated that EGF induced cell growth into the scratch area as soon as 8 h after treatment, with complete confluence at 24 h. However, VEGF-B did not influence reepithelialization of the scratched area. Data are expressed as mean ± SEM of three independent experiments. *$P \leq 0.01$. (Scale bar, 50 μm.)