

Critical Role of TrkB and Brain-Derived Neurotrophic Factor in the Differentiation and Survival of Retinal Pigment Epithelium

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In the vertebrate eye, the retinal pigment epithelium (RPE) and the neural retina arise from a single layer of neuroectoderm. Factors influencing the differentiation of retinal neurons have been identified; however, little is known about molecules directing the differentiation of the RPE. Here we have found that the neurotrophin brain-derived neurotrophic factor (BDNF) plays an autocrine role in the differentiation and survival of *Xenopus laevis* RPE. Fluorescent *in situ* hybridization studies showed a precise co-expression of BDNF and its receptor trkB in the retinal neuroepithelium and

actively differentiating RPE; *in vitro* studies demonstrated survival- and differentiation-promoting effects in serum-free explants and dissociated cultures. When a dominant negative mutant of the trkB receptor was expressed in developing embryos, severe arrest of RPE differentiation was seen with persistence of nestin- and *Notch*-positive neuroblasts.

Key words: BDNF; trkB; neurotrophin; retinal pigment epithelium; differentiation; retina; dominant negative

The vertebrate retina differentiates from a double layer of neuroepithelial precursors constituting the optic cup (for review, see Park and Hollenberg, 1993). Cells of the outer layer withdraw from the cell cycle to differentiate as a single layer of pigmented cuboidal epithelium, whereas cells of the inner layer continue proliferating and differentiating until the entire multilayered neural retina is generated. Numerous molecules, including basic fibroblast growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3, insulin-like growth factor I, laminin, retinoic acid, and mammalian *hairy* and *Enhancer of Split* Homolog 1 (Pittack et al., 1991; Cohen-Cory and Fraser, 1994; Kelley et al., 1994; Pittack and Reh, 1994; Bovolenta et al., 1996; Frade et al., 1996; Tomita et al., 1996), have been implicated in the differentiation of the neural retina; however, little is known about putative differentiation factors for the RPE.

The neurotrophins are a family of growth factors that have potent survival- and differentiation-promoting effects in neuronal and epithelial cell populations (Wheeler and Bothwell, 1992; Eide et al., 1993; Hallbook et al., 1993; Brill et al., 1995; Mitsiadis and Luukko, 1995). Receptors for the neurotrophins (the trks) are found throughout the visual system, including in germinal retinal neuroepithelium and most retinal cell groups (LaVail et al., 1992; Cohen-Cory and Fraser, 1994; Okazawa et al., 1994; Unoki and LaVail, 1994; Koide et al., 1995; Perez and Caminos, 1995; Ugolini et al., 1995; Cohen-Cory et al., 1996). BDNF became a particularly interesting candidate for trophic activity in the RPE because of its light inducibility (Okazawa et al., 1994) and its ability to protect photoreceptors from damage by light *in vivo* (LaVail et al., 1992). Among its many critical functions in vision,

the RPE protects photoreceptors from injury by strong light and regulates ion conductances for light–dark adaptation (for review, see Bok, 1993).

MATERIALS AND METHODS

cDNAs and probes. The cDNA clone for *Xenopus Notch* (AN119) was generously provided by R. I. Dorsky and W. A. Harris (University of California San Diego) (Dorsky et al., 1995). cDNAs for *Xenopus* BDNF and trkB (Cohen-Cory et al., 1994) were obtained by reverse transcription-PCR from oligo(dT)-primed reverse transcription of poly(A⁺) RNA isolated from stage 30 embryo heads. The trkB primers (5', GACATTAAGATGCCAGTGACAATG; 3', CGCGTTGC-CAGCAGCCGC) amplified a 661 bp region, which included the kinase-containing domain of trkB. The specificity of this probe for trkB has been described previously by Cohen-Cory and Fraser (1994). The primers for BDNF (5', CTCCTGACCCAGCCAGGCGT; 3', AGTGATACACAAGAAGTGTC) were based on the entire coding sequence for the mature protein (Isackson et al., 1991; 339 bp). The PCR products were ligated into PCR II (InVitrogen TA cloning kit), and then the sequences were confirmed by restriction analysis and double-stranded sequencing using Sequenase (United States Biochemicals, Cleveland, OH). *In vitro* transcription was used to generate antisense cRNA probes incorporating fluorescein-12-uridine-5-triphosphate (fluorescein-12-UTP; Boehringer Mannheim, Indianapolis, IN) or chromaridetetramethylrhodamine UTP (TMR-5-UTP; Molecular Probes, Eugene, OR). The plasmids containing the interested regions of genes for trkB, BDNF, and *Notch* were linearized and purified for use in the labeling reaction. After reactions, the labeled probes were precipitated with DEPC–NaO₃ and reconstituted in RNase-free water.

Tagged wild-type and mutant TrkB constructs. Construction of tagged wild-type and mutant trkB receptors has been described previously (Eide et al., 1996). Briefly, site-directed mutagenesis was used to introduce a Lys⁵⁶⁰ → Met⁵⁶⁰ substitution at a critical lysine in the ATP binding site of the full-length trkB receptor. Replacement by a methionine at this site resulted in a kinase-deficient receptor, which could interrupt signaling by forming nonfunctional heterodimers with wild-type receptors (Eide et al., 1996). To distinguish wild-type and mutant trkB receptors, small epitope tags were attached by PCR to the C termini of each receptor. We had previously found that the attachment of tags at this location had no interference with receptor activation or downstream signal transduction (Eide et al., 1996). The wild-type trkB receptor was tagged using an epitope from influenza virus hemagglutinin (Niman et al., 1983); the mutant receptor was tagged with an epitope from the c-myc proto-oncogene (myc) (Evan et al., 1985). Mutations were confirmed by DNA sequencing using the dideoxy chain termination technique (United States

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Biochemical). Both receptors were cloned under the control of the frog β -globin promoter in vector pSP64T (Krieg and Melton, 1984).

In situ hybridization. *Xenopus* embryos were harvested at various stages (stages 26–45) and then fixed in BOSCO (95:5:0.25% ethanol/acetic acid/chromium trioxide) as described previously (O'Keefe et al., 1991). After successive dehydration in alcohol and immersion in xylene, embryos were embedded in Paraplast according to the manufacturer's instructions (Oxford Labware). Six-micrometer sections were collected on the poly-L-lysine-coated RNase-free slides, deparaffinized, rehydrated, and then deproteinized by proteinase K (125 mg/ml). Sections were then treated with triethanolamine-acetic anhydride, dehydrated, and prehybridized in the presence of 0.3 M NaCl, 0.1 M NaPO₄, pH 6.8, 5 mM EDTA, 0.02% (w/v) Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin (fraction V), 40% formamide, 10% dextran sulfate, total yeast tRNA (0.1 mg/ml), and 0.01 M DTT at 50°C as described previously (Angerer et al., 1987). Sections were hybridized with fluorescein or rhodamine sense or antisense riboprobes of *Xenopus Notch*, *trk B*, and BDNF. After hybridizations, sections were washed in successive washes of 2 \times , 1 \times , and 0.5 \times SSC. Sections were mounted in 4:1 glycerol/2 \times SSC containing sodium azide (25 mg/ml).

Optic cup cultures. For optic cup explants, stage 26 *Xenopus* embryos were killed in 0.1% tricaine, and then optic vesicles were dissected aseptically in a modified HBSS described by Defoe and Easterling (1994). Explants were plated onto thin coats of growth factor-reduced Matrigel (Becton Dickinson, Mountain View, CA) in NCTC-135 media (Sigma, St. Louis, MO) containing 10 mg/ml insulin, 5.5 mg/ml transferrin, 6.7 ng/ml sodium selenite, 0.11 mg/ml sodium pyruvate, 1 mg/ml bovine serum albumin, 10 mg/ml linoleic acid-albumin, 20 nM hydrocortisone, 10 nM triiodothyronine, 0.3 mg/ml putrescine, 50 ng/ml epidermal growth factor (Sigma), and 50 mg/ml aprotinin. A BDNF-neutralizing antibody was purchased from R & D Systems (Minneapolis, MN) and used according to the manufacturer's instructions. The 50% neutralization dose (ND₅₀) for this antibody on dorsal root ganglion cultures had previously been estimated at 5–15 μ g/ml (R & D Systems). The ND₁₀₀ was 50 μ g/ml. Twenty-four explants were studied (eight controls, eight 15 μ g/ml BDNF antibody, and eight 50 μ g/ml BDNF antibody). All cultures were incubated at 23°C in a 5% CO₂ incubator.

For survival studies, fifty stage 45 optic cups were dissociated in 0.05% trypsin and 2.4% dispase and then distributed equally into to 16 10 mm wells (eight controls and eight BDNF-treated) coated with growth factor-reduced Matrigel as described above. Cells were allowed to attach overnight in the defined media described above plus 10% fetal bovine serum (FBS). After attachment was allowed to occur, cells were washed and maintained in serum-free media. Media were changed every 48 hr to remove cell debris. Only pigmented cells were counted. Attachment rates were compared using the binomial proportions test (Rosner, 1995). The life table method was used to estimate survival functions for the two groups each day (Collett, 1994). The log-rank test (Collett, 1994) was used to determine statistical differences in survival between control and BDNF-treated cultures.

In vitro transcription, generation, and injection of embryos. cRNA was prepared as described previously (Eide et al., 1996) using a kit from Promega (Madison, WI) and RNeasy columns from Qiagen (Chatsworth, CA). Embryos were generated in human chorionic gonadotropin-primed female *Xenopus* as previously described by MacNicol et al. (1993). Briefly, milked oocytes were fertilized *in vitro* in a 0.2 \times modified Barth's saline solution containing HEPES (MBSH). Zygotes were briefly treated with 2% cysteine to remove the gelatinous extracellular matrix and then transferred to 1 \times MBSH containing 5% Ficoll. Embryos were injected with RNase-free water or cRNA into one cell of a two-cell embryo.

Whole-mount immunohistochemistry, immunofluorescence, and Western blotting. For whole-mount immunohistochemistry, stage 32 embryos were fixed in Dent fixative overnight (Klymkowsky and Hanken, 1991) and then bleached in 10% hydrogen peroxide/Dent fixative for 3 d. After washing in TBS (20 mM Tris, pH 7.4, and 0.15 M NaCl), embryos were incubated in anti-c-myc antibody (9E10; Santa Cruz Biotechnology, Santa Cruz, CA) in 95% normal goat serum and 5% DMSO in the presence of 0.1 thiomersol. After extensive washing in TBS, embryos were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Amersham Life Sciences). Embryos were again extensively washed in TBS and then reacted with Sigma Fast DAB for 15–30 min. The reactions were stopped by dehydration in methanol and then cleared in benzyl alcohol and benzyl benzoate as described elsewhere (Klymkowsky and Hanken, 1991).

For immunofluorescence of sectioned tissue, embryos were fixed in

Dent fixative, embedded in Paraplast, and then collected in 6 μ m sections. After incubation in the anti-c-myc antibody (9E10; 1 μ g/ml), anti-*nestin* (a gift from R. D. McKay, National Institutes of Health) (Tohyama et al., 1992), XAP-1 (a gift from D. Sakaguchi (Iowa State University) (Harris and Messersmith, 1992), or anti-cellular retinaldehyde binding protein (CRALBP; a gift from J. Saari, University of Washington). Sections were incubated in FITC-conjugated anti-mouse or rabbit IgG, washed in PBS, and then mounted in 4:1 glycerol/PBS containing sodium azide (25 mg/ml). In immunofluorescent studies involving CRALBP, tissues were briefly treated with a bleaching solution consisting of 0.25% potassium permanganate and 5% oxalic acid.

For Western immunoblotting analysis, stage 26 embryos were lysed in 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 5 mM EDTA, 10 mM Tris, pH 7.4, 1% Triton X-100 containing 2 mM PMSF, 25 μ M leupeptin, 10 μ M pepstatin A, 0.2 U/ml aprotinin, and 1 mM vanadate. After clearing by centrifugation, lysates were precleared with normal goat serum, immunoprecipitated with an anti-c-myc antibody (9E10; 1 mg/ml), collected with protein G plus agarose (Santa Cruz Biotechnology), boiled in Laemmli's buffer (Laemmli, 1970), and then separated on 7.5% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (Amersham, Arlington Heights, IL), washed in TBS, and then blocked in 3% BSA and 5% dry milk in TBS with Tween 20 (TBST). After washing, membranes were incubated in 9E10 antibody (1 μ g/ml) diluted in blocking buffer for 45 min, washed in TBST, and then incubated in HRP-conjugated goat anti-mouse IgG diluted 1:3000 in blocking buffer. After washing in TBST, blots were developed using an enhanced chemiluminescence kit from Amersham.

Cresyl violet-eosin Y staining. For cresyl violet-eosin Y staining, embryos were fixed in Dent fixative and cleared in benzyl alcohol and benzyl benzoate as described above, embedded in Paraplast, and then collected in 6 μ m sections. Sections were then stained in 0.1% cresyl violet and eosin Y and then mounted in Permount (Fisher Scientific, Houston, TX).

RESULTS

Notch, BDNF, and TrkB mRNA in the early optic cup

To determine whether *trkB* transcripts were present in the early *Xenopus* optic cup, double fluorescent *in situ* hybridization (FISH) studies were performed using probes for the neuroepithelial marker *Notch* (Artavanis-Tsakonas et al., 1991) and the kinase-containing region of *trkB* (Cohen-Cory and Fraser, 1994). Figure 1, A–C, shows a single section (viewed *en face*) through a Nieuwkoop and Faber (1956) stage 26 optic cup probed with *Notch* (Fig. 1A), *trkB* (Fig. 1B), and *Notch* and *trkB* combined (Fig. 1C). A precise overlap is seen between *Notch*- and *trkB*-expressing cells, suggesting that, at least at this stage, full-length *trkB* transcripts are expressed homogeneously throughout the optic cup. Sense probes for *Notch* and *trkB* showed a complete absence of signal (data not shown). Figure 1, D–F, shows a stage 28 optic cup probed with *trkB* (Fig. 1D), BDNF (Fig. 1E), and *trkB* and BDNF (Fig. 1F). At this stage cells appear to have begun segregating into different differentiating cell populations. BDNF- and *trkB*-expressing cells can be seen in the outermost layer, the region of the presumptive RPE.

Patterns of TrkB, BDNF, and Notch transcripts in the RPE precursor layer and actively differentiating RPE

By stage 30, RPE progenitors appeared to have aligned into a more distinct (and single) layer of cells co-expressing *trkB* and BDNF (Fig. 1I). Examination of *Notch* transcripts at this stage showed persistent high levels in the outermost layers of the retina (Fig. 1J). By the time the first pigment granules were detected in the RPE (stage 32), however, *trkB* and *Notch* transcripts diverged; kinase-containing *trkB* transcripts remained at high levels in the actively differentiating RPE layer (Fig. 1K,L), whereas *Notch* had become downregulated (Fig. 1M,N).

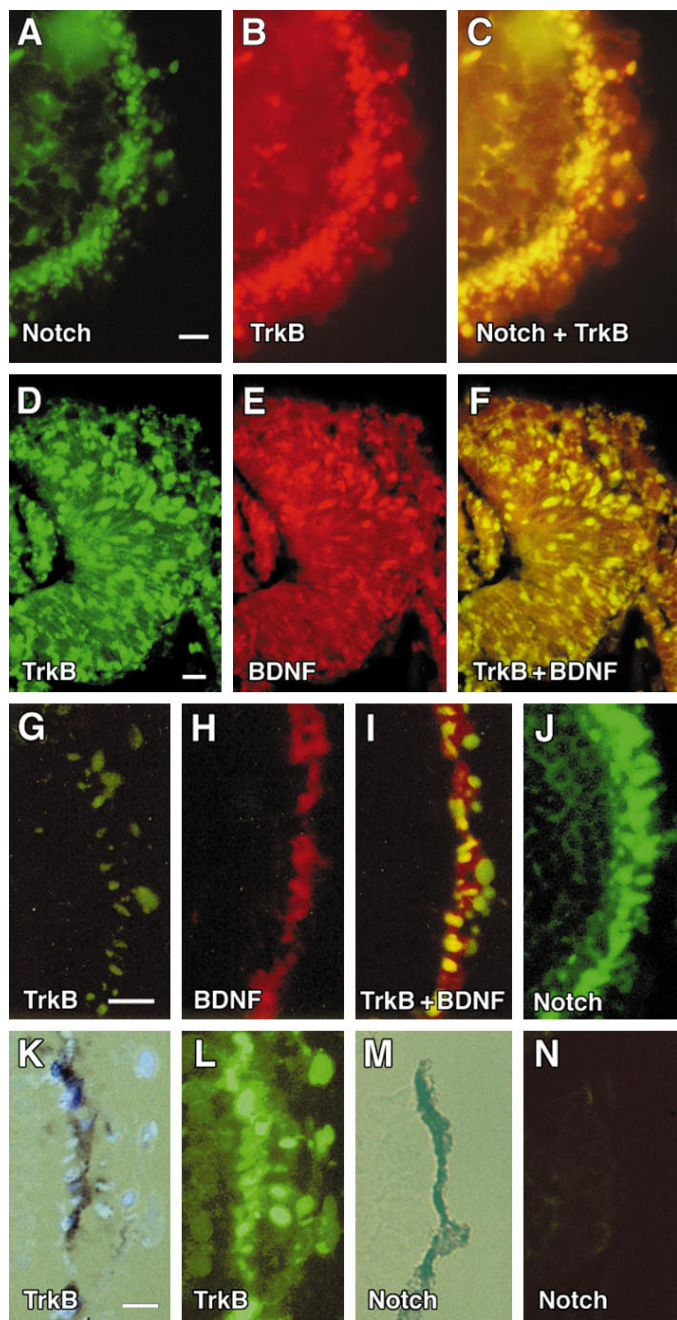


Figure 1. Co-localization of *Notch*, *TrkB*, and *BDNF* in the developing optic cup. *Top panels*, FISH of stage 26 *Xenopus* optic cups (sagittal sections, *en face*). In *A–C*, a single section was probed with cRNA antisense probes incorporating fluorescein-12-UTP (Boehringer Mannheim) for *Xenopus Notch* (*A*) or TMR-5-UTP (Molecular Probes) fluorescein isothiocyanate for the kinase-containing domain of *Xenopus trkB* (*B*) and *Notch* plus *trkB* (*C*). *D–F*, FISH of stage 28 retinas (coronal sections) probed with FITC antisense riboprobes for *trkB* (*D*), RITC probes for *BDNF* (*E*), and FITC-*trkB* plus RITC-*BDNF* (*F*). *G–J*, *en face* views of stage 30 *Xenopus* retinas probed with FITC-*trkB* (*G*), RITC-*BDNF* (*H*), FITC-*trkB* plus RITC-*BDNF* (*I*), and FITC *Notch* (*J*). *K*, Bright-field exposure of a stage 32 *Xenopus* retina probed with FITC-*trkB* (*L*). *M*, Bright-field exposure of a stage 32 *Xenopus* retina probed with FITC-*Notch* (*N*). Scale bar, 0.1 mm.

BDNF-blocking antibody inhibits RPE differentiation *in vitro*

To test an autocrine role for *BDNF* in the differentiation of the RPE, serum-free explants of stage 26 optic vesicles were cultured

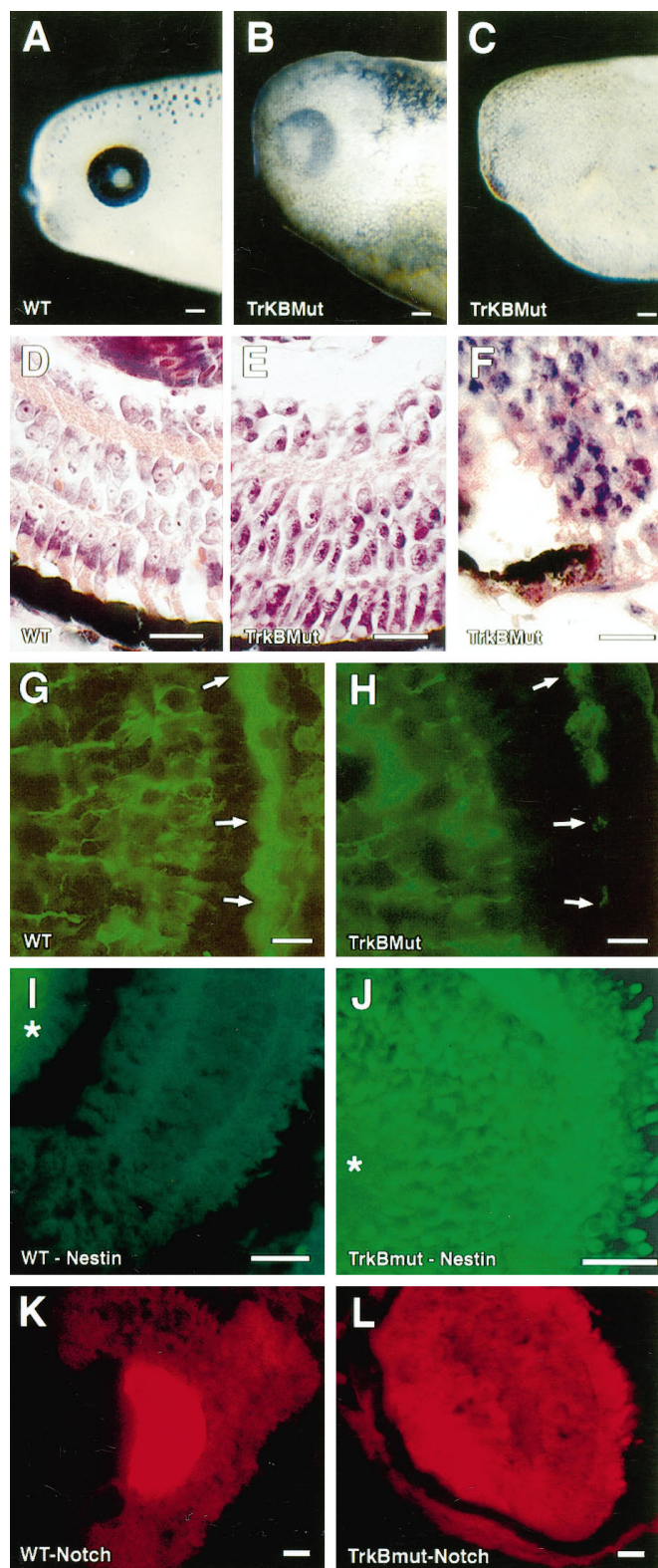


Figure 5. Retinal pigment abnormalities in dominant negative *TrkB* mutants. Representative photographs of the eyes of water-injected controls (*A*) and the *trkB* dominant negative mutants (*B*, *C*). *D–F*, Representative sections from the central retinas of cresyl violet-eosin-stained tissues. Embryos were generated simultaneously and killed at stage 45. A water-injected embryo is shown in *D*; mildly and severely affected embryos are seen in *E* and *F*, respectively. Scale bar, 50 μ m. *G*, *H*, Immunofluorescent sections from a stage 45 water-injected control embryo (*G*) or mildly affected mutant (*H*) probed with a (*Figure legend continues*)

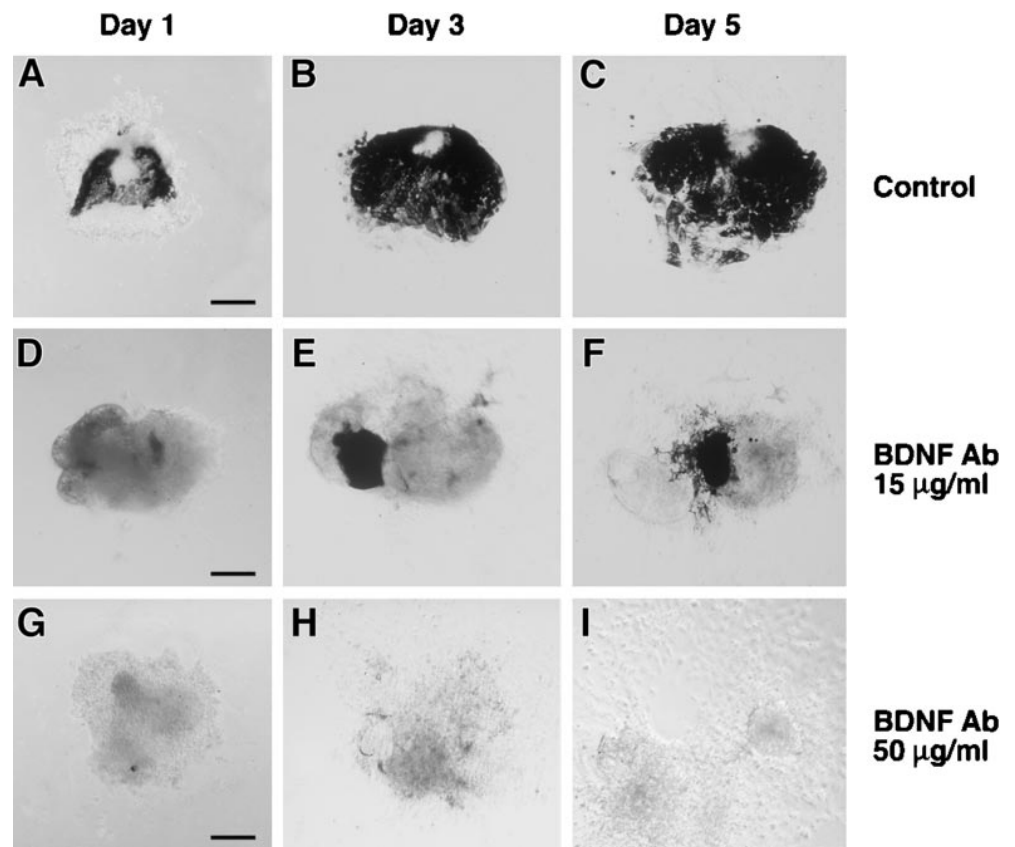


Figure 2. BDNF-blocking antibody prevents RPE differentiation *in vitro*. *A–C*, Representative control stage 26 explant at days 1, 3, and 5 after plating. *D–F*, Representative explant cultured in the presence of 15 $\mu\text{g/ml}$ BDNF antibody at days 1, 3, and 5. *G–I*, Representative explant cultured in the presence of 50 $\mu\text{g/ml}$ BDNF antibody.

in the absence or presence of a neutralizing antibody to BDNF (see Materials and Methods). Cultures were photographed on days 1, 3, and 5 after harvest. Representative photographs are shown in Figure 2. Control explants developed small clusters of pigmented cells by day 1 (Fig. 2*A*). By day 3, these cells had coalesced into a sheet of cuboidal pigmented epithelium (Fig. 2*B*), which diminished somewhat by day 5 (Fig. 2*C*) (consistent with some cell death of pigmented cells). Cultures incubated in the presence of 15 $\mu\text{g/ml}$ BDNF antibody (ND₅₀; see Materials and Methods) showed fewer clumps of pigmented cells (Fig. 2*D–F*) compared with controls (Fig. 2*A–C*). The death of pigmented cells was also noted between days 3 and 5 (Fig. 2*E,F*). At 50 $\mu\text{g/ml}$ BDNF antibody (ND₁₀₀), a near-complete block of RPE differentiation was seen (Fig. 2*G–I*). Interestingly, nonpigmented cells continued to grow exuberantly (Fig. 2*I*).

BDNF promotes survival of RPE *in vitro*

To test for a survival-promoting effect of BDNF on the RPE, stage 45 optic cups were dissociated and then plated in the presence or absence of BDNF. Cultures were allowed to attach in media containing 10% FBS overnight, and then they were washed and maintained in serum-free media (see Materials and Methods) \pm 100 ng/ml BDNF (3.7 nM).

←

polyclonal antibody to CRALBP (Bunt-Milam and Saari, 1983). Arrows indicate the RPE layer. *I, J*, Immunofluorescent sections from stage 45 water-injected controls (*I*) or a severely affected mutant (*J*). Sections were probed with a polyclonal antibody raised against the intermediate filament nestin (Tohyama et al., 1992). *K, L*, Sections from a stage 45 water-injected control (*K*) or severely affected *trkB* mutant (*L*) probed with RITC antisense riboprobe for *Xenopus Notch*. Scale bar, 0.1 mm.

Attachment rates (cell counts on day 2) were not significantly different between groups (control, 92% \pm 4; BDNF, 93% \pm 4; $p = 0.38$). Survival data were displayed graphically in Figure 3. Percent survival was determined by comparing daily cell counts to the number of cells attached on day 2. Each data point represents the average of eight wells. A significantly higher rate of survival was noted in BDNF-treated cultures compared with controls ($p < 0.001$).

Dominant negative *TrkB* mutant inhibits retinal differentiation *in vivo*: wild-type receptor rescues phenotype

To study the developmental contribution of the *trkB* receptor to RPE differentiation, a dominant negative mutant of the *trkB* receptor was expressed in developing *Xenopus*. In previous work, we had found that substitution of a single-base pair (Lys \rightarrow Met) at the ATP binding site of the *trkB* receptor could generate a noncatalytic receptor, which could dominantly inhibit BDNF signaling *in vivo* (Eide et al., 1996). This receptor was tagged at its C terminus with an epitope from the c-myc proto-oncogene (Evan et al., 1985) to facilitate its detection by Western blotting and immunohistochemistry. Wild-type *trkB* receptors used in rescue experiments were tagged with an epitope from influenza virus hemagglutinin (Niman et al., 1983). cRNAs encoding mutant and wild-type *trkB* receptors were generated by *in vitro* transcription and then microinjected into one-half of a two-cell *Xenopus* embryo as described previously by MacNicol et al. (1993). Figure 4 shows expression of the mutant receptor in embryo whole mounts (Fig. 4*A*), in paraffin section tissues (Fig. 4*B*), and in embryo lysates (Fig. 4*C*; Western blot).

Table 1 summarizes the results of the dominant negative trials. Embryos were allowed to develop until stage 45 and then scored

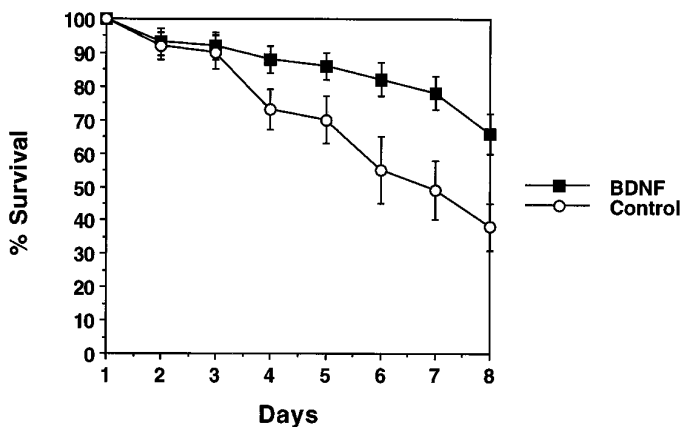


Figure 3. BDNF promotes RPE survival. The survival rates of dissociated RPE in the presence (*black squares*) or absence (*white circles*) of 100 ng/ml BDNF are shown. *Points* represent the average of eight wells. Error bars indicate SD.

for the presence of gross eye abnormalities. Three percent of uninjected embryos and 8% of water-injected embryos demonstrated some evidence of eye defects, compared with 40% of embryos injected with the *trkB* dominant negative mutant. There was no statistical difference between uninjected and water-injected controls ($p = 0.21$). On the other hand, eye abnormalities among the *trkB* mutants were significantly different from controls ($p < 0.0001$). Co-injection of wild-type *trkB* receptors at a 1:1 ratio (wild-type *trkB*/mutant *trkB*) rescued the eye phenotype (14%), supporting a specific effect of this mutant on *trkB*-mediated pathways. Interestingly, this rescue brought the incidence of eye abnormalities to the point that it did not significantly differ from that of water-injected controls ($p = 0.168$). An increased death rate (29%) was also noted among mutants compared with controls (5%) ($p < 0.0001$). Interestingly, co-injection of wild-type receptors also appeared to reverse this trend (13%) ($p < 0.002$).

To determine whether any delay or deficiency of eye pigmentation was seen as the result of the *trkB* mutant, stage 32 embryos were scored for the presence of eye pigment. Ninety percent (26 of 29) of uninjected controls and 82% (18 of 22) of water-injected controls showed at least some evidence of eye pigmentation by this stage, compared with 21% (4 of 19) of *trkB* mutants ($p < 0.0001$). The presence of eye pigmentation in 38% (8 of 21) of embryos co-injected with a 1:1 ratio of wild-type/mutant receptors suggested that this defect was also at least partially reversible.

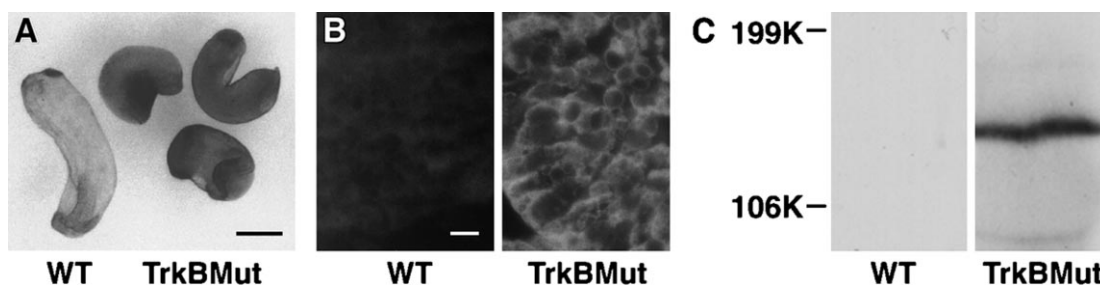


Figure 4. Expression of the Myc-tagged *TrkB* mutant. *A*, Whole-mount immunohistochemistry of stage 32 embryos using an anti-myc antibody (9E10). At left is an embryo injected with sterile water, at right, three mutants expressing the myc-tagged *trkB* mutant. Scale bar, 0.1 mm. *B*, Immunofluorescent staining (using the 9E10 antibody) of the eyes of stage 26 embryos injected either with water (*WT*) or cRNA encoding the *trkB* mutant (*TrkBMut*). Scale bar, 10 μ m. *C*, Western blot of stage 26 embryo lysates injected with either sterile water (*WT*) or cRNA encoding the myc-tagged *trkB* mutant (*TrkBMut*). A band at 145 kDa corresponds to the mutant *trkB* receptor.

Table 1. Eye abnormalities and deaths in dominant negative *trkB* receptor mutants: rescue by wild-type receptors

| cRNA injected | Eye abnormalities [% (n)] | Deaths (%) |
|---|---------------------------|------------|
| Uninjected controls (11/243) | 3 (3/99) | 5 |
| H ₂ O-injected controls (11/216) | 8 (8/100) | 5 |
| <i>TrkBMut</i> (2 ng/embryo) (108/378) | 40 (27/67) | 29 |
| <i>TrkBMut</i> :WT (2:2 ng/embryo) (51/396) | 14 (21/154) | 13 |

Figure 5, *A–C*, shows representative eyes of water-injected controls (Fig. 5*A*) and mildly affected (Fig. 5*B*) or severely abnormal (Fig. 5*C*) mutants. Some abnormality of RPE morphology (e.g., decreased pigmentation, decreased RPE thickness, and discontinuity) was noted in every abnormal *trkB* mutant examined at the light microscopic level (Fig. 5*E,F*). The most severely affected embryos showed complete absence of RPE (not shown).

Abnormalities of the neural retina were also quite prominent in the *trkB* dominant negative mutants. In mildly affected mutants (Fig. 5*E*), a laminar pattern of the neural retina was preserved; however, photoreceptors showed shortened outer segments and poorly formed synaptic pedicles, and cells of the inner nuclear layer retained an elongated columnar appearance, in addition to showing thinning of both plexiform layers. Immunofluorescent staining for CRALBP, a marker of differentiated RPE and muller glia (Bunt-Milam and Saari, 1983), showed weak staining of the RPE layer in mildly affected *trkB* mutants (Fig. 5*H*, arrows), consistent with the impaired differentiation of the RPE (Fig. 5*G*, arrows). Staining in muller glia was not significantly different from water-injected controls (Fig. 5*G,H*).

In severely affected embryos, most or all of the differentiated components of the retina appeared to be replaced by cells staining deeply with cresyl violet (Fig. 5*F*). These cells were identified as retinal neuroepithelia by the presence of nestin positivity (Fig. 5*J*) (Tohyama et al., 1992) and *Notch* mRNA (Fig. 5*L*). Some severely affected mutants showed rudimentary ganglion cell layers and photoreceptor rosettes in addition to dense collections of neuroblasts (data not shown). The identity of photoreceptors within rosette-like structures was confirmed by XAP1 staining (data not shown) (Harris and Messersmith, 1992).

DISCUSSION

Germinal retinal neuroepithelium consists of a large pool of cells competent for differentiation into diverse cell types (for review, see Austin et al., 1995; Cepko et al., 1996). As development

proceeds, intrinsic and extrinsic factors begin segregating subsets of cells that will differentiate along divergent pathways. Here we have found that BDNF and its receptor trkB play a critical part in that segregation by directing uncommitted neuroblasts to differentiate into the retinal pigment epithelium.

In stage 26 of *Xenopus*, BDNF and trkB can be found broadly expressed in the germinal retinal neuroepithelium (Fig. 1A–C). By stage 28, BDNF and trkB transcripts have clustered within subsets of cells appearing to represent different differentiating cell populations, including the presumptive RPE layer (Fig. 1D–I). By stage 32, *Notch* transcripts were seen to downregulate in this layer (Fig. 1M,N), perhaps heralding the onset of RPE differentiation. Other groups have shown that *Notch* regulates alternative fates within equivalence groups by inhibiting subsets of cells that have an equivalent neural fate potential (for review, see Heitzler and Simpson, 1991). Within the *Xenopus* retina, Coffman et al. (1990) and Dorsky et al. (1995) have suggested that *Notch* plays a similar role, inhibiting the differentiation of certain subsets of neuronal precursors until later inductive signals restrict their cellular identities.

Roles for BDNF and TrkB in RPE differentiation and survival

In this study, an autocrine role for BDNF in RPE differentiation was confirmed by incubating serum-free cultures of stage 26 optic vesicles with a blocking antibody to BDNF. RPE differentiation was blocked in a dose-dependent manner by this antibody, whereas the growth of at least some neuronal elements appeared to continue exuberantly (Fig. 2).

Survival analysis (Fig. 3) showed that BDNF also had some survival-promoting effects; however, because a significant decrease in cell numbers was seen by day 7 (control cultures, 38%; BDNF-treated cultures, 66%), additional survival-promoting factors for the RPE are likely to be discovered in the future.

In the dominant negative phase of our experiments, expression of noncatalytic trkB receptors caused severe abnormalities of RPE development, which included a loss of RPE pigmentation, discontinuity of the RPE layer, and decreased expression of the differentiated marker CRALBP (Fig. 5F,H). The most severely affected embryos showed near-complete arrest of retinal development with persistence of nestin- and *Notch*-positive neuroblasts (Fig. 5J,L). The trkB embryos shared a number of abnormalities in common with RPE-ablated transgenic mice (diphtheria toxin expressed under an RPE-selective promoter) (Raymond and Jackson, 1995), including the loss of pigmentation and discontinuity in RPE layers, shortening of photoreceptor outer segments, and disrupted lamination of the retina. The RPE-ablated animals did not show any loss of differentiated cell types or accumulation of neuroblasts. Interestingly, Campochiaro and colleagues have recently identified a splice variant of the full-length trkB receptor that preferentially binds BDNF in adult human and bovine RPE (P. Campochiaro, unpublished observations). The presence of survival- and/or differentiation-promoting effects of BDNF in mature RPE could have future implications for the treatment of degenerative retinal diseases such as retinitis pigmentosa.

BDNF, TrkB, and pigmented cells

The presence of pigment abnormalities in the skin of the trkB mutants also raised the question of whether trkB receptors could be regulating broader effects on the melanogenic cascade instead of or in addition to multiple local effects on discrete pigmented

cell groups. A complete answer to this question is beyond the scope of this paper; however, work from other groups at least suggests that BDNF and trkB mediate local trophic effects for diverse pigmented cell populations. In addition to mediating trophic effects in the RPE (present study), BDNF promotes the survival and/or differentiation of pigment-containing cells of the substantia nigra (Hyman et al., 1994), neural crest (Langtimm-Sedlak et al., 1996), and locus coeruleus (Sklair-Tavron and Nestler, 1995). Interestingly, the ability of BDNF to increase dopamine uptake and content in nigral neurons (Hyman et al., 1994) likely has implications for melanogenesis in these cells.

Although deficits of pigmentation appeared to occur early in the pathogenic process of the dominant negative mutants, we believe that the ocular phenotype in these embryos (e.g., loss of differentiated cell types and persistence of neuroblasts) is unlikely to result from a deficiency of pigmentation alone. Developmental defects associated with amelanotic retinas are well known to occur, but they have generally been limited to an absence of the fovea centralis (Klintworth, 1994), abnormalities of photoreceptor synapses and segments (Perez and Perentes, 1994; Szczesny et al., 1996), and projection of the temporal retina onto contralateral brain (Klintworth, 1994). Furthermore, the analysis of mildly affected trkB mutants in this study also showed an early loss of CRALBP expression (Fig. 5H), consistent with either an early impairment RPE differentiation or an early loss of differentiated RPE.

Comparison of *Xenopus* trkB dominant negative and transgenic mouse knock-outs

The severity of the eye abnormalities observed in the dominant negative mutants contrasted sharply with the normal ocular phenotypes reported in trkB, BDNF, and BDNF plus neurotrophin-4/5 knock-out mice (Klein et al., 1993; Jones et al., 1994; Conover et al., 1995). Reasons for this discrepancy are not entirely clear; however, fundamental differences between the paradigms could account for some significant differences in phenotypic outcomes. Murine knock-out experiments, for instance, cause permanent and complete disruptions of genes before the onset of conception, whereas dominant negative experiments in *Xenopus* cause transient incomplete inhibitions of gene function; the inhibition of gene function in *Xenopus* varies both in the time of onset as well as in its duration (Vize et al., 1991). A difference in any of these variables could result in a differential activation of compensatory growth factor pathways or other developmental pathways, causing significant differences in phenotypic outcomes. Perhaps the use of conditional or retinal layer-specific promoters in future experiments will help elucidate cell-specific contributions of trkB receptor pathways within the developing retina.

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