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Retinal TrkB receptors regulate neural development in the inner, but not outer, retina

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Abstract

BDNF signaling through its TrkB receptor plays a pivotal role in activity-dependent refinement of synaptic connectivity of retinal ganglion cells. Additionally, studies using TrkB knockout mice have suggested that BDNF/TrkB signaling is essential for the development of photoreceptors and for synaptic communication between photoreceptors and second order retinal neurons. Thus the action of BDNF on refinement of synaptic connectivity of retinal ganglion cells could be a direct effect in the inner retina, or it could be secondary to its proposed role in rod maturation and in the formation of rod to bipolar cell synaptic transmission. To address this matter we have conditionally eliminated TrkB within the retina. We find that rod function and synaptic transmission to bipolar cells is not compromised in these conditional knockout mice. Consistent with previous work, we find that inner retina neural development is regulated by retinal BDNF/TrkB signaling. Specifically we show here also that the complexity of neuronal processes of dopaminergic cells is reduced in conditional TrkB knockout mice. We conclude that retinal BDNF/TrkB signaling has its primary role in the development of inner retinal neuronal circuits, and that this action is not a secondary effect due to the loss of visual signaling in the outer retina.

Keywords

retina; photoreceptors; development; BDNF; TrkB; electroretinograms

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INTRODUCTION

Neurotrophins were initially identified and characterized as proteins that promote neuronal survival (Reichardt, 2006). It is now widely accepted that neurotrophins also modulate development and plasticity of synapses in the developing and mature central nervous system (CNS). Brain-derived neurotrophic factor (BDNF) and its receptor TrkB are of special interest in this regard, as neuronal activity promotes the expression and release of BDNF and cell surface expression of TrkB (Shieh *et al.*, 1998; Du *et al.*, 2000; Balkowiec and Katz, 2002). BDNF-mediated activation of TrkB modulates axonal and dendritic branching, promotes synapse formation and maturation, and regulates the activity of neuronal networks (Huang and Reichardt, 2001; Huang and Reichardt, 2003).

In the retina BDNF plays an important role in the experience-dependent development of retinal function (Liu *et al.*, 2007; Landi *et al.*, 2007a; Landi *et al.*, 2007b). Levels of BDNF rise during retinal development with expression promoted by early visual experience, and reach a peak at the same time as retinal development is completed (Pollock *et al.*, 2001; Seki *et al.*, 2003; Liu *et al.*, 2007). Retinal ganglion cells (RGCs) are important targets of BDNF: BDNF controls the development of their dendritic trees and enhances their axonal arborizations (Lom and Cohen-Cory, 1999; Alsina *et al.*, 2001; Lom *et al.*, 2002) and increases stability of developing synapses (Hu *et al.*, 2005). BDNF also modulates N-methyl-D-aspartate (NMDA) receptor levels in these cells (Ladewig *et al.*, 2004). BDNF is also involved in retrograde signaling from tectal neurons to RGCs, suggesting that BDNF provides a means for central regulation of retinal development (Du and Poo, 2004). By blocking BDNF with intraocular injections of antisense oligonucleotides Landi *et al.* (2007a) demonstrated that the morphological segregation of dendritic arbors of RGCs into ON and OFF sublaminae was inhibited. Using strains of BDNF-overexpressing mice and TrkB hypomorphic mice, Liu *et al.* (2007) showed that BDNF promotes the visual experience-dependent segregation of the RGC arbors into ON and OFF sublaminae that underlies functional segregation of ON and OFF pathways. In this process laminar organization of dendritic fields of RGCs is refined through the pruning of mistargeted/ectopic dendrites and through the addition of new branches to correctly targeted dendrites. In addition to RGCs, BDNF also has been shown to target dopaminergic amacrine cells. In BDNF knockout mice the development of the dopaminergic amacrine cell network is impaired, while in mice overexpressing BDNF there is hypertrophy of these neurons (Cellerino *et al.*, 1998). Dopamine secreted by these cells modulates the size of receptive fields of various retinal neurons, including RGCs (Witkovsky, 2004).

Several studies have implicated TrkB signaling in the development of photoreceptors and development and/or maintenance of synaptic communication between photoreceptors and second order retinal neurons. Suppression of TrkB function in the avian retina by expression of a truncated, tyrosine kinase-deficient TrkB splice isoform, known to exert a dominant negative effect on full-length TrkB, has indicated that TrkB controls the differentiation of photoreceptors (Turner *et al.*, 2006). In mice, the absence of TrkB in all tissues results in retarded postnatal rod development, and severe attenuation of synaptic communication from rods to the inner retina of *TrkB* knockout mice (Rohrer *et al.*, 1999). Notably, the b-wave of the ERG and c-fos expression in the inner retina could not be detected in this mutant (Rohrer *et al.*, 1999; Rohrer, 2001; Rohrer and Ogilvie, 2003; Rohrer *et al.*, 2004). In addition the retinas of *TrkB* mutant mice lacked recoverin-positive OFF-center cone bipolar cells. It was concluded from these previous studies that TrkB is required for the development of these retinal cells (Rohrer *et al.*, 1999; Rohrer and Ogilvie, 2003).

The results of prior studies on *TrkB* knockout mice raise two major questions regarding the origin and localization of BDNF/TrkB-mediated effects. First, does the reduced photoreceptor and synaptic development seen in the TrkB null mice secondarily regulate the actions in the

inner retina? Second, given that TrkB receptors are not expressed on photoreceptors, is the abnormal outer retinal development in TrkB null mice a consequence of BDNF activation of other retinal neuron classes or of cells in other tissues of the body? The possibility that the loss of TrkB activation in other regions of the body could indirectly control retinal development is suggested by the finding that mice lacking BDNF or TrkB rarely survive beyond early adulthood due to cardiac, sensory and motor deficits (Klein *et al.*, 1993; Donovan *et al.*, 2000; Luikart *et al.*, 2003). In the present paper, we have examined retinal development in a conditional mutant in which *TrkB* is deleted in the retina, but not in most other regions of the animal. We have investigated light-evoked photoreceptor function and synaptic transmission in the outer retina using the electroretinogram (ERG). We have also used cell-specific markers to assess how the deletion of TrkB in retina regulates development of neuron subtypes in the retina. We conclude that TrkB signaling within the retina is not required for photoreceptor development or for the establishment of synaptic transmission between photoreceptors and the second order retinal neurons. TrkB signaling, however, is required for the developmental reorganization of some synaptic networks within the inner retina.

RESULTS

Generation of conditional *TrkB* knock-out mutant

As the initial step of this study we prepared a mouse model in which *TrkB* was eliminated within the entire retina, but not in most other tissues of the animal. For this purpose we used a mouse line carrying a floxed *TrkB* allele in which the first coding exon of *TrkB*, including the signal sequence, is flanked with *loxP* sites. Cre-mediated deletion of this exon prevents expression of all TrkB isoforms (Fig. 1) (Liu *et al.*, 2007). The absence of a signal sequence prevents all surface expression of this receptor. In addition, the ATG initiation codons present in the second coding exon are not in phase, so activation of an mRNA surveillance mechanism that degrades mRNAs containing premature stop codons (Lykke-Andersen, 2001) is expected to result in the absence of TrkB mRNA (Liu *et al.*, 2007). To eliminate TrkB in the entire retina, we used a transgenic mouse line in which Cre recombinase is expressed under regulation of a 9 KB genomic DNA fragment of the mouse *Six3* homeobox gene (*Six3A1A2-cre*), that drives transgene expression in the eye and ventral forebrain. In early mouse embryos, *Six3* is expressed in the anterior-most neural plate, and subsequently also in the developing eye (Oliver *et al.*, 1995). The *Six3Cre* transgene drives recombination in the developing retina early, starting around embryonic day (E) 9-E9.5 (Furuta *et al.*, 2000). As a result, the entire process of retinal development, including cytodifferentiation, layering and synapse formation, is expected to occur in the absence of TrkB. *Six3Cre;TrkB^{fl/fl}* mutant mice were viable, fertile, and of normal size, compared to control littermates.

To characterize the extent of recombination driven by the *Six3Cre* transgene, we analyzed recombination of the *R26Rosa* locus at postnatal day (P) 30. Characterization of the pattern of recombination in the adult retina demonstrated very effective recombination in all retinal layers (Fig. 1A, *XGal staining*). In photoreceptors, LacZ stain accumulated in the inner segments and in the outer plexiform layer (OPL), consistent with prior data demonstrating that the LacZ reporter is localized to the axonal compartment and inner segment of photoreceptor cells (Chang *et al.*, 2000). To confirm that the *Six3Cre* transgene efficiently deletes the floxed *TrkB* allele, Western blots were analyzed for the presence of TrkB protein using antibodies directed to the extracellular domain of this protein. Results show that the kinase and truncated isoforms of TrkB receptors were below detectable levels in the mutant neural retina by Western blotting (Fig. 1B, *left panel*). In addition, expression of the non-kinase-containing truncated TrkB protein in the retinal pigment epithelium (RPE), where normally only this isoform is expressed (Hackett *et al.*, 1998), was dramatically reduced but not completely absent (Fig. 1B, *right panel*). Immunostaining of retinal sections agreed well with the results of Western

blotting. In retinal sections of control littermates, anti-TrkB strongly labeled the IPL and RPE, and weakly labeled the OPL. *Six3Cre*-mediated recombination resulted in complete loss of TrkB immunoreactivity from the neural retina and partial loss from the RPE, where patches of TrkB-expressing cells were observed in the mutant (arrows, Fig. 1C).

To validate that the *Six3Cre*-dependent conditional removal of *TrkB* from the retina preceded the development of photoreceptors and synaptogenesis in the OPL, we examined TrkB immunoreactivity in the P0 retina (Olney, 1968; Blanks *et al.*, 1974). In the control retina, TrkB antibody labeled the RGC layer and developing IPL, but did not label cells in the outer neuroblastic layer. This staining pattern agrees with previous studies of TrkB expression in the developing rodent retina (Ugolini *et al.*, 1995; Rohrer *et al.*, 1999). No TrkB expression was observed in the mutant neural retina at P0, in agreement with published data demonstrating that *Six3Cre* drives recombination early in retinal development (Furuta *et al.*, 2000) (Fig. 1D).

TrkB localization in the retina using a Cre-mediated reporter

Previous studies using antibodies from many sources and in situ hybridization analysis generally agree that TrkB is expressed in RGCs (Perez and Caminos, 1995; Koide *et al.*, 1995; Cellierino and Kohler, 1997; Suzuki *et al.*, 1998; Rohrer *et al.*, 1999). There is some disagreement whether TrkB is expressed by other classes of retinal neurons (Rohrer *et al.*, 1999; Wahlin *et al.*, 2000; Di Polo *et al.*, 2000; Rohrer *et al.*, 2004; Wahlin *et al.*, 2004). As an alternative strategy to identify which classes of retinal neurons expressed TrkB, we utilized a Cre-mediated reporter. For this purpose we used a different floxed *TrkB* allele, termed *fBZ*. With this allele, Cre-mediated recombination deletes a *TrkB* cDNA-transcription termination cassette, permitting expression of tau-lacZ under control of the endogenous *TrkB* promoter (Xu *et al.*, 2000). Tau-LacZ expression resulting from *Six3Cre*-mediated recombination of the *fBZ* locus in the retina was detected using immunofluorescence (Fig. 2). As expected, strong tau-LacZ expression was detected in the RGC layer, consistent with the expected expression of *TrkB* in retinal RGCs (Figure 2A,B,C). We also detected tau-LacZ expression in a sparse population of amacrine cells, including TH-immunoreactive dopaminergic amacrine cells (Figure 2D,E,F) in agreement with prior results of others (Cellierino and Kohler, 1997). Interestingly, tau-LacZ expression was also observed in a small subset of retinal bipolar cells in the innermost region of the inner nuclear layer (INL). We found that β -galactosidase immunoreactivity was not present in PKC-labeled rod bipolar cells (Figure 2G,H,I). Similarly, β -galactosidase did not colocalize with the recoverin-expressing subclass of OFF-type cone bipolar cells or with strongly PMCA1-expressing ON-type cone bipolar cells (Krizaj *et al.*, 2002; Haverkamp *et al.*, 2003). Consequently, *TrkB* does not appear to be expressed in rod bipolar cells. Instead its expression in the proximal part of the INL is limited to an unidentified, small population of cone bipolar cells (See Suppl. Fig. 1). No tau-LacZ expression was detected in the Müller glia, in agreement with the analysis of *TrkB* gene expression in isolated mouse Müller cells (Wahlin *et al.*, 2004). In summary, we conclude that TrkB is normally expressed by RPE cells, RGCs, subsets of amacrine cells, including TH-positive amacrine cells, and a subset of cone bipolar cells. In the conditional knockout mice, TrkB expression was eliminated in all classes of retinal neurons except a subset of RPE cells.

Adult mice with TrkB deleted in retina have normal ERGs

The ERG is a light-evoked mass potential composed of several waves generated by the sum of the responses of different cell types within the eye. The cornea-negative scotopic a-wave is generated by the termination of a dark current in the rods. The cornea-positive scotopic b-wave reflects the depolarization of rod bipolar cells in dark-adapted retinas and of cone ON bipolar cells in light-adapted retinas. The amplitude and timing of the b-wave is a measure of the

synaptic transmission of visual signals from photoreceptors to bipolar cells (Nusinowitz S. *et al.*, 2002).

To determine whether conditional deletion of *TrkB* from the retina affects synaptic communication between photoreceptors and the inner retina, we compared scotopic and photopic ERG responses from control and conditional mutant adult mice. Figure 3A illustrates scotopic ERG responses to a range of flash intensities from control and mutant mice. We did not observe significant abnormalities in the scotopic b-wave in the *TrkB* mutant retina. The scotopic a- and b-wave amplitudes increased comparably with the intensity of light stimulus in both the control and mutant mice (Fig. 3B, *left panel*). Implicit times of scotopic a-waves and b-waves decreased similarly with increasing light intensity in both control and mutant mice as well (Fig. 3B, *right panel*). These results indicate that BDNF/TrkB signaling within the retina plays no discernible role in the regulation of synaptic transmission between photoreceptors and bipolar cells in the scotopic pathway. In addition, the lack of any significant difference between the control and *TrkB* mutant mice in scotopic a-wave amplitudes and implicit times shows that rod photoreceptor function does not require the BDNF/TrkB signaling pathway in adult mice. Overall, the absence of any significant effect of TrkB deficit on ERG a- and b-wave parameters agrees with the normal retinal morphology observed in *TrkB* mutants, including normal rod photoreceptor morphology (Fig. 5).

We also compared control and mutant littermates for changes in oscillatory potentials (OPs), which are high frequency oscillations of electric potential present on the rising phase of the ERG b-wave. OPs are believed to reflect neuronal activity mostly in the inhibitory, rather than excitatory, neuronal pathways in the inner retina, and they strongly depend on dopaminergic, GABAergic and glycinergic transmission (Wachtmeister, 1998). We analyzed implicit times and amplitudes of scotopic ERG OP wavelets. Results of OP analysis did not reveal any difference between control and mutant mice (Fig. 3C), suggesting that the TrkB deficit does not critically affect the components of neuronal circuits activated in the rod pathway, at least to a degree detectable by ERG. TrkB function seems not to be essential for the maintenance of inhibitory neurotransmission involved in the generation of OPs. This is consistent with the observation that *TrkB* expression is restricted to a subset of amacrine cells in the inner retina.

In another series of experiments we tested whether the loss of TrkB from retina affected the cone-driven photopic pathway. Similar to the scotopic ERG responses, photopic ERG responses were not affected by conditional deletion of *TrkB* from the retina (Fig. 3D, *left panel*). Photopic ERG amplitudes increased comparably with flash intensity in both control and mutant mice (Fig. 3D, *right panel*).

Juvenile mice with deletion of retinal TrkB have slightly reduced scotopic but normal photopic b-waves

To determine whether absence of TrkB results in a developmental delay that exhibits ERG deficits in young animals but not in older animals, we examined whether ERG deficits reported for younger conventional *TrkB* knockout animals (Rohrer *et al.*, 1999) were evident in P16 conditional retinal-specific TrkB null animals. No comparable loss of ERG was found in the juvenile conditional knockout animals (Fig. 4), and implicit times for a- and b-waves (Fig. 4B, *right panel*) and oscillatory potentials were not different in the mutant and control mice (Fig. 4C). However, scotopic ERG b-wave amplitudes were slightly, but statistically significantly diminished in the retinal-specific *TrkB* mutants for all but the brightest intensity, where the difference approached statistical significance ($P = 0.058$) (Fig. 4B, *left panel*). This reduction was specific for scotopic, rod photoreceptor-dependent ERG responses; photopic cone-driven ERG responses were not different (Fig. 4D). It is unclear whether the reduced b-wave amplitudes resulted from slightly less effective synaptic transmission between rod photoreceptors and rod bipolar cells or from decreased function of photoreceptors. At the

highest flash intensities, where a-waves are measured most accurately, we detected a small but significant decrease in scotopic a-wave amplitudes (Fig. 4B, left panel). At these intensities the b- to a- wave ratio was not significantly different between the mutant and control retinas ($P = 0.89$). This would suggest that the reduced b-wave amplitudes result from reduced a-wave magnitudes, secondary to abnormal photoreceptor function.

Outer retinal anatomy in the absence of retinal TrkB

We investigated the effects of conditional *TrkB* deletion from the retina on retinal anatomy. Previous studies of the effects of conventional *TrkB* deletion on retinal development performed on juvenile mice indicated that TrkB played a role in the development of the outer segments of photoreceptors, as well as the development of subsets of cells in the inner retina Rohrer et al., 1999). By contrast, retinal histology was not affected by the conditional removal of *TrkB* (Fig. 5). We compared retinal morphology of control and conditional TrkB mice in retinal sections embedded with plastic. With this higher resolution microscopy, we could discern no differences in the retinal morphology. We compared equivalent sections from central, mid-peripheral and peripheral regions. In particular we found the ONL thickness in the retinas of mutant mice to be similar to that of the control retinas, indicating that photoreceptors developed normally and did not degenerate with a detectably higher rate in the absence of TrkB.

We asked whether the decreased scotopic a-wave amplitudes observed at the brightest flashes in the conditional mouse mutant at P16 could result from the abnormal development of photoreceptor outer segments. To determine whether retinal-specific loss of *TrkB* shortened the lengths of rod photoreceptor outer segments, we measured their average lengths in semi-thin plastic sections. Results indicate that TrkB deficit may result in marginally shortened photoreceptor outer segments. This decrease, however, is not statistically significant (superior retina: control, $17.3 \pm 1.2 \mu\text{m}$; mutant $16.0 \pm 0.9 \mu\text{m}$, $P=0.18$; inferior retina: control $14.8 \pm 1.1 \mu\text{m}$; mutant, $14.5 \pm 0.7 \mu\text{m}$, $P=0.23$. $N=4$ mice for both mutant and control).

Overall, these results suggest a rather limited, if any, role of TrkB in the development of photoreceptors and rule out the involvement of TrkB signaling within the neural retina in the development or maintenance of synaptic transmission between photoreceptors and bipolar cells. The absence of obvious anatomical abnormalities in the TrkB-deficient retinas is consistent with the results of our ERG evaluations.

Inner retinal anatomy in the absence of TrkB

In the next series of experiments we determined whether the TrkB deficit affects synapse density. Sections of the retina were stained with an antibody against vesicular glutamate transporter (VGLUT1) that labels excitatory synapses in the IPL and OPL (Fig. 6A,B). No differences between WT and mutant retinas were detected, indicating that TrkB-mediated signaling within the retina does not affect the overall density or distribution of glutamatergic synapses. Similarly, we demonstrated earlier that staining of the retina with an antibody specific for the inhibitory synaptic marker GAD65 did not detect differences between control and mutant retina (Liu et al., 2007).

BDNF has been reported to regulate the expression of calcium-binding proteins in the cerebral cortex and hippocampus, as well as in cultured cortical and hippocampal neurons (Ip et al., 1993; Widmer and Hefti, 1994; Pappas and Parnavelas, 1997; Fiumelli et al., 2000). We studied whether deletion of *TrkB* affected the expression of calretinin and calbindin, two calcium-binding proteins that label different classes of retinal cells. We found that the immunolocalization of calretinin was indistinguishable between the control and TrkB-deficient retinas. Calretinin-immunoreactive somata were observed in both the INL and RGC layer, and three immunopositive bands were seen in the IPL (Fig. 6C,D).

Calbindin D28 is highly expressed in many cell types in the INL and RGC layers and in horizontal cells. The distribution of calbindin in the INL and RGC layers was not affected by the mutation. Calbindin antibodies strongly labeled horizontal cells that have been reported to express TrkB (Rohrer *et al.*, 1999), and this staining was not affected by the loss of TrkB (Fig. 6E,F). The results indicate that, in contrast to the brain (Jones *et al.*, 1994), the expression of these two Ca²⁺-binding proteins in the retina is not promoted by BDNF/TrkB signaling.

Studies of retina from conventional *TrkB* knockout mice have reported the complete absence of recoverin-expressing OFF-cone bipolar cells (Rohrer *et al.*, 1999). We found that these cells were present in the retinas of *TrkB* conditional knockout mice (Fig. 6G,H), indicating that retinal expression of TrkB is not critical for the differentiation of the recoverin positive OFF-cone bipolar cells.

BDNF/TrkB signaling has been reported to exert time-specific effects on the survival of RGCs. BDNF or TrkB deficit was shown to increase the rate of developmental RGC death, however it did not affect the final number of RGCs (Pollock *et al.*, 2003). To determine whether absence of TrkB affects survival of two immunologically identified subsets of RGCs, we stained the retinas of P30 mice with anti-Brn3a that specifically labels murine RGCs (Quina *et al.*, 2005) and with anti-calretinin that labels both RGCs and amacrine cells (Haverkamp and Wässle, 2000). No reduction of cellular staining in the RGC layer was apparent in the adult retina (Fig. 6G,H). To further quantify the effect of TrkB deletion on RGC survival, we examined its effect on the survival of SMI-32 positive RGCs. SMI-32 is a neurofilament protein expressed in α -type RGCs, as well as in 2 or 3 other types of RGCs (Liu *et al.*, 2007). Vulnerability of SMI-32 positive RGCs to degeneration was shown to be similar to other RGC types in the mouse DBA/2J strain that serves as a model of genetically inherited glaucoma (Jakobs *et al.*, 2005). We counted the number of SMI-32 positive RGCs in the retinas from 3 control (*TrkB*^{f/f}) and 3 mutant mice at P50. We found that the average density of SMI-32 positive RGCs in the mutant retinas was similar to that in the controls: 145.04 ± 22.20 , and 133.54 ± 6.28 cells/mm², respectively, which was not a significant difference ($P = 0.64$) (Figure 7). These observations complement a finding from studies of juvenile conventional *TrkB* knockout mice that showed TrkB signaling is not essential for the early postnatal survival of RGCs (Rohrer *et al.*, 2001). Instead of being essential for the survival of RGCs during retinal development and in adults, TrkB may play a role in the long-term survival of RGCs by protecting them from oxidative stress or excitotoxic damage (Rohrer *et al.*, 2001). Our observation that RGC number is not reduced in the retinal-specific *TrkB* mutant is important for the interpretation of studies related to mechanisms of glaucomatous RGC death. Interruption of retrograde axonal transport of neurotrophic factors has been proposed as a mechanism contributing to RGC death in glaucoma (Quigley *et al.*, 2000; Pease *et al.*, 2000; Iwabe *et al.*, 2007). As RGC loss was not observed in the conditional TrkB mutant mice, our data do not support a role for either BDNF or TrkB in RGC loss. Our data do not eliminate the possibility that it may be more important in aged animals, or that it may be one of several trophic factors whose impaired transport contributes to apoptosis in glaucoma.

TrkB signaling is essential for the development of dopaminergic terminals the retina

Prior results have suggested that BDNF promotes the survival and development of midbrain dopaminergic neurons as well as of dopamine neurons in retina (Hyman *et al.*, 1994; Studer *et al.*, 1995; Cellierino *et al.*, 1998; Rohrer and Ogilvie, 2003). We analyzed the effect of TrkB absence on dopaminergic amacrine cells *in vivo*. Retinal dopaminergic neurons were visualized by staining for TH in whole-mounted retinas from adult mice. The absence of TrkB did not affect the localization of dopaminergic neurons or the laminar organization of their processes, which occupy the most distal sublamina in the IPL (Fig. 8A,B). TH expression level, as assessed by Western blot, was also not affected by the loss of TrkB (Fig. 8C). We found that the absence

of TrkB is not critical for determining the number of dopamine neurons in the retina during development (Fig. 8H). At P30, loss of TrkB did not result in a statistically significant decrease in number of dopaminergic amacrine cells (26.84 ± 3.12 cells/mm² in control, vs. 25.55 ± 1.15 cells/mm² in mutant, $P=0.71$; $N=4$ for control and mutant). All of these data indicate that TrkB does not play a significant role in the neurogenesis of retinal dopaminergic neurons *in vivo*. In addition, TrkB signaling was not essential for the assembly of the axonal rings or pericellular baskets that surround AII amacrine cells. However, the density of TH-immunopositive varicosities was significantly reduced in the TrkB deficient retinas (Fig. 8D,E,F,G,I). The reduction in varicosity number suggests that TrkB regulates the density of release sites for dopamine that are associated with varicosities on the axons of dopamine neurons. These varicosities are known to contain large numbers of dopamine-storing vesicles immunoreactive for vesicular monoamine transporter (VMAT2) (Witkovsky *et al.*, 2004). Next, we tested whether the reduction of synaptic varicosities observed in retinas lacking TrkB reflected a developmental delay or more permanent reduction. If the former, the developmental delay should be overcome with increasing age. We analyzed the plexus of TH positive processes in the retinas from mice at P60 and found that mutant retinas still had a 50% lower average density of varicosities than the density present in control retinas (Fig. 8I, right panel). We conclude that TrkB signaling is essential for the maturation of the dopaminergic network in the retina.

DISCUSSION

The data presented in this paper argue that BDNF to TrkB signaling affects retinal development both directly through control of RGC and dopaminergic amacrine cell development within the retina and indirectly through signaling outside of the retina that is essential for normal animal homeostasis. In particular, many of the deficits previously observed in cell development in the outer retina appear to reflect requirements for TrkB outside of the retina that have indirect effects on development of cell types within the retina. Recent studies of retinal development have clearly demonstrated that BDNF-activated TrkB signaling plays an essential role in the activity-dependent development of RGC dendritic arbor patterns (Landi *et al.*, 2007b). BDNF promotes the anatomical segregation of the dendrites of ON- and OFF-center RGCs in different sublaminae of the IPL (Liu *et al.*, 2007; Landi *et al.*, 2007a). Retinal-specific *TrkB* deletion strongly inhibits visual experience-dependent refinement of RGC dendritic trees (Liu *et al.*, 2007). Because these phenotypes are observed in the retinal-specific TrkB mutant, they appear to result from direct effects of BDNF to TrkB signaling. Since RGCs express substantial levels of TrkB, these functions of BDNF and TrkB are likely to be cell autonomous. BDNF expression in the retina is upregulated in a visual stimulation-dependent manner (Pollock *et al.*, 2001; Seki *et al.*, 2003; Mandolesi *et al.*, 2005). Thus, activity-dependent stimulation of BDNF expression appears to promote RGC development, which is probably the major direct role for BDNF in the development of RGC dendritic morphology. Subsets of amacrine cells, including dopaminergic neurons, are also known targets of BDNF (Cellerino *et al.*, 1998; Cellerino *et al.*, 1999), and development of dopaminergic amacrine cells also depends on visual experience (Morgan and Kamp, 1982; Melamed *et al.*, 1986; Spira and Parkinson, 1991; Shelke *et al.*, 1997). Visual experience-mediated enhancement of BDNF expression provides the most likely molecular basis through which neuronal activity promotes morphological changes in these cells, as well.

It has not been clear whether the role of BDNF in the maturation of synaptic connections in the inner retina reflects its function in the inner or outer retina. Several studies have reported that TrkB promotes the development of photoreceptors and synaptic communication between photoreceptors and the inner retina (Rohrer *et al.*, 1999; Rohrer, 2001; Rohrer and Ogilvie, 2003; Rohrer *et al.*, 2004). Studies of retinal anatomy and function in conventional *TrkB* deficient mutants have found the development of rod photoreceptors, and the development or maintenance of synaptic transmission between rod photoreceptors and bipolar cells, is

diminished or absent. In addition to defective photoreceptor development and function, the retinas of *TrkB* mutant mice lacked recoverin-positive OFF-center cone bipolar cells and dopaminergic neurons, indicating that TrkB is required for the development of these cells (Rohrer *et al.*, 1999; Rohrer and Ogilvie, 2003).

In this study we sought to identify which of the phenotypes observed in the BDNF and TrkB mutant mice reflect roles for TrkB within the retina through use of a floxed TrkB allele and a retinal-specific Cre line. First, we examined the expression of *TrkB* in different retinal cell types. In the murine retina, prior work has detected TrkB in RGCs and in some amacrine cells, notably in dopaminergic and nitric oxide synthetase-positive amacrine cells (Cellerino and Kohler, 1997; Cellerino *et al.*, 1998; Cellerino *et al.*, 1999). In later studies, TrkB has been also reported to be expressed in horizontal cells, Müller glia, rod bipolar cells, and the RPE (Rohrer *et al.*, 1999; Rohrer *et al.*, 2004). Our analysis using a TrkB-specific antibody detected TrkB immunoreactivity in the RPE, in the dendritic arbors of RGCs and in a few cells in the INL. In contrast to prior studies, we used as controls mice in which TrkB had been genetically deleted from all cells within the retina, which abolished immunoreactivity completely. Thus the TrkB immunoreactivity observed in our studies is almost certainly due to the presence of TrkB and not cross-reactive antigens. Consistent with this, using a tau-LacZ reporter cassette under control of *TrkB* genomic locus, we found strong expression in the RPE, RGCs and in a subpopulation of amacrine cells including dopaminergic neurons, as well as in a subpopulation of cone bipolar cells. We did not detect tau-lacZ expression in horizontal cells or Müller glia. Our data thus agree with the results of single-cell gene expression analyses which failed to detect TrkB in Müller glia (Wahlin *et al.*, 2004) and indicate that the elevated c-fos and ERK phosphorylation reported in these cells following BDNF injection is not a cell autonomous, direct response.

Second, our studies demonstrated that TrkB signaling within the mouse retina is not critical for the development and maintenance of photoreceptors. In our study, TrkB was eliminated before the development of rod photoreceptors and bipolar cells, so development of these cells and formation of functional synapses between them proceeded in the absence of TrkB signaling. Thus, our results indicate that TrkB has a more restricted, direct role within the retina than that inferred from previous studies utilizing conventional *TrkB* knockout mice, which appear instead to reflect roles for TrkB in animal homeostasis. Our observations are consistent with the restricted expression of *TrkB* within the retina that we found using both anti-TrkB and a tau-LacZ reporter under control of the *TrkB* genomic locus.

Our studies of retinal function in juvenile mice showed that TrkB signaling in the retina modestly, if at all, influences the interaction between rod photoreceptors and bipolar cells at young ages. Loss of *TrkB* slightly decreased the scotopic ERG b-wave amplitudes at P16, although this difference disappeared in adulthood. While the slight but significant reduction in scotopic b-wave amplitudes observed in the juvenile mice most likely reflects reduced photoreceptor function, we cannot rule out a very modest abnormality in rod to bipolar cell transmission in the younger animals. Since no difference in the scotopic ERG amplitudes was found in the retinas from adult mice, the observed difference may reflect a slight delay in maturation of rod photoreceptors and bipolar cells in mutant mice. In contrast to the conventional *TrkB* mutant (Rohrer *et al.*, 1999), we did not observe a significant reduction in rod photoreceptor outer segment length, nor did the retina-specific mutant lack recoverin-positive OFF-center bipolar cells. Since differentiated rod photoreceptors and their progenitors in the outer neuroblastic layer do not express TrkB, this phenotype most likely reflects a requirement for TrkB in the other cell types that provide trophic support to photoreceptors, such as the RPE. For example, BDNF up regulates expression of one known factor, bFGF, in the RPE (Hackett *et al.*, 1997). One caveat to mention is that we cannot absolutely rule out the possibility that the few RPE cells that expressed TrkB in the conditional knockout mice may

have provided all the trophic support needed for the outer retina. This seems unlikely given studies of chimeric RCS rat retina showing that trophic support provided to rods from each functional RPE cell extends less than 50 microns laterally across the retina (Mullen and LaVail, 1976). The average distance between each TrkB expressing RPE cell (Fig. 1) is several-fold larger.

Why have the studies of retinal anatomy and function in these two different models of TrkB deficiency in mouse produced such different results? Almost certainly the phenotypes observed in the conventional *TrkB* mutant mice must be due to deficits in organs or tissues outside of the retina. The severe deficits in the ERG responses and in the retinal anatomy in the conventional TrkB knockout mice may be secondary effects of developmental delay caused by poor cardiac function, deficits in organ homeostasis or malnutrition. It is known that BDNF/TrkB signaling is required for survival of cardiac vascular endothelial cells and normal cardiac function (Donovan *et al.*, 2000), as well as in neoangiogenesis after injury (Kermani *et al.*, 2005). TrkB also is required for survival of the sensory innervation to the carotid gland and other glands that control blood pH and organ homeostasis (Conover *et al.*, 1995). Finally, BDNF is required for development of a normal respiratory rhythm (Katz, 2003). Thus, the abnormal ERGs observed in the conventional TrkB mutant are likely to have been caused by impaired retinal development due to hypoxia or abnormal organ homeostasis. Indeed, ERG b-waves are known to be more sensitive than a-waves to impaired vascular perfusion and retinal ischemia (Howard and Sawyer, 1975; Kothe and Lovasik, 1990; Tinjust *et al.*, 2002).

In the present study we show an important role of TrkB in the patterning of networks formed by dopaminergic amacrine neurons. TrkB deficiency results in a markedly decreased density of TH-positive varicosities on the processes of dopaminergic neurons. In prior studies, the absence of BDNF has also been shown to result in a significantly decreased density of the TH-positive varicosities formed by these neurons (Cellerino *et al.*, 1998). In each instance, the number of varicosities is reduced, but not abolished. TrkB is expressed in the retinal dopaminergic neurons (Cellerino and Kohler, 1997), and retinal dopaminergic neurons can respond to TrkB stimulation by increasing dopamine secretion in response to BDNF application (Neal *et al.*, 2003). Thus, this phenotype appears to reflect a direct role for TrkB signaling within these neurons. Interestingly, maturation of dopaminergic transmission in the retina is regulated by visual experience (Morgan and Kamp, 1982; Melamed *et al.*, 1986; Spira and Parkinson, 1991; Shelke *et al.*, 1997). The onset of visual function after eye opening was proposed to induce maturation of dopaminergic neurons through activation of second messenger systems triggered by synaptic inputs (Witkovsky *et al.*, 2005). BDNF expression in the retina has been shown to be upregulated in a visual stimulation-dependent manner (Pollock *et al.*, 2001; Seki *et al.*, 2003; Mandolesi *et al.*, 2005). Taken together, these observations lead to the speculation that visual experience may promote the maturation of dopaminergic neuron functions through its regulation of BDNF expression. Our observations on the effect of TrkB deletion on development of retinal dopaminergic amacrine neuron varicosities are similar to the effects of *TrkB* deletion on the development of hippocampal Schaffer collateral synapses and cerebellar GABAergic synapses, where the absence of TrkB results in significant decreases in synapse density (Rico *et al.*, 2002; Luikart *et al.*, 2005).

In contrast, our experiments indicate that the loss of TrkB from the retina *in vivo* does not reduce the number of dopaminergic neurons. Thus, TrkB signaling is not essential for the neurogenesis or survival of retinal dopaminergic neurons. Similarly, recent studies have shown that there is no significant effect of the loss of TrkB signaling on the development or survival of midbrain dopaminergic neurons *in vivo* (Kramer *et al.*, 2007).

In summary, the results of this study demonstrate that TrkB is dispensable for the development of general retinal architecture, as well as for development and maintenance of synaptic

transmission between photoreceptors and bipolar cells. Our work demonstrates that BDNF/TrkB signaling is essential for normal development of the retinal dopaminergic network.

EXPERIMENTAL METHODS

Animals

The conditional elimination of *TrkB* from retina relied on a mouse line carrying the floxed *TrkB* allele (further denoted as *TrkB^{f/f}*), which was isolated in our laboratory. This murine line was generated using homologous recombination in embryonic stem (ES) cells. In this allele, a 0.7 kb region containing the first coding exon of the *TrkB* gene was flanked with *loxP* sites. Cre-recombinase mediated recombination in this allele prevents expression of both full-length and truncated isoforms of TrkB proteins. For conditional elimination of *TrkB* in the entire retina, we used a transgenic mouse line in which Cre recombinase was expressed under regulation of a genomic DNA fragment of the mouse *Six3* homeobox gene (*Six3A1A2-cre*), that contains regulatory elements that can drive transgene expression in the eye and ventral forebrain (Furuta *et al.*, 2000). This mouse *Cre*-line was kindly made available to us by G. Oliver (Department of Genetics, St. Jude Children's Research Hospital, Memphis, Tennessee). For genotyping, mouse genomic DNA was isolated from tail samples with the DirectPCR (tail) DNA isolation kit (Viagen). Genotyping for the *Six3Cre* allele was performed using a protocol described by Furuta and co-authors (Furuta *et al.*, 2000). Genotyping for floxed *TrkB* allele was performed using the following primers: TrkB-n2: 5'-ATG TCG CCC TGG CTG AAG TG-3', and TrkB-c8: 5'-ACT GAC ATC CGT AAG CCA GT-3'. Amplification of the wild-type allele generated a 369 bp PCR product, while amplification of the floxed allele generated a 450 bp product. To detect the pattern of expression of *TrkB* in retina we used a mouse that carried floxed *TrkB* allele termed *fBZ*, coupled with tau-LacZ reporter cassette (Callahan and Thomas, 1994), which was designed in our lab (Xu *et al.*, 2000). TrkB cDNA with polyA termination site, flanked with *loxP* sites, followed by tau-lacZ expression cassette, was introduced into the first coding exon of *TrkB*. In this allele Cre mediated recombination deletes *TrkB* cDNA and activates expression of tau-lacZ. All mouse strains were bred on C57BL/6 genetic background (Charles River Lab). Mice were maintained under a 12 hr light/dark cycle in the animal facilities of the University of California San Francisco, with food and water *ad libitum*. All procedures were approved by the University of California San Francisco IACUC and conformed to the guidelines for animal research of the Association for Research in Vision and Ophthalmology.

Semithin sections

Animals were deeply anesthetized with CO₂ and perfused transcardially with a fixative of 2% paraformaldehyde and 4% glutaraldehyde in PBS (phosphate buffered saline, 176 mM KH₂PO₄, 8.05 mM Na₂HPO₄, 136 mM NaCl, 2.68 mM KCl, pH 7.4). Eyes were isolated and hemisected through three landmarks (superior rectus muscle, optic nerve, and inferior rectus muscle) to guarantee the same orientation in all eyes. After tissue postfixation with osmium tetroxide, eyecups were embedded in an Epon-Araldite mixture. Semithin (1 µm) sections were cut with a glass knife and stained with toluidine blue solution (1% toluidine blue and 1% borax in distilled water). Micrographs were taken on a Nikon FXA photomicroscope (Tokyo, Japan).

Frozen sections and immunostaining

Animals were deeply anesthetized by intraperitoneal injection of avertin and intracardially perfused with 4% paraformaldehyde solution in PBS, pH 7.2. Eyes were enucleated and corneas and lenses were carefully removed. Eyecups were soaked in 30% sucrose in PBS overnight and immersed in a 1:1 mixture of OCT medium with 10% sucrose in PBS. Eyecups were frozen in blocks of OCT/sucrose mixture in transverse orientation, and 20-µm sections were cut at or near the vertical meridian of the eye using Jung Frigocut 2800 cryostat (Leica). Sections of the

eyecups were mounted and air-dried on Superfrost glass slides. Antigens were visualized using immunofluorescence. Nonspecific binding was blocked by incubating sections for 2 hrs in blocking solution (3% bovine serum albumin, 10% normal goat serum, and 0.4% Triton-X in PBS). Primary antibodies (see below) were applied overnight in blocking solution, followed by fluorescence-labeled secondary antibodies. For preparation of sections of retinas from mice at P0 animals were sacrificed by decapitation, and heads were flash-frozen in OCT medium. 20- μ m coronal sections were cut through the eyes, along the vertical meridian. Sections were air-dried and fixed in an acetone-methanol mixture (1:1) for 1 hr. For preparation of whole flat-mounted retinas, eyeballs of mice perfused with 4% paraformaldehyde solution in PBS were enucleated and corneas and lenses were carefully removed. Retinas were separated from the choroid in PBS with 5 mM Na-EDTA, and relaxation cuts were made. Tissue was incubated in blocking solution overnight, after which primary and secondary antibodies were applied sequentially at 4°C for 72 and 24 hr, respectively. Stained retinas were flat-mounted on microscopic slides, photoreceptor side down. Coverslips were mounted over specimens using Prolong Antifade Kit (Invitrogen). Specimens were examined by confocal microscopy (LCM 5 Pascal, Carl Zeiss).

Morphometric analysis

Rod outer segment lengths were measured in semithin sections, with 27 measurements in each of the superior and inferior hemispheres of the eye. The method was that described previously for the ONL thickness in mice (LaVail *et al.*, 1987), but in this case the measurements were made along the lengths of the outer segments. Means of the 27 measurements in each hemisphere were compared between mutant and control retinas. To analyze the effect of *TrkB* deletion on the density of TH-positive dopaminergic amacrine cells, we collected confocal optical sections through sublamina S1 of IPL and the innermost part of INL, from flat-mounted retinas. We collected a sample of 6 fields of 0.921 mm² from each retina. Fields were collected from the central retina. Cells were counted with help of ImageJ 1.34s software, and the average density of TH-positive cells per mm² was estimated. To quantify the density of TH-immunopositive varicosities, we sampled the central retina with several fields collected using confocal microscopy, with a 40X oil immersion objective. Stacks of 20 0.5- μ m thick Z-sections each were collected to cover a thickness of sublamina 1 and adjacent layers in the IPL and INL. Projections of the stacks were built and then processed using ImageJ. Images were thresholded automatically and the number of varicosities was counted using particle analysis function. Particles with size less than 0.25 μ m² and more than 7.58 μ m² were excluded from analysis. For each retina, 6 fields were collected and the average density was estimated. To count SMI-32-positive RGCs, we used the same method as for the counting of TH-positive neurons, except the mid-peripheral regions of retinas were sampled.

Analysis of recombination efficacy

To test the recombination efficacy within the adult retina *in vivo*, males carrying the *Six3Cre* transgene were crossed with females that carried the *ROSA26* reporter (*R26R*) allele (Soriano, 1999). Animals were sacrificed at P30 and β -galactosidase activity resulting from Cre-mediated recombination of the *R26R* locus was visualized by X-Gal staining in 20 μ m frozen sections. Sections were examined using the Nikon FXA photomicroscope.

Antibodies

The polyclonal antibody recognizing TrkB was raised against the extracellular domain of TrkB in rabbits and affinity purified. It does not distinguish between the kinase and the truncated forms of TrkB (1:400; obtained from G. Wilkinson, University of California San Francisco; (Meyer-Franke *et al.*, 1998)). The following antibodies were also used: rabbit anti-recoverin (1:1000; Chemicon); rabbit anti-calretinin and anti-calbindin (1:1000; Chemicon); rabbit anti-

β -actin (1:5000; Sigma); mouse anti- β -galactosidase (1:5000, Promega); rabbit anti- β -galactosidase (1:2000, Biochemicals); mouse anti-TH monoclonal (1:200; Chemicon); SMI-32 anti-neurofilament monoclonal (1:1000; Steinberg Monoclonals); rabbit anti- Brn3a (1:100; Chemicon); Guinea pig anti-glutamate vesicular transporter (VGLUT1) (1:5000; Chemicon); rabbit anti- Glutamic acid decarboxylase GAD65 (1:200, Chemicon); rabbit anti-PKC (1:1000, Chemicon). Rabbit PMCA1 antibody was kindly provided by D. Krizaj.

Western blots

Eyeballs were enucleated and corneas and lenses were carefully removed. After enucleation, all steps were performed at 4°C. Neural retinas were separated from the RPE in PBS with 5 mM Na-EDTA. Tissue was then homogenized by trituration in the lysis buffer (20 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1% Triton X-100), with the CompleteMini protease inhibitor cocktail (Roche Diagnostics). To prepare protein extracts from the RPE after removal of the neural retina, eyecups were filled with lysis buffer and buffer was carefully triturated. Lysates were collected and clarified by centrifugation. Total protein content was measured using Bio-Rad D_C Protein Assay kit. Protein extracts were separated on 4%-15% gradient SDS-PAGE precast gels (BioRad) and transferred to PVDF membranes.

Electroretinogram (ERG) recordings

ERG responses were recorded as previously described (Bok *et al.*, 2002) Briefly, mice were dark-adapted overnight and then anesthetized in dim red light with intramuscular injections of xylazine (13 mg/kg) and ketamine (87 mg/kg). Pupils were dilated with topical atropine (1%) and phenylephrine (2.5%), and one drop of methylcellulose (1%) was applied to the cornea. Custom-made contact lenses for mice with gold wire loops (Bayer *et al.*, 1999) were placed on both corneas with a drop of methylcellulose. A silver wire electrode was placed in the nose and a ground electrode was placed subcutaneously in the tail. Bilateral full-field electroretinogram responses were elicited with 10 μ s-long white light flashes and were recorded using a UTAS-E 3000 Visual Electrodiagnostic System (LKC technologies, Inc., Gaithersburg, MD, USA). Stimuli were presented in order of increasing luminance from -4.6 to $+0.4$ log cd sec/m². Several responses at each intensity were computer averaged. Interstimulus intervals ranged from 5 seconds at the lowest intensities to 120 seconds at the highest intensities, and between 2 and 15 repeated responses were recorded at each stimulus intensity. The amplitude and implicit time (time to peak) of the a- and b-waves were measured. The scotopic b-wave threshold criterion amplitude was 20 μ V, the scotopic a-wave threshold criterion amplitude was 10 μ V, and the photopic b-wave threshold criterion amplitude was 10 μ V. Below a-wave threshold, b-wave amplitudes were measured from baseline to peak; at intensities where measurable a-waves were present, b-wave amplitudes were measured from the a-wave trough to the b-wave peak. Given the interference of post-receptoral activity to the dark-adapted a-wave, (Robson *et al.*, 2003) a-wave amplitudes were measured at a specific point in time corresponding to the trough of the a-wave at the brightest intensity measured in all mice, $+0.4$ log cd sec/m². The implicit time and amplitude of scotopic ERG oscillatory potential (OP) wavelets measured in response to a $+0.4$ log cd sec/m² flash were extracted using FFT filtering at 75 Hz in the control and mutant mice. The mice were then light-adapted to 30 cd/m² background light for 10 min, and photopic responses were elicited with -0.6 , $+0.4$, $+1.4$ and $+2.4$ log cd sec/m² stimuli presented at 2 Hz. Responses to 20 successive flashes were averaged. Responses were amplified at a gain of 4000, filtered between 0.3 and 500 Hz and digitized at a rate of 2000 Hz on two channels. Because photopic a-waves are negligible in mice (Peachey *et al.*, 1993), the amplitude and implicit time of the photopic b-waves were measured. The photopic b-wave amplitude was measured from the first negligible cornea-negative trough after the baseline to the earliest cornea-positive peak.

Statistical analysis

ERG responses from control and mutant mice, as well as data from morphometric measurements were compared using a Student's *t*-test. Values are presented as mean $\pm$ SEM, and *P*-values <0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A. In vivo recombination of the *R26R* locus mediated by *Cre* transgene product in retina. X-Gal staining pattern of a 20-μm section of the retina of a mouse hemizygous for the *Six3Cre* and *R26R* alleles at P30. Sections were stained with X-Gal (blue) and counterstained with a general nuclear marker Vector Nuclear Fast Red (Vector) (pink). Recombination was detected in photoreceptors, and cells in the INL and retinal ganglion cell (GCL) layers. Note, that in photoreceptors, LacZ stain accumulates in the inner segments and in the OPL but not in the cell bodies, as a result of transport of the LacZ reporter to their axons and inner segments (Chang *et al.*, 2000). *Left panel*: 4X magnification. *Right panel*: 40X magnification. RPE, retinal pigment epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. *B.* Western blot analysis of TrkB protein in control *TrkB^{fl/fl}* and mutant *Six3Cre;TrkB^{fl/fl}* retina. *Left panel*: Protein extracts were prepared from the neural retina of P30 mice. The blot was probed with antibodies to TrkB and β-actin simultaneously. TrkB-TK, the 145 kDa tyrosine-kinase isoform of TrkB. TrkB-T, 95 kDa truncated splice isoform of TrkB. *Right panel*: Protein extracts were prepared from the RPE of P30 mice, by the detergent extraction of the interior chamber of eyecup after removal of the neural retina. *C.* Pattern of *Six3Cre*-mediated deletion of the floxed *TrkB* allele in the retina. Expression of TrkB was determined by immunofluorescence. *Left panel*: Pattern of expression of TrkB in the retina of a control mouse homozygous for the floxed *TrkB* allele (no *Cre* expression). *Central panel*: No expression of TrkB is detected in the neural retina of *Six3Cre;TrkB^{fl/fl}* mutant. *Right panel*: Continued expression of TrkB in a few scattered cells in

the RPE of *Six3Cre;TrkB^{ff}* mutant retina (white arrows). *D.* *Six3Cre*-mediated deletion of the floxed *TrkB* allele in the retina of newborn (P0) mouse. Expression of TrkB was determined by immunofluorescence. Upper panels: Pattern of expression of TrkB in the retina of a control mouse at P0. Lower panels: *Six3Cre*-dependent recombination eliminates TrkB expression is at P0. Left panel: TrkB immunostaining. Central panel: Nuclear labeling with ToPro3 dye. Right panel: Merge. ONBL, outer neuroblastic cell layer; GCL, retinal ganglion cell layer. Scale bar, 40 μ M.

Figure 2.

Detection of activity of *TrkB* gene in the retina utilizing *Six3Cre, TrkB^{fBZ/+}* cross. In this cross Cre-mediated recombination activates expression of tau-lacZ under control of *TrkB* genomic locus. Tau-LacZ expression resulting from the recombination of *fBZ* locus in the retina was detected using immunofluorescence. *A, B, C.* tau-LacZ expression is detected in the RGCs, identified by Brn3a. *D-F.* Optical section through the low margin of INL of flat-mounted *Six3Cre, TrkB^{fBZ/+}* retina. Expression of tau-LacZ is detected in the population of amacrine cells, including TH-positive dopaminergic amacrine cells (*E*, white arrowheads). *G, H, I.* *TrkB* locus driven tau-LacZ expression is absent from rod bipolar cells. White arrowheads show two types of β -galactosidase immunopositive cells in the INL – bipolar and amacrine cell. Rod

bipolar cells are identified by high levels of PKC immunoreactivity. *J,K,L*. TrkB locus driven tau-LacZ expression is absent from Müller glia. In *A-I* tau-LacZ was detected using mouse anti- β -galactosidase antibody (Promega), and in *J-K* tau-LacZ was detected using rabbit anti- β -galactosidase antibody (Biochemicals). Scale bar, 20 μ M.

Figure 3.

Effect of TrkB deficit on the ERG in adult mice. **A.** Representative scotopic ERG responses of *Six3Cre;TrkB^{f/+}* control (left panel) and *Six3Cre;TrkB^{f/f}* mutant mice (right panel). ERG responses were elicited by short flashes of increasing light intensity as follows (from bottom to top traces): $-4.6, -3.6, -2.6, -1.6, -0.6, +0.4$ log cd sec/m². Arrow indicates time of stimulus. **B.** Dose-dependency of scotopic ERG a-wave and b-wave amplitude (left panel) and implicit time (right panel) on flash intensity. Responses with amplitudes greater than the scotopic b-wave threshold criterion amplitude of 20 μV are plotted. **C.** Absence of the effect of TrkB deficit on oscillatory potentials. Left panel: OP wavelets extracted using FFT filtering at 75 Hz in control (above) and mutant (below) adults. Right panel: Amplitudes and implicit times of scotopic ERG OP wavelets in the control (white bars) and mutant (black bars) mice. Error bars = standard error of the mean (SEM), n = 4 mice for each group. **D.** Effect of TrkB deficit on the photopic ERG in adult mice. Left panel: Representative photopic ERG responses of *Six3Cre;TrkB^{f/+}* control (left) and *Six3Cre;TrkB^{f/f}* mice (right). Intensities of flashes were as follows (from bottom to top traces): $+0.4, +1.4$ and $+2.4$ log cd sec/m². Right panel: Dependence of photopic ERG b-wave amplitude on light flash intensity. ○ - control, ■ - mutant. Error bars = standard error of the mean (SEM), n = 4 mice for each group.

Figure 4.

Effect of TrkB deficit on the ERG in juvenile (P16) mice. **A.** Representative scotopic ERG responses of control (*left panel*) and mutant animals (*right panel*). ERG responses were elicited by short flashes of increasing light intensity as follows (from bottom to top traces): -4.6 , -3.6 , -2.6 , -1.6 , -0.6 , $+0.4$ log cd sec/m². Arrow indicates time of stimulus. **B.** Dose-dependency of scotopic ERG a-wave and b-wave amplitude (*left panel*) and implicit time (*right panel*) on flash intensity. The mutant b-wave amplitudes were significantly lower than the control responses at this age at all but the brightest stimulus intensity, where the difference approached significance ($P = 0.058$). Mutant a-wave amplitudes were significantly lower than control at the brightest intensity tested, where the a-wave amplitude is measured most reliably. The mutant response to the dimmest intensity was below the scotopic b-wave threshold criterion amplitude and was not plotted. **C.** Absence of the effect of TrkB deficit on oscillatory potentials. *Left panel*: OP wavelets extracted using FFT filtering at 75 Hz in control (above) and mutant (below) adults. *Right panel*: Amplitudes and implicit times of scotopic ERG OP wavelets in the control (white bars) and mutant (black bars) mice. **D.** Effect of TrkB deficit on the photopic ERG in the P16 mice. *Left panel*: Representative photopic ERG responses of control (*left*) and mutant mice (*right*). *Right panel*: TrkB deficit has no effect on the relationship between photopic ERG b-wave amplitude and flash intensity. ○ - control. ■ - mutant. Error bars = standard error of the mean (SEM), $n = 4$ mice for mutant mice and $n = 3$ mice for controls.

Figure 5.

General morphology of the *Six3Cre;TrkB^{fl/+}* control (+/-) and *Six3Cre;TrkB^{fl/fl}* mutant retina (-/-). *Upper panels*, Central inferior retina. *Medium panel*, Mid-peripheral superior retina. *Low panels*, Far peripheral superior retina at the ora serrata. Semi-thin 1 μ m plastic sections of retinas at P67 are shown. RPE, retinal pigment epithelium; ROS, rod photoreceptor outer segments; RIS, rod photoreceptor inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, retinal ganglion cell layer. Scale bar, 40 μ m.

Figure 6.

TrkB deficit does not affect the expression of excitatory and inhibitory markers in the retina. Retinal sections of the *Six3Cre;TrkB^{f/f}* control (A, C, E, G) and *Six3Cre;TrkB^{f/f}* mutant retina (B, D, F, H) at P30 are shown. A, B. VGLUT1 immunoreactivity, labels excitatory synaptic terminals. C, D. Calretinin immunoreactivity, labels amacrine cells and RGCs. E, F. Calbindin immunoreactivity, labels horizontal and amacrine cells. G, H. OFF-center recoverin-positive bipolar cells (Green) and Brn3a positive RGCs (Red). White arrows point to recoverin-positive bipolar cells. Scale bar, 20 μ m.

Figure 7.

TrkB deficit does not result in the loss of retinal RGCs in adult mice. SMI-32 positive RGCs in the whole-mounted retinas. A. $TrkB^{+/+}$ control, B. $Six3Cre;TrkB^{+/+}$ mutant retina at P50. C. Average density of SMI-32 positive cells per mm^2 , $P=0.64$. Control = white bar, mutant = black bar. Error bars = SEM. N = 3 for each group.

Figure 8.

TrkB deficit inhibits maturation of the retinal dopaminergic network. *A,B.* TrkB deficit does not affect the lamination pattern of processes of retinal dopaminergic cells. Sections of *Six3Cre;TrkB^{f/+}* control (*A*) and *Six3Cre;TrkB^{f/f}* mutant (*B*) retina at P30 are shown. TH-positive dopaminergic amacrine cells are labeled red, calbindin immunopositive cells are labeled green. Arrowheads indicate dopaminergic amacrine cells. *C.* Western blot analysis of TH protein in control *TrkB^{f/+}* and mutant *Six3Cre;TrkB^{f/f}* retina. Protein extracts were prepared from the neural retina of mice at P30. 5, 10, 20 and 40 μ g protein of control (*left*) and mutant (*right*) retina and fractionated by SDS-PAGE. *D,E.* Plexus of dopaminergic innervation in TrkB control (*D*) and TrkB deficient (*E*) retina of adult mouse. *F, G.* Enlargements of boxed

areas in D and E. Scale bar, 20 μ M. *H*. Average density of dopaminergic neurons is not reduced by TrkB deficit. P30 retinas were used for evaluation. N=4 for control (white bars) and mutant (black bars). Error bars = SEM. $P=0.7$. TrkB deficit results in the reduction of the density of synaptic varicosities of dopaminergic neurons. *I*. *Left panel*. Mean number of densities of TH-positive varicosities in the retinas of P30 animals. N=5 for control (white bars) and N=4 for mutant (black bars) animals. *Right panel*. Mean number of densities of TH-positive varicosities in the retinas of P60 animals. N=3 for control (white bars) and N=3 for mutant (black bars) animals. Error bars = SEM. $*P=0.023$, $**P=0.004$.