Functionally intact glutamate-mediated signaling in bipolar cells of the TRKB knockout mouse retina

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Abstract

In the juvenile trkB knockout (trkB<sup>−/−</sup>) mouse, retina synaptic communication from rods to bipolar cells is severely compromised as evidenced by a complete absence of electroretinogram (ERG) b-wave, even though the inner retina appears anatomically normal (Rohrer et al., 1999). Since it is well known that the b-wave reflects light-dependent synaptic activation of ON bipolar cells via their metabotropic glutamate receptor, mGluR6, we sought to analyze the anatomical and functional integrity of the glutamatergic synapses at these and other bipolar cells in the trkB<sup>−/−</sup> mouse. Although rod bipolar cells from wild-type juvenile mice were determined to be immunopositive for trkB, postsynaptic metabotropic and ionotropic glutamate receptor-mediated pathways in ON and OFF bipolar cells were found to be functionally intact, based on patch electrode recordings, using brief applications (“puffs”) of glutamate or its analog, 2-amino-4-phosphonobutyric acid (APB), a selective agonist for mGluR6 receptors. Ionotropic glutamate receptor function was assayed in OFF-cone bipolar and horizontal cells by applying exogenous glutamatergic agonists in the presence of the channel-permeant guanidinium analogue, 1-amino-4-guanidobutane (AGB). Electron-microscopic analysis revealed that the ribbon synapses between rods and postsynaptic rod bipolar and horizontal cells were formed at the appropriate age and appear to be structurally intact, and immunohistochemical analysis did not detect profound defects in the expression of excitatory amino acid transporters involved in glutamate clearance from the synaptic cleft. These data indicate that there does not appear to be evidence for postsynaptic deficits in glutamatergic signaling in the ON and OFF bipolar cells of mice lacking trkB.

Keywords: Patch clamp recording, Agmatine uptake, Electronmicroscopy, Synapse, Excitatory amino acid transporter

Introduction

Tyrosine Kinase (TrkB) receptors are a family of three closely related receptor tyrosine kinases, each of which is activated by one or more of the neurotrophins (Huang & Reichardt, 2003). The Trk receptors mediate most of the survival-promoting and differentiation-promoting actions of the four neurotrophins (Kaplan & Miller, 2000; Huang & Reichardt, 2001; Patapoutian & Reichardt, 2001; Hempstead, 2002). TrkB is strongly activated by brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NT-4) (Huang & Reichardt, 2003), and in some cases it is also activated by NT-3. Both trkB and its ligands are expressed in the mouse retina where they regulate neuronal differentiation (Di Polo et al., 2000; Wahlin et al., 2001; Lom et al., 2002). BDNF and NT-3 are expressed in retinal ganglion and inner nuclear layer cells (Bennett et al., 1999; Cusato et al., 2002; Pollock & Frost, 2003), whereas trkB is widely expressed in the retina. Cell-specific labeling has been reported in dopaminergic amacrine, retinal ganglion, horizontal, and Müller glial cells (Cellerino & Kohler, 1997; Llamosas et al., 1997; Rohrer et al., 1999; Vecino et al., 2002).

Rod photoreceptor development is regulated by trkB-mediated signaling <i>in vivo</i> and in organ culture (Rohrer et al., 1999; Rohrer & Ogivie, 2003). Using ERG recordings, we have shown that in trkB knockout mice, functional and structural development of rods lags in time relative to development in wild-type mice and up to 16 postnatal days (oldest time point studied; limited by animal’s survival) there is no evidence of a functional synaptic connection between rods and rod bipolar cells. These findings are surprising.
because rods do not express trkB receptors (Rickman & Brecha, 1995; Ugolini et al., 1995; Rohrer et al., 1999; Di Polo et al., 2000; Harada et al., 2000). Our findings, furthermore, suggest that rod (and possibly rod bipolar cell) development depends on remote-acting molecules controlled by trkB-receptor expressing cells. Indeed, recent experiments suggest that BDNF may modulate postnatal photoreceptor survival indirectly, by controlling the release of growth factors from Müller glia (Harada et al., 2002).

Our previous studies demonstrated that trkB knockout mice lack a detectable ERG b-wave at all ages tested (Rohrer et al., 1999). Since it is now widely accepted that the ERG b-wave is a direct electrical manifestation of rod bipolar cell activity (reviewed by Pugh et al., 1998), the collective a-wave and b-wave results imply that these mice suffer from a profound defect in the signaling pathway between rods and rod bipolar cells. The specific locus of the defect, whether presynaptic in the rod terminal, or postsynaptic in the rod bipolar cells, is not known, and cannot be investigated using ERG recordings.

The present study was undertaken with the objective of examining the anatomical and functional integrity of the glutamate synapse between photoreceptors and bipolar cells in trkB+/− mice. Although immunocytochemical studies demonstrated that trkB receptors are normally present on rod bipolar cells of wild-type (wt) mice, electrophysiological experiments and AGB mapping studies demonstrated that depolarizing (ON) and hyperpolarizing (OFF) bipolar cells from the trkB−/− retina respond to exogenous applications of glutamate analogs. Using electron microscopy (EM), no differences were observed in the ultrastructure of the rod ribbon synapse in trkB−/− mice when compared to wt mice, and immunocytochemical experiments failed to show profound differences in the expression of excitatory amino acid transporters, required for glutamate clearance. The absence of a profound defect in postsynaptic receptor-mediated signaling suggests that the trkB-dependent remote-acting molecules that control rod development do not mediate ribbon synapse morphology, or rod bipolar cell development and signaling.

Material and methods

Animals

The trkB knockout mouse line (trkB+/−; Rohrer et al., 1999) was generated by deletion of the first coding exon that is shared by all trkB isoforms, so it expresses neither the kinase nor the non-kinase generated by deletion of the first coding exon that is shared by all isoforms of trkB. TrkB+/− homozygous mutants die within 3 weeks of birth. The original heterozygous founders of the trkB+/− mice (mixed 129 × C57/B16) were outcrossed into an ICR (Institute of Cancer Research) background for improved survival. Experiments were performed on littermates raised under cyclic light conditions (12 h light, 12 h dark) at the University of California San Francisco animal care facility. Genotyping utilized mouse tail DNA samples and PCR analyses. All experiments were in accordance with the Association for Research in Vision and Ophthalmology guidelines for animal research, using protocols approved by the UCSF Committee on Animal Research.

Patch-clamp recordings

Retinal slices were prepared from eyes of trkB+/− mice and wt littermates between the ages of P12 and P19. Cells included in this study had a mean “age” of P15.0 (wt) and P14.8 (trkB+/−). Mice were deeply anesthetized with CO₂ and decapitated. The eyes were removed and dissected in oxygenated Ames medium [(Ames III & Nesbett, 1981); Sigma, St. Louis, MO]. The retina was removed from the sclera and retinal pigment epithelium, trimmed, and cut into two pieces. One piece of retina was transferred to a glass coverslip, ganglion cells-side-up, in a bead of medium. After gently sucking off excess medium, a dry filter (Millipore, Bedford, MA) was placed on top of the retina. The filter paper and adherent retina were then removed to a custom-made recording and slicing chamber, and sliced into ~150 μm-thick sections using a custom-made tissue slicer. Finally, several slices were transferred to slots in the recording portion of the chamber and rotated 90 deg to expose the retinal layers.

The recording chamber was placed on the fixed stage of an upright microscope (Zeiss Axioskop, Thornwood, NY) equipped with epifluorescence and Nomarski optics. Slices were perfused continuously (at ~1 ml/min) using a gravity-fed system, and maintained at 37°C by warming the chamber bottom and the inflowing perfusate (Cell MicroControls, Norfolk, VA). A manually operated Hamilton valve (Hamilton Co., Reno, NV) was used to select between Ames’ solution or a calcium-free Locke’s solution containing 2–6 mmol/l CoCl₂ to block endogenous neurotransmitter release. Perfusing the slices for about 30 min prior to recording helped remove damaged cells from the surface of the slice.

Cells were studied using conventional whole-cell recording techniques. Electrodes were typically filled with a Cs-gluconate-based solution (in mmol/l: 130 CsOH, 120 p-gluconic acid, 10 CsCl, 3 MgCl₂, 10 Hepes-NaO₄, 15 glucose, 3 ATP-Na₂, 1 GTP-Na₃; pH 7.2). EGTA (5–10 mmol/l) was included in some experiments, and in some early experiments K-gluconate was used instead of Cs-gluconate. Lucifer yellow (0.02–0.04%) was usually included to label and identify cells using fluorescence, although filling was not always successful. Cells were held at a potential of approximately −40 mV. Signals were acquired with an Axopatch 1D amplifier (Axon Instruments, Union City, CA), filtered with a 4-pole Bessel anti-aliasing filter (Warner Instruments, Hamden, CT), and digitized using an Instutech ITC-16 (Institutech, Port Washington, NY) and Pulse Control software package (freeware developed and distributed by the Bookman Laboratory at the University of Miami, FL). Subsequent analysis was done using Igor (Wavemetrics, Oswego, OR).

Cell somas in the outer half of the inner nuclear layer (INL) were targeted for study. This would be expected to enrich the cell sample pool for ON bipolar to the exclusion of OFF bipolar and amacrine cells. Horizontal and Müller cells were easily identified instead of-Cs-gluconate. Lucifer yellow (0.02–0.04%) was usually included to label and identify cells using fluorescent, although filling was not always successful. Cells were held at a potential of approximately −40 mV. Signals were acquired with an Axopatch 1D amplifier (Axon Instruments, Union City, CA), filtered with a 4-pole Bessel anti-aliasing filter (Warner Instruments, Hamden, CT), and digitized using an Instutech ITC-16 (Institutech, Port Washington, NY) and Pulse Control software package (freeware developed and distributed by the Bookman Laboratory at the University of Miami, FL). Subsequent analysis was done using Igor (Wavemetrics, Oswego, OR).

A Picospritzer (General Valve, Fairfield, NJ) under computer control was used to pressure eject solutions (i.e. puff) onto the outer plexiform layer (OPL) through a glass pipette (2–4 μm diameter tip). Puff durations were 100–400 ms, and puff solutions consisted of glutamate (0.3 to 1 mmol/l) or L(+)-2-amino-4-phosphonobutyric acid (APB; 100 μmol/l) in a solution matching the extracellular solution. In a few experiments the recording electrode was used to gently “pull” a cell from the slice, and puffs were then applied to the isolated cell.

A small optical bench delivered light from a light-emitting diode (LED) source, through a glass fiber optic cable, and up through the microscope condenser to the slice. An adjustable aperture con-
jugate to the specimen controlled the size of the spot stimulus, always >200 μm diameter in these experiments. The intensity and timing of stimuli were computer controlled using the Pulse software, the ITC-16, and custom-built LED driver circuitry. LED intensity was controlled by modulating the driving current. A small portion of the LED light was used in a feedback circuit to maintain LED linearity. Stimuli were brief flashes, 10 ms in duration.

**AGB mapping**

WT and trkB<sup>−/−</sup> retinas were rapidly isolated, flat-mounted on pieces of nitrocellulose filter (5 μm pore size, Whatman) and incubated in oxygenated Ames medium at 35°C (95% O₂, 5% CO₂). After a short rest period (10 min), the medium was replaced with pre-warmed, oxygenated medium for 180 sec, in which 8 pieces of nitrocellulose filter (5 μm pore size, ultrathin retinal sections taken from P16 mice per genotype were analyzed for overall morphology of the Geigy, Basel, Switzerland). Ultrathin sections for electron microscopy were performed in the presence or absence of 30 μmol/l kainic acid (KA) on the two eyes of each animal. After 30 min of incubation, reactions were stopped by fixation in Karnovsky’s fixative (see electron microscopy section) and tissue was processed for epoxy resin embedding and immunohistochemical visualization (Marc, 1999b). Sections (250 μm) were deplasticized, probed with antibody at 20°C overnight (in 1% goat serum in 0.1 mol/l phosphate buffer, pH 7.4 and 0.05% thimerosal, GSPBT), followed by secondary antibody treatment (1 nm gold conjugated anti-rabbit IgG (Marc et al., 1999)) and silver intensification (0.11% silver nitrate and 0.4% Triton-X in TBS). Primary antibodies were applied overnight in blocking solution, followed by biotinylated secondary antibodies and the avidin and biotinylated horseradish peroxidase complex (ABC, Vector Laboratories Inc.; Burlingame, CA) for 1 h each. Slides were developed in DAB (0.05% diaminobenzidine in 0.1 mol/l Tris pH 7.5, and 0.003% hydrogen peroxide) for 1–5 min, dehydrated, and mounted in DPX. Sections were photographed using a Zeiss Axiophot, equipped with a digital camera and Spot acquisition software. For double-labeling immunohistochemistry, tissue sections or cells were first incubated in blocking solution for 1 h (see above), followed by the application of both primary antibodies which were raised in different species, for 4 h (single cells) or overnight (tissue sections). Antibody binding sites were visualized using fluorescently labeled secondary antibodies conjugated to Cy3 and Cy5 (Molecular Probes, Eugene, OR). Retinal sections were examined by confocal microscopy whereas single cells were photographed using a Zeiss Axio phot, equipped with fluorescence. Images were false-colored and superimposed using the Adobe Photoshop software.

The following antibodies were used in this study: a rabbit polyclonal antibody against the extracellular domain of trkB (Meyer-Franke et al., 1998), a rabbit polyclonal against the kinase domain of trkB (a generous gift by D. Kaplan, University of Montreal; Rohrer et al., 1999); a monoclonal antibody against PKCa/β (Amersham, Arlington Heights, IL) to identify rod bipolar cells; and two polyclonal antibodies against the two main excitatory amino acid transporters in the retina: GLAST (a generous gift by M. Watanabe, Hokkaido University School of Medicine, Sapporo, Japan) and GLT-1 (a generous gift by J.D. Rothstein, Johns Hopkins University).

**Electron microscopy**

Animals were deeply anesthetized with CO₂ and perfused cardially with Karnovsky fixative (2% paraformaldehyde, 4% glutaraldehyde in phosphate-buffered saline; pH 7.4). The posterior portion of each eye was cut into quadrants, fixed additionally for 1 h with 1% osmium tetroxide (0.1 mol/l phosphate buffer, pH 7.2), dehydrated, and embedded in Araldite 502 (Ciba Geigy, Basel, Switzerland). Ultrathin sections for electron microscopy section were stained with uranyl and lead salts. Sections from three mice per genotype were analyzed for overall morphology of the ribbon synapses.

Synaptic ribbon length was measured from digital images of ultrathin retinal sections taken from P16 wt and P16 trkB<sup>−/−</sup> mice. Two wt and two trkB<sup>−/−</sup> mice were included in the analysis. Synaptic ribbons in rod photoreceptor synaptic terminals that were sectioned at right angles to the plane of the ribbon were imaged with a KeenView digital camera (Soft Imaging System, Lakewood, CO) fitted to a Zeiss EM 910 electron microscope (Oberkochen, Germany). The microscope magnification was set at 10,000× for all of the images. The length of each ribbon was measured using analySIS™ software (Soft Imaging System, Lakewood, CO). A total of 40 synaptic ribbons were measured for the P16 wt retinas and 47 for the P16 trkB<sup>−/−</sup> retinas. Means and standard deviations from the mean were computed and the level of significance determined by the Student two-tailed t-test.

**Immunohistochemistry**

For immunohistochemistry on retinal sections, animals were decapitated, and eyes were enucleated and immersion-fixed in Car-}

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{Intact ON-pathway in trkB<sup>−/−</sup> mice}

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{jugate to the specimen controlled the size of the spot stimulus, always >200 μm diameter in these experiments. The intensity and timing of stimuli were computer controlled using the Pulse software, the ITC-16, and custom-built LED driver circuitry. LED intensity was controlled by modulating the driving current. A small portion of the LED light was used in a feedback circuit to maintain LED linearity. Stimuli were brief flashes, 10 ms in duration.

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primer: CCC ATC CCA GAG TCA GAA AA, reverse primer: CTC TTC TCC GTT GCT TTT GG, amplicon size: 142 bp; β-actin: forward primer: GCT ACA GCT TCA CCA CCA CA, reverse primer: TCT CCA GGG AGG AAG AGG A T, amplicon size: 123 bp). Real-time PCR were performed in triplicate in a GeneAmp® 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) using the following cycling conditions: 50°C for 2 min, 94°C for 15 min, then 40 cycles of 94°C for 15 s and 58°C for 1 min. Quantitative values were obtained by the cycle number (Ct value), which is inversely proportional to the amount of a specific mRNA species. Relative gene expression levels were calculated using the equation \[ y = (1 + AE) \Delta \Delta Ct \], where \( AE \) is the amplification efficiency of the target gene (set at 1.0 for all calculations), and \( \Delta \Delta Ct \) the difference between the mean experimental and control \( Ct \) values. The \( \Delta Ct \) value is the difference between the \( Ct \) value for a retina-associated gene and the β-actin internal reference control gene. Data were expressed as mean ± standard error of the mean (SEM) and a Z-test was used to test for a difference from zero (i.e., no difference between wt and trkb−/− mice).

**Results**

**Rod bipolar cells express TrkB receptors**

We had shown previously that the distribution of TrkB receptors follows the development and maturation of the mouse retina (Rohrer et al., 1999). Here, using fluorescently labeled antibodies, TrkB was localized to cell bodies in the distal part of the outer nuclear layer as well as the outer plexiform layer of P14 wt retinas. Double-labeling immunohistochemistry for PKCa/β (Fig. 1A) and TrkB (Fig. 1B) revealed that rod bipolar cells account for some of the TrkB immunoreactivity in the inner nuclear layer and outer plexiform layer. Labeling of dissociated retinal cells obtained from P14–P16 wt retinas confirmed that all PKCa/β-positive cells were co-labeled with antibodies against TrkB (Fig. 2). It appears that rod bipolar cells grown in short-term cultures, like isolated retinal ganglion cells (Meyer-Franke et al., 1998), rapidly internalize TrkB receptors presumably due to the absence of appropriate external signals. Thus, out of the two cell types that make up the through-pathway for visual information processing, rod photoreceptors and rod bipolar cells, one is negative (i.e., rods), the other one is positive (i.e., rod bipolar cells) for the TrkB receptors.

**Normal response to glutamate of postsynaptic metabotropic receptors**

In normal mammals, the bipolar cells respond to the modulation of glutamate released by photoreceptor cells. Rod and cone ON bipolar cells possess mGluR6 glutamate receptors that mediate a...
hyperpolarizing response to glutamate (Masu et al., 1995), whereas cone OFF bipolar cells possess ionotropic glutamate receptors (iGluRs) that lead to depolarizing responses. The absence of a recordable ERG b-wave in *trkB*<sup>−/−</sup> mice raises the possibility that bipolar cells, particularly rod ON bipolars, do not respond properly to glutamate. To test this idea, we made patch electrode recordings from bipolar cells in a retinal slice preparation and tested whether they responded to brief applications (“puffs”) of glutamate or its analog APB, a selective agonist for mGluR6 receptors (Slaughter & Miller, 1985; Euler et al., 1996; McGillem & Dacheux, 2001).

In wt mice, 25 cells were studied. Based on responses to glutamate or APB puffs, nine of these were classified as ON bipolar and 11 were classified as OFF bipolar cells (Figs. 3B & 3D). Five were unresponsive to either APB (4 cells) or glutamate (1 cell). In *trkB*<sup>−/−</sup> mice, 22 of 44 cells tested responded to glutamate or APB with outward currents, and so were classified as ON-type bipolar cells (Fig. 3A). Seven cells, presumably OFF bipolar cells, responded to glutamate with inward currents (Fig. 3D). Fifteen cells were unresponsive, but most of these (10 of the 15) were tested only with APB, so some may have been OFF cells.

Strongest evidence for the integrity of the ON pathway comes from experiments using APB puffs, or glutamate puffs when synaptic transmission was blocked with Co<sup>2+</sup> and 0 Ca<sup>2+</sup> in the perfusate (Fig. 3A). Peak responses to 100 μmol/l APB were similar in *trkB*<sup>−/−</sup> and wt mice (12.6 ± 11.3 pA, n = 8, in *trkB*<sup>−/−</sup>; 12.1 ± 19 pA, n = 4, in wt). ON responses to glutamate (0.8–1 mmol/l) were more variable (23 ± 32 pA, n = 3, in *trkB*<sup>−/−</sup>; 15.5 ± 5.0 pA, n = 2, in wt). OFF-bipolar cells also gave similar peak responses (inward currents) to glutamate puffs (0.8–1 mmol/l) in *trkB*<sup>−/−</sup> and wt mice (−25 ± 25 pA, n = 4 in *trkB*<sup>−/−</sup>; −34 ± 27 pA, n = 9 in wt).

A significant portion of putative rod bipolar cells in both wt and *trkB*<sup>−/−</sup> retinas did not respond vigorously to exogenous puffs of glutamate or APB. Euler and colleagues (Euler et al., 1996) similarly reported responses from only 50% of rod bipolar cells in their study using rat slices. In contrast, light-evoked (glutamate-mediated) responses from wt ON bipolar cells could be consistently recorded.
(Fig. 3C; D.M. Schneeveis and R. Blanco, unpublished observations, 2000). This difficulty with puffs is probably in part an access problem, since mGluR6 receptors are localized to the tips of ON bipolar dendritic processes that reside deep within the invaginating photoreceptor synapse. Furthermore, in mouse, light-evoked rod bipolar responses represent a summed input from ~20 rods (Tsukamoto et al., 2001), many below the surface of the slice, whereas exogenous puffs will be most effective close to the surface where the fragile dendritic terminals are most likely to be damaged. In contrast, GABA_A and GABA_C receptors are known to be more widely distributed over the cell surface of bipolar cells (Haverkamp & Wassle, 2000), and robust responses to GABA puffs were reliably obtained (data not shown).

In summary, we conclude that there does not appear to be a postsynaptic deficit in glutamatergic signaling in the bipolar cells of mice lacking trkB.

Normal responses to glutamate of postsynaptic ionotropic receptors

To look further into whether the ionotropic glutamate receptors that are present on mouse cone bipolar cells respond normally to glutamate in the trkB mutant retina, we examined the accumulation of AGB, a guanidinium analogue, which permeates open ionotropic glutamate-gated channels associated with α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate, and N-methyl-d-aspartate (NMDA) receptors (Marc, 1999b). AGB is endogenously expressed at low levels in the mammalian brain (Li et al., 1994, 1995), where it is thought to act as a neurmodulator, but when applied exogenously in the presence of AGB-selective antiserum it is a useful tool for demonstrating the glutamatergic drive in the retina (Marc, 1999b).

Similar to previous observations on rabbit retina (Marc, 1999b), no endogenous AGB was detected in the mouse retina (B. Rohrer, unpublished observations, 2000). Acute (3 min) incubation of tissue in the presence of AGB alone (25 mmol/l) did not result in significant neuronal labeling in either the wt or the trkB−/− retinal tissue (Figs. 4A & 4C). However, following a 3-minute incubation in the presence of 100 μmol/l KA, a relatively specific agonist for AMPA and kainate receptors, a pattern of AGB labeling was observed in horizontal, bipolar, amacrine and ganglion cells that was indistinguishable between wt and trkB−/− retinas (Figs. 4B & 4D). The strength of the signal varied both between and within cell classes due to differences in receptor type and expression pattern (Marc & Jones, 2002). Kainate-induced labeling of Müller cells in wt and trkB−/− retinas is likely due to activation of glial stretch receptors (Marc, 1999b) following kainate-activated retinal swelling in vitro. Müller cells in rabbits and fish never show significant kainate-activated signals (Marc, 1999a,b; Marc & Jones, 2002), but they are structurally more robust in vitro than the fragile mouse retina.

In mouse, as in other mammals, cell bodies of the ON and OFF subtypes of bipolar cells are segregated to the distal and middle portions of the INL, respectively (Haverkamp & Wassle, 2000). AGB labeling did appear to be specific to OFF bipolar cells as might be expected since ON bipolar cells receive their primary input via metabotropic glutamate receptors. There have been reports that mammalian ON bipolar cells too, can possess iGluR subunits (Hughes, 1997; Vardi et al., 1998); however, see the report by Haverkamp et al. (2000) (Haverkamp et al., 2000). Regardless, the similar AGB labeling patterns in the trkB−/− and wt retinas, especially in the distal INL, are not consistent with a profound difference in ionotropic receptor-mediated glutamatergic signaling in cells postsynaptic to photoreceptors (i.e. bipolar and horizontal cells) in the trkB−/− mouse retina.

Morphological analysis of the rod-bipolar synapse

As the electrophysiological studies suggest that glutamatergic signaling is not impaired in ON or OFF bipolar cells, we also investigated the morphology of the rod-bipolar synapse in trkB knockout mice. The ribbon synapses that are formed between rods, rod bipolar cells and horizontal cells were compared in P16 wt and trkB−/− retinas (Fig. 5). Rod ribbon synapses are characterized by an electron-dense ribbon in the cytoplasm of the presynaptic process (arrows in Figs. 5B & 5F), and invaginating contacts made by a central rod bipolar cell dendrite (asterisks in Figs. 5B & 5F) and two lateral horizontal cell dendrites (arrowheads in Figs. 5B & 5F). Representative results presented in Fig. 5 depict examples of rod ribbon synapses found in the outer plexiform layer of P16 wt and trkB−/− retinas. We found no obvious structural deficits either presynaptically or postsynaptically in the trkB−/− ribbon synapse. In both control (wt) and trkB−/− synapses, there were (1) presynaptic ribbons, without any obvious reduction in number and size (see morphometry of ribbon lengths below); (2) synaptic vesicles along the ribbon as well as throughout the rod spherele, making up the reserve pool; (3) postsynaptic densities; and (4) the triads. The synapses between cones and cone bipolar cells also showed no defect in the trkB−/− mouse retina as judged by the same criteria (data not shown). The mean length for the wt and trkB−/− ribbons were similar and there was no statistical difference in average length (P > 0.11). Mean ribbon length was 0.46 μm ± 0.21 (n = 47) for the trkB−/− ribbons and 0.39 μm ± 0.16 (n = 40) for the wt ribbons. The standard deviations were relatively large due to the fact that synaptic ribbons are crescent-shaped structures and the measured “length” is a function of the position at which the section is taken through the crescent. The number of docked vesicles was not counted in these ribbons, because we reasoned that if the observed complete absence of synaptic signaling were due to an anatomical defect detectable at the EM level, it had to be a drastic, obvious effect such as the absence of docked vesicles.

Excitatory amino acid transporters in the OPL

Normal synaptic signaling depends critically on the efficient clearance of glutamate from the synaptic cleft. In the OPL, this may be accomplished by (1) glutamate transporters; (2) reuptake into vesicles; (3) presynaptic uptake into vesicles; (4) the triads. The transporters play a key role in maintaining the appropriate concentration of glutamate in the synaptic cleft, which is critical for the proper functioning of the photoreceptor-to-bipolar cell synapse. In the ON bipolar cells, the glutamate transporters that are expressed are GLT-1 (EAA T2; expressed in ON bipolar cells) and GLAST (EAA T1; expressed in OFF bipolar cells). The expression of GLT-1 and GLAST was examined in wt and trkB−/− retinas. We found no obvious structural deficits either presynaptically or postsynaptically in the trkB−/− ribbon synapse. In both control (wt) and trkB−/− synapses, there were (1) presynaptic ribbons, without any obvious reduction in number and size (see morphometry of ribbon lengths below); (2) synaptic vesicles along the ribbon as well as throughout the rod spherele, making up the reserve pool; (3) postsynaptic densities; and (4) the triads. The synapses between cones and cone bipolar cells also showed no defect in the trkB−/− mouse retina as judged by the same criteria (data not shown). The mean length for the wt and trkB−/− ribbons were similar and there was no statistical difference in average length (P > 0.11). Mean ribbon length was 0.46 μm ± 0.21 (n = 47) for the trkB−/− ribbons and 0.39 μm ± 0.16 (n = 40) for the wt ribbons. The standard deviations were relatively large due to the fact that synaptic ribbons are crescent-shaped structures and the measured “length” is a function of the position at which the section is taken through the crescent. The number of docked vesicles was not counted in these ribbons, because we reasoned that if the observed complete absence of synaptic signaling were due to an anatomical defect detectable at the EM level, it had to be a drastic, obvious effect such as the absence of docked vesicles.

Discussion

The experiments described above were motivated by our previous dark-adapted ERG work, demonstrating that trkB−/− mice possess...
Fig. 4. AGB mapping in isolated wild type (wt; A, B) and trkB<sup>+/−</sup> (C & D) mouse retinas. Control retinas were probed with 25 mmol/l AGB (1-amino-4-guanidobutane) alone, while activated retinas were also exposed to 100 μmol/l kainate (KA). Background signals characteristic of the basal permeation of AGB through cation channels is evident in both wt (A) and trkB<sup>+/−</sup> retinas (C). Passive diffusion of AGB into cut vessels (V in Panel A) provides an internal calibration for presence of 25 mmol/l AGB. KA activation triggered massive AGB permeation into an array of neuron types known to express AMPA and/or KA receptors (B & D): wt and trkB<sup>+/−</sup> retinas are indistinguishable. Neurons varied in their strength of responses to KA, as described by Marc (Marc, 1999b) for rabbit retina. Clusters of amacrine cells (ACs, boxed in Panels B & D) range from high to moderate responsiveness in both wt and trkB<sup>+/−</sup> retinas. Similarly, strong (GC hi, black arrows, in Panels B & D) and weak (GC lo, white arrows, in Panels B & D) ganglion cell responses coexist in wt and trkB<sup>+/−</sup>, as they do in the rabbit. Bipolar cell signals are more dramatically differentiated, being either clearly responsive (BC+, black arrows, probably OFF-center cells, in Panels B & D) or unresponsive (BC−, white arrows, probably ON-center cells, in Panels B & D). elm: external limiting membrane; gcl: ganglion cell layer; inl: inner nuclear layer; ipl: inner plexiform layer; onl: outer nuclear layer; and opl: outer plexiform layer. Scale for all panels (shown in Panel D) = 20 μm.
comparatively normal rod-mediated \( a \)-waves, but no \( b \)-waves, up to at least day P16, the approximate life expectancy of these mice (Rohrer et al., 1999). Since convincing data from several mammalian species now supports the hypothesis that the dark-adapted \( b \)-wave is generated by rod bipolar cells (Robson & Frishman, 1995, Green & Kapoustu-Bruneau, 1999; Karwoski & Xu, 1999; Lei & Perlman, 1999), our observations indicate that a functional defect exists in the rod bipolar cell signaling pathway at some point proximal to the locus of phototransduction in the rods. The present study pursues this point further. Based on our finding that rod bipolar cells express trkB receptors, we carefully examined the integrity of the signaling pathway postsynaptic to the photoreceptor-bipolar cell synapse. Using multiple techniques to probe the glutamatergic drive, we demonstrated that postsynaptic bipolar cells can respond to exogenously applied glutamate. Additional experiments suggest that the nature of the \( trkB^{+/−} \) defect is unrelated to obvious structural abnormalities of the rod ribbon synapse, and not due to a drastic downregulation of glutamate transporter expression around the photoreceptor synaptic terminal. We cannot, however, rule out the possibility that a combination of subtle effects involving the elements we investigated account for the \( trkB^{+/−} \) phenotype.

**Integrity of postsynaptic signaling pathways**

Studies using transgenic or knockout mice have demonstrated that communication between rods and rod bipolar cells can be compromised by defects in any of several aspects of synaptic communication. Thus, defective glutamate release from rods (Ball et al., 2002), an absence of glutamate receptors on ON bipolars (Dhingra et al., 2002; Masu et al., 1995), and reduced glutamate re-uptake (Harada et al., 1998; Barnett & Pow, 2000) can all cause substantially reduced or altered \( b \)-wave. In this report, we focus on the postsynaptic and inner retina components. Our data indicate that deficits in postsynaptic neurons cannot explain the deficits observed in the \( trkB^{+/−} \) mice. Of particular importance is our observation that ON bipolar cells responded to glutamate with outward currents of similar amplitude in both control and \( trkB^{+/−} \) animals.

**Fig. 5.** Representative examples of ribbon synapses found in the outer plexiform layer in wt (A–C) and \( trkB^{+/−} \) retinas (D–F). No obvious structural differences were found in the two genotypes based on four criteria: (1) normal number and size of presynaptic ribbons (arrows); (2) the presence of synaptic vesicles along the ribbon and throughout the rod spherule; (3) the presence of postsynaptic densities; and (4) the presence of triads (asterisks, rod bipolar cell dendrites; arrowheads, horizontal cell dendrites). Please note that rod pedicles in C and E were chosen to highlight the synaptic vesicles along the ribbons, whereas B and F were picked to illustrate the triad. Scale bar in Panel D for the overview images = 1 \( \mu \)m. Scale bar in Panel F for the details = 0.5 \( \mu \)m.
Intact ON-pathway in trkB<sup>−/−</sup> mice

**Fig. 6.** Analysis of excitatory amino acid transporters at the protein (A–D) and mRNA level (E). Immunohistochemical profiles for excitatory amino acid transporters GLAST (A & B) and GLT-1 (C & D) in wt (left column) and trkB<sup>−/−</sup> retinas (right column). No difference in staining was observed with the GLAST antibody, whereas GLT-1 reactivity was reduced in the inner retina of the trkB<sup>−/−</sup> retina. Quantitative real-time PCR analysis confirmed the immunohistochemical observations (n = 5) (E). ELM: external limiting membrane; RGC: ganglion cell layer; INL: inner nuclear layer; IPL: inner plexiform layer; ONL: outer nuclear layer; and OPL: outer plexiform layer. Scale bar for all panels (shown in Panel D) = 20 μm.

In addition, ON bipolar cells appear to be present in normal numbers, and the mGluR6 receptors are localized to their dendritic terminals (Rohrer et al., 1999).

Our results also indicate that, in the trkB<sup>−/−</sup> mice, OFF bipolar cells, which are known to express ionotropic AMPA receptor subunits (Haverkamp et al., 2000; Haverkamp & Wassle, 2000), are able to respond normally to glutamate with inward currents of normal amplitude and kinetics. In addition, horizontal cells and OFF-cone bipolar cells were shown to accumulate AGB following glutamate application, indicating that the ionotropic glutamate receptors known to be present in these neurons were activated normally (Haverkamp et al., 2000; Haverkamp & Wassle, 2000). In summary, our results indicate that glutamate activates receptors normally in each of the cells in direct synaptic contact with photoreceptors. It is possible, however, that the glutamate receptors being activated by AGB, or by glutamate agonists in puff application experiments, are largely extrasynaptic. In that case, there would remain the possibility that the trkB<sup>−/−</sup> mouse has a defect in the postsynaptic localization of glutamate receptors. To answer this question would require immunogold localization of the glutamate receptor, which is beyond the scope of this study.

**Anatomically intact ribbon synapses**

The ERG deficit observed in the trkB<sup>−/−</sup> mouse is similar to, but more global than, that observed in the mGluR6 knockout mouse (Rohrer et al., 1999). While ERGs of both knockout mouse genotypes are characterized by a lack of a recordable b-wave, mGluR6<sup>−/−</sup> mice showed immediate early gene expression in some cells in the inner retina in response to stroboscopic illumination [presumably activated through the OFF pathway (Yoshida et al., 1998)], whereas no such response was observed in the trkB<sup>−/−</sup> mice (Rohrer et al., 1999). These results need to be seen in the context of the data presented here, showing functional metabotropic and ionotropic glutamate receptors in the trkB<sup>−/−</sup> retina. The most parsimonious explanation would be that no functional synapses were formed in the outer plexiform layer to allow for synaptic transmission in these mice. Yet, our results show that the ribbon synapses between photoreceptor and bipolar cells form at the appropriate age and appear morphologically normal in EM. Direct testing of presynaptic photoreceptor function will be necessary to determine whether exocytosis or only light-mediated regulation of exocytosis is impaired. We are initiating experiments to examine regulated endocytosis and exocytosis, using FM1–43 in an effort to distinguish between these possibilities (Betz & Bewick, 1992; Kay et al., 1999).

**A role for defects in glutamate transporters**

As discussed above, synaptic transmission can also be compromised by defects in mechanisms responsible for the removal of neurotransmitter from the synaptic cleft (Harada et al., 1998). Mice deficient in GLAST, the major glial glutamate transporter in the retina, have a significantly reduced and slowed b-wave (Harada et al., 1998; Barnett & Pow, 2000), whereas elimination of GLT-1 only affected the onset kinetics of the b-wave, but not its amplitude (Harada et al., 1998). As the b-wave is triggered by the cessation of glutamate release from the photoreceptors in response to light, the slower kinetics of the b-wave indicate that regulation of glutamate removal from the synaptic cleft is defective, but not abolished in each of these mutants. As the dark-adapted trkB<sup>−/−</sup> ERG resembles an isolated PIII wave, without any indication of a b-wave (a much stronger phenotype than observed in the absence of either GLAST or GLT-1), it is very unlikely that a defect in glutamate transporters alone can be responsible for the lack of signaling in trkB<sup>−/−</sup> animals.

**Possible mechanisms of neurotrophin action**

Neurotrophin-mediated signaling has previously been shown to regulate both synapse development and function (e.g. Gonzalez et al., 1999; Huang et al., 1999; Alsina et al., 2001; Rico et al., 2002), exerting its effect through presynaptic or postsynaptic
signaling, depending upon the synapse (Kovalchuk et al., 2002; Zhang & Poo, 2002). In each of these examples, the effect on synapse development, maturation, or maintenance is presumably direct, because trkB is strongly expressed in at least one of the two cells that synapse with each other. In the case of the mouse ribbon synapse described here, the presynaptic cells (photoreceptors) express no or undetectable levels of trkB ([Rohrer et al., 1999] and Fig. 1), whereas the postsynaptic cells (rod bipolar cells) appear to express significant amounts of trkB receptors (Figs. 1 & 2); yet a bipolar cell defect could not be detected (Figs. 3–5).

At least two possible scenarios could account for the defect in synaptic communication observed in the trkb−/− mouse retina; first, a trkB-mediated retrograde signal derived from rod bipolar cells might control presynaptic signaling in the rod ribbon synapse; or second, the defect in synaptic signaling may instead reflect an indirect effect via remote-acting molecules, on the cells that form this synapse. In support of the first possibility, Micheva and colleagues (2003) have shown that the retrograde signaling molecule nitric oxide can regulate synaptic vesicle endocytosis and participate in synaptic vesicle recycling in hippocampal cells. In addition, nitric oxide (although maybe acting in a cell-autonomous way) has been shown to increase L-type Ca2+ channel-gating properties in salamander rod photoreceptors, providing a potential mechanism whereby the rod signal can be enhanced in postsynaptic neurons (Kurennyy et al., 1994). These two mechanisms have not yet been addressed in the mouse retina. Alternatively, in previous studies, neurotrophins have been shown to regulate photoreceptor degeneration presumably through control of growth factor secretion from Müller glial cells (Harada et al., 2000).

To identify the cells through which trkB signaling controls synaptic communication in the retina will require cell-specific trkB deletions. Recently developed techniques, such as “caged BDNF” and temporally regulated gene deletion should also make it possible to eventually determine whether trkB regulates a developmental or a functional process at the photoreceptor-rod synapse (Kossel et al., 2001).

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References


