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Retarded outer segment development in TrkB knockout mouse retina organ culture

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Purpose: To determine the effects of trkB deficiency in the mouse retina on photoreceptor development and retinal organization, in the absence of confounding systemic effects.
Methods: Newborn mice that carried two null trkB alleles ($trkB^{-/-}$) and their wild type (WT) littermates were used for retinal organ cultures. On Day 21, rod development was assessed histologically in plastic sections (outer segment length) and retinal organization was analyzed using retinal cell-type specific antibodies. Anatomical data obtained from the organ cultures were compared to previously published histological results from in vivo data.
Results: (1) Rod outer segment length was significantly shorter in retinas from $trkB^{-/-}$ mice in the presence of normal numbers of rods. (2) No dopaminergic amacrine cells were observed in the knockout retina. (3) Unlike in the in vivo condition, recoverin-positive OFF-cone bipolar cells were present in $trkB^{-/-}$ retinas grown in culture.
Conclusions: (1) These results demonstrate that rod outer segment development is compromised in the absence of trkB in the retina. (2) This study further supports our previous conclusion that the elimination of trkB expression alters rod development, because the presence of trkB receptors within the retina is essential for normal rod maturation and not because of confounding systemic effects. (3) More generally, this study stresses the importance of investigating complex phenotypes in gene knockout mice under conditions that isolate the organ under investigation from unrelated systemic variations.

Mouse photoreceptors are born starting at embryonic day 13, with the peak of production at postnatal day 1 (P1). By P10, the differentiating photoreceptors have migrated into the outer nuclear layer (ONL), after which they start their final process of maturation, the growth of the outer segment (OS), and the establishment of functional synapses. The outer plexiform is complete by about P14 [1]. Thus, when testing visual function in young mice using scotopic electroretinogram recordings (ERG), a-waves (reflecting rod responses) can be recorded as early as P10 [2], whereas b-waves (reflecting the photoreceptor-driven postsynaptic response of the bipolar cells) can be recorded by about P11 ([3]; unpublished observation). Several candidate factors that control rod photoreceptor differentiation have been identified, such as the transcription factors nrl [4] and crx [5,6], or the basic fibroblast growth factor FGF-2 [7].

In addition to their role in development, growth factors, neurotrophic factors, and cytokines have been implicated in maintaining photoreceptor health and function, as well as in preventing them from undergoing light-induced cell death. For example, adult mouse rods can be protected from light-induced cell death and stimulated to regenerate their OS by a single dose of brain-derived neurotrophic factor BDNF [8]. If this were a direct effect, it would imply that rods express trkB receptors, and cells in the retina or the retinal pigment epithelium (RPE) provide the neurotrophin BDNF. Interestingly, it has now been demonstrated that rods themselves do not express detectable levels of TrkB by either immunohistochemistry [9,10] or RT-PCR [11]. However, other cells such as a subset of cones [12], horizontal, some bipolar, amacrine and ganglion cells as well as Mueller glial cells in the retina and the RPE, do express TrkB [10]. BDNF has been shown to be present in retinal ganglion cells and secretory cells overlying the ciliary body, as well as cells of the superior colliculus [13,14],which can deliver BDNF to the retina via neurotrophin-receptor-dependent retrograde transport (for review see: [15]). Thus, candidate cells in the retina that could respond to BDNF, and thereby produce the indirect effect, include all the cell types that express trkB receptors, in particular the Mueller glial cells. Mueller glial cells have been shown to secrete factors important for photoreceptor survival in culture [16]. They are thought to participate in a push-pull mechanism that regulates photoreceptor survival and apoptosis in vivo [17] and they respond to exogenous BDNF with c-fos expression and ERK phosphorylation [10,18]. The identification of these molecules acting on the photoreceptors, and the cells involved, will be an important milestone for understanding the control of photoreceptor neurodegeneration and protection.

We have investigated the requirement of TrkB in the development of the retina by analyzing transgenic mice in which all isoforms of trkB have been eliminated [10]. Migration and

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differentiation of rods occurs, albeit slightly delayed, but their OS fails to develop properly. At any age up to P16 (when most trkB$^{-/-}$ animals die) the OS in the mutant mouse is shorter than that in age matched normal animals. When rod function was assessed in ERG recordings, it was determined that the P16 mutant rods elicited hyperpolarizing a-waves of amplitudes and kinetics that were comparable to those of P12 wildtype animals. Interestingly, the ERG also revealed that at any age studied, the knockout photoreceptor responses did not elicit a rod bipolar cell-driven depolarizing b-wave due to a presynaptic deficit [10]. By conducting a gene-dosage study in which trkB was varied from 0-100% in steps of 25%, we confirmed that the knockout of trkB expression altered rod development not because of alternative indirect mechanisms, but because this gene product is essential for normal rod maturation [19].

Rod photoreceptor structure and function are susceptible to systemic variations such as blood flow [20,21]. In order to determine whether differences can be seen in photoreceptor development at longer time points and in the absence of systemic effects caused by the lack of trkB, we have grown wild type and trkB deficient retinas in organ culture for 21 days postnatal. No difference in overall structure of the retina and photoreceptor survival could be detected between wild type and trkB$^{-/-}$ mice. However, the shortened OS was maintained under in vitro conditions, arguing again for a true role for trkB signaling in photoreceptor development.

METHODS

Organ culture: Animals were handled in accordance with institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Retinas were harvested and established in organ culture as previously described [22]. Briefly, neonatal mice were anesthetized on ice, their eyes were enucleated within the first 24 h after birth, incubated in Dulbecco's Modified Eagle's Media (DMEM, Gibco 11965, Rockville, MD) with 0.5% proteinase K (Boehringer Mannheim, Indianapolis, IN) for 7 min followed by a rinse and removal of the sclera, choroid and anterior segment. The retina was subsequently incubated in DMEM with 10% fetal calf serum (FCS, Summit Biotechnology, Ft. Collins, CO) and 1.25 µg/mL Fungizone (Sigma, St. Louis, MO) for 30 min at 37 °C, and then separated from the RPE. The isolated retina was placed photoreceptor side down on a Millicell-CM culture insert (Millipore, Bedford, MA). The media (DMEM, 10% FCS, and 1.25 µg/mL Fungizone) was maintained at the level of the membrane interface. Organ cultures were grown at 37 °C, 5% CO_2 for up to 29 days in complete darkness. We found it unnecessary to provide cyclic light, as we have determined previously that light-conditions do not influence photoreceptor development or outer segment length in culture (Mosinger-Ogilvie J., et al. Survival of photoreceptors in organ cultures of *rd* mouse retina. Invest. Ophthalmol. Vis. Sci. 1996; 37(Suppl):S624).

Histology: For quantitative analysis, organ cultures were harvested at P29. Tissue was fixed in 2.5% glutaraldehyde and 2% paraformaldehyde overnight, postfixed in 1% osmium tetroxide followed by 1% uranyl acetate, rinsed, dehydrated and embedded in Epon-Araldite. One micron sections were stained with toluidine blue.

For immunohistochemical analysis, organ cultures (harvested at postnatal day 21) were fixed in 4% paraformaldehyde, rinsed, cryoprotected in 30% sucrose overnight, frozen in OCT (Sakura, Torrance, CA) and cut into 8-10 µm cryostat sections. Immunohistochemistry was performed as published previously [10], using the following primary antibodies: glial fibrillary acidic protein or GFAP (1:1000; Sigma), recoverin (1:5000; a generous gift by A. Dizhoor, University of Washington, Seattle, WA, [23]), tyrosine hydroxylase or TH (1:200; Pelfreeze, Rogers, AR), calbindin (1:2000; Swant, Bellinzona, Switzerland) and rhodopsin (1:10000; Robert Molday, University of British Columbia, Vancouver, BC, Canada, [24]). For visualization of single antigens, we used the peroxidase method, whereas for double labeling immunohistochemistry, fluorescently labeled secondary antibodies were used [10]. Each staining was performed on at least two slides, which contain 4-6 different sections throughout the entire organ culture (WT: n=3; trkB$^{-/-}$: n=3).

Sections were photographed using a Nikon microscope equipped with a digital camera and Axioscope software. Double-labeled fluorescent images were examined by confocal microscopy (Olympus Fluoview) and images were false-colored and superimposed using the Fluoview software.

Data Analysis: Quantitative analysis was performed by a trained observer, blind to the experimental conditions, using a grid reticule at 40x magnification as previously described [22]. Briefly, the thickness of the outer nuclear layer (ONL) was determined by averaging the number of ONL cells in a vertical column touching a single grid line on the reticule for 5 columns in each of 2 randomly selected regions. The total number of ONL nuclei within the 2 regions was also counted to determine the density of cells/10 µm length of ONL. Statistical significance for thickness and density was determined using a Student t-test. In all cases, density corresponded to the thickness of the ONL. Inner and outer segment development was ranked on a scale of 0-3, where 3 represented healthy inner segments (IS) with numerous, elongated outer segments (OS); 2 represented IS and some, shorter OS; 1 represented shortened IS with no OS; and 0 represented very few or no IS (Figure 1). Statistical significance was determined using a Mann-Whitney test.

RESULTS

TrkB$^{-/-}$ mice have a maximum lifespan of approximately 18 days postnatal. The gross histological appearance of the in vivo retina in these animals is similar to that of wild type littermates; however, development appears to be delayed such that outer segments appear significantly shortened at P16 in trkB mutants compared to wild type littermates [10]. Although recent experiments have suggested that this delay is unrelated to the overall poor health of the mutant animals [19], we wished to determine whether differences could be seen in photoreceptor development at longer time-intervals without systemic effects, by growing trkB$^{-/-}$ retinas in organ culture for 21 days postnatal.

Retinas in organ culture maintained their structural integrity, despite a thinning of all nuclear and plexiform layers (Figure 2), when compared to retinas developed in the intact animal [22]. Wild type retinas in organ culture maintained an ONL thickness of 5.7±1.7 cells and a density of 13.6±4.8 cells/10 μm length of ONL (n=8). No significant difference was seen in the ONL of the trkB deficient retinas with a thickness of 5.5±1.8 cells and a density of 11.5±5.2 cells/10 μm length of ONL (n=7). Evaluation of IS/OS development, however, did show a significant deficit in trkB deficient retinal organ cultures. IS/OS development was ranked on a scale of 0-3, where 3 represents the best morphology with healthy IS and elongated OS, and 0 represents a retina that has very few or no IS (see Methods for more details and Figure 1). According to this ranking scale, IS/OS development of trkB$^{-/-}$ retina organ cultures scored at 1.6±0.4 (mean±SD) compared to wild type (2.5±0.2; p=0.046). This observation suggests that the shortened OS seen in vivo at P16 is not due to systemic effects in vivo, but rather is a true indicator of a role for trkB signaling in photoreceptor development.

Retinas collected from both wild type and trkB$^{-/-}$ animals were compared using antibodies specific for different identified retinal cell types to further analyze the overall organization of the retina in organ culture and secondly to examine whether changes originally described in vivo [10] could be confirmed in vitro (Figure 3). GFAP, a marker for reactive Mueller cells, was found to stain both wild type and trkB$^{-/-}$ retinas when grown in organ culture (Figure 3A), consistent with previous observations of reactive Mueller glial cells in organ culture [25]. The calbindin antibody, a marker for horizontal cells (located in the outer half of the INL), amacrine (located in the inner half of the INL), and displaced amacrine cells (located in the RGC layer) in the mouse retina [26], produced similar labeling in the knockout and wild type organ cultures. In the ONL, the calbindin-labeling revealed horizontal cell dendritic sprouting in both genotypes, which appeared more pronounced in the culture from the trkB$^{-/-}$ animals (Figure 3B, arrows). Dopaminergic amacrine cells, which express trkB receptors and depend on BDNF stimulation for maintenance of their phenotype ([27]; unpublished observation), were identified, using an antibody against tyrosine hydroxylase (TH). TH staining identified cells in the inner-half of the inner nuclear layer of the wild type retina, but staining was completely eliminated in trkB$^{-/-}$ retina organ cultures (Figure 3C). Finally, we had reported that one class of bipolar cells, the recoverin-positive cone OFF-bipolar cells failed to express recoverin in the trkB$^{-/-}$ mouse in vivo [10]. The anti-recoverin antibody, which labels rod photoreceptors and the cell bodies and dendrites of cone OFF-bipolar cells [10], revealed strong rod photoreceptor staining in both wild type and knockout organ cultures. In addition, a few immunopositive cell bodies were labeled in the INL, but not their respective dendrites (Figure 3D). In order to examine whether these cell bodies are misplaced rod photoreceptors or cone OFF-bipolar cells, we used double-labeling immunohistochemistry with antibodies against recoverin (which labels both rod and cone OFF-bipolar cells) and rhodopsin (which only labels rods). The double-labeling experiment, however, revealed that in all cases these recoverin-positive cell bodies in the INL belong to recoverin-positive bipolar cells and thus are not misplaced rod nuclei, as they failed to co-label with rhodopsin (Figure 3D).

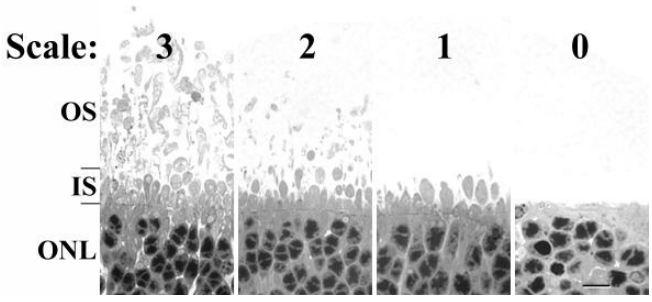

Figure 1. Inner and outer segment development ranking scale. Photoreceptor inner and outer segments were ranked on a scale of 0-3. Healthy inner segments (IS) with numerous, elongated outer segments (OS) were given a rank of 3. Healthy IS with some, shorter OS were given a rank of 2. Shortened IS with no OS were given a rank of 1. Photoreceptor with very few or no IS were given a rank of 0. Scale bar represents 10 μm. The outer nuclear layer (ONL) is labeled.

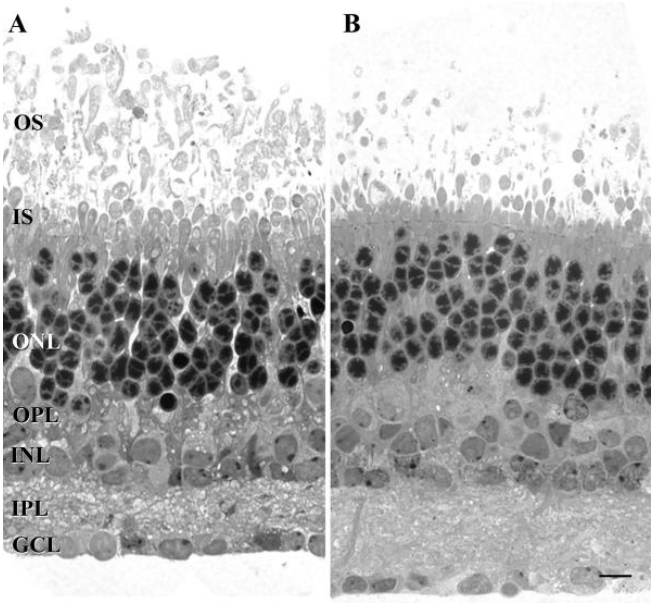

Figure 2. Representative plastic sections of retina organ cultures. Outer segment development was perturbed in the trkB$^{-/-}$ mice, as demonstrated by the significantly shorter outer segment lengths (**B**) when compared to those of their age matched littermates (**A**). Scale bar represents 10 μm. The ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), inner segments (IS), and outer segments (OS) are labeled.

DISCUSSION

The goal of this study was to address whether retinal changes reported for trkB$^{-/-}$ mice in vivo could also be seen under culture conditions and thus under conditions in which potential systemic effects were eliminated, and consequently to strengthen our understanding of trkB in retinal and/or photoreceptor development. We report here that, first rod outer segment development from trkB$^{-/-}$ mouse retinas was found to be perturbed under culture conditions, further supporting our previous suggestion that trkB activity plays an important role in the developmental maturation of mouse rod photoreceptors. Secondly, we confirmed previous in vivo results that trkB expression is necessary for the maintenance of the TH-positive phenotype of a subset of amacrine cells; and thirdly, we found evidence that unlike the intact trkB$^{-/-}$ retina, recoverin-positive OFF-cone bipolar cells are being generated in the trkB$^{-/-}$ retina culture.

Since the beginning of the development of transgenic animals, it has become clear that the phenotype in a particular organ may not be due to the loss of the gene that was knocked-out, but rather due to deficits in other organs or brain regions [28]. And thus, the changes seen in the retina of the trkB$^{-/-}$ mice may not be caused by a direct effect on retinal cells, but may be due to alterations in the circulatory system or poor nutrition, which are known to affect retinal function [20,29], or changes in retrograde signaling [30,31]. To eliminate the presence of confounding variables, and to isolate the direct effects of the gene knockout, retinas obtained from 1-day-old littermates (trkB$^{-/-}$ or wild type) were cultured under identical conditions.

As reported previously [10], the developmental sequence leading to the layered organization of the retina does not appear to be perturbed in the trkB$^{-/-}$ mouse retina. Retina organ cultures analyzed at P21 demonstrated that the number of rows of photoreceptors and photoreceptor packing density is similar in wild type and trkB$^{-/-}$ retinas. While rod outer segment lengths were reduced by about 2.5-fold in the knockout mouse in vivo at P16, they were short or absent by P21 in the organ culture. These results support our original hypothesis that rod outer segment development requires trkB. Furthermore, they suggest the RPE is an unlikely candidate for the trkB-positive cell(s) that control photoreceptor development and maturation, as our cultures are grown after the removal of the retinal pigmented epithelium. However, residual RPE cells divide and

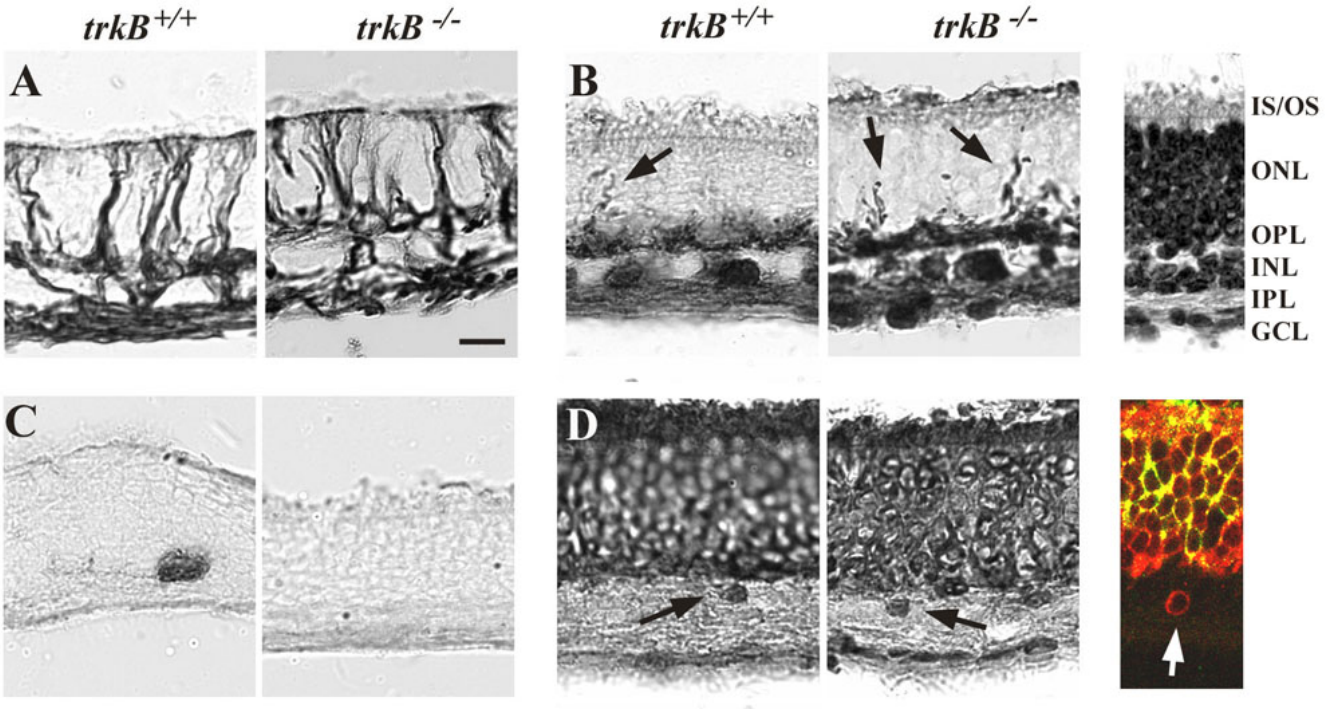

Figure 3. Analysis of retinal organization in retina organ cultures. Retinal layers are indicated on the Toluidine blue stained section in the upper right panel. Retinas grown in organ culture expressed an elevated level of glial fibrillary acid protein in the radial Mueller glial cells (**A**), irrespective of the genotype of the animal. Calbindin-antibodies (**B**) identified amacrine (inner half of the INL), displaced amacrine (RGC layer) and horizontal cells (outer half of the INL), and revealed sprouting of horizontal cell dendrites into the outer nuclear layer (arrows). Dopaminergic amacrine cells were identified by tyrosine hydroxylase immunohistochemistry in the wild type retina, but were absent from the knockout retina (**C**). Recoverin-antibodies labeled both photoreceptors and some cell bodies in the INL (arrows) in trkB$^{+/+}$ and trkB$^{-/-}$ retinas with equal intensity (**D**). To distinguish whether the cell bodies in the INL belong to cone OFF-bipolar cells or to migrating rod photoreceptors, double labeling immunohistochemistry with antibodies against rhodopsin (FITC; rod specific) and recoverin (Texas Red; recoverin is expressed in both rods and cone OFF-bipolar cells) confirmed that these cell bodies were indeed bipolar cells (color inset of **D**). Scale bar represents 20 µm. The ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), inner segments (IS), and outer segments (OS) are labeled.

begin to regrow by 21 DIV, signifying that RPE cannot be completely eliminated from consideration. Our results leave open the possibility that dopaminergic interplexiform cells, which provide paracrine feedback to the photoreceptor cells [32-34], could contribute to the development of the rod phenotype. While one of us has shown that dopamine depletion blocks photoreceptor degeneration in the *rd* mouse [35], making dopamine not a likely candidate to contribute to outer segment elongation, it has been reported that dopamine stimulation is required for normal disc shedding [36], Na^+, K^+-ATPase activity [37], modulation of photoreceptor calcium currents [38], or regulation of photoreceptor cAMP metabolism [39], all of which when misregulated could contribute to outer segment loss. Finally, the results from the recoverin-positive OFF-cone bipolar cells support the warning summarized by Lathe [28] and voiced by others, that an observed phenotype (i.e., the lack of recoverin staining in bipolar cells of the P16 $trkB^{-/-}$ retina) may not be due to the genotype (i.e., $trkB^{-/-}$), but due to confounding circumstances (i.e., deficits in other organs or brain regions). Alternatively, the presence of serum in the organ culture medium could stimulate alternate pathways leading to recoverin expression in bipolar cells.

In summary, the present study demonstrated trkB-dependent deficits in rod outer segment development in retina organ cultures, and localized the trkB-positive cell within the retina. These experiments lend further support to the notion that complex phenotypes, which affect multiple target organs, can be addressed in gene knockout mice, if precautions are taken to eliminate confounding variables. Future experiments, using cell-specific Cre-recombinase (e.g., [40]) together with floxed trkB mice [19,41], will allow further characterization of the retinal circuits that control photoreceptor development and function.

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