Gene dosage effect of the TrkB receptor on rod physiology and biochemistry in juvenile mouse retina

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Purpose: To strengthen our understanding about the role of trkB in rod development by correlating functional and biochemical retinal phenotypes with levels of trkB expression in two independently created trkB transgenic lines.

Methods: Juvenile mice that carried two hypomorphic trkB alleles (trkB<sup>hi<sup>hi</sup></sup>) expressing roughly 25% of normal trkB, and their heterozygous (trkB<sup>++<sup>/-</sup></sup>) and wild type (WT) littermates were tested with electroretinographic (ERG) protocols that isolate rod driven responses. Rod development was assessed histologically (outer segment length) and spectrophoto-metrically (rhodopsin content). Functional and biochemical data were compared to those obtained from mice that have trkB removed completely (trkB<sup>-/-</sup>).

Results: (1) Retinal rod function and morphology was unaffected by a loss of up to 50% of trkB. (2) However, reducing trkB below a critical threshold (<50%) significantly reduced rhodopsin content and outer segment length, resulting in reduced a- and b-wave amplitudes and slower kinetics. (3) A second threshold was determined for rod to bipolar cell signaling, which requires the presence of at least 25% of wild type trkB levels.

Conclusions: (1) These results demonstrated that rod biochemistry, physiology and synaptic signaling are compromised in a gene dosage dependent manner when the expression of trkB is reduced in transgenic mice. (2) This study confirmed our previous conclusion that the knockout of trkB expression altered rod development, because this gene product is essential for normal rod maturation and not because of alternative indirect mechanisms. (3) More generally, this study showed that the specificity of complex phenotypes can be investigated in gene knockout mice, if a gene dosage study is performed.

Neurotrophins and their receptors play defined roles in neuronal development and function [1-3]. In the vertebrate retina three neurotrophins are expressed: brain derived neurotrophic factor (BDNF, [4,5]), neurotrophin-3 (NT-3, [4]), and nerve growth factor (NGF, [6]), together with the neurotrophin receptors trkB and trkA (which are tyrosine kinase containing receptors for BDNF and NGF, respectively) and p75 (the low affinity neurotrophin receptor that binds all neurotrophins) [7].

Although the rod photoreceptors themselves do not express trkB, expression of this gene in yet unidentified retinal cells appears to be crucial for normal photoreceptor development. In mice where trkB is knocked out (trkB<sup>-/-</sup>), development of rod photoreceptor structure and phototransduction function is slowed down relative to wild type (WT) animals, and photoreceptor synaptic function fails to develop [8]. Photoreceptor maturation (as determined by rhodopsin content), outer segment elongation and phototransduction function are delayed by 4 days relative to wild type mice. Therefore, postnatal day 16 (P16) trkB<sup>-/-</sup> photoreceptors correspond to P12 WT photoreceptors. P16 trkB<sup>-/-</sup> photoreceptors fail to activate rod-bipolar cells, although the bipolar cells in the same retina do respond to applied, exogenous glutamate [9].

Conclusions from our experiments in knockout mice, however, can be challenged because the complex phenotype we observed can arise from nonspecific or indirect effects of gene suppression. For example, the lack of synaptic development may not be absolute, but simply a reflection of a generally slowed down rod development. Knockout mice have limited survival (maximum P18); because of this, it is possible that synaptic development might eventually occur. Also, since trkB is not ubiquitous to the retina (but is widely distributed throughout both the peripheral and central nervous system) systemic effects not limited to the retina could have indirectly caused the effects seen in the eye.

The problem of the potential lack of specificity when using knockout animals in studies of postnatal development, has been widely discussed elsewhere [10-14]. Apart from the issue of indirect effects due to deficits in organs not directly targeted, Gerlai [13] noted the possible effects of hitchhiking genes. These are genes surrounding the targeted gene in the embryonic stem cell strain (typically strain 129), which might adversely affect the animal through processes unrelated to those affected by the gene knockout. Two recommendations have been made to experimentally address these potentially confounding problems. First, is to always use as controls the littermates of the affected animals, since all will share a similar genetic make up. And second, is to characterize more than one independently created animal line. This varies the potential hitchhiking genes and, in addition, it should allow the study

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and xylazine (25 mg/kg) under dim red light, their pupils dilated with 1% atropine and 2.5% phenylephrine and placed on a heating pad held at 37 °C in a light tight Faraday cage. ERG recordings were performed on 7 wild type, 4 trkB<sup>+</sup> and 7 trkB<sup>+</sup> mice. In previous experiments [8], such group sizes were found appropriate to generate statistically reliable results.

**Methods**

**Animals:** TrkB hypomorphic animals (trkB<sup>C</sup>/<sup>C</sup>) and knock-out mice (trkB<sup>C</sup>*) together with their respective wild type (WT) and heterozygous littermates (trkB<sup>+</sup>/<sup>C</sup> and trkB<sup>C</sup>/<sup>B</sup>, respectively) were compared in this study [8,15]. TrkB hypomorphic animals were created by inserting rat trkB cDNA followed by an SV40 polyA sequence into the first coding exon (exon S, which contains the start site and the signal peptide for trkB and is shared by all trkB isoforms). Therefore, the homozygous trkB<sup>B</sup>/<sup>B</sup> animals do not express any truncated isoforms of trkB, and the expression of full length trkB is reduced to approximately 25%. The results of this study confirm and extend our previous conclusions; that is (1) signals from trkB-positive cells control the rate of development of structure and phototransduction functions of rods, and (2) in their absence, development of synaptic function fails to occur.

**Histology:** Animals were sacrificed by cervical dislocation, after which enucleated eyes were fixed by immersion in Carnoy’s fixative for 2 h [8]. Eyes were then dehydrated and transversely oriented in paraffin. Sections (7 µm) were cut, dried on poly-L-lysine coated glass slides, dewaxed and rehydrated for Toluidine Blue staining, coverslipped using DPX medium, and photographed using a Nikon microscope (Tokyo, Japan) with a photographic attachment.

**ERG analysis—Animals:** For electroretinogram (ERG) analysis, mice were anaesthetized with ketamine (10 mg/kg) and xylazine (25 mg/kg) under dim red light, their pupils dilated with 1% atropine and 2.5% phenylephrine and placed on a heating pad held at 37 °C in a light tight Faraday cage. ERG recordings were performed on 7 wild type, 4 trkB<sup>+</sup> and 7 trkB<sup>+</sup> mice. In previous experiments [8], such group sizes were found appropriate to generate statistically reliable results.

**ERG Instrument:** Our photostimulation provided full field homogeneous illumination using a device adapted from Lyubarsky and Pugh [16] and previously described in detail [8]. In short, a clear plastic rod, shaped at its end to match the shape of the cornea, was gently placed against the cornea and electrical contact was made through a drop of methylcellulose. The recording electrode (0.2 mm platinum wire) was glued to the side of the rod. The back end of the rod was placed against the end of a fiber optic light guide and thus illuminated with light of known duration, color and intensity. Tungsten needle electrodes were placed in the neck and tail and served as reference and ground electrodes, respectively. Responses were amplified 2000 fold, bandpass filtered between 0.1-1000 Hz (2 pole Butter-worth filter) and digitized with a 12 bit analog to digital converter at 2 Hz. Data was stored, displayed and analyzed with a PC interface and Acquire software.

**Light stimulus:** A single channel optical bench was used for light stimulation. The optical pathway consisted of a 150 W halogen lamp, manually operated neutral density filters, lenses to focus the light beam to the end of the light guide, a 500 nm bandpass filter and a mechanical shutter. Absolute light intensity (photons/mm²) delivered to the plane of the mouse’s pupil was calibrated with a photodiode placed in the...
position of the eye. ERGs were recorded in response to two protocols: (A) 10 ms flashes of increasing light intensities; and (B) constant photon flux steps of increasing duration. Time between flashes was set to allow recovery of the b-wave between flashes and two to four responses were averaged.

Data analysis: Data was analyzed with respect to several parameters: a- and b-wave amplitudes, slopes of the initial rise of the a- and b-wave, duration of the b-wave and amplitudes of oscillatory potentials. Peak a-wave amplitude was measured from baseline to the initial negative going voltage, whereas peak b-wave amplitude was measured from the trough of the a-wave to the peak of the positive b-wave. The initial slopes of a- and b-waves were measured as the slope of the tangent that best fit the initial rise of the normalized waveforms (expressed in $\mu V/\text{ms}$; [8]). The duration of the b-wave was determined at 1/2 band width, which is at the point in the decay of the b-wave when the amplitude is half maximal. In order to determine the amplitude of the oscillatory potentials (OP1-OP4), the ERG was low pass filtered at 16.5 Hz (red trace in Figure 1) and subtracted from the original ERG wave. Amplitudes were measured from the preceding trough to the peak of the oscillatory potential (Figure 1, inset). This method was originally developed for cone OPs [17], and thus tends to

Figure 2. Electroretinogram responses from wild type (WT) and hypomorphic (trkB fbz/fbz) mice. Electroretinogram responses were elicited by short light flashes of increasing light intensities from P20 WT and trkB fbz/fbz mice. In the P20 WT (A), the electroretinogram consists of the negative a-wave, which is clipped by the fast positive b-wave and the overlying oscillatory potentials. The trkB fbz/fbz (B) photoreceptor responses (a-wave) are somewhat smaller and slower than the WT control responses (C); but more importantly, they elicit a rod bipolar cell driven b-wave characterized by a slower onset time and a significantly reduced amplitude (D). Light intensities in A and B were $2.37 \times 10^9$, $9.42 \times 10^9$, $8.32 \times 10^{10}$ and $3.85 \times 10^{11}$ photons/mm$^2$ as measured at the level of the cornea. p values: *p<0.01; **p<0.005; # p<0.06; ## p<0.003. For additional statistical analyses refer to Table 1.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>a-wave Amplitude</th>
<th>a-wave Slope</th>
<th>b-wave Amplitude</th>
<th>b-wave Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100.0 ± 25.4</td>
<td>101.6 ± 13.5</td>
<td>100.0 ± 41.0</td>
<td>111.39 ± 39.6</td>
</tr>
<tr>
<td>trkB (+/fbz)</td>
<td>104.4 ± 9.6</td>
<td>(not done)</td>
<td>122.9 ± 17.7</td>
<td>111.39 ± 39.6</td>
</tr>
<tr>
<td>trkB (+/-)</td>
<td>105.6 ± 17.0</td>
<td>(not done)</td>
<td>93.3 ± 24.5</td>
<td>(not done)</td>
</tr>
<tr>
<td>trkB (fbz/fbz)</td>
<td>74.8 ± 12.2*</td>
<td>75.8 ± 25.3</td>
<td>53.8 ± 35.9**</td>
<td>38.2 ± 23.2**</td>
</tr>
<tr>
<td>trkB (-/-)</td>
<td>25.4 ± 12.1**</td>
<td>52.4 ± 22.0**</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

Data was combined from the two sets of animals, normalized to their respective wild type (WT) controls and expressed in [% WT levels]. The retina appears to be able to compensate for a loss of up to 50% of trkB (trkB+/fbz and trkB+/−) with respect to amplitudes, light sensitivity and kinetics; whereas any further loss (trkB fbz/fbz and trkB−/−) resulted in a dose dependent deterioration of sensitivity and kinetics. p values: *p<0.01; **p<0.003; #p<0.05; ##p<0.005.
overestimate the absolute amplitudes of OP1 and OP2 in scotopic ERGs due to the large rod a-wave. For statistical purposes, a standard t-test was employed, accepting a significance level of \( p<0.05 \).

Rhodopsin measurements in animals: Mice were killed by cervical dislocation under dim red light, the eye excised and the anterior portion removed. After the removal of the lens, whole retinas were pulled from the eyecup by simply turning the eyecup inside out. Individual retinas were stored in centrifuge tubes at \(-80^\circ\text{C}\) until the day of the assay. Five retinas were analyzed per genotype.

**RESULTS**

ERG waveform analysis of trkB fbz/fbz mice: Rods in the retina of P16 trkB -/- mice respond to light with less sensitivity and

Rhodopsin measurement assay: All procedures were done under dim red light. The samples were prepared according to Dodge and coworkers [18], with slight modifications. Retinas were allowed to thaw out and were triturated in 200 \( \mu \)L of distilled water, using 200 \( \mu \)L eppendorf pipette tips and gel loading tips for reduced diameter (20 motions each). The samples were then incubated in the dark at room temperature for 1 h on a rocking platform, and centrifuged at 14,000x g for 20 min at room temperature. The resulting pellet was carefully resuspended in 200 \( \mu \)L of 1% Emulphogene (Sigma) in 50 mmol/L Tris-acetate buffer, pH 6.9 and incubated for 2 h as above for solubilization of membrane bound rhodopsin. Unsolubilized material was then removed by centrifugation at 14,000x g for 30 min, after which aliquots of the supernatant were removed for analysis on the SpectroPette microspectrophotometer (World Precision Instruments). The microspectrophotometer was set to an integration time of 132 ms. Absorbance spectra were taken from 380 to 760 nm first in the dark and again after light exposure to calculate the difference spectra. Light exposure of 1 min was sufficient due to the small volume of the pipette (60 nL/mm lightpath length, or 720 nL in total, [19]). Each sample was measured three times pre- and post-bleach, using a fresh sample each time due to the small amount of bleaching caused by each scan [20]. Data were exported into Excel (Microsoft) for further analysis.
slower kinetics than rods in their WT littermates [8]. They also fail to signal to bipolar cells, as evidenced by the lack of b-waves in the ERG. Unfortunately, these mice die within a couple of days of the recording (~P18), thus it is possible that the lack of synaptic function is not absolute, and function would appear had animals survived longer. To investigate this possibility, a hypomorphic line (trkB<sup>fbz/fbz</sup>) in which homozygous mice express approximately 25% of their normal wild type trkB levels, was tested.

Electroretinograms (ERGs) were measured on juvenile trkB<sup>fbz/fbz</sup>, and their heterozygous (trkB<sup>fbz/+</sup>) and WT littermates. At lower light intensities (<9.42x10<sup>9</sup>photons/mm<sup>2</sup>) a-wave amplitudes and kinetics were indistinguishable between the three different genotypes. Whereas a-waves were significantly reduced in amplitudes in the trkB<sup>fbz/fbz</sup> mice compared to their WT littermates at maximum light intensities (p = 0.01), the differences in slopes of the a-waves (kinetics) did not reach statistical significance (p=0.06; Figure 2B,C; Table 1). Unlike the trkB<sup>-/-</sup> mice [8], the ERG in trkB<sup>bsh/bsh</sup> mice did exhibit a b-wave, however, b-waves were characterized by slower kinetics and significantly reduced amplitudes (Figure 2D; Table 1) when compared to those recorded in WT mice (Figure 2B) at most light intensities.

It was thought that the lowered amplitudes of the b-wave could possibly reflect a compromised blood supply to the inner retina [21]. This possibility was tested by analyzing the amplitudes of the oscillatory potentials (OPs), which are an indicator for retinal perfusion [22]. OPs (which reach mature levels in the rodent retina by P17 [23]) were measured at maximum light intensity in the dark adapted ERG (3.8511<sup>10</sup> photons/mm<sup>2</sup>). The first four OPs (Figure 1, inset) were measured for this analysis and were found not to differ significantly between the WT and the trkB<sup>bsh/bsh</sup> mice. OP1 and OP4 reach amplitudes of approximately 10-12 µV (OP1: 12.52±6.1 vs. 10.5±4.6 µV; p=0.5; OP4: 11.6±6.5 vs. 9.8±5.8 µV; p=0.6), whereas OP2 and OP3 are more prominent (OP2: 68.2±23.6 vs. 46.8±34.6 µV; p=0.2; OP3: 41.2±19.8 vs. 34.0±16.5 µV; p=0.5).

_Slower b-wave kinetics in trkB<sup>bsh/bsh</sup> mice:_ To reiterate, the trkB<sup>-/-</sup> retina is characterized by complete rod synaptic failure, whereas in the trkB<sup>bsh/bsh</sup> mice, synaptic function was not normal, but also was not entirely absent, resulting in a slow rate of rise of the b-wave. It is not possible, then, to reach an unequivocal conclusion based on the ERG data. However, it was previously speculated that the underlying cause of the complete lack of a b-wave in the trkB<sup>-/-</sup> mice might be a presynaptic defect in either or both, synaptic transmitter release from the photoreceptors, or clearing of glutamate from the synaptic cleft. These assumptions were based on the findings that both ON- and OFF-bipolar cells in slices from trkB<sup>-/-</sup> mice respond normally to glutamate puffs [9].

For this study, a model to interpret the new ERG data that is consistent with the previous findings was developed. In a mass measurement such as the ERG, one can expect the sum

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**Table 2. Rhodopsin concentration per eye**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Rhodopsin Concentration [mole per eye]</th>
<th>Percent of Genotype WT</th>
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<tbody>
<tr>
<td>P16 WT</td>
<td>0.1720 ± 0.0150</td>
<td>100 ± 8.72</td>
</tr>
<tr>
<td>P20 WT</td>
<td>0.0777 ± 0.0177</td>
<td>100 ± 18.12</td>
</tr>
<tr>
<td>trkB&lt;sup&gt;(+/-)&lt;/sup&gt;</td>
<td>0.0764 ± 0.0135</td>
<td>78.20 ± 13.82</td>
</tr>
<tr>
<td>trkB&lt;sup&gt;(fbz/fbz)&lt;/sup&gt;</td>
<td>0.0425 ± 0.0123</td>
<td>43.5 ± 12.59</td>
</tr>
<tr>
<td>trkB&lt;sup&gt;(-/-)&lt;/sup&gt;</td>
<td>0.0563 ± 0.0050</td>
<td>32.73 ± 1.91</td>
</tr>
</tbody>
</table>

Data were combined from trkB<sup>-/-</sup> [8] and trkB<sup>fbz/fbz</sup> mice (data reported here). It should be noted that the absolute amounts of rhodopsin (middle column) differ between the two sets of experiments due to the different methods and instruments used. Data was again normalized to their respective wild type (WT) controls to allow comparison of the different genotypes. Again, a 75% or greater loss of trkB (trkB<sup>fbz/fbz</sup> and trkB<sup>-/-</sup>) resulted in a dose dependent decrease in retinal rhodopsin, which was reflected in the electroretinogram data analysis. Abbreviations: P16: postnatal day 16; P20: postnatal day 20. p values: *p<0.04; **p<0.003.

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Figure 5. Difference spectra and outer segment development. In all three genotypes, the absorption maximum is at 504 nm (A). Heterozygous animals (trkB<sup>fbz/+</sup>) contain slightly, but insignificantly less rhodopsin in comparison to the wild type (WT) littermates, whereas rhodopsin levels are reduced by 56% in the homozygous (trkB<sup>fbz/fbz</sup>) mutants. Outer segment development is slowed down in the trkB<sup>fbz/fbz</sup> mice, as demonstrated by the significantly shorter outer segment lengths (C) when compared to those of their age matched littermates (B). Abbreviations: IS: inner segments, OS: outer segments. The scale bar represents 20 µm.
of synapses of different efficiencies to cause bipolar cells responses (the actual generators of the b-wave) to each have a variable delay and a variable duration, in contrast to their synchronous behavior in the WT retinas. The reduced synaptic activity and consequent asynchrony should cause b-waves of reduced amplitude and modified kinetics.

To test this hypothesis, ERG responses were recorded in WT and trkB<sup>flh</sup>/+ mice in response to steps of constant photon flux (2.37x10<sup>9</sup> photons/mm<sup>2</sup>) and varying duration (from 10 to 220 ms), as the slower b-wave kinetics were predicted to be more pronounced in response to longer test flashes. At this flash intensity, the ERG response was comprised of an a- and b-wave followed by a slow positive c-wave. Even at the longest flash duration (220 ms), the test flash offset did not produce an OFF-component (Figure 3 insets, arrows), which in rat and mouse appears to involve cone driven cells [24,25]. As expected, with increasing stimulus duration the time to peak of the b-wave was delayed, and the duration of the b-wave was prolonged (Figure 3A). These features were exacerbated in the trkB<sup>flh</sup> mice (Figure 3B). The duration of the b-wave, to the point in the decay where the amplitude is half maximal, was measured in responses to 100 and 220 ms steps; and found to be significantly increased in the trkB<sup>flh</sup> mice when compared to age matched WT controls. For 100 ms steps, the response in WT mice was 117.4±13.5 ms in duration, while it was 158.4±2.53 ms in the trkB<sup>flh</sup> response, an increase of 35%. The response elicited by 220 ms steps was prolonged by 55% from 134.4±21.9 ms in WT mice to 208±27.2 ms in trkB<sup>flh</sup> mice (p<0.02).

The prediction, that a slow rise of the b-wave is expected when the photoreceptor to bipolar synapse is less efficient than that observed in WT mice, was tested quantitatively. If rod synaptic function were controlled by trkB in an all or none fashion, the reduction in trkB in the trkB<sup>flh</sup> mice would cause some synapses to function properly while others would fail completely; then the delay in the b-wave should be reproducible by scaling the WT b-wave to 54% (Table 1). However, scaling delays the onset only slightly, but not sufficiently to reproduce the trkB<sup>flh</sup> b-wave slope (data not shown), which argued for a synaptic delay in the rod ribbon synapse. To simulate the effects expected on the ERG from a model that assumes variable synaptic delays, ERG waveforms measured in WT mice were successively added, introducing a delay between each added waveform. The synaptic delay in the photoreceptor synapse is approximately 1 ms [26], thus, in the simulations normal WT b-wave waveforms were added to each other, starting each with a 1 ms delay with respect to the preceding one. This computational method recreated ERG waveforms that matched the experimentally recorded ones. For example, in Figure 4, the first peaks in the WT and the trkB<sup>flh</sup> b-wave differ by approximately 16 ms. The green trace illustrates the waveform obtained by adding a total of 16 WT waveforms, each delayed by 1 ms with respect to the preceding one and scaling the amplitude by 54% (Table 1). The computed waveform reproduced the slower rate of onset, the slower decay of the b-wave as well as the reduced amplitude of the trkB<sup>flh</sup>b-wave.

The signal summation used in the computation eliminated the oscillatory potentials since these are synchronous with respect to the stimulus flash. In the intact trkB<sup>flh</sup> mice, OPs are not affected because their occurrence in the animals is independent of the rate of rise of the b-wave. In general, the rising phase of the trkB<sup>flh</sup> b-wave can be modeled by (1) introducing a synaptic delay approximately 5-20 times longer than that in the WT mice, depending on the WT-trkB<sup>flh</sup> pair used for the comparison together with (2) a scaling factor for the b-wave amplitude. Taken together, these two parameters reproduced the characteristics of the mutant b-wave, which argues for a reduced gain and an increased delay in the trkB<sup>flh</sup> rod-ribbon synapse.

Anatomical and biochemical correlates: We [8] have previously shown that the reduced a-wave amplitude generated by the trkB<sup>+/-</sup> photoreceptors is correlated with shorter outer segments, and a reduced concentration of rhodopsin. If these features were linearly related to the level of trkB expression, one would expect to find a dependence of rod outer segment length and retinal rhodopsin concentration on the level of trkB gene expression (gene dosage). To assess this possibility, rhodopsin content in trkB<sup>flh</sup> mice, and their heterozygous (trkB<sup>flh</sup>) and WT littermates were measured.
Difference visible absorption spectra of retinas of the experimental mouse retinas were recorded and the maximum absorption was determined at 504 nm (Figure 5A). Rhodopsin concentrations were not significantly lower in the heterozygous trkB+/fbz mice when compared to the WT controls (p=0.46), which was expected, as the a-wave parameters were indistinguishable between those two groups (Table 1). On the other hand, the trkB+/fbz retinas contained only 44% of the WT levels of rhodopsin (p=0.04; Table 2). These changes in rhodopsin concentration per retina were reflected by the shorter outer segment lengths found in the trkB+/fbz retinas (Figure 5B,C). The outer segment length in the heterozygous trkB+/fbz mice was not affected (data not shown).

Retinal physiology and biochemistry are affected by trkB in a gene dosage dependent manner: Lowering trkB to 25% of the WT levels significantly reduced a- and b-wave amplitudes and kinetics. How do these findings compare to the ERG data in trkB-/- mice [8]? In Table I, ERG data from both the trkB hypomorphic and the trkB knockout mouse line were compared. The retina was able to compensate for a loss of up to 50% of trkB with respect to amplitude, light sensitivity and kinetics of both a- and b-waves (Table 1, rows 1-3). No changes in any of these parameters could be determined. However, when the gene dosage of trkB was reduced below a critical threshold to 25% (i.e., trkB+/fbz) or 0% (i.e., trkB-/-), rod responses, as reflected by a-wave amplitudes and slopes, were significantly reduced (Table 1, rows 4 and 5, columns 2 and 3). Whereas rod synaptic signaling to the inner retina (b-wave) was completely abolished in the trkB-/- mice (Table 1, row 5, columns 4 and 5), 25% of trkB (i.e., trkB+/fbz) resulted in b-waves of reduced amplitudes with a very slow onset time (Table 1, row 4, columns 4 and 5).

As expected from the a-wave analysis, rhodopsin content was found not to be significantly different in animals with 100% (WT) and 62.5% of trkB (trkB+/fbz; Table 2, rows 1-3), however, lowering trkB levels to 25% (trkB+/fbz) and 0% (trkB-/-) resulted in rhodopsin levels of 44 and 33%, respectively (Table 2, rows 4 and 5). And consequently, outer segment lengths were only affected in the homozygous animals of the two trkB mutant lines (trkB+/fbz; Figure 5B and C; trkB-/-: for more information on the histology of the trkB-/- mouse retina, please refer to Figure 2 of Rohrer et al., [8]).

What is the normal correlation between the physiological and anatomical parameters during the development of the rodent retina? Fulton and coworkers [27] reported that rod outer segments elongate faster than they accumulate rhodopsin, such that mature length is achieved prior to mature levels of rhodopsin. The maturation of the b-wave amplitude appears to correlate well with the increase in rhodopsin levels, whereas that of the a-wave parallels, but lags behind, the growth of the outer segments.

In the two lines of trkB mutant mice, retinal rhodopsin levels were correlated with trkB expression levels (R=0.99297; Figure 6 inset), thus shifting the biochemical age of the mutant retina to a younger age. When the a- or b-wave parameters (amplitudes and/or slopes) were plotted against either trkB levels (Figure 6) or rhodopsin concentration (data not shown), the polynomial curves that describe the a-wave behavior in both cases were shifted to the a lesser gene dosage of trkB, or a lesser retinal rhodopsin concentration in comparison to the b-wave, confirming Fulton and coworkers’s data [27] that the a-wave maturation precedes that of the b-wave. That is, 50% of the WT levels for a-wave parameters were reached at rhodopsin or trkB levels that are 10 and 15% below those necessary for 50% wild type levels for b-wave parameters, respectively.

DISCUSSION
The scope of this work was two fold: (1) to address possible shortcomings of the knockout mouse technology that can occur when a gene of interest (i.e., trkB) is expressed not only in the tissue of interest (retina) but in more than one location during development; and (2) to strengthen our understanding of the role of trkB in rod development. Both questions were addressed by investigating the structural and functional development of rods in two independently created transgenic lines with various levels of trkB expression. Two points were made by this study: (1) the results provided conclusive evidence that complex phenotypes can be analyzed using classical gene knockout technology, if a gene dosage study can be performed; and (2) the results further confirmed our previous suggestion that trkB-positive cells play an important role in the developmental maturation of mouse rod photoreceptors and are essential for the full functional development of the rod-bipolar cell synapse.

Gene dosage effect of trkB on photoreceptor signaling and development: Although classical gene knockout animals are being used widely, it may not be possible to attribute the phenotype in a particular organ to the loss of the gene that was knocked out [14]. In the case of the complete trkB knockout mice (trkB-/-), it may not be possible to attribute the observed retinal phenotype to a lack of trkB, as trkB is widely distributed in the central and peripheral nervous system, and systemic complications (e.g., those caused by alterations in the circulatory system or poor nutrition) could affect retinal function [21,28]. Thus, it was important to confirm the results obtained from the trkB-/- mice in a second line of trkB mutant mice, provided of course that the issue of systemic side effects is addressed. Crisvo [11] argued, that if the animals from two separate lines with different numbers of copies yielded consistent results, that one “might be reasonably certain that the observed effects were due to the manipulated gene and not to genetic contamination by residual donor genes.”

The two independently created transgenic trkB mouse lines, which were used in this study, had the same genetic background (126/C57BL/6/ICR), which is important, due to large anatomical and behavioral variations between inbred mouse strains [10,29,30]. If the elimination of trkB could be correlated with rod photoreceptor development, a gene dosage dependent effect on retinal rhodopsin, outer segment development and ERG characteristics was expected. It was observed that once a critical threshold of 50% trkB levels was reached, a further stepwise reduction in the trkB gene led to a gene dosage dependent decrease in ERG amplitudes, kinetics,
rhodopsin concentration and outer segment length (Table 1, Table 2; Figure 5).

The effect of trkB gene dosage on photoreceptor development is consistent with previous information on normal photoreceptor development. A-wave amplitudes and slopes, when plotted against trkB levels (Figure 6) could be fitted by one polynomial curve, suggesting that both parameters were affected together by the gene knockout. The same is true for the b-wave parameters (Figure 6). The polynomial describing the a-wave parameters is offset toward the lower trkB levels, and therefore to lower rhodopsin levels, in comparison to that describing the b-wave parameters, which is in concordance with the observations by Fulton et al. [27], regarding normal retina development.

To eliminate the possibility of compromised retinal perfusion or malnutrition as confounding factors in retinal function, spontaneous runs with bodyweights similar to those of the trkB−/− mice were examined [8] and OP amplitudes in trkB<sub>fl</sub>/<sup>+/−</sup> mouse b-waves were measured. Mice with decreased bodyweight were found to have no defect in their b-wave [8], and the OP amplitudes were not significantly lower in the trkB<sub>fl</sub>/<sup>+/−</sup> ERGs (see Section entitled “ERG waveform analysis of trkB<sub>fl</sub>/<sup>+/−</sup> mice”).

Taken together, these new findings suggest that retinal phenotypes observed in the trkB mutant mice are specific. They also confirm that using multiple mutant lines when analyzing a complex phenotype is an effective means to test for the presence of genetic artifacts. While the experiments have carefully addressed problems of gene knockout experiments, they still do not rule out entirely that compensatory changes in related genes or possibly a higher susceptibility to environmental factors could contribute to the observed phenotype in one or both trkB mutant lines (trkB<sub>fl</sub>/<sup>−/−</sup>; trkB<sup>−/−</sup>).

**Synaptic delay at the rod ribbon synapse:** Development of rod synaptic signaling, as assayed through the gain and kinetics of the electroretinographic b-wave, appeared to depend on retinal trkB signaling pathways. In the absence of trkB signaling, the light generated membrane voltage in trkB<sup>+/−</sup> rod photoreceptor cannot elicit a b-wave [8], although in the same retinas, bipolar cells respond to exogenously applied glutamate [9]; whereas in the trkB<sub>fl</sub>/<sup>+/−</sup> ERG, the rising phase of the b-wave was significantly delayed and the absolute amplitude of the b-wave was reduced. Thus, while specific mechanisms are yet to be identified, it is clear that trkB-positive cells specifically control the maturation and function of the rod presynaptic terminal and that this process can be dissociated from the maturation of the phototransduction molecular machinery.

Based on the constraints placed by the previous and current results, a presynaptic deficit model was presented (see Section entitled “Slower b-wave kinetics in trkB<sub>fl</sub>/<sup>+/−</sup> mice” and Figure 4) that was found to be consistent with all data sets ([8,9] and the data presented here). However, due to their complex nature, the ERG analyses do not provide proof of the proposed model.

Although both the data and the model lend further support to the possibility of a presynaptic deficit in the rods, the two different possible underlying mechanisms cannot yet be resolved: a deficit in glutamate clearance from the synaptic cleft or a defect in neurotransmitter release. Defects in either of the two components of synaptic signaling have been shown in both mice and humans to result in major reductions of b-wave amplitudes and/or kinetics [31-34]. Additional experiments are underway to attempt to distinguish between the two possibilities.

In summary, the present study demonstrates gene dosage dependent deficits in the rod signaling pathway in mice with knockout and hypomorphic trkB alleles. The experiments affirm that complex phenotypes can be addressed in gene knockout mice, if enough data points (i.e., gene dosages) can be analyzed. The b-wave analysis of trkB<sub>fl</sub>/<sup>+/−</sup> mice confirmed the initial conclusion that synaptic signaling is defective in the absence of trkB, but further experiments are needed to pinpoint the exact defect. Comparing the trkB<sub>fl</sub>/<sup>+/−</sup> animals with progenies of trkB<sub>fl</sub>/<sup>+/−</sup> crossed with (future) retina specific or cell specific Cre-recombinase mice will allow further characterization of the retinal deficits.

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