Intravitreal Ciliary Neurotrophic Factor Transiently Improves Cone-Mediated Function in a *CNGB3*$^{-/-}$ Mouse Model of Achromatopsia

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Purpose. Ciliary neurotrophic factor (CNTF) was recently shown to augment cone function in CNGB3 mutant achromat dogs. However, testing CNTF-releasing implant in human CNGB3 achromats failed to show benefit. We evaluated the effects of CNTF protein on the retinal function in an additional achromatopsia model, the CNGB3$^{-/-}$ mouse.

Methods. Fifty-nine CNGB3$^{-/-}$ mice (postnatal day [PD] ± SD = 30 ± 7) received a unilateral intravitreal injection of 1 or 2 μg CNTF protein, and 15 wild-type (WT) mice (PD = 34 ± 3) received 1 μg CNTF. Retinal function was evaluated by flash ERG and photopic flicker ERG (fERG) at 7 and 14 days after treatment.

Results. Seven days post CNTF, the photopic b-wave V_{max} was significantly increased in CNGB3$^{-/-}$ mice ($P < 0.01$), whereas it was reduced in WT mice ($P < 0.05$). Ciliary neurotrophic factor significantly increased the amplitude of photopic fERG and the photopic oscillatory potentials (OPs) in CNGB3$^{-/-}$ mice. Ciliary neurotrophic factor did not alter the scotopic a-wave in either CNGB3$^{-/-}$ or WT mice, but it increased the scotopic b-wave *k* ($P < 0.01$) in CNGB3$^{-/-}$ mice, indicating diminished scotopic sensitivity, and reduced the scotopic b-wave V_{max} in WT mice ($P < 0.05$). No difference was found in ERG parameters between 1 or 2 μg CNTF. Fourteen days after CNTF injection the ERG changes in CNGB3$^{-/-}$ mice were lost.

Conclusions. Intravitreal bolus CNTF protein caused a small and transient improvement of cone-mediated function in CNGB3$^{-/-}$ mice, whereas it reduced rod-mediated function. The increase in photopic OPs and the lack of changes in scotopic a-wave suggest a CNTF effect on the inner retina.

Keywords: achromatopsia, CNGB3, CNTF, intravitreal injection, cone photoreceptors

Achromatopsia is an inherited retinal condition characterized by substantial or even total loss of cone photoreceptor function. It has an estimated prevalence of 1:30,000 in the general population[1] and it is transmitted by autosomal recessive inheritance. Mutations in the *CNGA3* and *CNGB3* genes, which encode for the two structural subunits of the cone cyclic nucleotide-gated (CNG) channels, represent the main cause of the disease and account for almost 25% and 50% of the human achromats, respectively.[2]

Localized in cone and rod outer segments, CNG channels represent the main source of Ca^{++} in the photoreceptors and play a critical role in the phototransduction cascade by regulating the dark current.[3,4] Abnormal CNG channels impair the photoreceptor light response and eventually, through a mechanism that involves endoplasmic reticulum stress,[5] they result in photoreceptor death.

Cyclic nucleotide-gated channels are formed of A and B subunits. The rod channel consists of CNGA1 and CNGB1 subunits while the cone channel is formed by CNGA3 and CNGB3 subunits. Studies have indicated that the A-subunits are required for the ion-conducting activity of the channel, while the B-subunits act as modulators.[3] Furthermore, while A-subunits alone can reconstitute a partially functional homomeric channel, isolated B-subunits cannot.[4,6] As a consequence, mutations that selectively affect either the A- or the B-subunit have a different impact on the retinal phenotype.

This difference has been clearly documented in the CNGA3$^{-/-}$ and CNGB3$^{-/-}$ mouse models.[7–9] Compared with the CNGA3$^{-/-}$ murine model, CNGB3-deficient mice exhibit a slower progression of cone photoreceptor degeneration. At postnatal day 30, even though the cone ERG amplitude is reduced by 70%, cone density is decreased only by 20% and after 1 year 50% of the cones still remain in the CNGB3$^{-/-}$ retina.[9,10] Similarly, in CNGB3 mutant achromat dogs, the Alaskan Malamute and the German Shorthaired Pointer breeds, only approximately 25% of cone photoreceptors are lost at 1 year of age.[11] As the photoreceptor loss is slow and many cone photoreceptors are still present at an older age, the CNGB3$^{-/-}$

mouse represents a suitable model for testing the effects of a neuroprotective approach on cones.

Ciliary neurotrophic factor (CNTF) is a neurotrophic agent that has been shown to slow rod photoreceptor loss in several animal models of inherited retinal degeneration.[12] Photoreceptors protection by exogenous CNTF relies on initial signaling through gp130 receptors on Müller cells.[13] However, despite promoting photoreceptor survival, CNTF also significantly suppresses rod photoreceptor function, as demonstrated by reduced scotopic ERG responses following its administration.[14]

Recently, a role for CNTF as a neuroprotective factor for cone photoreceptors has been proposed. Ciliary neurotrophic factor promoted cone outer segment regeneration in transgenic rats carrying the rhodopsin mutation S334ter.[15] Sustained CNTF expression in a mouse model of retinitis pigmentosa led to life-long preservation of cone photoreceptors and, despite suppression of the ERG responses, preserved vision until the end-stages of degeneration.[16] Quite surprisingly, CNTF was found to improve residual cone function in dogs with CNGB3-related achromatopsia.[11]

Based on these promising preclinical studies, human CNTF, secreted into the vitreous through controlled release devices (encapsulated cell technology implants), was tested in clinical trials for the treatment of retinitis pigmentosa,[17,18] AMD,[19,20] and more recently, achromatopsia.[21] Unlike achromat dogs however, CNTF showed no evidence of improving cone function for human achromats, suggesting that human cones respond to CNTF differently than canine cones. This raised the question of whether CNTF effects on retinal function might be species dependent.

Due to the contrasting results of the preclinical studies with dog and the human clinical trial, we conducted the present study to assess whether CNTF could improve residual cone function in an additional animal model of achromatopsia, the CNGB3$^{-/-}$ mouse. We administered single intravitreal injections of CNTF to CNGB3$^{-/-}$ mice as was done in the canine model, and we assessed changes in retinal function by ERG analysis over the following 2 weeks. Ciliary neurotrophic factor was also tested for effects on the wild-type (WT) murine retina.

Materials and Methods

Expression and Purification of Recombinant Human CNTF Protein and Biological Essay

The cDNA open reading frame of human CNTF was cloned into an expression vector pQE30 (Qiagen, Valencia, CA, USA), and fused to a 6× His tag at the N terminus to generate plasmid pQE–CNTF. Recombinant human CNTF (hCNTF) protein was expressed in *Escherichia coli* (XL-blue; Stratagene, La Jolla, CA, USA) and purified by immobilized-metal affinity chromatography on Ni-NTA agarose columns (Qiagen) under native conditions. Eluted protein was buffer exchanged to PBS. The purified recombinant protein is approximately 23 kDa on acrylamide gel electrophoresis. To test the biological activity of the hCNTF protein, one eye each of 14 Sprague Dawley rats received intravitreal injection of 10 μg hCNTF, and retinal function was tested one week later by ERG. Scotopic b-wave amplitudes were reduced approximately 35% in injected eyes, confirming bioactivity and also the previous findings that CNTF reduces the scotopic function in WT rats.[14]

Animals and Rearing Conditions

A breeding pair of CNGB3$^{-/-}$ mice (on C57BL/6 background) was purchased from Deltagen, Inc. (San Mateo, CA, USA) and progeny were born and raised at National Institutes of Health (NIH; Bethesda, MD, USA). The original line was engineered by targeted deletion of the mouse CNGB3 mRNA (GenBank accession number AJ243572) sequence from base 733 to base 749. Wild-type C57BL/6 mice were obtained by Jackson Laboratory (Bar Harbor, ME, USA). All tested animals were reared in dim (8–20 lux) cyclic light (12-hour light/dark). This research was conducted in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and received protocol approvals by Animals Care and Use Committee at NIH.

Intravitreal Injection

Mice were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg), and hCNTF (1 and 2 μg in 1 μL PBS) or PBS (1 μL) were delivered by intravitreal injections through a 35-G needle on a 10 μL Hamilton microsyringe (Hamilton, Reno, NV, USA). Injection of CNTF or PBS was interchanged randomly for the right or left eyes of animals. Triple antibiotic ophthalmic ointment (neomycin, polymyxin, and bacitracin) was applied after injections. No change in pupil size or reactivity to light was observed in injected eyes.

Western Blot Analysis

The retina was dissected and total protein obtained by lysing in RIPA buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). Total protein of each sample was determined by the Pierce Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific) using bovine serum albumin as a standard. Total protein (40 μg) from each sample was electrophoresed on SDS-PAGE (Thermo Fisher/Life Technologies, Carlsbad, CA, USA), followed by wet transfer to polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were incubated for 1 hour in ready to use blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) and then incubated overnight with the appropriate antibodies. Antibodies to phospho-STAT3 (rabbit polyclonal; 1:1000; Cat# 9145; Cell Signaling Technology, MA, USA), and β actin (mouse mAb, 1:4000; Cat# 3853; Sigma-Aldrich Corp., St. Louis, MO, USA) were used in this study. After overnight incubation the membranes were rinsed in PBST (PBS+0.1% Tween-20), followed by incubation with IR-Dye 800CW conjugated goat anti-rabbit IgG (Cat# 926-32211; LI-COR, Lincoln, NE, USA) or with Alexa Fluor 680-conjugated anti-mouse IgG (Cat# A 21058; Thermo Fisher Scientific, Rockville, MD, USA). Blots were scanned on LI-COR Odyssey Infrared Imaging System (Model 9120; LI-COR Biosciences).

Electroretinography

Electroretinogram measurements were performed using an Espion E^2 electrophysiology system with a ColorDome Ganzfeld (Diagnosys LLC, Lowell, MA, USA). All animals were dark adapted for at least 12 hours before ERG recording. Mice were anesthetized under dim red light with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Pupils were dilated with topical 0.5% tropicamide and 0.5% phenylephrine HCl. The cornea was kept moist with methylcellulose solution. A gold wire was placed on the moistened cornea as a recording electrode and a gold reference electrode was placed in the mouth. A needle electrode inserted subcutaneously close to the tail served as ground. Electroretinograms were recorded simultaneously from both eyes at bandwidths of 0.1 and 500 Hz.

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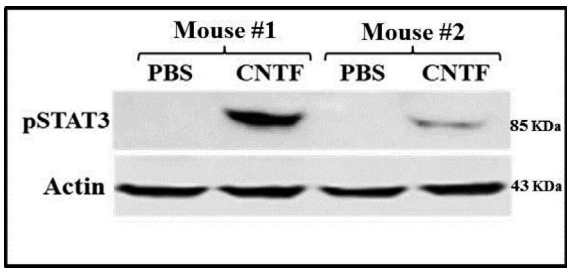

FIGURE 1. Ciliary neurotrophic factor induced STAT3 phosphorylation (pSTAT3) in the mouse retina. Two CNGB3$^{-/-}$ mice (mouse #1 and mouse #2) received 1 μg CNTF in one eye and 1 μL PBS in the fellow eye. Retinas were harvested and STAT3 phosphorylation was examined by Western blot. Significantly high pSTAT3 levels were detected in the CNTF-treated retinas of both CNGB3$^{-/-}$ mice, whereas phosphorylation of STAT3 was not detectable in the PBS fellow eyes.

Full-Field ERG

Full-field scotopic ERGs were evoked by achromatic flashes (−5.8 to 2.7 log scotopic candela-sec/meter^2 [sc cd-s/m^2]). Interflash interval ranged from 3 seconds for the dimmest intensity up to 30 seconds for the flash intensity of −1.3 log sc cd-s/m^2. For higher flash intensities (−0.8 to 2.7 log sc cd-s/m^2), the interflash interval ranged from 60 to 90 seconds. Electroretinogram a-wave amplitude was measured from baseline to trough. For flash intensities from −5.8 to −1.3 log sc cd-s/m^2, ERG b-wave amplitude was measured from either baseline or from the trough of the a-wave (when present) to the peak, after subtraction of oscillatory potentials (OPs), which were isolated with a digital band-pass filter (45–500 Hz). The Naka-Rushton function[22,23] was fitted to the scotopic b-wave intensity-response curve between −5.8 and −1.3 log sc cd-s/m^2 to derive the maximum b-wave amplitude, V_{max} (μV) and the flash intensity that produces the half-maximum response, k (sc cd-s/m^2) or the reciprocal of the scotopic b-wave sensitivity ($1/k$). To quantify photoreceptor kinetics, a

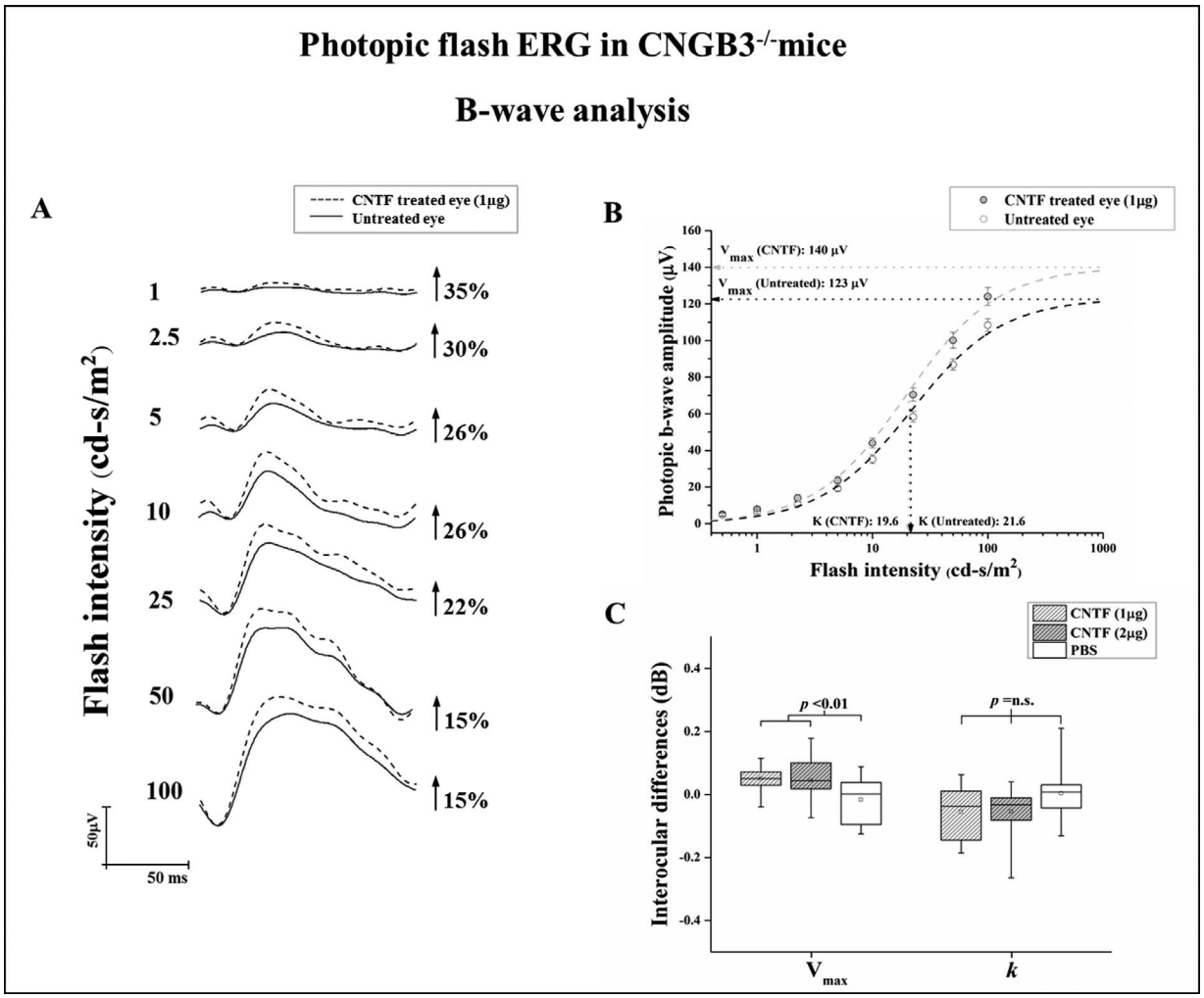

FIGURE 2. Analysis of cone-mediated function in CNGB3$^{-/-}$ mice 7 days after the intravitreal injection of CNTF (1 and 2 μg) and PBS. (**A**) Photopic b-waves to increasing flash intensities recorded from a representative CNGB3$^{-/-}$ mouse. *Dasbed lines* represent the CNTF-injected eye and *solid lines* are the uninjected eye. Numbers on the right of each waveform indicate percentage increase in b-wave amplitude following CNTF in the treated eye respect to the untreated eye. (**B**) Averaged photopic b-wave amplitudes from both eyes of 24 CNGB3$^{-/-}$ mice after unilateral injection of 1 μg CNTF, plotted as function of flash intensity. *Error bars* indicate standard errors. The intensity-response function was fitted using the Naka-Rushton function (*dasbed lines*) to derive the maximum photopic b-wave amplitude V_{max} (μV) and the flash intensity that produces the half maximum response, k (cd-s/m^2). (**C**) Box-whisker plots of log interocular differences (treated eye–untreated eye in decibels) in photopic b-wave V_{max} and k of CNGB3$^{-/-}$ mice injected with CNTF at 1- or 2-μg dose versus PBS. In each diagram the *symbol* is the mean, the *box* indicates the median and interquartile range, and *bars* indicate the 95 percentiles.

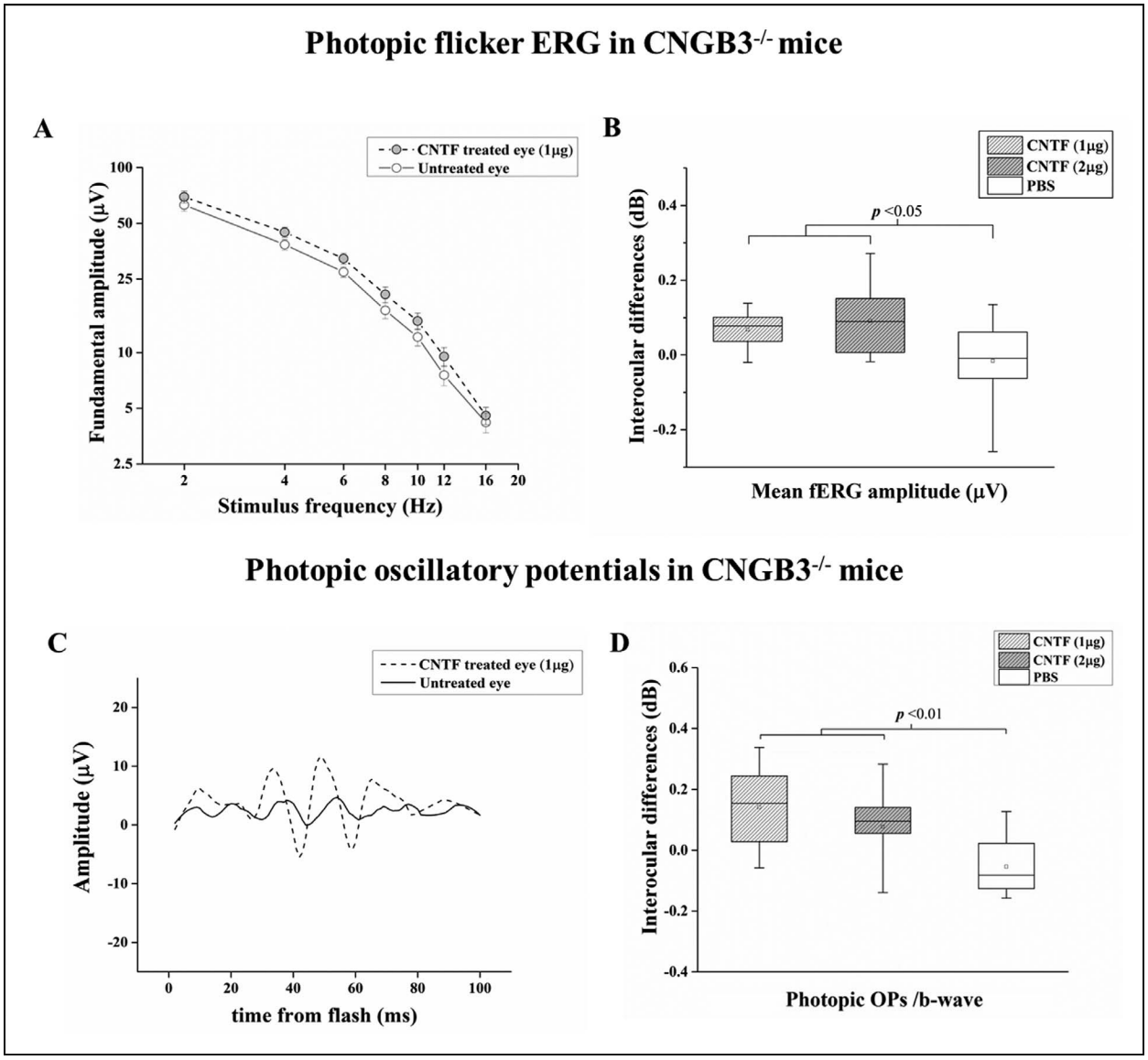

Figure 3. Analysis of photopic fERG and photopic oscillatory potentials (OPs) in CNGB3$^{-/-}$ mice 7 days after the intravitreal injection of CNTF (1 and 2 μg) and PBS. (**A**) Averaged photopic fERG amplitudes from both eyes of 24 CNGB3$^{-/-}$ mice receiving 1 μg CNTF in one eye, plotted as a function of the logarithm of the flicker frequency. *Dashed lines* represent the CNTF-injected eyes and *solid lines* are the uninjected eyes. *Error bars* indicate standard errors. (**B**) Box-whisker plots of log interocular differences (treated eye–untreated eye in decibels) in mean fERG amplitude in CNGB3$^{-/-}$ mice injected with 1 or 2 μg CNTF versus PBS. (**C**) Photopic OPs from both eyes of a representative CNGB3$^{-/-}$ mouse injected in one eye with 1 μg CNTF. The *dashed waveform* represents the OPs extracted from the photopic b-wave of the CNTF injected eye; the *solid waveform* represents OPs from the uninjected eye. (**D**) Box-whisker plots of log interocular differences (treated eye–untreated eye in decibels) in the photopic OPs amplitude/photopic b-wave amplitude ratio in CNGB3$^{-/-}$ mice injected with 1 or 2 μg CNTF versus PBS. In each diagram the *symbol* is the mean, the *box* indicates the median and interquartile range, and *bars* indicate the 95%.

model of the activation phase of the rod phototransduction cascade[24,25] was ensemble fitted to the leading edges of ERG a-waves recorded for flash intensities from –0.8 to 1.0 log sc cd-s/m^2. The "leading edge" was determined as all points up to 80% of the a-wave peak for each flash intensity. The 80% value was chosen to avoid the influence of postreceptoral components that may contribute to the a-wave near its peak.[26] Derived parameters were: R_{max} (μV) the maximal rod response, S ([sc cd-s]$^{-1}$ m^{-2}) the rod sensitivity parameter that scales flash intensity and t_d (ms), the delay contained in both the response and instrumentation. During fitting, R_{max} was fixed at the maximal a-wave amplitude recorded and S and t_d were allowed to vary.

Full-field photopic ERGs were evoked with achromatic flashes of increasing intensity (0.0 to 2.0 log sc cd-s/m^2) presented against a steady background of 34 sc cd/m^2, after 5 minutes exposure to a background of the same intensity. The interflash interval was set at 3 seconds for all flash intensities. Photopic b-wave amplitude was measured, after subtracting the OPs by digital filtering, from the baseline or from the trough of the a-wave when present, to the peak. The Naka-Rushton function was fitted to the photopic b-wave to derive the parameters V_{max} and k, and gave a good fit (mean R^2 = 0.99). Photopic OPs were isolated by band-pass filtering (45–500 Hz) the wave of the photopic flash ERGs evoked by a flash of 2.0 log sc cd-s/m^2. The amplitude of the OPs was calculated

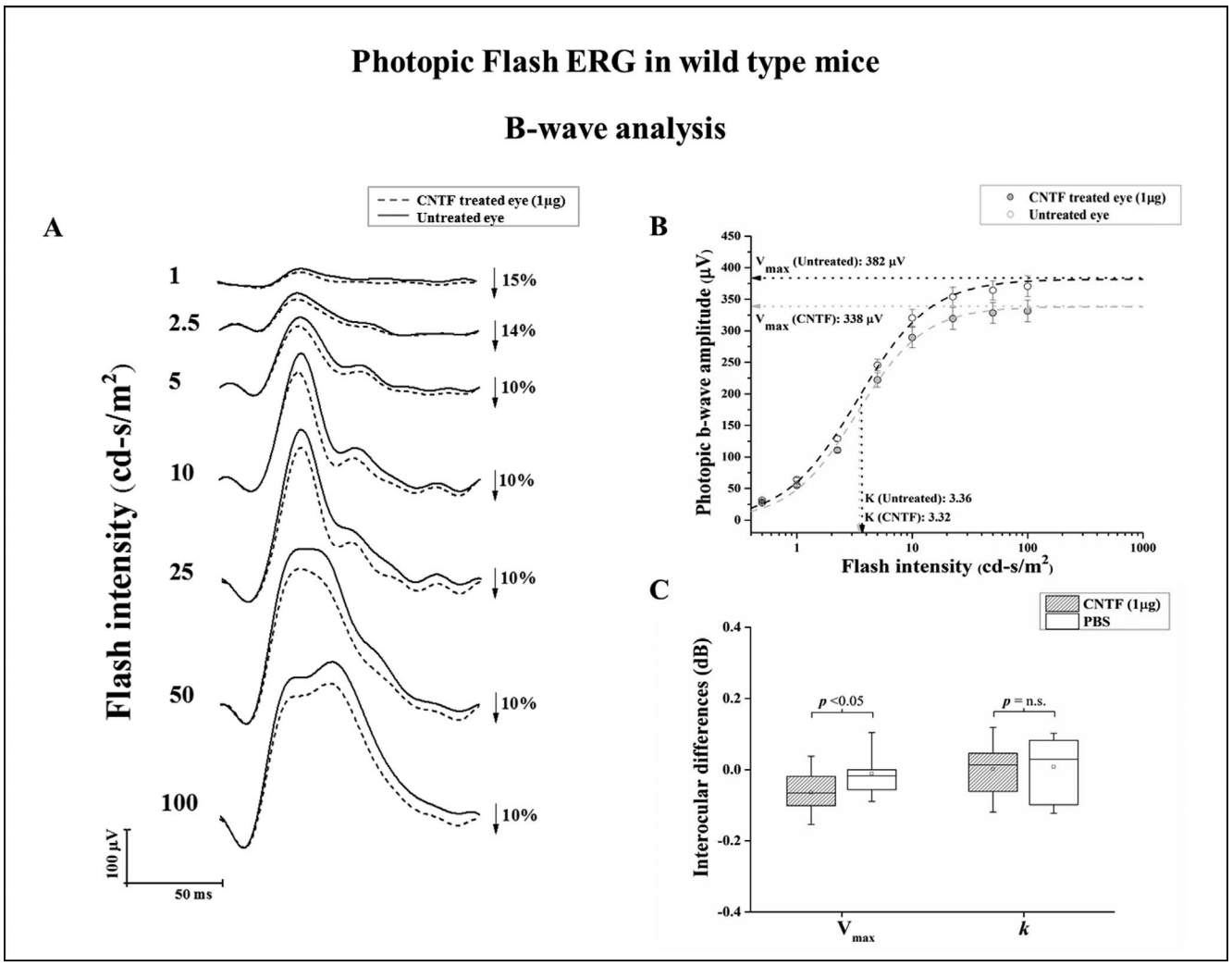

Figure 4. Analysis of cone-mediated function in WT mice 7 days after the intravitreal injection of 1 µg CNTF and PBS. (**A**) Photopic b-waves to increasing flash intensities recorded from a representative WT mouse. *Dashed lines* represent the CNTF-injected eye and *solid lines* are the uninjected eye. On the right of each waveform, numbers indicate the percentage decrease in b-wave amplitude of the CNTF-treated eye respect to the untreated eye. (**B**) Averaged photopic b-wave amplitudes from both eyes of 15 WT mice unilaterally injected with 1 µg CNTF, plotted as function of flash intensity. *Error bars* indicate standard errors. The response-intensity function was fitted using the Naka-Rushton function (*dashed lines*) to derive the maximum photopic b-wave amplitude V_{max} (µV) and the flash intensity that produces the half maximum response, k (cd-s/m^2). (**C**) Box-whisker plots of log interocular differences (treated eye–untreated eye in decibels) in photopic b-wave V_{max} and k in WT mice injected with 1 µg CNTF and PBS. In each diagram the *symbol* is the mean, the *box* indicates the median and interquartile range, and *bars* indicate 95%.

by the sum of all peaks and then normalized to the amplitude of the corresponding photopic b-wave to obtain the OPs/b-wave ratio.

Photopic Flicker ERG (fERG)

Photopic fERG was elicited by full-field stimuli delivered by green light-emitting diodes (LEDs) with a peak wavelength of 517 nm, built into the ColorDome Ganzfeld (Diagnosys LLC). Light intensity was sinusoidally modulated at 13 temporal frequencies between 2 and 50 Hz, at 95% contrast and a mean luminance of 800 sc cd/m^2. At this mean luminance rods are saturated,[27] and ERG responses reflect the activity of the cone-mediated neural pathways in the retina. Each stimulus was presented for approximately 10 seconds and the recording was synchronized with the stimulus onset and offset. The initial and final 500 ms of each recording were removed before data analysis. The amplitude (µV) and phase (degrees) of the fundamental harmonic at each temporal frequency was extracted by discrete Fourier analysis. Background noise was estimated from the average of the amplitude of the six frequencies (three on each side) that flanked the fundamental frequency. At each temporal frequency only the fERG responses with a signal to noise greater than or equal to 3 were accepted for statistical analysis. Responses were then averaged to derive the mean fERG amplitude.

Statistical Analysis

Changes in flash ERG parameters were evaluated between treatment groups by one-way ANOVA. A logarithmic transformation was applied to each parameter value to better approximate normal distribution. As the variability in ERG amplitude between individual animals is usually greater than between eyes of each animal,[28] the effect of a monocular treatment is best expressed as the average ratio of the treated eye to the untreated fellow eye, as reported previously.[29] A post hoc Tukey test was used for multiple between-group

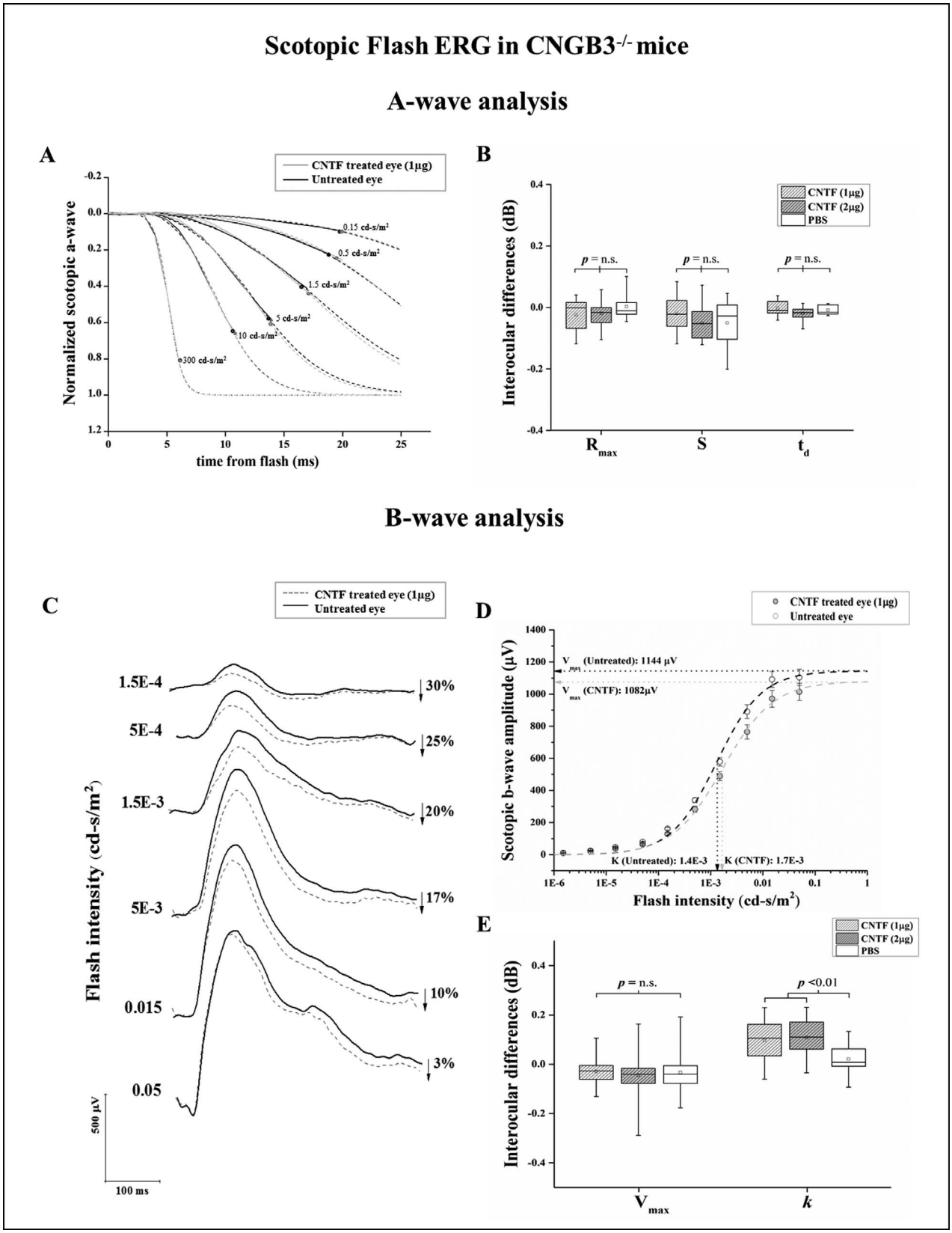

Figure 5. Analysis of rod-mediated function in CNGB3$^{-/-}$ mice 7 days after the intravitreal injection of CNTF (1 and 2 μg) and PBS. (**A**) A family of a-waves from a representative CNGB3$^{-/-}$ mouse injected with 1 μg CNTF in one eye (*black lines*). The uninjected eye (*gray lines*) served as control. A-waves were normalized by the amplitude of the response to a bright flash that produced a saturating response (300 cd-s/m^2) and fitted with the transduction cascade activation model (*dashed traces*) up to the time point marked with the filled circle (80% of each a-wave peak amplitude). Responses to flash intensities from 0.15 to 10 cd-s/m^2 were fitted simultaneously after fixing R_{max} at the saturated a-wave amplitude to derive the amplification constant (S) and the time delay parameter (t_d). (**B**) Box-whisker plots of log interocular differences (treated eye–untreated eye in

decibels) in phototransduction parameters R_{max}, S, and t_d in CNGB3$^{-/-}$ mice treated with CNTF at two doses (1 and 2 μg) and 1 μL PBS. (**C**) Scotopic b-waves at increasing flash intensities recorded from the same CNGB3$^{-/-}$ mouse in (**A**). *Dashed lines* represent the CNTF-injected eye and *solid lines* represent the uninjected eye. On the right of each waveform, numbers indicate the percentage decrease in b-wave amplitude of the CNTF-treated eye respect to the untreated eye. (**D**) Averaged scotopic b-wave amplitudes from both eyes of 24 CNGB3$^{-/-}$ mice injected with 1 μg CNTF in one eye, plotted as function of flash intensity. *Error bars* indicate standard errors. The response-intensity function was fitted using the Naka-Rushton function (*dashed lines*) to derive the maximum scotopic b-wave amplitude V_{max} (μV) and the flash intensity that produces the half maximum response, k (cd-s/m^2). (**E**) Box-whisker plots of log (treated eye–untreated eye in decibels) in scotopic V_{max} and k in CNGB3$^{-/-}$ mice treated with CNTF at two doses (1 and 2 μg) and 1 μL PBS. In each diagram the *symbol* is the mean, the *box* indicates the median and interquartile range, and *bars* indicate the 95 percentiles.

comparisons. Unpaired *t*-test analysis was used to compare changes in ERG parameters in WT mice, as well as 2 weeks ERG changes in CNGB3$^{-/-}$ mice. Photopic fERG results were analyzed by two-way ANOVA with repeated measures, with the dependent variable the log interocular difference in fERG amplitude and with stimulus temporal frequency and treatment as independent variables. We considered P less than 0.05 as threshold for significance.

Results

CNTF Activities of the JAK/STAT Pathway in CNGB3$^{-/-}$ Mice

Ciliary neurotrophic factor activates STAT3, a major downstream signaling molecule, by inducing its phosphorylation through Jak kinases. We observed a marked increase in STAT3 phosphorylation in CNGB3$^{-/-}$ retinas 2 days after intravitreal injection of CNTF, confirming the biologic viability and activity of the CNTF used in this study. STAT3 phosphorylation was not observed in the PBS-treated retinas (Fig. 1).

CNTF Improved Cone-Mediated Function in CNGB3$^{-/-}$ Mice by 7 Days

Photopic flash ERGs were elicited with flashes of increasing intensity presented on a light adapted background that suppresses rod-mediated responses. Due to the small number of cone photoreceptors in the murine retina (3%) the a-wave is scarcely represented in the mouse photopic ERG, and the waveform consists mainly of a positive-going photopic b-wave that originates primarily from activity of the cone depolarizing bipolar cells.[30] Figure 2A illustrates a series of photopic b-waves to increasing flash intensities from a representative CNGB3$^{-/-}$ mouse, 7 days after the intravitreal injection of 1 μg CNTF. Compared with the untreated eye recorded simultaneously, CNTF augmented the b-wave amplitude at all flash intensities. The amplitude increase was larger for dimmer flashes and progressively less for brighter flashes, resulting in a 15% increase at the maximum intensity of 100 sc cd-s/m^2. Averaged photopic b-wave amplitudes from 24 CNGB3$^{-/-}$ animals were plotted as a function of the flash intensity, and the intensity-response function was fitted using the Naka-Rushton equation to derive the parameter V_{max} and the half-maximal intensity parameter k (Fig. 2B). Ciliary neurotrophic factor increased V_{max} by 13% and shifted the intensity-response curve slightly to the left, reducing the parameter k, which indicates an increase in the cone-mediated sensitivity. Analysis of log interocular differences between CNTF- and PBS-injected CNGB3$^{-/-}$ mice showed that CNTF significantly increased the photopic b-wave V_{max} ($P < 0.01$), whereas there was no significant difference in retinal sensitivity k (P = n.s.; Fig. 2C).

To evaluate whether CNTF could improve the temporal sensitivity of the cone system in the CNGB3$^{-/-}$ mice (i.e., increase responses to stimuli at higher frequency), a photopic fERG was recorded with sinusoidal stimuli of different frequencies (Fig. 3A). Injection of 1 μg CNTF significantly augmented the mean fERG amplitude in CNGB3$^{-/-}$ mice (Fig. 3B). However, two-way ANOVA did not indicate a significant interaction between CNTF and the temporal frequency (P = n.s.), indicating that the increase in fERG amplitudes was constant across all the temporal frequencies and that CNTF did not improve the sensitivity of the cone system in a frequency dependent manner.

Figure 3C shows photopic OPs isolated from the photopic b-wave of a representative CNGB3$^{-/-}$ mouse injected with 1 μg CNTF. Photopic OPs were larger in the CNTF-treated eye respect to the untreated eye. To learn whether this was simply due to an increase in the underlying photopic b-wave amplitude, the photopic OPs were normalized to the amplitude of the corresponding b-wave and the OPs/b-wave ratio was calculated. Compared with PBS, CNTF significantly increased the OPs/b-wave ratio ($P < 0.01$) of CNGB3$^{-/-}$ mice, indicating selective increase in OPs in the CNTF-treated eyes (Fig. 3D).

Conversely from what was observed in CNGB3$^{-/-}$ mice, CNTF reduced cone-mediated ERG responses in WT mice. As shown in a representative WT mouse (Fig. 4A), photopic b-wave amplitude was reduced by almost 10% at all flash intensities in the CNTF-injected eye respect to the untreated eye. Naka-Rushton fitting of the response-intensity function, averaged from 15 WT mice, showed that photopic b-wave V_{max} was reduced by 12% in the CNTF-treated eye, whereas k was unchanged (Fig. 4B). Statistical comparison of interocular differences between WT mice treated with CNTF or PBS confirmed this finding, and indicated a significant decrease of the photopic b-wave V_{max} in the CNTF treated eyes ($P < 0.05$), whereas k did not change (P = n.s.; Fig. 4C).

CNTF Decreased Rod-Mediated Function in CNGB3$^{-/-}$ Mice

Rod-pathway function was assessed by dark-adapted flash ERGs recorded 7 days after CNTF. The negative ERG component immediately following the flash stimulus, namely the a-wave, originates mainly from the suppression of the circulating dark current in rod outer segments. The following positive component, called the scotopic b-wave, derives mainly from the activity of rod depolarizing bipolar cells.[26,31]

A family of scotopic a-waves to increasing flash intensities for a CNGB3$^{-/-}$ mouse treated with 1 μg CNTF is shown in Figure 5A. A-waves were normalized to the amplitude of the response to the most intense flash eliciting a saturating response (R_{max}). The dashed lines show the fit of the model of the activation phase of the rod phototransduction cascade to the a-waves. Normalized a-waves from the CNTF-treated and the untreated eyes were nearly superimposed for all intensities. Analysis of interocular differences between CNTF and PBS eyes (Fig. 5B) did not detect any significant statistical difference in phototransduction parameters R_{max}, S, or t_d, indicating that CNTF did not affect the kinetics of the activation phase of the rod phototransduction cascade in CNGB$^{-/-}$ mice.

Figure 5C shows scotopic b-waves recorded at increasing flash intensities from the same representative CNGB3$^{-/-}$

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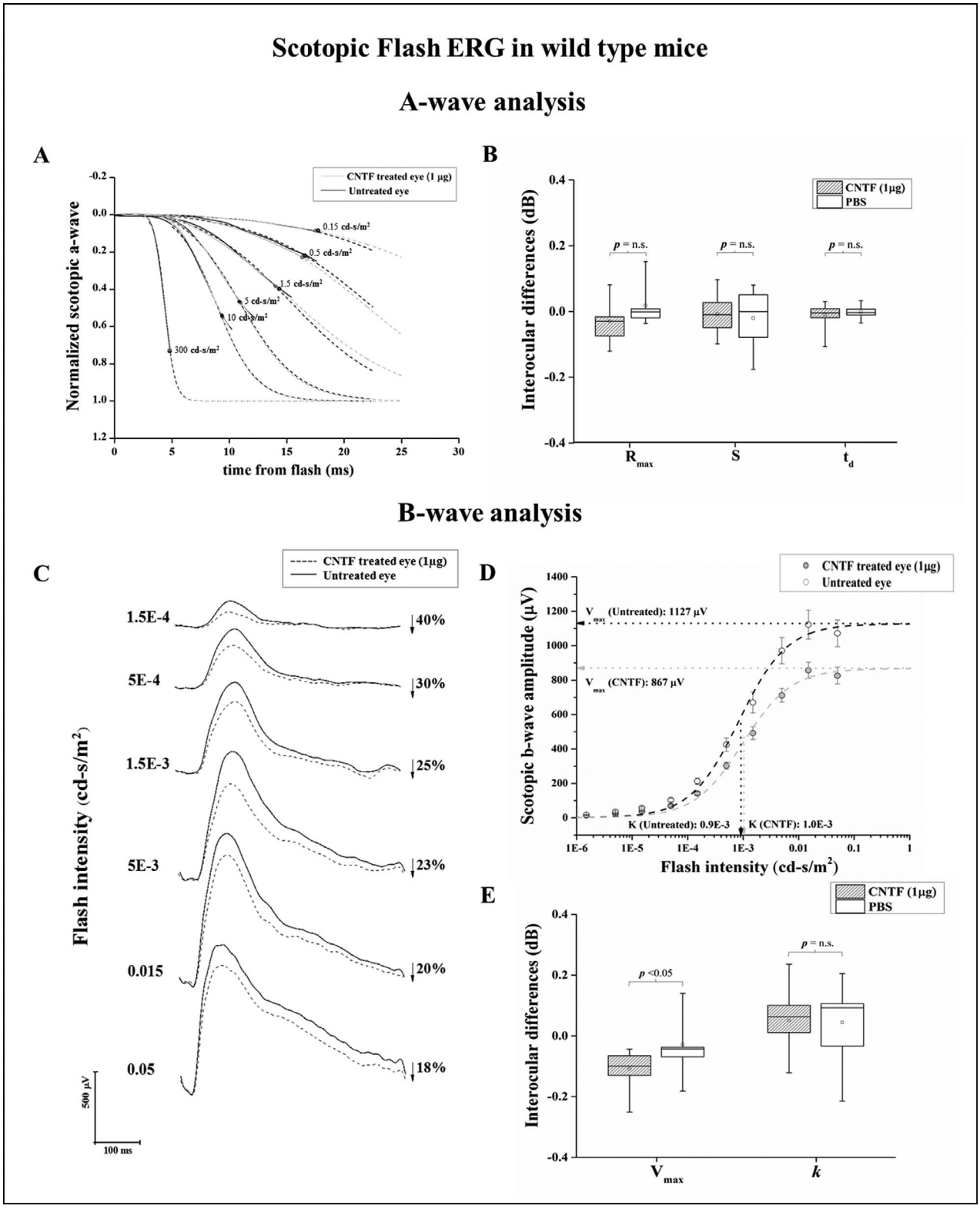

Figure 6. Analysis of rod-mediated function in WT mice 7 days after the intravitreal injection of 1 µg CNTF and PBS. (**A**) A family of a-waves from a representative WT mouse injected with 1 µg CNTF in one eye (*black lines*). The uninjected eye (*gray lines*) served as a control. A-waves were normalized by the amplitude of the response to the most intense flash producing a saturating response (300 cd-s/m^2) and fitted with the transduction cascade activation model (*dashed traces*) up to the time point marked with the filled circle (80% of each a-wave peak amplitude). Responses recorded for flash intensities from 0.15 to 10 cd-s/m^2 were fitted simultaneously fixing R_{max} at the saturated a-wave amplitude to derive the amplification constant (S) and the time delay parameter (t_d). (**B**) Box-whisker plots of log interocular differences (treated eye–untreated eye in decibels) in phototransduction parameters R_{max}, S, and t_d in WT mice treated with 1 µg CNTF and 1 µL PBS. (**C**) Scotopic b-waves at increasing flash intensities recorded from the same WT mouse in (**A**). *Dashed lines* represent the CNTF-injected eye and *solid lines* represent the uninjected eye. On the right of each waveform, numbers indicate the percentage decrease in b-wave amplitude of the CNTF-treated eye respect to the untreated

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eye. (**D**) Averaged scotopic b-wave amplitudes from both eyes of 15 WT mice injected with 1 μg CNTF in one eye, plotted as function of flash intensity. *Error bars* indicate standard errors. The response-intensity function was fitted using the Naka-Rushton function (*dashed lines*) to derive the maximum scotopic b-wave amplitude V_{max} (μV) and the flash intensity that produces the half maximum response, k (cd-s/m^2). (**E**) Box-whisker plots of log interocular differences (treated eye–untreated eye in decibels) in scotopic V_{max} and k in WT mice treated with 1 μg CNTF and 1 μL PBS. In each diagram the *symbol* is the mean, the *box* indicates the median and interquartile range, and *bars* indicate the 95 percentiles.

mouse shown in Figure 5A. Ciliary neurotrophic factor caused a reduction in the scotopic b-wave amplitude that was more pronounced at dimmer flashes. Amplitude reduction became progressively smaller as the flash intensity increased, and at 0.05 sc cd-s/m^2 the scotopic b-wave in the CNTF-treated eye was reduced only by 3% respect to the untreated eye. Figure 5D displays Naka-Rushton fitting of the intensity-response function derived from averaged scotopic b-wave amplitudes of 24 CNGB3$^{-/-}$ animals injected with 1 μg CNTF. The figure shows a reduction (5%) in the average V_{max} of the CNTF-treated eyes with respect to the untreated eyes and a shift of the intensity-response curve to the right, indicating a decrease in rod-mediated sensitivity. Interocular differences comparison between CNGB3$^{-/-}$ mice receiving CNTF or PBS (Fig. 5E) showed that CNTF significantly increased the parameter k ($P < 0.01$), confirming a reduction in scotopic retinal sensitivity, whereas V_{max} was not significantly affected.

Injection of 1 μg CNTF reduced the rod-mediated function of WT mice (Fig. 6). Similarly to what observed in CNGB3$^{-/-}$ mice, CNTF suppressed the scotopic b-wave amplitude without affecting the scotopic a-wave kinetic or amplitude (Figs. 6A, 6B). In contrast to CNGB3$^{-/-}$ mice, CNTF decreased the scotopic b-wave amplitude of WT mice across all flash intensities, including the highest stimulus (0.05 sc cd-s/m^2) where the b-wave amplitude was reduced by 18% (Fig. 6C). Statistical analysis of the scotopic b-wave parameters, derived from the Naka-Rushton fitting of the intensity-response function from all the 15 WT mice (Fig. 6D), showed a significant decrease in V_{max} ($P < 0.05$) after CNTF, without a significant change in the parameter k (P = n.s.; Fig. 6E).

Greater CNTF Dose Did Not Further Increase the Effect in CNGB3$^{-/-}$ Mice

Compared with the PBS control group, 2 μg CNTF significantly increased the photopic b-wave V_{max}, the mean fERG amplitude, and the OPs/b-wave ratio in CNGB3$^{-/-}$ mice. However, these changes were not different from those observed after 1 μg CNTF. Similarly, 2 μg CNTF did not cause a greater reduction in the rod-mediated responses in CNGB3$^{-/-}$ mice versus 1 μg. One week after CNTF treatment, scotopic a-wave parameters remained unchanged from control, as did the scotopic b-wave V_{max}. Parameter k was significantly increased ($P < 0.01$), but the change was not greater than for 1 μg CNTF (Table).

Injection of 4 μg CNTF in an additional group of CNGB3$^{-/-}$ mice ($n = 7$) produced comparable trends in the tested ERG parameters. However, data from this group were not included in the statistical analysis because the small sample size did not permit sufficient power for statistical comparison with the other treatment groups.

CNTF Effects Were Lost by 14 Days

A separate group of 18 CNGB3$^{-/-}$ was injected with 1 μg CNTF and tested 14 days after treatment to assess persistence of the effect. Twelve CNGB3$^{-/-}$ mice were injected with PBS as control. No significant change in interocular differences were found between the two groups of CNGB3$^{-/-}$ mice for all the ERG tested parameters (Table), indicating that the CNTF effects on retinal function was transient and reversible. Note that we tested this separate group at 14 days, as fragility of mice to repeated anesthesia prevents robust serial ERG recordings at baseline, 7 and 14 days.

Discussion

This study evaluated the effects of CNTF protein on the retinal function of CNGB3$^{-/-}$ achromat mice. A single bolus intravitreal injection of CNTF exerted a beneficial effect on the residual cone function in CNGB3$^{-/-}$ mice, as shown by a small but significant improvement of the cone-mediated ERG responses 1 week after treatment. As previously reported,[14] CNTF effects on retinal function were transient and reversible. Two weeks after treatment, the improvement in the cone-mediated ERG was no longer detectable, and ERG responses reverted to pretreatment levels.

The CNTF effects on CNGB3 mutant retina appear to depend on the species. A study by Komáromy et al.[11] showed that CNTF protein, given by bolus intravitreal injection, partially and transiently augmented the residual cone-ERG responses in CNGB3-mutant dogs. Similar results of transient augmentation of cone function were also reported in three achromat dogs during continuous release of CNTF by means of an encapsulated cells technology (ECT) implant in the vitreous (Komáromy AM, unpublished observation, 2013). On the basis of these results, a human phase I/II clinical trial was conducted using intravitreal CNTF secreting devices in five CNGB3-achromat patients who were evaluated across a 12-month period.[21] Although there was clear evidence of a CNTF effect on the study eyes, no objective indication of an improvement in the cone function was identified in any of the treated individuals. These results indicate that human cones appear to respond differently or have a different sensitivity to CNTF administration than do canine cones. Hence, the present demonstration that CNTF can augment cone-mediated function in CNGB3$^{-/-}$ mouse is further evidence of a species difference in the neurotrophic effects of CNTF.

The present study in mouse essentially confirms what was reported in the CNGB3 mutant dogs. However, due to the difference in vitreous volume between the two species,[32,33] the 1 μg CNTF dose injected into the mouse eye was proportionally 50-fold higher than that given to the dog (12 μg). Despite the dosing difference, the CNTF effect was more durable in canine retina. The improvement of the cone-ERG in mouse was lost by 2 weeks, whereas in dog the CNTF effect was still detectable at 2.5 weeks and disappeared only after 5 weeks. Given the short half-life of CNTF in vivo (2.9 minutes in plasma of rats)[34] it is unlikely that this difference results from longer persistence of CNTF in the canine vitreous but rather it could be ascribed to a different sensitivity to CNTF between the canine and the murine CNGB3-retina.

Ciliary neurotrophic factor dose-dependent effects on retinal function have been previously described.[35] In normal rats, increasing the dose of CNTF from 1 to 10 μg, (the latter equivalent to 1 μg in the mouse when corrected by vitreous volume[36]) had a larger effect on the photopic ERG responses. In an attempt to further increase the improvement in the photopic function that we observed after 1 μg CNTF, we injected a higher dose of CNTF into the CNGB3$^{-/-}$ mouse

Table. Study Design and ERG Data From CNTF- and PBS-Treated CNGB3$^{-/-}$ and WT Mice

				Scotopic Flash ERG										Photopic Flash ERG				Photopic Flicker ERG		Photopic Oscillatory Potentials	
				a-wave						b-wave				b-wave							
				R_{max}		S		t_d		V_{max}		k		V_{max}		k		Amplitude		Amplitude	
CNGB3$^{-/-}$ Mice, n	Age, d	ERG Test, d	Eye Treatment	µV	log IO diff	log s^{-1}	log IO diff	ms	log IO diff	µV	log IO diff	log cd-s/m^2	log IO diff	µV	log IO diff	cd-s/m^2	log IO diff	µV	log IO diff	µV	log IO diff*
24	28 ± 7	7	CNTF (1 µg)	744 ± 22	−0.02 ± 0.01	3.50 ± 0.02	−0.02 ± 0.05	4.05 ± 0.06	0.00 ± 0.01	1082 ± 49	−0.03 ± 0.02	−2.78 ± 0.02	0.10 ± 0.02	140 ± 6	0.05 ± 0.01	19.6 ± 1.2	−0.04 ± 0.02	29 ± 2	0.07 ± 0.01	46 ± 5	0.14 ± 0.03
			Untreated	788 ± 26		3.52 ± 0.02		4.08 ± 0.07		1144 ± 36		−2.87 ± 0.02		123 ± 4		21.6 ± 1.1		25 ± 2		32 ± 4	
17	28 ± 7	7	CNTF (2 µg)	773 ± 26	−0.03 ± 0.02	3.45 ± 0.01	−0.05 ±	3.95 ± 0.05	−0.02 ± 0.01	949 ± 62	−0.04 ± 0.02	−2.89 ± 0.04	0.11 ± 0.02	133 ± 7	0.04 ± 0.02	19.6 ± 1.2	−0.06 ± 0.02	32 ± 2	0.09 ± 0.02	43 ± 3	0.08 ± 0.03
			Untreated	842 ± 40		3.50 ± 0.01	0.01	4.13 ± 0.06		1057 ± 78		−3.00 ± 0.09		122 ± 6		22.7 ± 1.4		25 ± 2		32 ± 2	
18	31 ± 3	7	PBS	1034 ± 46	−0.00 ± 0.02	3.43 ± 0.03	−0.05 ± 0.03	3.80 ± 0.12	−0.01 ± 0.01	1187 ± 52	−0.03 ± 0.02	−2.96 ± 0.03	0.02 ± 0.01	106 ± 8	−0.03 ± 0.02	23.2 ± 1.8	0.00 ± 0.04	20 ± 2	−0.02 ± 0.03	25 ± 4	−0.05 ± 0.02
			Untreated	1027 ± 43		3.48 ± 0.02		3.94 ± 0.11		1282 ± 52		−2.94 ± 0.03		111 ± 8		24.2 ± 2.5		22 ± 2		33 ± 8	
Multiple comparison analysis of log interocular differences between groups, P value																					
			CNTF (1 µg) vs. PBS		*0.57*		*0.58*		*0.82*		*0.99*		*0.008*†		*0.005*†		*0.07*		*0.016*†		*0.000*†
			CNTF (2 µg) vs. PBS		*0.41*		*0.99*		*0.59*		*0.94*		*0.004*†		*0.02*†		*0.11*		*0.003*†		*0.006*†
			CNTF (1 µg) vs. CNTF (2 µg)		*0.44*		*0.44*		*0.12*		*0.88*		*0.88*		*0.95*		*0.98*		*0.67*		*0.222*
18	32 ± 7	14	CNTF (1 µg)	879 ± 37	0.02 ± 0.01	3.46 ± 0.02	−0.04 ± 0.03	3.86 ± 0.04	0.00 ± 0.00	1067 ± 74	0.00 ± 0.03	−2.83 ± 0.03	0.03 ± 0.03	114 ± 11	−0.02 ± 0.02	27.7 ± 3.4	0.00 ± 0.08	19 ± 1	0.00 ± 0.12	25 ± 2	0.06 ± 0.04
			Untreated	820 ± 26		3.49 ± 0.02		3.88 ± 0.05		1062 ± 63		−2.86 ± 0.02		117 ± 10		29.1 ± 3.4		19 ± 2		27 ± 5	
12	36 ± 8	14	PBS	912 ± 63	−0.03 ± 0.02	3.50 ± 0.03	−0.01 ± 0.04	4.17 ± 0.07	−0.01 ± 0.00	1046 ± 110	−0.05 ± 0.04	−2.93 ± 0.03	−0.02 ± 0.03	125 ± 11	−0.04 ± 0.02	20.1 ± 1.6	−0.03 ± 0.01	27 ± 2	−0.02 ± 0.01	25 ± 2	0.01 ± 0.03
			Untreated	968 ± 62		3.50 ± 0.05		4.25 ± 0.07		1155 ± 106		−2.91 ± 0.02		134 ± 8		21.5 ± 1.7		26 ± 2		26 ± 2	
t-test analysis of log interocular differences between groups at 14 d, P value																					
			CNTF (1 µg) vs. PBS		*0.08*		*0.65*		*0.32*		*0.32*		*0.34*		*0.52*		*0.68*		*0.74*		*0.48*

Table. Continued

				Scotopic Flash ERG										Photopic Flash ERG			
				a-wave						b-wave				b-wave			
				R_{max}		S		t_d		V_{max}		k		V_{max}		k	
WT Mice, *n*	Age, d	ERG Test, d	Eye Treatment	µV	log IO diff	log s^{-1}	log IO diff	ms	log IO diff	µV	log IO diff	log cd-s/m²	log IO diff	µV	log IO diff	cd-s/m²	log IO diff
15	34 ± 3	7	CNTF (1 µg)	715 ± 28	−0.03 ± 0.01	3.53 ± 0.01	−0.01 ± 0.01	3.57 ± 0.08	−0.01 ± 0.01	867 ± 47	−0.11 ± 0.01	−3.00 ± 0.03	0.05 ± 0.02	338 ± 15	−0.06 ± 0.01	3.32 ± 0.11	0.00 ± 0.02
			Untreated	771 ± 39		3.54 ± 0.02		3.66 ± 0.10		1127 ± 79		−3.06 ± 0.03		382 ± 16		3.36 ± 0.11	
10	34 ± 2	7	PBS	799 ± 75	−0.00 ± 0.02	3.58 ± 0.03	0.04 ± 0.03	4.17 ± 0.08	0.01 ± 0.01	953 ± 137	−0.03 ± 0.03	−2.90 ± 0.07	0.04 ± 0.04	325 ± 24	−0.01 ± 0.02	3.58 ± 0.35	0.01 ± 0.03
			Untreated	798 ± 37		3.54 ± 0.03		4.11 ± 0.09		965 ± 59		−2.95 ± 0.07		329 ± 16		3.49 ± 0.27	
t-test analysis of log interocular differences between groups, *P* value																	
			CNTF (1 µg) vs. PBS		*0.09*		*0.7*		*0.5*		*0.02*†		*0.88*		*0.04*†		*0.83*

All values are expressed as mean ± SE except for age, expressed as mean ± SD. All ERG parameters are described in the Materials and Methods. *P* values are given in italics. IO diff, interocular differences (treated eye–untreated eye in decibels).

* IO difference in photopic oscillatory potentials amplitude/photopic b-wave amplitude.

† Statistically significant value.

vitreous. However, neither 2 nor 4 μg (data not shown) of CNTF induced an additional change in retinal function in either photopic or scotopic conditions.

It may be that 1 μg CNTF is sufficient to saturate the retinal CNTF receptors in the mouse retina, so that increasing the dose further does not produce a greater effect.

The improvement of ERG photopic responses in achromat mice seemed to occur by a mechanism that is specific for CNGB3$^{-/-}$ retina. When administered to WT mice, CNTF reduced conversely the photopic b-wave amplitude, indicating that CNTF effects on cone-mediated function may depend on the functional/structural integrity of components of the cone pathway, which is preserved in the WT mouse but altered in the CNGB3$^{-/-}$ mouse in which cones are degenerating.

It is difficult to discern whether CNTF exerted its effects on cone photoreceptors or on postreceptoral components along the cone pathway. The photopic b-wave contains contributions from bipolar cells, mainly depolarizing bipolar cells, and Müller cells, but it also reflects cone function to the degree that cones drive the depolarizing bipolar cells activity.[26,31] Similarly, photopic flicker responses include an amalgam of components from cone photoreceptors, to depolarizing and hyperpolarizing bipolar cells.[37] Therefore, changes in the photopic b-wave amplitude and/or the flicker response can reflect modifications in the functional/anatomical integrity of any of these components.

Evidence exists that CNTF can act on photoreceptors, directly[38] or through the activation of Müller cells.[13] Ciliary neurotrophic factor administration[14] transiently "deconstructed" photoreceptors in Long Evans rats, reducing their outer segment length, downregulating the expression of proteins involved in the phototransduction cascade and consequently suppressing the ERG responses. The reduction in the photopic b-wave amplitude that we observed in WT mice after CNTF could therefore be explained by a "deconstruction" of cone outer segments. In CNGB3-mutant dogs injected with CNTF,[11] the re-elaboration of the cone outer segments after the deconstruction phase resulted in the assembly and translocation of partially functional CNGA3 homomeric channels to the cone outer segments. If this mechanism applies also to CNGB3$^{-/-}$ mice, it would explain the partial recovery in photopic b-wave amplitude that we observed, and why CNGB3$^{-/-}$ mice responded differently from WT mice to CNTF administration.

Alternatively, the changes in photopic b-wave could derive from CNTF action on postreceptoral components such as bipolar cells. Trophic factors, including brain-derived neurotrophic factor[39,40] and others,[41] are known to be bioactive on proximal retinal cells. The increase in amplitude of photopic oscillatory potentials in CNGB3$^{-/-}$ mice supports this hypothesis, as oscillatory potentials arise from circuitry in the inner retina involving amacrine, bipolar, and retinal ganglion cells.[42]

Our analysis of the rod-mediated function further supports the hypothesis that CNTF is active on retinal cell types other than photoreceptors.

Ciliary neurotrophic factor decreased the rod-mediated responses of both CNGB3$^{-/-}$ and WT mice, as evidenced by a reduction in the scotopic b-wave amplitude. However, we did not detect any change in the scotopic a-wave, indicating that CNTF did not affect rod photoreceptor function. These results concur with what we reported in the human achromat trial,[21] that CNTF decreased rod scotopic b-wave amplitude in all patients but not the scotopic a-wave amplitude, suggesting that the suppression of the scotopic responses was due to an effect on postreceptoral components of the rod-mediated pathway. In CNGB3$^{-/-}$ mice the CNTF effect on scotopic function was greater at lower stimulus intensities. Ciliary neurotrophic factor reduced the scotopic b-wave amplitudes at the lowest flash intensities, decreasing the sensitivity of the scotopic system, but did not affect ERG responses at brighter saturating stimuli. Several studies[43,44] have shown that the activity of cells in the proximal retina contributes proportionally more to the ERG responses at very low intensities. Thus it appears likely that the reduction of scotopic sensitivity in CNGB3$^{-/-}$ mice derives from CNTF action on the proximal retina.

The WT mouse response was somewhat different, as CNTF reduced the scotopic b-wave amplitude at both the lowest and highest flash intensities and decreased the saturated response without any significant change in the scotopic sensitivity. Why this is different from CNGB3$^{-/-}$ mice is not clear. However, because rod signals enter the cone pathway at several stages during visual processing,[45] it could be that an altered cone system, caused by abnormal signaling from degenerating CNGB3$^{-/-}$ cones, might influence the sensitivity of the rod-mediated responses to CNTF.

In conclusion, we found that bolus administration of CNTF transiently improved the residual cone-mediated function in CNGB3$^{-/-}$ mice. This confirms what was previously observed in the achromat dog. The mechanism by which CNTF produces these functional changes in achromatopsia requires further clarification. Though prior reports showed that CNTF effects photoreceptor structure and function, the present study indicates that CNTF modulates retinal function by acting on cells along the retinal signaling pathway proximal to photoreceptors.

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