Intraocular CNTF Reduces Vision in Normal Rats in a Dose-Dependent Manner

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PURPOSE. CNTF is a neuroprotective agent for retinal degenerations that can cause reduced electroretinogram (ERG) amplitudes. The goal of the present study was to determine the effects of intraocular delivery of CNTF on normal rat visual function.

METHODS. Full-field scotopic and photopic ERG amplitudes and spatial frequency thresholds of the optokinetic response (OKR) of adult Long-Evans rats were measured before and after intravitreal injection of CNTF or subretinal delivery of adeno-associated virus-vectorized CNTF (AAV-CNTF) into one eye. Visual acuity was also measured by using the Visual Water Task in AAV-CNTF-injected animals. Multiunit luminance thresholds were recorded in the superior colliculus after CNTF injection, and the eyes were examined histologically.

RESULTS. In eyes injected with a high dose of CNTF, ERG amplitudes and OKR thresholds measured through CNTF-injected eyes were decreased by 45% to 70% within 6 days after injection. ERG amplitudes had begun to recover by 21 days, whereas OKR thresholds only began to recover after 56 days. Neither OKR thresholds nor ERG amplitudes fully recovered until 90 to 100 days. When measured in the superior colliculus at 2 weeks after CNTF injection, luminance thresholds were elevated by 0.35 log units. In AAV-CNTF-injected eyes, OKR thresholds, and visual acuity were reduced by approximately 50% for at least 6 months, and scotopic and photopic ERG b-waves were reduced by 30% to 50%. Photoreceptor loss occurred in the injected regions in some of the eyes. By contrast, comparison of dose–response analysis with a dose–response study of light damage strongly suggests that therapeutic doses of CNTF exist that do not suppress ERG responses.

CONCLUSIONS. Intraocular delivery of CNTF, which preserves photoreceptors in animal models of retinal degeneration, impairs visual function in normal rats at very high doses, but not at lower doses that still provide protection from constant light damage. (Invest Ophthalmol Vis Sci. 2007;48:5756–5766) DOI:10.1167/iovs.07-0054

Several neuroprotective agents slow photoreceptor (PR) cell loss in animal models of inherited retinal degenerations (RDs).1-13 Ciliary neurotrophic factor (CNTF) appears to be mutation independent and the most universally effective thus far, as it slows PR degeneration in 12 different inherited RDs in mice,14-17 rats,18,19 dogs (Pearce-Kelling S et al., unpublished observations, 2004),25 and cats (Chong NHV et al. IOVS 1997;38:ARVO Abstract 1445)21 and protects PRs from the damaging effects of light in rats2 and mice.15 The relative lack of specificity of CNTF in its survival-promoting activity is potentially advantageous and has led, in part, to a recent phase I (safety) clinical trial using implants of encapsulated cells releasing CNTF in patients with retinitis pigmentosa22 using encapsulated cell technology described previously in the rd11 canine model of retinitis pigmentosa.20

A worrisome property of CNTF is that, whereas it rescues PRs from degeneration when delivered continuously after adenoassociated virus (AAV) vector transduction, it reduces scotopic (rod-dominated) and photopic (cone-dominated) electroretinographic (ERG) amplitudes,17,18 which are typically a measure of PR and retinal function. This effect appears to occur in a dose-dependent manner.17 The continuous delivery of CNTF also results in a reduction of some or all ERG responses in normal mice,23 rabbits,24 and rats (Liang FQ et al. IOVS 2003;44:ARVO E-Abstract 3585). Continuous delivery of CNTF has also been associated with structural changes in PRs17,24-25 and inner retinal cells,25,26 with outright loss of PR cells in some cases (Liang FQ et al. IOVS 2003;44:ARVO E-Abstract 3585), and these changes also appear to occur in a dose-dependent manner in mice (LaVail MM et al., unpublished observations, 2004).25

In contrast to continuous delivery, the intravitreal injection of CNTF or Axokine (Regeneron, Tarrytown, NY), a mutant form of CNTF, has also been shown to reduce ERG amplitudes transiently in dogs with cone–rod dystrophy (Luthert PJ, personal communication, 2001) and in normal rats.27 In both of these cases, the ERG suppression was evident by approximately 1 to 2 weeks after injection (rat and dog, respectively), but at 3 to 4 weeks after injection, the ERG amplitudes had...
returned to match those of the control eyes in the same animals. In the study with rats, transient changes in PR structure and PR phototransduction proteins also occurred.17,27

An important unanswered question about the suppression of ERG responses by CNTF is whether it reflects functional change in vision. In most instances, the reduction of ERG amplitudes follows the loss of vision in patients with inherited RDs, and so the suppression of ERG amplitudes by CNTF may be seen as a negative effect. In some cases, though, reduced or absent ERG amplitudes seem to have little effect on vision, such as in Duchenne muscular dystrophy.28,29 Moreover, it is not clear that CNTF-induced ERG alterations are a negative effect, since the agent can, in fact, protect PRs from cell death not clear that CNTF-induced ERG alterations are a negative effect, since the agent can, in fact, protect PRs from cell death.17,18,23 To understand the effect of CNTF-induced reduction of ERG responses on vision in the normal, mature rat eye, we used a virtual optokinetic system (VOS; Optomotry; CerebralMechanics, Lethbridge, Alberta, Canada30,31) to quantify spatial frequency thresholds of the optokinetic response (OKR), before and after intravitreous injection of CNTF, which results in transient suppression of ERG responses, and subretinal injection of AAV- vectored CNTF, which results in sustained suppression of ERG responses. In addition, we used the Visual Water Task32 to evaluate the effects of the AAV-vectored CNTF on perceptual spatial vision. The central physiological consequences of CNTF injection were also examined by measurement of luminance thresholds in the superior colliculus. After behavioral testing, the retinas were examined for the morphologic phenotypic features some have found to be associated with the application of CNTF.17,24,25 and the behavioral findings were correlated with measurements of ERG amplitudes.

MATERIALS AND METHODS

Animals and Injections

All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the institutional animal care committees at the University of Lethbridge, University of Utah, and University of California, San Francisco. Adult Long-Evans rats were maintained in a 12:12-hour light–dark cycle at an in-cage illuminance of <150 lux. Before injections, ERG measurements or physiological recording from the superior colliculus, rats were anesthetized with intramuscular injection of ketamine (13 mg/kg) and xylazine (87 mg/kg). The corneas were anesthetized with 0.5% proparacaine, and the pupils were dilated with 2.5% phenylephrine hydrochloride followed by 1.0% atropine.

To study transient effects of CNTF, intravitreous monocular injections of recombinant rat CNTF (R&D Systems, Minneapolis, MN) were made using a transcleral approach as described elsewhere,35 and the dose of 10 μg (2 μg/mL in 5 μL of PBS) was injected to be consistent with that used previously by Wen et al.27 After our initial study, we performed a dose–response analysis of the effects of intravitreously injected recombinant rat CNTF at doses of 1000, 100, 10, and 1 ng, each in a volume of 2 μL.

For comparison of the dose–response analysis of rat CNTF effects on the ERG responses and OKR thresholds to therapeutic doses of CNTF, we included the morphologic results of a dose–response study of the protective effect of rat CNTF performed several years ago by one of us (MML) in normal albino Sprague-Dawley rats 3 to 4 months of age. The recombinant rat CNTF for this study was a gift of Regeneron Pharmaceuticals and was injected intravitreously into one eye of rats at doses of 400, 200, 100, 50, 25, 10, 2, and 0.2 ng, each in a volume of 1 μL. Two days after the injection of CNTF, the rats were exposed to constant fluorescent light for 7 days at an illuminance level of 125 to 175 foot-candles, as described elsewhere.34

To study long-term effects of constitutively secreted CNTF, we made subretinal injections of AAV-vectored CNTF into the superior hemisphere of rat eyes as described.17 The AAV vector (serotype 2) with chicken β-actin promoter resulted in the expression of a secreted (DH) variant of human CNTF with an increased affinity toward CNTF receptors, which was the most potent survival-promoting form of several AAV-CNTF types in rescuing PIs in rd17/–p210L mice.17 Two microliters of the rAAV-CB-sDH-CNTF vector (hereafter, AAV-CNTF) with a titer of 1011 infectious particles per milliliter17 were injected into one eye of each rat. In initial experiments, we found that both intravitreous and subretinal PBS-injected eyes gave indistinguishable results from noninjected eyes, as measured by histology, ERG, and OKR testing. Therefore, noninjected eyes served as control samples for most of the experimental study.

Electroretinography

The rats were dark-adapted overnight and then, in dim red light, were anesthetized as described earlier. Bilateral, simultaneous, full-field scotopic ERGs were elicited with 10-μs flashes of white light, and responses were recorded with contact lens electrodes36 (UTAS-3000 Visual Electrodiagnostic System; LKC Technologies, Inc., Gaithersburg, MD). Scotopic stimuli were presented at an intensity of 0.4 log cd·s/m2 at 2-minute intervals, and the response to two successive flashes was averaged, followed by a single stimulus at 2.4 log cd/s/m2. The rats were then exposed to a background light of 29 cd/m2 for 10 minutes before photopic responses were recorded to stimuli presented at a rate of 2 Hz at 0.4 log cd·s/m2; 20 successive flashes were averaged. Responses were amplified at a gain of 4000, filtered between 0.3 and 500 Hz, and digitized at a rate of 2000 Hz on two channels. The amplitude of the a- and b-waves was measured. Scotopic a-waves were measured from the baseline to the peak in the cornea-negative direction in response to a stimulus of +2.4 log cd·s/m2, and the b-waves were measured from the cornea-negative peak to the major cornea-positive peak in response to a stimulus of +0.4 log cd·s/m2. ERG responses from the treated eye were compared to responses from the contralateral control eye for each animal.

Virtual Optokinetic System

OKR spatial frequency thresholds were measured once before injection and repeatedly after injection. The VOS measures the threshold of optokinetic tracking response to moving gratings.30,35 The apparatus consisted of four computer monitors positioned around a square testing arena. A sine wave grating was drawn on a virtual cylinder projected in three-dimensional coordinate space on the monitors (OptoMotry; CerebralMechanics), and the cylinder was rotated. In brief, an unrestrained rat was placed on a platform in the center of the arena. A video camera provided real-time video feedback from above, and the position of the head on each frame was used to center the hub of the cylinder continually at the rat’s viewing position. The cylinder was rotated at a constant speed (12°/s). On each trial an experimenter judged whether the rat made tracking movements with its head and neck to follow the drifting grating. The spatial frequency threshold, the point at which animals no longer tracked, was obtained by incrementally increasing the spatial frequency of the grating at 100% contrast. Thresholds through each eye were measured separately by reversing the rotation of the cylinder.30

Visual Water Task

The visual water task (VWT) is a visual-perception task described elsewhere in detail.37–40 Briefly, the apparatus consisted of a trapezoidal–shaped pool with two computer monitors facing through a clear glass wall into the wide end of the pool, and a midline divider extended into the pool from between the monitors, creating a Y-maze with a stem and two arms. Animals were trained to discriminate between a sine wave grating and gray of the same mean luminance displayed on the screens by a computer program (Vista; CerebralMechanics). Training and testing followed the same procedure as described elsewhere.37–39 except the animals were tested only once at approximately 180 days postinjection (PI) of AAV-CNTF. The animals were
tested in blocks of trials at progressively incrementing spatial frequencies until they could no longer distinguish between the visual stimuli at a minimum of 7 of 10 correct. Grating acuity was calculated as the 70% correct point on a cumulative normal curve fit to the data. Monocular testing required placing a small occluder over one eye during each trial, which was removed immediately after that trial. All testing was performed early in the light phase of the circadian cycle. Thresholds through each eye were obtained in sequence.

**Luminance Threshold Responses Recorded in the Superior Colliculus**

We measured luminance thresholds by recording multiunit neuronal responses in the superior colliculus contralateral to the tested eye, as described elsewhere. In short, for each position recorded in the superior colliculus, a discrete receptive field was localized and the brightness of a flashing spot, 3° in diameter projected on a hemisphere, was varied with neutral-density filters until a response was obtained that was double the background activity. This response was defined as the luminance threshold level. The measurements were performed 2 weeks after CNTF injection at the initial high dose of 10 μg (2 mg/mL in 5 μL of PBS). The luminance thresholds were measured separately in both colliculi, contralateral to treated and untreated eyes in the same animal in one recording session, to compare both eyes in the same individual rat.

**Histology and Morphometric Analysis**

After behavioral testing, animals received a lethal dose of sodium pentobarbital (Euthansol; Schering-Plough Animal Health, Pointe-Claire, Quebec, Canada; 0.3 mL/kg), and those killed after ERG testing or constant light exposure were killed with an overdose of carbon dioxide and then were immediately perfusion fixed with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in phosphate-buffered saline. Eyes were marked for orientation, removed, and then bisected along the vertical meridian and embedded in an Epon-Araldite mixture, with sections cut at a 1-μm thickness as described. Measurements of the outer nuclear layer (ONL) thickness, taken as an index of the number of PNs and rod outer segment lengths, were obtained from 54 locations around the retina as described. These 54 measurements were either averaged to provide a single value for each retina to allow statistical comparison of treatment and control groups or plotted as a distribution of thickness across the retina. For morphologic assessment of the therapeutic dose-response of CNTF in the light-dark experiments, the relative scale of 0 to 4+ rescue based on number of PNs surviving and PR integrity was used, as has been described.

**Statistical Analysis**

In most cases, the data between CNTF-injected and contralateral control eyes were compared by two-tailed paired Student’s t-test. OKR spatial frequency thresholds were compared at each time point for each rat by repeated-measures analysis of variance (ANOVA), with the Bonferroni post hoc test for ANOVA (P < 0.05) used to compare means at individual time points.

**RESULTS**

**Effects of Intravitreous Injection of CNTF**

The most thorough analysis of transient effects of intravitreously injected CNTF is that by Wen et al. To compare our findings with that work, in most of our studies described herein, we used the same large dose of 10 μg as was used by Wen et al. These findings are followed by dose-response analyses of CNTF injection.

**Functional Changes Measured with the ERG.** A group of 11 rats monocularly injected with 10 μg CNTF was initially followed for 28 days PI, since we anticipated an initial suppress-
Scotopic b-wave amplitudes remained below control values through 91 days PI, and were significantly different through 49 days and at 70 days PI (Fig. 1A). Photopic b-waves were the most severely affected both in the degree and duration of suppression. These remained below control levels throughout the test period and were significantly different from those of controls through 84 days PI, except at 63 days PI. Thus, the first time when all three ERG waves recovered from CNTF-induced suppression was at 91 days PI.

Optokinetic Response. The spatial frequency threshold of the OKR averaged 0.53 cycles per degree (cyc/deg) before injection (Fig. 1B), which was comparable to previously published values from the same strain.\(^\text{30}\) PI measures revealed that thresholds through CNTF-injected eyes dropped slightly to 0.51 cyc/deg after 2 days, and by 3 days the thresholds had declined to 0.45 cyc/deg (Fig. 1B), the first PI interval at which the thresholds were significantly different from those of controls \((P < 0.001)\). This decline continued until day 8 when the threshold reached 0.21 cyc/deg where it remained until PI day 56. Thereafter, the threshold began to recover. The spatial frequency threshold had recovered to near control values by 85 and 94 days PI, but still differed significantly \((P < 0.05)\). Full recovery was seen at 105 days (Fig. 1B) and beyond (data not shown).

Luminance Threshold Responses Measured in the Superior Colliculus. Multiunit recordings were made in five animals 2 weeks after intravitreous injection of CNTF in the right eye using the same doses as those used for the ERG and behavioral testing in the initial high-dose study. The left eye was an intact control. In each animal, the thresholds were measured at four to six points in each colliculus. In sum, we collected data from 29 points related to the injected eyes, and from 23 points related to the untreated eyes. The corresponding mean values (±SEM) of the thresholds were 0.64 ± 0.085 log units for the injected eyes, and 0.29 ± 0.023 log units for the untreated eyes. Thus, CNTF injection elevated the visual thresholds in treated eyes by 0.35 log units (∼2.2 times) in comparison with untreated eyes with a high level of statistical significance \((P < 10^{-7})\).

Morphologic Changes. We examined the retinas of three or more rats before and 6, 21, 28, and 199 days after intravitreous injection of 10 µg CNTF. At 6 days PI, the PR outer segments were clearly damaged, being irregular in caliber and somewhat tattered (Fig. 2B) compared with the smooth profiles of normal outer segments (Fig. 2A). The rod outer segments were also much shorter than normal, reduced to approximately 54% of their normal length (preinjection length, 23.6 ± 1.1 µm, mean ± SD; 6 days PI, 12.7 ± 0.8 µm; \(n = 3\); \(P < 0.0005\)). By 21 days PI (Fig. 2C) and thereafter, the outer segments had returned to their normal appearance and length (preinjection 23.6 ± 1.1 µm; 21 days PI, 23.8 ± 0.8 µm, \(n = 3\), \(P = 0.85\)). The outer segments were normal at the later ages, as well (data not shown). These changes are virtually identical with those reported by Wen et al.\(^\text{27}\) at 6 and 21 days PI.

The rod PR nuclei appeared normal at all PI times (Figs. 2B, 2C), and at no time did they show dispersed heterochromatin, giving a conelike appearance such as that seen with continuous delivery of CNTF in mice\(^\text{17}\) and rabbits.\(^\text{24}\)

Dose–Response Analysis of Transient Changes Induced by Intravitreous Injection of CNTF. Persistent suppression of ERG responses and reduction of spatial frequency thresholds of the OKR resulted from injection of 10 µg CNTF. Since the doses being tested for potential therapeutic use in humans are several orders of magnitude lower than the 10 µg used in the present study and by Wen et al.,\(^\text{27}\) we performed a log-unit step reduction dose–response analysis of ERG responses and OKR threshold measurements at 6 and 21 days PI.

As already noted, the 10-µg (10,000 ng) injection resulted in a large reduction in the OKR threshold and in all ERG responses 6 days PI, most of which had not returned to normal values at 21 days PI (Fig. 3A). After a 1000-µg dose (Fig. 3B), all the measured parameters were similarly affected at 6 days PI, but by 21 days PI, all three of the ERG responses had recovered to levels not significantly different from those of control eyes. The OKR threshold, while not as reduced as it was with the higher dose, nevertheless was still affected at 21 days PI (Fig. 3B). At a dose of 100 ng (Fig. 3C), the ERG responses at 6 days PI appeared somewhat reduced, but were not significantly different from control values, and by 21 days PI, all the ERG responses were at normal levels (Fig. 3C). At doses of 10 ng (Fig. 3D) and 1 ng (Fig. 3E), the ERG responses were unaffected by the CNTF injection.

The OKR thresholds were reduced at lower doses of CNTF than those affecting the ERG responses. At a dose of 1000 ng, for example, the OKR threshold was still reduced from normal at 21 days PI (Fig. 3B), whereas the ERG responses no longer were significantly different from that of the control. Similarly, at both PI intervals at a dose of 100 ng (Fig. 3C) and after 6 days PI at a dose of 10 ng (Fig. 3D), OKR thresholds were still reduced, whereas the ERG responses were not different from control values. Neither OKR thresholds nor ERG responses were affected by the dose of 1 ng (Fig. 3E).

Dose–Response Analysis of Protection of the Retina from Constant-Light Damage by Intravitreous Injection of CNTF. The injection of 100, 200, and 400 ng CNTF gave maximum protection from the damaging effects of a 7-day constant light exposure (Fig. 4). The dose of 50 ng was slightly less protective, but was still significantly indistinguishable from that produced by 400 ng. The dose of 25 ng provided significantly less protection than the maximum dose \((P < 0.005)\), but it gave a relative score of 2+ for the degree of protection (Fig. 4), which is considered moderate protection.\(^\text{35}\) Lower doses of 10, 2, and 0.2 ng gave progressively less rescue, but while the protection afforded by 10-ng dose was minimal, it was significantly greater than that provided by the 0.2-ng dose \((P < 0.05)\).

Effects of Subretinal AAV-CNTF Injection

Functional Changes Measured with the ERG. Rats injected subretinally with AAV-CNTF were examined by ERG at 42 and 95 days PI. As shown in Figure 5A, all ERG amplitudes were approximately 20% to 40% lower than in contralateral control eyes at 42 days PI and approximately 20% to 50% lower at 95 days PI, although at the latter PI interval, the scotopic
a-waves were not significantly different from control values. As with the intravitreous CNTF injections, the photopic b-waves were the most significantly depressed of the different ERG responses (Fig. 5A).

**Optokinetic Response.** Spatial frequency thresholds were obtained before injection, and all eyes tested returned normal values (0.53 cyc/deg, n = 12). Thresholds obtained after injection were very close to normal through 11 days PI (0.50 cyc/deg; Fig. 5B) but were already significantly lower than normal by 11 days PI (P < 0.01). By 14 days PI, however, the thresholds had fallen rapidly to 0.390 cyc/deg (Fig. 5B). The thresholds continued to decrease rapidly to 0.210 cyc/deg by 18 days to 50% of normal (P < 0.003), where they remained unchanged until at least 180 days PI, the longest PI interval examined (Fig. 5B).

A second group of animals with subretinal AAV-vectored CNTF was tested at 120 days PI and also showed a significant impairment in acuity (0.300 cyc/deg), although slightly less than the previous group (data not shown). Noninjected eyes of all rats had normal thresholds (0.53 cyc/deg) at all points tested.

**Acuity.** The perceptual visual acuity of five animals that received AAV-vectored CNTF was measured binocularly and monocularly through each eye separately at 6 months PI by using the VWT. All animals learned the task readily and had no obvious behavioral deficits in task performance. Acuity measured binocularly and monocularly from noninjected eyes was approximately 1.0 cyc/deg (Fig. 6), which is comparable to previous data generated in Long-Evans rats. However, acuity measured monocularly through the injected eye (Fig. 6) was significantly lower (0.500 cyc/deg, P < 0.003).

**Morphologic Changes.** A group of five rats that had been injected subretinally in the superior hemisphere with AAV-CNTF and that showed suppressed ERG amplitudes (Fig. 5A) and reduced spatial frequency thresholds at 180 days PI (Fig. 5B) was examined histologically at 194 days PI. The retinas of two of the injected eyes appeared normal throughout the retinal section along the vertical meridian (cf. Figs. 7A, 7B; Fig. 8). However, three of the retinas showed loss of PRs across at least half of the superior hemisphere (Fig. 8), with reduction of the ONL to one to three rows of nuclei (Figs. 7C, 8). Despite the significant loss of PRs in some regions near the subretinal injections, the overall ONL thickness of the five injected eyes (25.8 ± 2.6 μm, mean ± SEM) was only slightly less than that of noninjected controls (25.3 ± 0.6 μm) and did not differ significantly (P = 0.16).
Most of the PRs in the severely depleted areas of the ONL showed dispersed heterochromatin, giving them a conelike appearance, and these cells were also present in the transition zones between the most degenerated and normal regions of retina (Fig. 7D). However, none were present in regions of relatively normal retina, such as that shown in Figure 7B or at the far right of Figure 7C. Since continuous delivery of CNTF results in an increased thickness of the inner retina in dogs, we measured the inner retina of the eyes injected with AAV-CNTF as we did for the ONL. The thickness of the inner retina in the eyes injected with AAV-CNTF was indistinguishable from that in the control eyes, regardless of whether the entire retina was compared from all five injected animals or from the three injected animals that showed loss of the ONL, measuring the INL only in the regions of the ONL loss (data not shown).

Using immunohistochemistry, Rhee et al. have found that the same AAV-CNTF vector used in the present study causes an increase in the number and a change in the distribution of bipolar and Müller cell nuclei in the degenerating retinas of rd11/rd11 transgenic mice. Although we could not use cell-specific staining with the plastic-embedded sections in the present study, we found changes in the inner nuclear layer of the normal rats injected with AAV-CNTF that were consistent with those found by Rhee et al. at least for Müller cell nuclei that can be identified in plastic sections. Whereas most Müller cell nuclei in normal rat retinas are situated in the middle or middle-to-out outer part of the INL (Fig. 7A), many were found distributed throughout the INL, both in regions of otherwise normal retina (Fig. 7B) and in partially degenerated regions (Fig. 7C).

**DISCUSSION**

We found that intravitreous injection of a high dose of CNTF (10 μg) results not only in transient suppression of ERG responses, but also functional changes in vision in normal rats as demonstrated by transient reduction of OKR thresholds and reduction of luminance threshold responses measured in the superior colliculus. In addition, continuous delivery of AAV-vectorized CNTF results in sustained reduction in ERG responses and OKR thresholds for at least 6 months, as well as reduced visual acuity measured with the VWT.

The precision with which the decline in OKR thresholds paralleled the suppression of the ERG responses after CNTF injection (Fig. 1) was remarkable, and although the ERG responses showed a more rapid partial recovery than did the spatial frequency thresholds, both returned to normal at about the same time, 91 (for all ERG responses) and 105 days after injection, respectively. The reduction in spatial frequency thresholds after subretinal AAV-CNTF injection occurred between 11 and 18 days after injection, which is precisely the time required for full gene expression of AAV2-vectorized markers such as green fluorescent protein. Thus, the OKR can be

**FIGURE 4.** Dose–response analysis of protection of the retina of albino Sprague-Dawley rats from constant light damage due to intravitreous injection of rat CNTF. CNTF was injected 2 days before a 7-day exposure to light. Doses of 50, 100, and 200 ng were not significantly different from the highest dose of 400 ng, and thus all gave maximum protection. A dose of 25 ng gave moderate protection, but was significantly less effective than the maximum dose (P < 0.005). Doses of 10 ng and lower gave progressively less protection, but protection from the 10-ng dose was significantly greater than the 0.2-ng dose (P < 0.05). For each dose, n = 5 or 6.

**FIGURE 5.** Changes in the ERG and OKR frequency thresholds after subretinal injection of AAV-CNTF. (A) Maximum ERG amplitudes of injected eyes compared with control eyes measured simultaneously. At 42 and 95 days PI, all the ERG waves in the injected eyes were reduced compared with those of control eyes, although scotopic a-waves were not significantly different (NS) at 95 days. (B) OKR spatial frequency thresholds measured through eyes injected subretinally with AAV-CNTF compared with noninjected control eyes. The thresholds of the injected eyes appeared almost normal until 11 days PI, although they were already significantly lower than normal (P < 0.01) and dropped rapidly between 11 and 18 days PI, after which they remained consistently at approximately 0.210 to 0.233 cyc/deg for up to 180 days PI. The measurements up to 49 days PI are from a group of nine rats, and the measurements at 180 days are from five different rats. At 18 days PI and thereafter, the injected eyes differed from control values significantly (P < 10⁻⁵).
used with high precision and sensitivity to rapidly and noninvasively assess the positive and negative effects of vision-based therapies.

CNTF signaling involves binding of the cytokine to receptor-associated Jak kinases, leading to phosphorylation of members of the signal transducer and activator of transcription (STAT) family, STAT1 and -3, as well as the triggering of signal-related kinase (p42/44 ERK).47,48 When the same AAV-CNTF used in the present study was injected subretinally into transgenic *rds*+/−/P216G mice, persistent cytokine signaling was seen 65 days later in the retina, as elevated levels of phospho-STAT1 and -3 and phospho-ERK1 and -2 were demonstrated by immunocytochemical staining and Western blot analyses.26 Moreover, Bok et al.17 have shown in these same mice that all ERG responses were significantly and persistently reduced after AAV-CNTF injection. Thus, in the present study, it was not surprising to find persistent ERG and visual behavioral changes in the rats injected with AAV-CNTF. However, it was most unexpected to find an extremely long recovery time of 91 to 105 days after intravitreal injection of CNTF, particularly since previous studies have shown CNTF to be cleared from the retina between 2 and 4 days87,49 and only transient elevation of relevant signaling molecules. After intravitreal injection of CNTF, pERK is elevated for less than 6 hours,50 STAT1 and -3 phosphorylation signals are seen for only up to 4 days,47 and phosphorylated MAPK (a member of the ras-MAPK signaling pathway) was only activated at 1 hour in rats.47 With the same high dose (10 μg) of intravitreally injected CNTF that we used, Wen et al.27 found an increase in STAT3 phosphorylation for as long as 6 days, with a gross reduction by 3 weeks, far less than the recovery time for the ERG and OKR (Fig. 1). In each of these and other studies and in studies showing that CNTF receptors are localized primarily on Müller cells and not on PRs in rodents,51 it is strongly suggested that Müller cells are the target of CNTF in the retina and that these cells initiate neuroprotective signaling of undetermined nature.27,47 This same undefined and perhaps long-lived signal may also mediate the suppression of ERG responses and spatial frequency threshold.
We have performed a formal dose–response study of intravitreally injected CNTF (Fig. 3), and the results were instructive in several ways. First, unlike the high (10,000 ng) dose, it was apparent that the ERG suppression was transient and short lived at a dose of 1,000 ng, and that none of the ERG waves was suppressed at doses of 100 ng or less. Second, the study demonstrated a clear segregation of the effect of CNTF on the ERG and OKR. The OKR was more sensitive to the effects of CNTF than was the ERG, with significant reduction in spatial frequency thresholds of the OKR at a 1-to 2-log-unit lower dose than that affecting the ERG. This greater effect on the OKR in the dose–response study is consistent with that seen with the very high dose (Fig. 1), in which the OKR thresholds remained at a very low level for a prolonged period, whereas the ERG responses partially recovered sooner. The separation of ERG responses and OKR thresholds after CNTF injection indicates that the two measures may not be tightly linked, but the mechanisms for this remain to be explored.

Third, the dose–response study provides a possible explanation of the prolonged recovery time after high-dose CNTF injection compared with the full recovery seen by 21 days by Wen et al.27 We have used recombinant rat CNTF rather than recombinant human CNTF used by Wen et al., and rat CNTF has four to five times the potency in survival-promoting activity than the human form.59 In our present study, reduction of CNTF dose by 1 log unit (to 1000 ng) resulted in full recovery...

**Figure 7.** Light micrographs of the retinas of Long-Evans rats at 194 days PI that were either normal, uninjected eyes (A) or had received a subretinal injection of AAV-CNTF into the superior hemisphere of the eye (B–D). (A) The control retina had the normal complement of PR nuclei in the ONL, and most of the densely staining Müller cell nuclei that typically conformed to the shape of the other nuclei of the INL were located in the middle or middle-to-outer part of the INL (arrowheads). (B) Example of one of the AAV-CNTF-injected retinas that had no apparent loss of PRs, but had some Müller cell nuclei located in the inner aspect of the INL (arrowheads). (C) Retina that had lost most of the PRs (left side) with a transition to almost normal retina (right side) and that showed atypical distribution of Müller cell nuclei (arrowheads). (D) Higher magnification of a transition zone where some rod PRs had the typical single large clump of heterochromatin with “moth-eaten” edges and densely staining euchromatin (arrows), but where most rod PR nuclei had dispersed heterochromatin, giving a spotty, cone-like appearance. True cones comprised only approximately 1.5% of the PR population in the normal rat. Quantitative measures and distribution of PR loss are presented in Figure 8.
one hand, these doses are probably below the threshold for ERG suppression, even in the present study, but in addition, these doses in the human trials were diluted approximately 200 times more than in the rat eye due to differences in eye size (vitreous volume approximately 4 mL in human eye, 20 μL in rat eye). Moreover, rat CNTF was used in the present study, which is four to five times more potent than human CNTF50 in the clinical trial. On the other hand, the CNTF in the human trials is released continuously, and the consequences of even low-dose continuous release is difficult to assess. Second, there are species differences reported in the localization of CNTF-Ro to PR cells, and rodent retinas may differ from human retinas in this regard.51 Third, it is probable that degenerating PR cells respond differently to various agents than do normal PRs. Zeiss et al.25 found that continuous application of CNTF caused greater morphological alterations in red-1 dog retinas than in wild-type dog retinas. However, although AAV-vectored CNTF suppresses ERG responses in wild-type mouse retinas,23 we have found that the same AAV-CNTF vector used in the present study can cause overt PR degeneration in the same strain (C57BL/6) of wild-type mice in the region of injection (and presumably highest concentration of CNTF; LaVail MM et al., unpublished observations, 2004). The same vector never resulted in this cell loss in rd8+/v−P216G mouse retinas,17 adding further to the idea that normal retinas may be more sensitive to the negative effects of high doses of CNTF than are degenerating retinas. Fourth, in the recent human clinical trial, in contrast to our findings several patients receiving CNTF-releasing implants actually showed improved visual acuity that was maintained for 6 months after the trial.22 Thus, there is still much to learn about the protective and possible negative actions of CNTF and how they relate to retinas undergoing inherited RDs. It is clear that very high doses of CNTF applied to the retina can cause reduction in ERG response amplitudes, elevation of luminance thresholds as measured at the superior colliculus, reduction in visual acuity and behavioral tracking responses and, in some cases, outright loss of PRs. Thus, therapeutic application of CNTF in RDs should be closely monitored to prevent these negative effects, should they occur in the human retina. However, our dose–response studies strongly support the notion that therapeutic doses of CNTF exist that do not suppress ERG responses.24

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References

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FIGURE 8. ONL thickness is shown across individual retinal sections through the vertical meridian of one normal, noninjected control eye (solid symbols) and four eyes injected subretinally with AAV-CNTF. One of the retinas showed no reduction in ONL thickness across the retina (open squares), and the retina from another rat in this cohort had a similar pattern (data not shown), despite having reduced ERG amplitudes and behavioral responses. The injected retinas from three of the rats had regions of reduced ONL thickness (i.e., loss of PRs) for variable lengths of retina in the superior hemisphere (open circles, triangles, and diamonds).
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