CNTF Attenuates Vasoproliferative Changes Through Upregulation of SOCS3 in a Mouse-Model of Oxygen-Induced Retinopathy

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Purpose. Retinal vascular disease represents a major cause for vision loss in the Western world. Recent research has shown that neuronal and vascular damage are closely related in retinal disease. Ciliary neurotrophic factor (CNTF) is a well-studied neurotrophic factor that is currently being tested in clinical trials for the treatment of retinal degenerative diseases and macular telangiectasia. However, little is known about its effect on retinal vasculature. In this study, we investigate the effects of CNTF in retinal neovascular disease using the mouse model of oxygen-induced retinopathy (OIR).

Methods. Newborn pups were exposed to 75% oxygen from postnatal day (P)7 to P12 and subsequently returned to room air. Ciliary neurotrophic factor was injected intravitreally at OIR P12 and the vaso-obliterated and neovascular areas were quantified at OIR P17. Immunohistochemistry, RNA, and protein analysis were used to identify CNTF-responsive cells. In vitro experiments were performed to analyze the effect of CNTF on endothelial and astroglial cells.

Results. In the OIR model, CNTF facilitated capillary regrowth and attenuated preretinal neovascularization in a dose-dependent manner. The protective effect of CNTF was mediated via activation of the JAK/STAT3/SOCS3 signaling pathway. Immunohistochemical studies identified endothelial cells among others as CNTF-responsive cells in the retina. In vitro studies confirmed the anti-angiogenic effect of CNTF on endothelial cell sprouting.

Conclusions. This study provides evidence for a therapeutic potential of CNTF beyond degenerative retinal disease. Vasoproliferative retinopathies may benefit from a CNTF-dependent and SOCS3-mediated angiomodulatory effect.

Keywords: CNTF, OIR model, revascularization, angiogenesis, proliferative retinopathy

Retinal vascular diseases are a major cause of vision loss in the Western world and the most common cause of childhood blindness worldwide.[1] Recent research demonstrated that neurovascular crosstalk is crucial for retinal development and to maintain retinal health.[2–5] Thus, disease concepts and therapeutic approaches focusing only on either the neuronal or vascular origin of retinal diseases are likely to be incomplete. Diabetic retinopathy and macular telangiectasia, for example, were originally understood as primarily vascular diseases. Accumulating evidence of impaired neuronal and glial cell function now emphasizes the neurodegenerative component of these diseases.[6–10] Future therapeutic concepts should therefore address vascular as well as neuroprotective aspects of retinal diseases.

Ciliary neurotrophic factor (CNTF) is one of the best studied neurotrophic agents in retinal disease.[11] It belongs to the Il-6 family of neuropoietic cytokines like leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin 1 (CT-1). Ciliary neurotrophic factor was first isolated from chick eyes after observing that chick embryo extract supported ciliary neuron survival in 1979.[12,13] During retinal development, CNTF is expressed by Müller cells.[14] In addition, CNTF is expressed by various glial cells in the central nervous system in response to retina injury.[15–17] Ciliary neurotrophic factor has potent trophic effects on neurons and oligodendrocytes, as well as mesenchymal cells, including adipocytes, muscle, and bone cells (for review see Ref. 18). Due to its neurotrophic properties, CNTF has been tested in clinical trials for the treatment of Huntington disease[19] and amyotrophic lateral sclerosis (ALS).[20] Treatment inefficacy and side effects like nausea and weight loss represented fundamental problems in systemic administration of CNTF. However, local administration of CNTF in the eye has yielded better results. Preclinical studies in more than 12 animal models from 4 different species provide strong support for the neuroprotective effect of CNTF on photoreceptors and ganglion cells in the retina[21–25] (for review see Ref. 11). Intravitreal CNTF is currently tested in clinical trials for its effect in retinal degenerative disease[26–28] and macular telangiectasia.[29] However, many questions regarding the cellular mechanisms mediating CNTF-triggered neuroprotection in

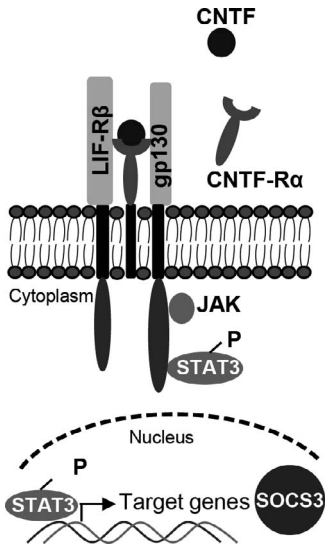

Figure 1. Schematic diagram of CNTF signaling. CNTF-induced intracellular signaling is mediated through a heterotrimeric receptor complex consisting of CNTF-Rα, LIF-Rβ and gp130. Ciliary neurotrophic factor–Rα exists in form of a membrane-bound or soluble receptor. Following formation of the CNTF/CNTF-Rα complex LIF-Rβ and gp130 are recruited and STAT3 is phosphorylated (modified after Wen et al.[11]). Activity of JAK/STAT3 induces transcription of target genes including the negative feedback regulator SOCS3.[35,43] Signaling of CNTF can be activated in all cell types expressing LIF-Rβ and gp130, if soluble CNTF-Rα is present in the tissue and incorporated into the cell membrane (see soluble CNTF-Rα in the diagram).

diseased retina remain open. Recent work by Rhee et al.,[30] for example, showed that CNTF-mediated photoreceptor rescue requires initial activation of Müller cells. Ciliary neurotrophic factor may hence affect more cell types in the retina than just neurons. More research is needed to fully evaluate the potential benefits of CNTF treatment, including its effects on the glio-/neurovascular unit.

Ciliary neurotrophic factor activates intracellular signaling through a heterotrimeric receptor complex consisting of CNTF receptor α (CNTF-Rα), leukemia inhibitory factor receptor β (LIF-Rβ), and glycoprotein 130 (gp130)[31] (Fig. 1). While LIF-Rβ and gp130 are expressed in many different cell types, CNTF-Rα expression is primarily observed in nervous tissue and skeletal muscle. The receptor CNTF-Rα itself lacks an intracellular signaling domain. It can be anchored to the plasma membrane via a glycosylphosphatidylinositol linkage.[32] Alternatively, CNTF-Rα can be released by cleavage through phospholipase C and serve as a soluble mediator of the effects of CNTF in vivo.[33] The receptor complex of CNTF activates the Janus tyrosine kinase/signal transducers and activators of transcription (JAK/STAT) pathway.[34] Signal transducer and activator of transcription 3 (STAT3) is preferentially phosphorylated and subsequently translocates to the nucleus to induce gene expression, including expression of the negative feedback regulator suppressor of cytokine signaling-3 (SOCS3).[35] Immunohistochemical localization of CNTF-induced phosphorylation of STAT3 (pSTAT3) thus represents a widely used technique to identify CNTF-responsive cells in vivo.[11,34]

In this study, we investigate the therapeutic potential of CNTF for retinal vascular disease. The mouse model of oxygen-induced retinopathy (OIR)[36] is used as a disease model to analyze vessel loss, physiologic vessel regrowth, and pathologic neovascular proliferation. In this model, intravitreal injection of CNTF led to a significant decrease of retinal neovascularization at postnatal day (P)17. The underlying mechanisms were found to be in part due to pSTAT3-/SOCS3-mediated direct antiangiogenic effects of CNTF on endothelial cells.

Materials and Methods

Animal Models

We treated C57BL/6 mice in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision research and experiments were approved by the local animal welfare committee. We used the OIR model as a model for neovascular retinopathy as previously described.[36] In brief, mice were exposed to hyperoxia (75% oxygen) between P7 and P12 and subsequently transferred to room air. Mice were euthanized by cervical translocation and eyes collected between P12 and P17 for analysis. Quantification of the vaso-obliterated (VO) and neovascular (NV) area were performed according to established techniques.[37] Vaso-obliterated and NV rescue were determined per mouse, comparing the amount of VO and NV of the CNTF-treated eye to the control-treated eye ([1—VO CNTF-treated eye/VO control treated eye] × 100). In all experiments, CNTF (rrCNTF, #557-NT-050; R&D Systems, Wiesbaden-Nordenstadt, Germany) was administered intravitreally once using a 33-gauge needle. The dose of CNTF used for intravitreal injection varied as described in the text. Fellow eyes injected with 0.1% BSA in PBS served as intraindividual controls. Statistically significant changes in VO and NV were determined using unpaired two-tailed Student's *t*-tests with correction for multiple testing where appropriate. Values of $P < 0.05$ were considered statistically significant.

Immunohistochemistry, RNA, and Protein Analysis

For flatmount preparation, eyes were fixed in 4% paraformaldehyde and stained with commercial glygoprotein (Isolectin Gs-Ib4, #I21412; Life Technologies, Darmstadt, Germany) overnight at 4°C. Cryosections were fixed for 10 minutes in ethanol. The following primary antibodies were used: goat anti-CNTF-Rα (#sc-1914; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); rabbit anti-collagen IV (#ab19808; Abcam, Cambridge, MA, USA); and pStat3 rabbit mAb (#9145; Cell Signaling Technology, Inc., Danvers, MA, USA). For quantitative (q)PCR analysis, RNA was isolated from single retinas or cells grown in 6-well plates. We reverse transcribed RNA into cDNA for qPCR with the primers listed in the Supplementary Methods section. For Western blot analysis, retinal lysates were blotted onto nitrocellulose membranes and incubated with the following antibodies: anti-bActin Ab, mouse monoclonal (#A1978; Sigma-Aldrich Corp., St. Louis, MO, USA); Stat3 (79D7), rabbit mAb (#4904; Cell Signaling Technology, Inc.); pStat3 (Tyr705), and rabbit mAb (Cell Signaling Technology, Inc.). Retinal CNTF levels were determined using an ELISA set (Rat CNTF DuoSet ELISA, #DY557; R&D Systems).

Endothelial Spheroid Sprouting Assay

The endothelial spheroid sprouting assay was performed as previously published.[38,39] In brief, human umbilical vein endothelial cells (HUVECs, #C-12203; PromoCell, Heidelberg, Germany) were suspended in endothelial growth medium (EGM, #PB-MH-100-199; Pelo Biotech, Planegg, Germany) containing 10% FBS and 0.25% (wt/vol) carboxy-methylcellulose (#M0512, Sigma-Aldrich Corp., Darmstadt, Germany). A total of 500 cells formed one endothelial spheroid in a hanging drop. Spheroids were harvested the next day and 30 spheroids seeded into 0.5 mL collagen I (#354236; Corning, Wiesbaden,

Germany; final concentration of 1.5 mg/mL) in 24-well plates. Spheroid-containing gels solidified at 37° and 5% CO_2 for 1 hour. Endothelial spheroids were photographed 24 hours after stimulation with 12.5 ng/mL hVEGF 165 (#293-VE; R&D Systems), 833 ng/mL rrCNTF, and/or 1667 ng/mL rrCNTF-Rα diluted in 0.1 mL serum-free endothelial basal medium. Endothelial cell sprouting was quantified using ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Results are expressed in percent, normalized to the average sprout length of VEGF-treated controls.

In Vitro CNTF Stimulation Experiments

HUVECs, human retinal microvascular endothelial cells (HRMVECs, # PB-CH-160-8511, Pelobiotech); and human fetal astrocytes (#PB-882-05f; Pelobiotech) were seeded into six-well plates and grown to confluency. Cells were stimulated with rrCNTF (100 ng/mL), rrCNTF-Rα (#558-CR, 200 ng/mL, R&D Systems) or CNTF plus rrCNTF-Rα for 6 hours before RNA was collected as described above.

Results

CNTF Promotes Capillary Regrowth and Attenuates Retinal Neovascularization in OIR

Recombinant rat CNTF (rrCNTF) was injected intravitreally at OIR P12 to evaluate its effect on preretinal neovessel formation and capillary regrowth in a model of hypoxia-induced proliferative retinopathy. Figure 2A shows the effect of three different doses of rrCNTF (0.5 ng, 50 ng, and 500 ng) on the VO and NV area at OIR P17. Intravitreal injection of all doses resulted in a significant reduction in preretinal NV at OIR P17 while only 50 ng and 0.5 ng rrCNTF significantly decreased the VO area. Overall, six different doses of rrCNTF were tested to determine the maximum treatment effect of recombinant CNTF on VO and NV in the OIR model (see Supplementary Fig. S1 for bar graphs of all treatment groups). We determined VO and NV rescue by comparing CNTF-injected eyes to control-treated eyes (Fig. 2B). Intravitreal injection of 5, 50, or 250 ng of rrCNTF yielded the strongest rescue effects with a maximum of 43% rescue in VO and 96% in NV area. Higher or lower CNTF doses resulted in less pronounced NV rescue.

Since human recombinant CNTF protein is known to have a relatively short half-life when administered systemically (120–400 minutes following subcutaneous injection, 2.9 minutes following intravenous administration),[40,41] we next analyzed retinal CNTF levels following one single intravitreal injection of rrCNTF (Fig. 2C). In analogy to the previous experiment, one of three different doses of CNTF (0.5, 50, or 500 ng CNTF) was injected intravitreally at OIR P12 and retinas were collected 4, 24, or 48 hours later. Eyes injected with PBS/BSA served as controls to determine endogenous mouse CNTF levels. Endogenous baseline CNTF levels were 0.28 to 0.33 ng CNTF/mg protein for the three time points tested. Intravitreal injection of 500 and 50 ng rrCNTF strongly increased retinal CNTF levels to 26.4 and 3.3 ng CNTF/mg protein, respectively, 4 hours postinjection. Twenty-four hours after intravitreal injection, retinal CNTF levels were still increased to 1.0 and 0.8ng CNTF/mg protein. At 48 hours, retinal CNTF was at 0.45 ng CNTF/mg protein for both treatment groups. Intravitreal injection of 0.5 ng rrCNTF, a dose that only resulted in 40% rescue of NV compared with a 95% rescue with higher CNTF doses (see Fig. 2B), did not significantly increase retinal CNTF levels. However, it has to be noted that CNTF was injected intravitreally and CNTF levels were measured in total retinal explants since the mouse vitreous does not yield enough material for analysis. Taken together, these data suggest that intravitreal rrCNTF has a high potency in preventing pathological NV development and improving capillary regrowth (Fig. 2A). Furthermore, rrCNTF showed a relatively broad therapeutic range yielding maximal rescue effects at treatment doses between 5 and 250 ng (Fig. 2B). An increase in retinal CNTF levels was detectable for up to 48 hours after injection of doses above 50 ng.

Endogenous CNTF and CNTF-Rα Levels Are Significantly Upregulated in Response to Retinal Hypoxia

In order to better understand the role of endogenous CNTF under hypoxic retinal stress, we measured CNTF and CNTF-Rα expression during OIR. During the initial hyperoxic phase (P10 and P12, 0 hours), CNTF and CNTF-Rα levels remained unchanged (Fig. 3A). Within the first hours of hypoxia, however, mRNA levels of CNTF-Rα increased significantly (P12, 6 hours), followed by an upregulation of CNTF expression at OIR P14. Immunohistochemical stainings revealed a granular signal for CNTF-Rα at the level of the outer photoreceptor segments and the ganglion cell layer in sections of OIR P14 retinas (Fig. 3B). Higher magnification images localized CNTF-Rα expression in part to retinal endothelial cells. However, PCR analysis of cultured human endothelial cells and astrocytes showed that, in vitro, both cell types expressed LIF-Rβ and gp130 but not CNTF-Rα (Fig. 3C). This difference in CNTF-Rα expression between in vivo and in vitro might be explained by a change in gene expression patterns of both cell types in 2D-culture systems in vitro. Alternatively, both cell types might also lack CNTF-Rα expression in vivo and incorporate soluble CNTF-Rα or CNTF/CNTF-Rα complexes provided by their surroundings.[33]

CNTF Activates STAT3 Signaling and SOCS3 Negative Feedback Inhibition in the Inner Retina

Activation of the JAK/STAT3 signaling pathway represents a common downstream effector of CNTF receptor activation.[34,42] Retinas injected intravitreally with 0.5, 50, or 500 ng rrCNTF at OIR P12 displayed significant increases in pSTAT3 levels at OIR P13, confirming the STAT3 pathway as important mediator of CNTF in ischemic retinas (Fig. 4A). Retinas treated with 50 ng rrCNTF displayed the strongest activation of the STAT3 signaling pathway, matching the dose-response effect observed in CNTF-treated eyes (Fig. 2B). Importantly, low-dose rrCNTF (0.5 ng) also induced a significant 3-fold increase in pSTAT3, explaining why low-dose rrCNTF injections resulted in partial OIR recovery, although no increase in overall retinal CNTF-levels had been detected in retinal time-course experiments with this dose (Fig. 2C).

We next aimed to identify CNTF responsive cells and STAT3 downstream targets modulated by CNTF treatment in ischemic retina. Immunohistochemical staining in OIR P13 retinas localized positive pSTAT3 signals to the level of the retinal ganglion cell layer and the superficial vascular plexus 24 hours after intravitreal CNTF injections (Fig. 4B). Recently, SOCS3 has been described to play an important role as endogenous inhibitor of pathologic angiogenesis in endothelial,[43] neuronal, and glial cells.[44] At the same time, SOCS3 represents an established target of the STAT3 signaling pathway.[45] We hence hypothesized that the antiangiogenic effect of CNTF in the retina may be mediated by JAK/STAT3/SOCS3 signaling. Quantitative PCR analysis 24 hours post-CNTF treatment (OIR P13) confirmed significant upregulation of retinal SOCS3

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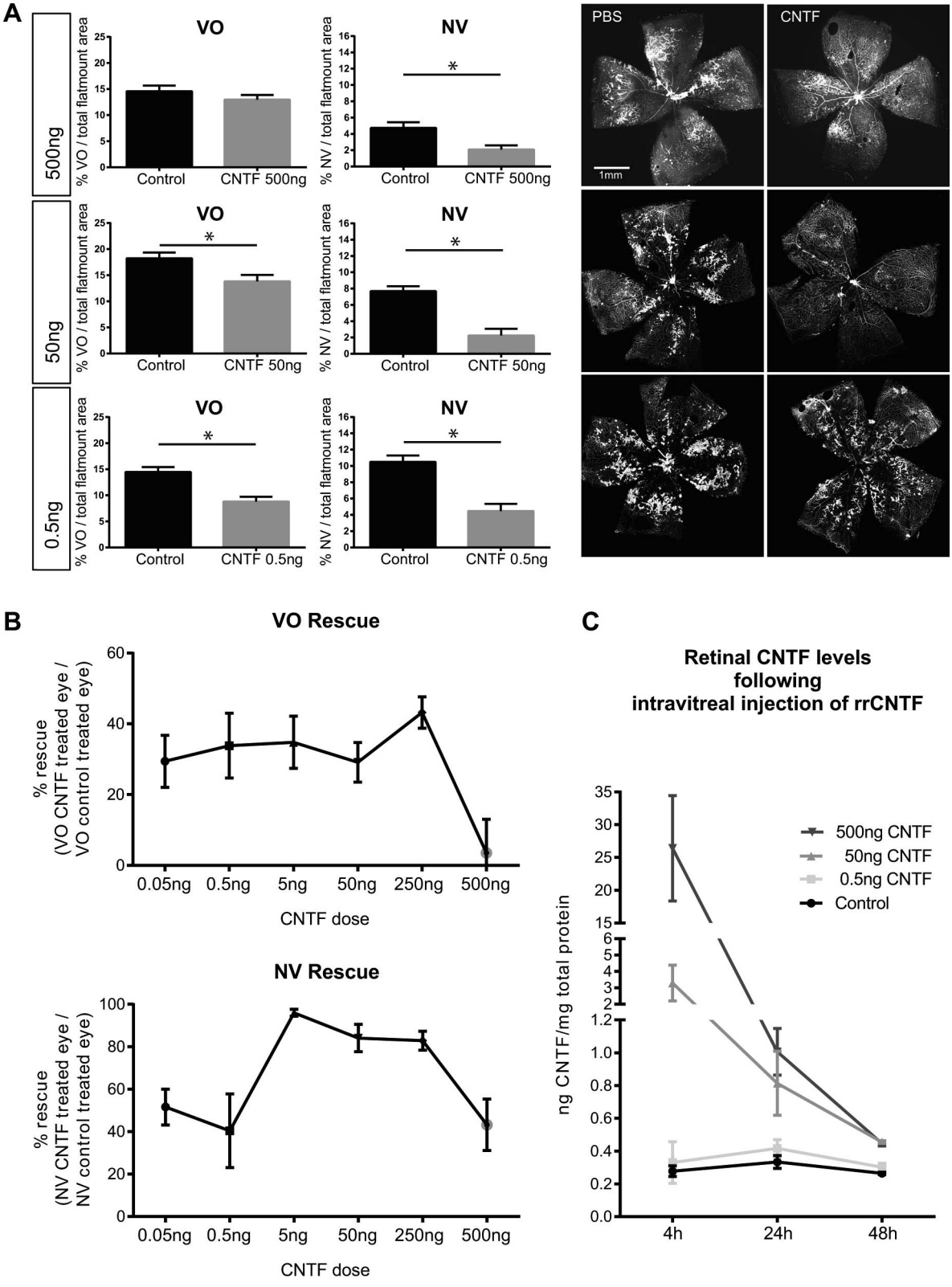

Figure 2. Ciliary neurotrophic factor promotes capillary regrowth and attenuates pathologic angiogenesis in OIR. Recombinant rat CNTF was injected intravitreally at different doses (0.05–500 ng) at OIR P12. Treatment with rrCNTF significantly decreased the area of VO and NV at OIR P17 at almost all doses tested (statistical significance is indicated by asterisk with $^*P < 0.05$; *Error Bars*: SEM). (**A**) Shows VO and NV values for a selection of three out of six dosages tested. For bar graphs of additional doses see Supplementary Figure S1. All treatment groups showed a significant decrease in preretinal NV. In addition, VO area was also significantly improved by rrCNTF treatment in all but the highest rrCNTF dose group. (**B**) Dose-response curve for the effect of rrCNTF on retinal VO and NV. Doses between 5 and 250 ng rrCNTF showed the strongest rescue effects. Graphs represent the VO and NV rescue effect as a function of rrCNTF dose: $n = 0.05$ ng, 10 mice; $n = 0.5$ ng, 12 mice; $n = 5$ ng, 11 mice; $n = 50$ ng, 14 mice; $n = 250$ ng, 12 mice; $n = 500$ ng, 16 mice. *Error bars*: SEM. (**C**) Time-course analysis of retinal CNTF levels measured 4, 24, and 48 hours after intravitreal injection of rrCNTF at OIR P12 (rat CNTF ELISA; $n = 3$ eyes per time and dose). *Error bars*: SEM. Intravitreal injection of 500

and 50 ng rrCNTF increased retinal CNTF levels for up to 48 hours. Intravitreal injection of 0.5 ng rrCNTF did not result in a significant increase in retinal rrCNTF measurements. It should be noted that retinal not vitreal levels were measured. Eyes injected with PBS/BSA served as control to determine baseline retinal CNTF.

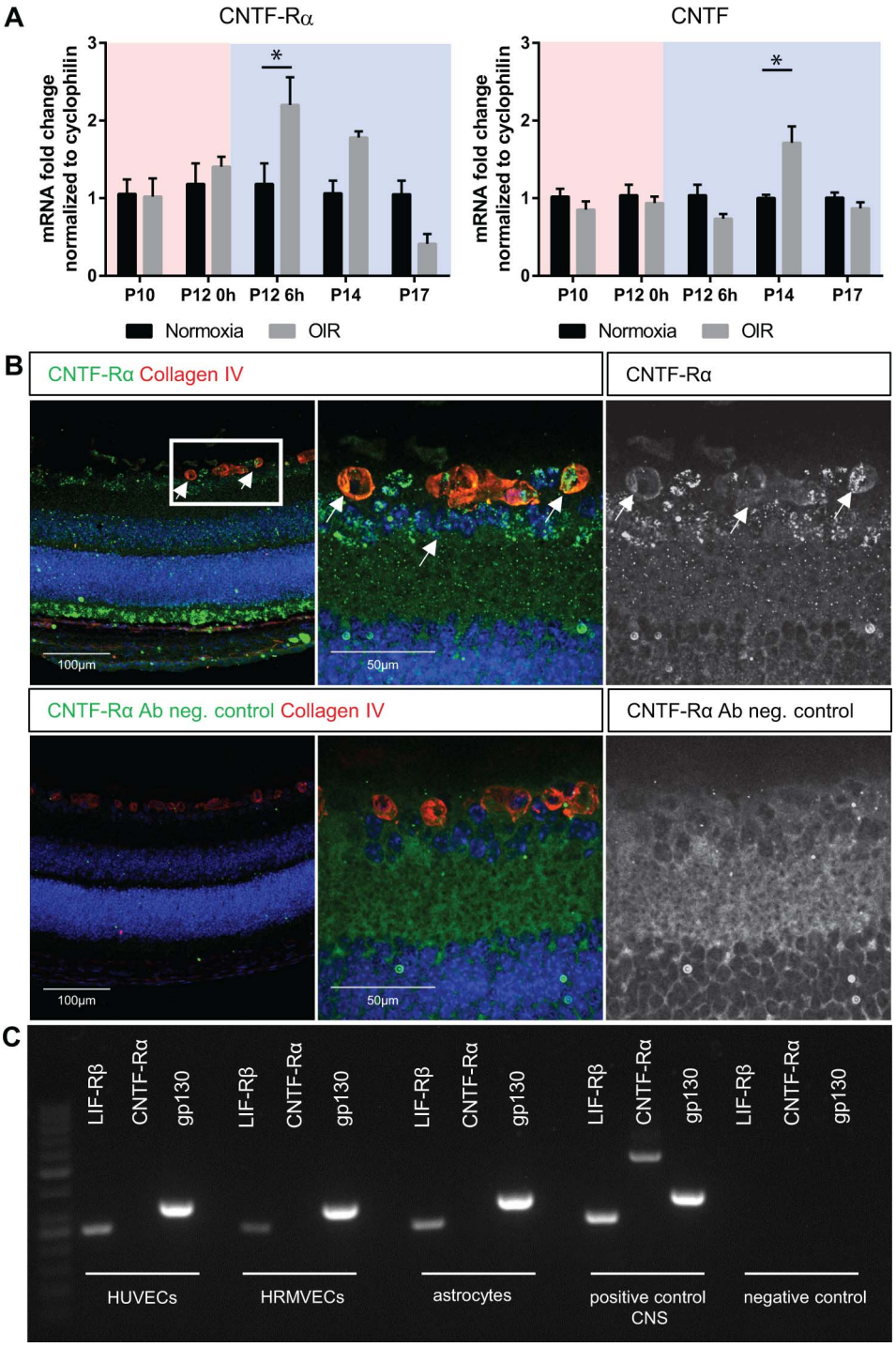

Figure 3. Endogenous CNTF and CNTF-Rα levels are significantly upregulated during the hypoxic phase of OIR. (**A**) Retinal CNTF and CNTF-Rα levels remained unchanged during the hyperoxic phase (P7-P12) of OIR. However, after onset of retinal hypoxia, both CNTF-Rα and CNTF were significantly upregulated. This finding indicates that retinal tissue increases endogenous CNTF and CNTFRα expression in response to hypoxic tissue injury (normoxia/OIR P10, P14, P15: n = five mice per group and time point; normoxia P12: n = six mice; OIR P12, 0 hours and OIR P12, 6 hours: n = four mice per time point; t-test corrected for multiple testing). *$P < 0.005$. (**B**) In cross-sections of OIR P14 retinas, expression of CNTF-Rα was detected at the level of photoreceptor outer segments and the inner retina. In the inner retina, CNTF-Rα signal localized to the superficial vascular plexus (*arrows*) as well as to surrounding cells. This staining pattern suggests that retinal endothelial cells are able to directly respond to CNTF. (**C**) Cultured human vascular endothelial cells and cerebral astrocytes were tested for the expression of components of the trimeric CNTF receptor complex, LIF-Rβ, gp130 and CNTF-Rα by PCR and gel electrophoresis. Samples of CNS served as positive controls. All cultured cell types expressed LIF-Rβ and gp130. However, neither HUVECs, HRMVECs nor astrocytes expressed CNTF-Rα in vitro. See text for more detail on the difference between in vivo and in vitro expression patterns.

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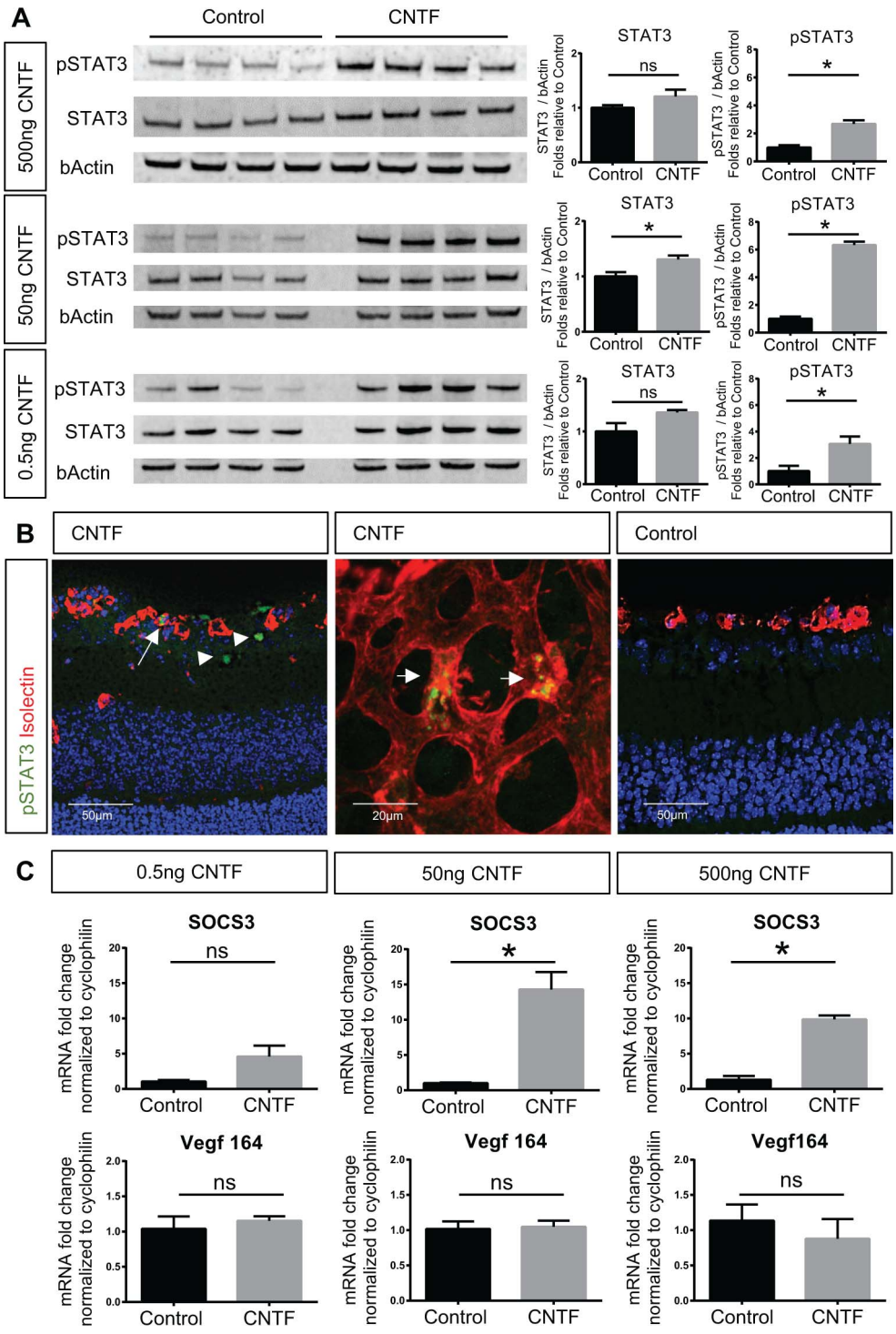

Figure 4. Ciliary neurotrophic factor activates STAT3 signaling in the inner retina in a dose-dependent manner. (**A**) Intravitreal rrCNTF injections at OIR P12 induced significant increases in phosphorylated STAT3 levels in the retina at OIR P13 compared with control-injected eyes ($n = 4$ mice per dose). *Error bars*: SEM. A dose of 50 ng rrCNTF most strongly activated STAT3 signaling through both increased expression and phosphorylation of STAT3. (**B**) Phosphorylation of STAT3 was localized to the ganglion cell layer (*arrowheads*) and the superficial vascular plexus (*arrows*), suggesting that multiple cell types, including vascular endothelial cells, respond to exogenous rrCNTF. (**C**) Intravitreal injection of rrCNTF leads to upregulation of SOCS3 expression. While the increase in SOCS3 expression following 0.5 ng rrCNTF injection was not statistically significant ($n = 4$ per dose, $P = 0.06$), doses of 50 and 500 ng rrCNTF induced high SOCS3 mRNA levels. From the doses investigated, 50 ng rrCNTF had the strongest effect on SOCS3 upregulation, which is in line with the pSTAT3 results. No changes in VEGF expression were observed at any dose, suggesting that the phenotypic changes induced by exogenous application of rrCNTF in the OIR model are independent of VEGF.

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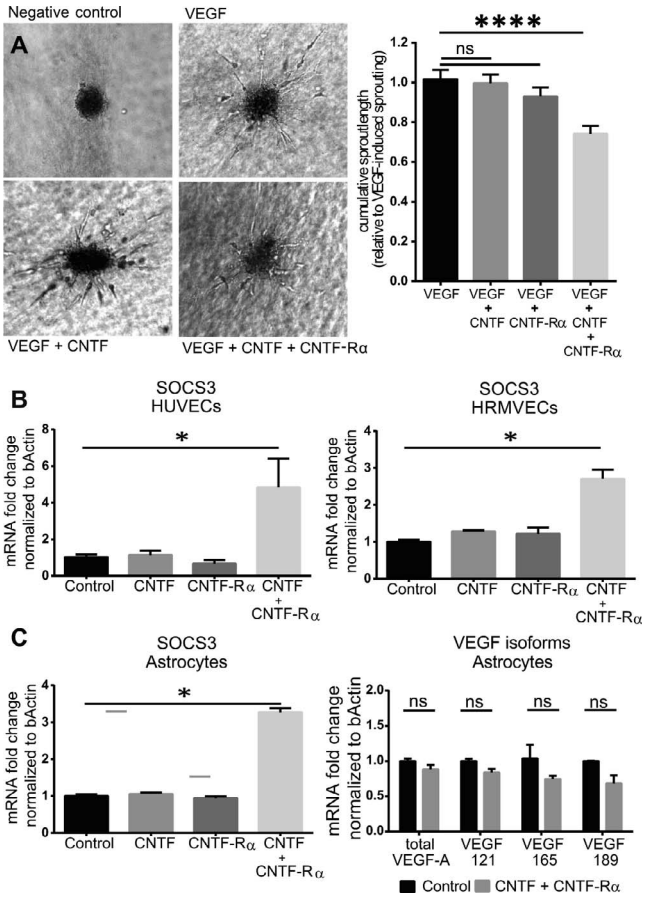

Figure 5. Ciliary neurotrophic factor has an anti-angiogenic effect on sprouting endothelial cells in the presence of CNTF-Rα. (**A**) Recombinant rat CNTF decreases VEGF-induced endothelial spheroid sprouting in the presence of CNTF-Rα (1-way ANOVA: $^{****}P < 0.0001$). (**B**) In the presence of CNTF-Rα, both HUVECs and HRMVECs upregulate SOCS3 in response to rrCNTF (1-way ANOVA: $^{*}P < 0.05$). (**C**) In vitro, astrocytes also respond with SOCS3 upregulation to rrCNTF stimulation in the presence of rCNTF-Rα. However, this SOCS3 increase in astrocytes does not alter total VEGF-A expression or expression of any of the VEGF-A isoforms investigated.

levels while VEGF expression remained unchanged (Fig. 4C, Supplementary Fig. S2), Upregulation of SOCS3 was strongest in response to 50 ng rrCNTF, which is in line with both the intensity of STAT3 activation and OIR rescue.

CNTF Inhibits Endothelial Sprouting and Induces Upregulation of SOCS3 in the Presence of CNTF-Rα

In order to validate the proposed mechanism of SOCS3-mediated antiangiogenic effects of CNTF, we performed in vitro experiments with endothelial and glial cells. As shown in Figure 3C, endothelial cells as well as astrocytes did not express CNTF-Rα in vitro. It is known, however, that CNTF/CNTF-Rα complexes can be incorporated into cell membranes of cells not intrinsically expressing CNTF-Rα, thus allowing CNTF signaling in these cells.[33,46] Therefore, all in vitro experiments were performed using four conditions: negative control, rrCNTF alone, rCNTF-Rα alone, and rrCNTF + rCNTF-Rα.

We first tested if CNTF exerts direct antiangiogenic effects on endothelial cells in a spheroid sprouting assay (Fig. 5A).[39,47] Recombinant rat CNTF alone did not reduce VEGF-induced endothelial cell sprouting in vitro. In contrast, when rrCNTF was administered together with soluble rCNTF-Rα, endothelial cell sprouting was significantly reduced, confirming the inhibitory effect of CNTF on VEGF-induced angiogenesis in the presence of CNTF-Rα.

Next, we tested whether exposure of endothelial cells to CNTF, CNTF-Rα, or a combination of rrCNTF and rrCNTF-Rα changes endothelial SOCS3 expression. In both HUVECs and HRMVECs, combinatory treatment with rrCNTF and rCNTF-Rα indeed resulted in a significant upregulation of SOCS3 mRNA in vitro (Fig. 5B). Similarly, upregulation of SOCS3 expression was induced in astrocytes by combined stimulation with CNTF plus CNTF-Rα (Fig. 5C). These results confirm the observed retinal upregulation of SOCS3 upon CNTF treatment in vivo (Fig. 4C). Similarly in line with results from CNTF-treated retina samples (Fig. 4C), expression of total VEGF-A and VEGF isoforms remained unaltered in human astrocytes upon stimulation with rrCNTF plus rCNTF-Rα (Fig. 5C). Together, these results confirm upregulation of SOCS3 as one of the mechanisms by which the anti-angiogenic effect of CNTF can be mediated.

Discussion

This study demonstrates that intravitreal injections of recombinant CNTF promote capillary regrowth and attenuate preretinal neovascularization in a mouse model of oxygen-induced retinopathy.[36] With its pronounced vascular phenotype that includes vaso-obliteration and preretinal tuft formation, the OIR model has developed into one of the most widely used vascular disease models.[48] Many studies have shown that neuronal[2,3] and glial[49–51] cells are strongly involved in the development of retinal vascular disease. The results from this study add a novel angioregulatory role for CNTF to this emerging picture of complex neurovascular interplay in the retina.

Ciliary neurotrophic factor and CNTF-Rα have been shown to be strongly expressed during rat retinal development, with Müller cells being a major source for CNTF expression and ganglion/amacrine cells the origin of CNTF-Rα synthesis.[31] In addition, CNTF has been identified as an important mediator for photoreceptor differentiation.[52–54] Beyond development, multiple studies have shown that retinal CNTF levels are also upregulated in response to injury.[55–57] No studies have been performed to date on the role of CNTF in the OIR model. In this study, we observed significant upregulation of endogenous CNTF-Rα and CNTF at OIR P12 (6 hours) and OIR P14 (Fig. 3A). The timing of CNTF upregulation corresponds to the onset of intraretinal hypoxia unfolding in the central avascular area at OIR P12 after transferring pups from hyperoxic atmosphere to normoxia. This finding indicates that the initial upregulation of CNTF-Rα may represent a response of the inner retina to ischemic stress. However, activation of the downstream signaling pathway JAK/STAT3 of CNTF was low at OIR P13 in control-treated eyes, suggesting that endogenous activation of the CNTF signaling pathway during the early hypoxic phase is minor, thus resulting in limited efficacy of this potential repair response (Fig. 4A). Supplementation of recombinant CNTF in the early hypoxic phase strongly upregulated the downstream signaling cascade, leading to a pronounced protective effect of CNTF against ischemic vascular changes in the retina (Figs. 2, 4).

The strong angiomodulatory effect of CNTF observed in our study renders CNTF a promising therapeutic approach for ischemic retinopathies. Best OIR rescue effects were achieved with CNTF doses between 5 and 250 ng (Fig. 2A). Both lower and higher doses of CNTF resulted in lower STAT3 phosphorylation, reduced SOCS3 upregulation and ultimately less protective effect. Studies testing CNTF in rodent models for

treatment of retinitis pigmentosa have repeatedly discussed potential dose-dependent effects of CNTF.[58–60] In our model, a comparably low dose of 50 ng CNTF yielded the best effects. This is important with regard to potential side effects. High CNTF doses from continuous delivery of CNTF through adeno-associated virus (AAV) vector transduction were reported to reduce electroretinogram (ERG) amplitudes.[61,62] A study by McGill, however, found that while an intravitreal injection of high CNTF doses (up to 10 μg) as well as subretinal delivery of AAV-vectored CNTF in rat resulted in reduced ERG amplitudes, this was not the case with low doses of CNTF treatment (1–100 ng).[59]

While CNTF is a well-characterized neuroprotective agent capable of rescuing both photoreceptors and ganglion cells from apoptosis,[11,21–25] the underlying mechanisms of the neuroprotective action of CNTF are still incompletely understood.[30] What is known, however, is that astroglia,[63] microglia,[46] and ganglion cells[64] have all been reported to respond to CNTF treatment. In our study, CNTF is shown to have a therapeutic angiomodulatory effect in vivo that is partially mediated through a direct effect on endothelial cells. We confirmed the direct antiangiogenic effect on endothelial cells by using an endothelial spheroid sprouting model that resembles the pathologic neovascular tuft formation in the retina driven by high amounts of VEGF. In this context, CNTF has a potent and direct antiangiogenic effect on endothelial cells. It is important to note that our spheroidal sprouting model contains only endothelial cells. In this exclusive endothelial cell milieu, CNTF-Rα has to be added exogenously in order to observe CNTFs antiangiogenic effects since neither HUVECs nor HRMVECs express CNTF-Rα endogenously in vitro. It is well established that CNTF signaling can occur in cell types that do not express the CNTF-Rα by incorporation of soluble CNTF-Rα into their cell membrane.[33] Further mechanistic workup in our study identified SOCS3 as one of the downstream mediators conveying the CNTF effect in endothelial cells. While knockdown of SOCS3 in endothelial cells has been shown to increase proliferation and sprouting in these cells,[43] its role in mediating CNTF effects in endothelial cells has not yet been described.

Beside the direct anti-angiogenic effect of CNTF on endothelial cells, it cannot be discounted that indirect angiomodulatory effects of CNTF via other cell types also play a role in the observed protective in vivo phenotype. Therefore, we tested astrocytes as potential cotarget cells of CNTF treatment. Similar to endothelial cells, human astrocytes do not express CNTF-Rα in vitro (Fig. 3C). Upon stimulation with CNTF plus CNTF-Rα, however, they upregulate SOCS3 similar to endothelial cells, suggesting that the beneficial effect of CNTF may partially be mediated through glial cells. Sun et al.[44] recently reported that SOCS3 deficiency in glial and neuronal cells increases VEGF expression, which in turn results in increased pathologic neovascularization in OIR. However, CNTF-mediated induction of SOCS3 did not alter VEGF expression, suggesting that the CNTF-induced protective effect in the OIR model is independent of VEGF. As a cautionary note, analysis of CNTF effects in cell culture is reliable only if the cell cultures used are confirmed not to be contaminated with other cell types.

Taken together, our results demonstrate that CNTF can have differential effects on retinal angiogenesis under hypoxic conditions, improving capillary regrowth while at the same time inhibiting the aberrant formation of new vessels. The magnitude of the effect of CNTF on preventing pathologic NV is comparable with that of established anti-VEGF agents in the OIR model. We have identified both direct effects on endothelial cells as well as indirect effects of CNTF on glial cells as important mechanistic pathways conveying these beneficial results. More work will be required to further elucidate the fine-tuned interplay of glial, neuronal, and endothelial cells in proliferative retinopathy. Another open question lies in the duration of the treatment effect achieved by the use of recombinant CNTF. In the OIR model, the timeframe between intravitreal injection of CNTF and analysis of the vascular phenotype spans only 5 days. While encapsulated cell implants[22,65,66] provide a potential solution for continuous delivery of recombinant CNTF in patients with degenerative retinal disease, it would be important to determine how long the angiomodulatory effect of a single intravitreal CNTF injection lasts in patients with angioproliferative retinal disease.

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