Retinal degeneration in Arylsulfatase G-deficient mice and neural stem cell-based neuroprotective approaches in mouse models of retinal degeneration.

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Quotation:

Anyone who thinks science is trying to make human life easier or more pleasant is utterly mistaken

(*New York Times*, by Albert Einstein, 1931)

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Abstract

Visual impairment as a result of retinal disorders may result from the dysfunction of loss of different retinal cell types such as photoreceptors, the photosensitive cells, or retinal ganglion cells (RGCs), which relay the visual signals through their axons to the brain. Among inherited degenerative retinal disorders with an involvement of photoreceptor cells, retinitis pigmentosa (RP) is the most frequent retinopathy. The retinal cells affected in glaucomatous neuropathies are the RGCs and their axons in the optic nerve. An elevated intraocular pressure (IOP) is considered as the major risk factor to develop glaucoma, and lowering the IOP is the only clinically proven treatment of glaucoma. Despite the successful reduction of the elevated IOP, some patients present with progressive glaucomatous changes, and a significant proportion of patients with glaucoma never develop an elevated IOP. Another group of diseases with a frequent involvement of the retina are the lysosomal storage disorders (LSDs). LSDs are characterized by mutations in genes encoding lysosomal transporters or enzymes, subsequent intracellular accumulation of metabolites and eventually cell death. For all these retinal dystrophies, there are currently no effective therapies available.

The present thesis has analyzed the retinal phenotype of a novel LSD mouse model of mucopolysaccharidosis (MPS) type IIIE. MPSs are caused by systemic and neural accumulation of glycosaminoglycans (GAGs). The seven subtypes of MPS are classified according to the specific enzyme involved and the clinical manifestation. In MPSIII, also termed Sanfilippo syndrome, retinopathies are moderate to severe with retinal dysfunction caused by the deposition of GAGs within the retinal pigment epithelium (RPE) and the photoreceptor cell layer. The new mouse model of MPSIIIE carries a mutation in the Arylsulfatase G (*Arsg*) gene. Analyses of the retina of *Arsg* knockout mice revealed a progressive degeneration of photoreceptors starting between 1 and 6 months of age. At the age of 24 months more than 50% of photoreceptor cells were lost. Photoreceptor loss was accompanied by reactive astrogliosis and microgliosis and elevated expression levels of some lysosomal proteins. Expression of ARSG protein in wild-type mice was restricted to the RPE. However, RPE cells in the knockout mouse appeared normal at the ultrastructural level, and evidence for the presence of storage vacuoles was not found.

Neurotrophic factor deprivation is among the various stress signals that may lead to the apoptotic cell death of retinal cells. Neurotrophic factors are diffusible molecules that confer survival effects on degenerating central nervous system neurons. The administration of exogenous neurotrophic factors aims to delay retinal degeneration and offers a widely applicable therapy across a range of conditions. Therefore, a cell-based

intraocular delivery system for a sustained administration of neurotrophic factors was established in two mouse models of RP and a CLN6 mouse model of juvenile neuronal ceroid lipofuscinosis (for photoreceptor degeneration) and an optic nerve crush (ONC) mouse model (for RGC degeneration). Murine neural stem (NS) cells were modified with a polycistronic lentiviral vector to express a secretable variant of ciliary neurotrophic factor (CNTF), a fluorescent reporter protein and a resistance gene. Because expression levels of the reporter protein and the neurotrophic factor from the polycistronic lentiviral vector are proportional to each other, clonally derived NS cell lines with high expression levels of the neurotrophic factor could be established by selection of single cells with high expression levels of the reporter gene using fluorescence activated cell sorting and subsequent clonal expansion. These modified clonal NS cell lines were transplanted into the vitreal cavity of the different mouse models prior to the onset of photoreceptor degeneration or one day after the optic nerve crush. The reporter protein enabled the analysis of the differentiation behaviour and the survival of the grafted cells. In all mouse models analyzed, CNTF-secreting NS cells significantly protected photoreceptors or ganglion cells. Furthermore, the CNTF-secreting NS cells stimulated long distance regeneration of the axotomized RGCs in the ONC mouse model. In addition to CNTF, the protective effects of vascular endothelial growth factor factor-B (VEGF-B) secreting NS cells were analyzed in the ONC mouse model. The VEGF-B-secreting NS cells significantly protected the lesioned RGCs for up to 2 months post-lesion but did not induce regeneration of RGC axons in the optic nerve. Despite the fact that members of the VEGF family promote vascularization, retinas from eyes with grafted VEGF-B expressing NS cells showed no pathological alterations of their vasculature.

In summary, this thesis has analyzed the retinal phenotype of a novel MPS mouse model, and thus provides further insights into the pathomechanisms of retinal disorders associated with LSDs. In addition, the present thesis has demonstrated that a sustained NS cell-based intraocular administration of CNTF attenuates photoreceptor loss in two mouse models of RP and a mouse model of CLN6 disease. The present work has also demonstrated that a NS cell-based intraocular administration of CNTF or VEGF-B attenuates the loss of axotomized RGCs in a mouse model of glaucoma. These combined data demonstrate that a sustained NS cell-based administration of neurotrophic factors might serve as a valuable tool for preclinical studies aimed at evaluating the protective effects of known or novel neurotrophic factors or factor combinations on retinal cells *in vivo*.

Zusammenfassung

Retinale Dystrophien können zu Beeinträchtigungen des Sehvermögens bis hin zur Erblindung führen. Dabei sind je nach Art der retinalen Dystrophie unterschiedliche Zellen betroffen, wie z.B. die Licht-sensitiven Photorezeptoren oder die retinalen Ganglienzellen (RGCs), die die visuellen Signale von der Retina über ihre Axone im optischen Nerven hin zum Gehirn leiten. Innerhalb der Retinopathien ist Retinitis pigmentosa (RP) die häufigste erblich bedingte retinale Erkrankung mit einer Degeneration der Photorezeptoren. Beim Glaukom, einer weiteren retinalen Erkrankung, degenerieren primär die RGCs und deren Axone. Ein erhöhter Augeninnendruck (IOP) gilt als Haupt-Risikofaktor für die Entwicklung eines Glaukoms. Daher ist die Reduktion des IOPs momentan die einzige Erfolg versprechende Therapie für Betroffene. Trotz einer erfolgreichen Reduktion des IOPs schreitet bei einigen Glaukompatienten die Krankheit unverändert voran. Hinzu kommt, dass eine beträchtliche Anzahl von Patienten ein sogenanntes Normal-Druck-Glaukom entwickelt, bei dem es trotz eines normalen IOPs zu einer fortschreitenden Degeneration der Gangleinzellen kommt. Lysosomale Speichererkrankungen (LSDs) bilden eine weitere Gruppe von Erkrankungen, bei denen oftmals eine retinale Degeneration zu den typischen Symptomen gehört. LSDs werden durch Mutationen in Genen verursacht, die für lysosomale Transporters oder Enzyme kodieren. Bei diesen Erkrankungen kommt es zu einer intrazellulären Akkumulation von Metaboliten und eventuell zum Zelltod. Für alle retinalen Dystrophien sind derzeit keine effektiven Therapien verfügbar.

Im Rahmen dieser Arbeit wurde der retinale Phänotyp eines neuen LSD Mausmodells für die Mukopolysaccharidose (MPS) IIIE analysiert. Ursache für MPSs ist eine systemische und neurale Akkumulation von Glykosaminoglykanen (GAGs). Dabei werden sieben Subtypen von MPS anhand der dysfunktionalen Enzyme und des klinischen Krankheitsbildes unterschieden. Bei MPS III, auch als Sanfilippo-Syndrom bezeichnet, sind Retinopathien mittelschwer bis stark ausgeprägt, wobei die retinale Fehlfunktion durch die Ablagerung von GAGs innerhalb des retinalen Pigmentepithels (RPE) und der Photorezeptoren verursacht wird. Das untersuchte Mausmodell für die MPS Variante IIIE weist eine Mutation im Arylsulfatase G (*Arsg*) Gen auf. Die Analyse der Retina von *Arsg* knockout Mäusen zeigte eine progrediente Photorezeptordegeneration mit einem Beginn, zwischen dem ersten und sechsten postnatalen Monate. In 24 Monate alten Tieren waren mehr als 50% der Photorezeptoren degeneriert. Begleitend zum Photorezeptorverlust waren eine reaktive Astrogliose und eine reaktive Mikrogliose, sowie eine erhöhte Expression von verschiedenen lysosomalen Proteinen nachweisbar. Wie in gesunden Mäusen durch immunhistochemische Untersuchungen gezeigt werden

konnte, beschränkt sich die Expression des ARSG Proteins auf das RPE. Ultrastrukturelle Analysen des RPEs von *Arsg* knockout Mäusen zeigten jedoch keine pathologischen Veränderungen, und typische Speichervakuolen waren ebenfalls nicht nachweisbar.

Innerhalb der verschiedenen Stresssignale, die zur Apoptose von retinalen Zellen führen können, wurde in der vorliegenden Arbeit primär der Mangel an neurotrophen Faktoren untersucht. Neurotrophe Faktoren sind kleine diffusible Moleküle, die eine protektive Wirkung auf Neurone des zentralen Nervensystems ausüben. Die Zufuhr neurotropher Faktoren hat zum Ziel, die retinale Degeneration zu verzögern und bietet somit ein breites Anwendungsfeld für viele degenerative Erkrankungen der Netzhaut. Zu diesem Zweck wurde ein zellbasiertes intraokuläres Applikationssystem für eine kontinuierliche Versorgung der Netzhaut mit neurotrophen Faktoren entwickelt, und dessen Anwendbarkeit in zwei RP Mausmodellen und dem CLN6 Mausmodel der juvenile neuronalen Ceroid-Lipofuszinose (für degenerierende Photorezeptoren) und in einem Mausmodell für das Glaukom (mit degenerierenden Ganglienzellen) analysiert. Hierzu wurden murine neurale Stammzellen (NS-Zellen) mit einem polycistronischen lentiviralen Virus modifiziert. Dieser Vektor kodierte eine sezernierbare Variante des *ciliary neurotrophic factor* (CNTF), ein fluoreszierendes Reporterprotein und ein Resistenzgen. Da die Expressionsstärke des neurotrophen Faktors und des Reportergens vom polycistronischen Vektor proportional zueinander sind, konnte durch eine Selektion einzelner stark fluoreszierender Zellen mittels *fluorescence activated cell sorting* und einer anschließenden klonalen Expansion dieser Zellen klonalen Zelllinien mit einer starken Expression des neurotrophen Faktors abgeleitet werden. Diese modifizierten NS-Zellen wurden in den Vitreus der unterschiedlichen Mausmodelle vor Beginn der Photorezeptordegeneration oder einen Tag nach der Läsion des optischen Nervs injiziert. Das Reportergen ermöglichte, die Differenzierung und das Überleben der transplantierten Zellen *in vivo* zu analysieren. In allen verwendeten Mausmodellen resultierte die kontinuierliche Applikation von CNTF in einer signifikanten Protektion der Photorezeptoren bzw. RGCs. Zusätzlich stimulierte CNTF im Läsionsmodell die Regeneration der RGC Axone über weite Strecken in den distalen Stumpf des optischen Nervs. Neben CNTF wurde der protektive Effekt von *vascular endothelial growth factor*-B (VEGF-B) exprimierenden NS-Zellen auf degenerierende RGCs im einem Mausmodell für das Glaukom analysiert. Die VEGF-B sezernierenden NS-Zellen übten eine signifikante Protektion auf axotomierte RGCs über einen Zeitraum von bis zu zwei Monaten nach der Läsion aus, induzierten aber keine Regeneration der RGC Axone. Obwohl die Mitglieder der VEGF Familie für ihre pro-angiogene Aktivität bekannt sind,

waren keine Veränderungen des Gefäßsystems in den mit VEGF-B behandelten Augen nachweisbar.

Zusammengefasst wurde in der vorliegenden Arbeit der retinale Phänotyp eines neuen Mausmodels für MPS analysiert, um weitere Einblicke in die Pathomechanismen von Retinopathien zu gewinnen, die mit LSDs assoziiert sind. Zusätzlich konnte diese Arbeit zeigen, dass eine kontinuierliche zellbasierte okuläre Applikation des neurotrophen Faktors CNTF eine protektive Wirkung auf degenerierende Photorezeptoren in zwei RP-Mausmodellen und einem Mausmodell für CLN6 hat. Des Weiteren konnte gezeigt werden, dass CNTF und VEGF-B die Degeneration axotomierter RGCs in einem Mausmodell für das Glaukom verlangsamte. Diese Daten demonstrieren, dass eine kontinuierliche NS-Zell-basierte Applikation neurotropher Faktoren eine geeignete Methode für präklinische Studien darstellt, die zum Ziel haben, die protektiven Effekte bekannter und neuer neurotropher Faktoren und Faktorkombinationen *in vivo* zu untersuchen.

1. Introduction and Summary

This thesis describes a new mouse model of retinal degeneration and a novel neural stem cell-based neuroprotective therapeutic approach that was evaluated in various mouse models of retinal degeneration. The following introduction provides an overview of different neurodegenerative retinal disorders in mice which are related to similar retinal dystrophies in humans, and proceeds with the description of the molecular mechanisms of photoreceptor and ganglion cell degeneration as well as possible therapeutic approaches that are currently in preclinical and clinical studies and aimed at delaying or preventing progressive retinal degeneration.

1.1. Retinal disorders and animal models for preclinical studies

To better understand retinal disorders and the retinal cells affected in different retinal diseases it is first necessary to understand the visual pathway and the function of the retinal cell types involved. In humans, vision is one of the primary senses to perceive the environment. Vision is a complex sensory process which starts in the retina (a neural tissue which is a compartment of the central nervous system) where light is transduced into a pattern of electrical signals that provides information to the brain (Gaillard and Sauve 2007).

The structure of the mammalian retina is composed of six different neuronal cell types and one type of glia. The retinal pigment epithelium (RPE) represents the outer margin of the retina. The RPE is a monolayer of cells that connects to the outer segments of photoreceptors. The photoreceptors form the outer nuclear layer (ONL), which is composed of rod and cone photoreceptors. Rod photoreceptors are responsible for scotopic vision while three different types of cones are responsible for photopic and colour vision (Yau and Hardie 2009). Photoreceptors transduce visual stimuli into electrical signals which are further processed by interneurons in the inner nuclear layer (INL). Therefore, cells of the outer nuclear layer are synaptically connected with the cells of the inner nuclear layer and these connections form the outer plexiform layer (OPL). The INL is composed of the cell bodies of three types of interneurons (horizontal cells, bipolar cells and amacrine cells) and the cell bodies of the Müller glia. Müller glia cells in the retina perform all the classical functions normally performed by astrocytes in the brain, and additionally serve as “living optical fibres” that conduct the light from the vitreous through all retinal cell layers to the outer segments of the photoreceptor cells. The processed visual information in the INL is further transmitted to the dendrites of the retinal ganglion cells (RGCs) in the inner plexiform layer (IPL). Ganglion cells bodies are

located at the vitreal side of the retina and the axons of RGCs relay the visual information to the brain (Fig. 1.1) (Karl and Reh 2010).

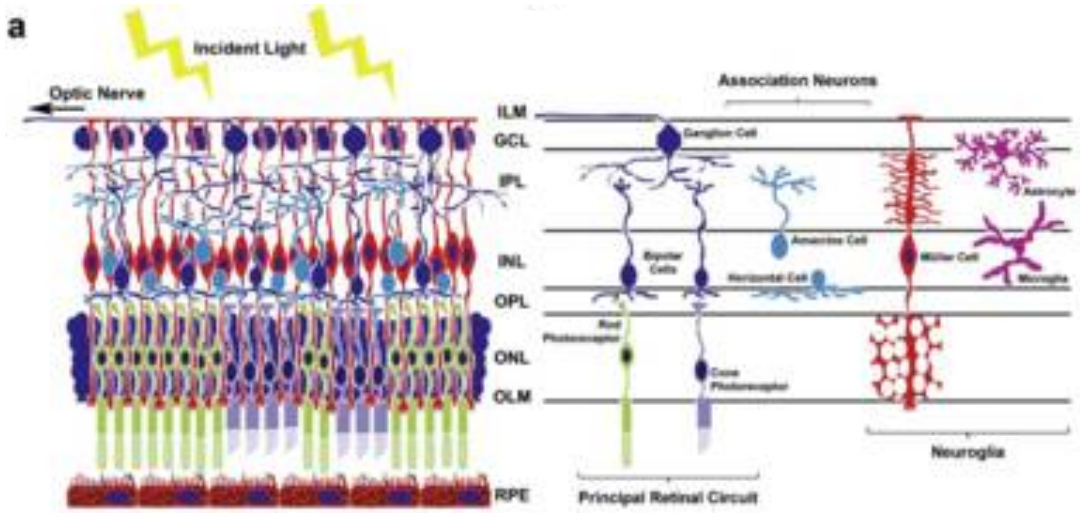


Figure 1.1: Cross section of a human retina illustrating the different retinal layers and cell types.
Müller cells serve as "living optical fibres" and guide the light from the vitreal side of the retina to the outer segments of photoreceptor cells at the outer margin of the retina. Photosensitive rods and cones transform light photons into electrical signals. The visual information is conducted through the inner nuclear layer to the ganglion cells located at the vitreal side of the retina. Finally electrical impulses are transmitted via the ganglion cell axons to the brain. Retinal pigment epithelium (RPE) phagocytes shed outer segments and restores the photopigments of photoreceptors, whereas microglia and astrocytes are supportive cell types within the retina. GCL: ganglion cell layer; ILM: inner limiting membrane; INL: inner nuclear layer; IPL: inner plexiform layer; OLM: outer limiting membrane; ONL: outer nuclear layer; OPL: outer plexiform layer (from West et al. 2009).

Dysfunction of any of these retinal cell types can lead to degeneration of these and possibly also other related retinal cell types, resulting in visual impairment or even blindness. The leading causes of visual impairment and blindness in patients over 40 years are cataracts (a clouding of the lens) and different retinal dystrophies. Age-related macular degeneration (AMD) is the most common form of retinal dystrophies, and is characterized by the primary dysfunction of RPE cells or defects of the Bruch's membrane, a thin limiting structure composed of extracellular matrix constituents and located between the RPE and the choroidea. The macula is a specialized area of the human retina and encloses the fovea centralis, the retina region with the highest visual acuity and the highest density of cone photoreceptor cells. Since photoreceptor metabolic activity is highly dependent on a proper function of the RPE, dysfunction or loss of RPE cells leads to a secondary degeneration of photoreceptors. Another frequent retinopathy is the diabetic retinopathy (DR), characterized by the degeneration of retinal cells as a result of long term diabetes, followed by glaucoma, a group of degenerative retinal disorders characterized by optic nerve damage and subsequent apoptotic RGC loss, and retinitis pigmentosa (RP), a clinically and genetically heterogeneous group of

degenerative retinal disorders caused by mutations in a large number of different genes (Gaillard and Sauve 2007). Glaucoma and RP are described in more detail below.

Glaucoma is the most common cause of irreversible blindness worldwide and estimated to affect about 80 million people by 2020 (Quigley and Broman 2006). This optic neuropathy is characterized by progressive optic disc cupping, which is caused by thinning of the neuroretinal rim of the optic nerve. The cupping is the result of the progressive loss of RGC axons, along with supporting structures like glia and vasculature and subsequent degeneration of RGC cell bodies. Patients with glaucoma typically experience visual field loss, starting in the periphery, and may become blind if not treated (You et al. 2013). Glaucoma is subdivided into three major subclasses: open-angle glaucoma (OAG), angle closure glaucoma (ACG) and developmental glaucomatous neuropathies. The predominant form is the OAG, where the outflow of the aqueous humor through the iridocorneal angle (the angle formed by the iris and cornea) is impaired, resulting in an elevated intraocular pressure (IOP). Elevated IOP is considered as a major cause of neurodegeneration in the inner retina. Mutations in the gene encoding myocilin (*MYOC*) have been identified to cause OAG. However, the genetic basis of glaucoma is not well understood. ACG can result from inflammation or can have neovascular causes (Fingert et al. 1999; Kwon et al. 2009). While the pathomechanisms of this complex and multifactorial optic neuropathy are only poorly understood, there are several risk factors and molecular pathways that have been implicated in optic nerve damage and RGC loss in glaucoma. Clinically, an elevated IOP is the major and only proven risk factor for glaucoma. However, some glaucoma patients never develop an elevated IOP and some patients show progressive glaucomatous alterations despite a successful reduction of the IOP (Caprioli 1997; Agarwal et al. 2009). Reduced ocular blood-flow (Butt et al. 1995; Kaiser et al. 1997; Zeitz et al. 2006) and a decreased central cornea thickness are other risk factors implicated in glaucoma (Medeiros et al. 2003; Brandt 2004; Brandt et al. 2008). The molecular changes associated with glaucoma, and the signalling and apoptotic pathways involved in RGC survival or death are discussed below.

Retinitis pigmentosa has a prevalence of 1 in 3,000, and is the most common cause of inherited photoreceptor degeneration in 20-64 year old patients (Buch et al. 2004). Mutations in more than 60 different genes and more than 3,000 pathogenic mutations in these genes have been identified to cause RP (RetNet: http://www.sph.uth.tmc.edu/RetNet/; Human Gene Mutation Database: http://www.hgmd.cf.ac.uk/) (Sahni et al. 2011). The highest number of pathogenic mutations causing RP have been identified in the genes encoding GFPase regulator (RPGR) (Breuer et al. 2002), rhodopsin (RHO) (Briscoe et al. 2004) and usherin

(USH2A) (Hartong et al. 2006). Typical clinical features of affected patients include poor night vision in early or middle life which progresses to loss of mid-peripheral field of vision. In late stage RP patients, a small area of central vision remains because of a long-term preservation of macular cone photoreceptor cells. The most common variant of RP is the rod-cone dystrophy, where rod photoreceptors degenerate in the first place resulting in poor night vision. This almost invariably leads to secondary cone photoreceptor degeneration late in the course of the disease. In other variants of RP, rods and cones are affected to a similar content, or cones are more severely affected than rods or is the only photoreceptor cell type involved (Wright et al. 2010).

Another heterogeneous group of rare inherited diseases with a frequent involvement of the retina are the lysosomal storage diseases (LSDs). LSDs are metabolic disorders that are usually inherited in an autosomal recessive manner. The mutations result in dysfunctional lysosomal transporters or enzymes and consequently in an intracellular accumulation of metabolites and eventually cell death (Klein and Futerman 2013). LSDs comprise at least 50 distinct genetic diseases and the incidence of single forms ranges from 1 in 57,000 for Gaucher disease to as low as 1 in 4.2 million for sialidosis, but is calculated to be 1 in 7,700 for LSDs as a group. As additional LSDs are likely to be discovered, it is expected that the incidence for LSDs might increase up to 1 in 5,000. Based on the accumulated substances, LSDs have been subdivided into oligosaccharidoses, mucopolysaccharidoses, lipidoses and glycogenoses. Although LSDs comprise an extremely heterogeneous group of metabolic disorders, many of them share clinical features like central nervous system dysfunctions, organomegaly, bone abnormalities and visual impairment due to retinal degeneration (Fuller et al. 2006).

The mucopolysaccharidoses (MPSs) are LSDs caused by systemic and neuronal accumulation of glycosaminoglycans (GAGs) which are ubiquitously present on the cell surface and extracellular matrix. There are seven subtypes of MPS (MPS I, II, III, IV, VI, VII and IX) which are subdivided in 13 variants according to the specific enzyme involved and the clinical manifestations. All types show diverse phenotypes, ranging from those with a fatal outcome in the first months of life to forms that are compatible with a normal lifespan. The overall incidence for all MPS subtypes is approximately 1 in 20,000 live births (Ganesh et al. 2013). Clinical symptoms common to all subtypes include hepatosplenomegaly, recurrent umbilical and inguinal hernia, middle ear disease and deafness, dental caries and abscesses, with variable skeletal, cardiac, respiratory, and central nervous system manifestations. Of interest in the present context are the frequent ocular manifestations of MPS patients (Ashworth et al. 2006a). The ocular involvement usually occurs early in MPS and depending on the MPS subtypes can include hyperopia, corneal clouding, ocular hypertension, retinal degeneration, optic disc swelling and optic

nerve atrophy. These changes may impair visual function, eventually leading to vision loss. Corneal opacification due to abnormal GAG deposition, is a prominent feature in MPS I and MPS VI but can appear in patients of all MPS subtypes (Azevedo et al. 2004; Ashworth et al. 2006b). The accumulation of GAGs in the angle of the anterior chamber may interfere with the outflow of the aqueous humor thereby elevating the IOP and eventually causing open-angle glaucoma. Because of GAG deposition a thickening of the iris and peripheral cornea can result in secondary angle-closure glaucoma in some patients. Furthermore, optic nerve abnormalities may occur due to an infiltrative swelling of GAG accumulation within the nerve and the meningeal sheath (Schumacher et al. 2008). Retinopathies are abundant in the MPS I, MPS II Hunter and MPS III subtypes, where progressive photoreceptor degeneration and retinal dysfunction are caused by the deposition of GAGs within the RPE and the ONL. In MPS III, also termed Sanfilippo-syndrome, retinopathies are moderate to severe and typically present with reduced scotopic electroretinograms (ERGs) because of rod photoreceptor degeneration. Secondary cone degeneration may be seen with a corresponding decrease in photopic ERG responses (Azevedo et al. 2004; Ashworth et al. 2006b; Valstar et al. 2008).

Neuronal ceroid lipofuscinosis (NCL or CLN) comprises a group of inherited neurodegenerative disorders with an onset mainly in childhood and youth. These lysosomal storage disorders are also known as Batten disease, according to a British pioneer of the field. So far, mutations in 14 different genes have been shown to cause NCL, and NCL subtypes have been classified accordingly from CLN 1 to CLN 14. The NCL-associated genes encode either soluble lysosomal enzymes or membrane proteins located in lysosomes or the endoplasmic reticulum, depending on the NCL variant. The different NCL forms all together represent the most common cause of inherited neurodegenerative disorder in childhood, with an incidence between 1:12,500 and 1:100,000 (Faller et al. 2015). Despite the heterogeneity of the disease-associated genes, several clinical symptoms are common to most of the storage diseases with childhood-onset, including progressive loss of vision, mental and motor deterioration, epileptic seizures and premature death. Adult-onset variants, in comparison, are much rarer with dementia as the predominant clinical symptom. Typically autofluorescent, electrondense material, resistant to lipid solvents and closely resembling lipofuscin, accumulates in the cytoplasm of most nerve cells and, to a lesser extent, in other cell types (Haltia and Goebel 2013). So far loss of vision has been described for CLN 1, CLN 2 and CLN 3, and the late-infantile variants CLN 5, CLN 6 and CLN 7. However, only little information is available on the progression of visual impairment in NCL patients, and no information is available on a retinal involvement in CLN 8, CLN 9 and CLN 10 (Anderson et al. 2013; Schulz et al. 2013).

1.2. Pathomechanisms and apoptotic pathways in retinal degeneration

The possible causes of photoreceptor degeneration are extremely diverse. For instance, photoreceptor cell death may occur as a result of a separation of the ONL from the underlying RPE and the choroidal vessels, which provide the metabolic support to photoreceptors. Retinal detachment is characteristic for retinal disorders such as AMD (Dunaief et al. 2002) or DR (Barber et al. 1998). Mutations in genes implicated in a variety of different functions might also result in photoreceptor degeneration, as exemplified in retinitis pigmentosa (Murakami et al. 2013). Other mechanisms shown to cause photoreceptor and RGC degeneration include mitochondrial dysfunction, excitotoxic damage caused by activated glial cells, and nitric oxide mediated toxicity or oxidative stress (Almasieh et al. 2012; Murakami et al. 2013; You et al. 2013). Cell death of photoreceptors and RGCs almost invariably occurs by apoptosis (Portera-Cailliau et al. 1994; Travis 1998; Buch et al. 2007). Increased oxygen free radicals (OFR) levels have also been implicated for both, RGC and photoreceptor degeneration, because OFR are known to induce the release of cytochrome c, which is involved in pro-apoptotic signalling cascades (Raha and Robinson 2001; Almasieh et al. 2012; Murakami et al. 2013). There are three major forms of cell death defined by morphological appearance: autophagy, necrosis, and apoptosis (Murakami et al. 2013). Autophagy is a degradation of organelles and macromolecules by the lysosome. Macroautophagy is the major pathway and involves the formation of membranes around cytoplasmic substrates resulting in the formation of organelles known as autophagosomes (Mizushima et al. 2002; Mizushima et al. 2008). Although autophagy mediates cell death in specific circumstances, there is also evidence that autophagy is crucial for cell survival by regulating the turnover of intracellular contents (Mizushima and Levine 2010). Necrosis was long believed to be an uncontrolled process of cell death. Studies on death receptor-induced cell death found that tumor necrosis factor-alpha (TNFα) is not only involved in apoptotic pathways but also induces necrosis (Laster et al. 1988). Death receptor-induced necrosis is mediated by the activation of receptor-interacting protein 1 (RIP1) (Holler et al. 2000), which in turn is regulated by RIP3 (Cho et al. 2009; He et al. 2009; Zhang et al. 2009a). However, there are also RIP kinase-independent mechanisms to induce necrosis (Murakami et al. 2013). Apoptosis, also termed programmed cell death, is the best characterized form of cell death and the apoptotic pathways are discussed in more detail below.

1.2.1. Apoptotic pathways

Similar to other neurons in the central nervous system (CNS) two major apoptotic pathways are involved in RGC and photoreceptor loss, the extrinsic or receptor mediated pathway and the intrinsic or mitochondrial pathway. Both pathways are triggered by stress stimuli and can occur independently or interact with each other (Qu et al. 2010; Almasieh et al. 2012; Murakami et al. 2013; You et al. 2013).

The intrinsic apoptotic pathway is triggered by mitochondrial damage caused by a variety of stress signals such as neurotrophic factor deprivation or oxidative stress which increase the permeability of the mitochondrial membrane (Scaffidi et al. 1998). First stress signals activate the apoptosis regulating kinase 1 (ASK1), which plays a key role in human neurodegenerative diseases (Hattori et al. 2009). Both the pro-apoptotic mitogen-activated protein kinases (MAPKs) c-jun n-terminal kinase (JNK) and p38 are direct targets of ASK1. Activation of BH3-only members of the Bcl-2 family such as Bax and Bak promote the release of cytochrome c and play a pivotal role in the regulation of RGC death (Li et al. 2000; Almasieh et al. 2012). Some Bcl-2 family members antagonize the pro-apoptotic molecules. Among these proteins Bcl-XL is the predominant anti-apoptotic protein in rat retina (Levine et al. 2008). When the concentration of pro-apoptotic proteins exceeds the concentration of anti-apoptotic Bcl-2 family members the mitochondrial membrane permeability increases and cytochrome c is released into the cytosol. Cytochrome c then binds to the apoptosis protease-activating factor 1 (APAF1) to form the apoptosome, which activates the procaspase-9 which in turn activates caspase-3 and -7. These caspases activate other caspases and induce the digestion of cellular contents, resulting in cell death (Li et al. 1997; Qu et al. 2010; Almasieh et al. 2012; You et al. 2013). Other cell death mediators released from mitochondria into the cytosol include second mitochondrial-derived activator of caspases and *Drosophila melanogaster* homologue (SMAC/DIABLO), apoptosis inducing factor (AIF), endonuclease G (EndoG) and high temperature requirement serine protease 2 (HTRA2/OMI) (Fig. 1.2) (Benn and Woolf 2004).

The extrinsic apoptotic pathway is initiated by death mediating receptors which are activated by ligands such as TNFα, Fas ligand (FasL) or TNF-related apoptosis-inducing ligands (TRAIL). Studies have shown that TNFα is upregulated in a mouse model of glaucoma (Nakazawa et al. 2006) and that under different stress conditions RGC death is mediated by increased expression of TNFα in glial cells (Tezel and Wax 2000). Activation of these receptors results in an intracellular adaptor Fas-associated death domain (FADD) recruitment, leading to the activation of caspase-8, subsequent activation of caspase-3 and -7, and finally cell death (Fig. 1.2) (Qu et al. 2010; Almasieh et al. 2012; Murakami et al. 2013; You et al. 2013).

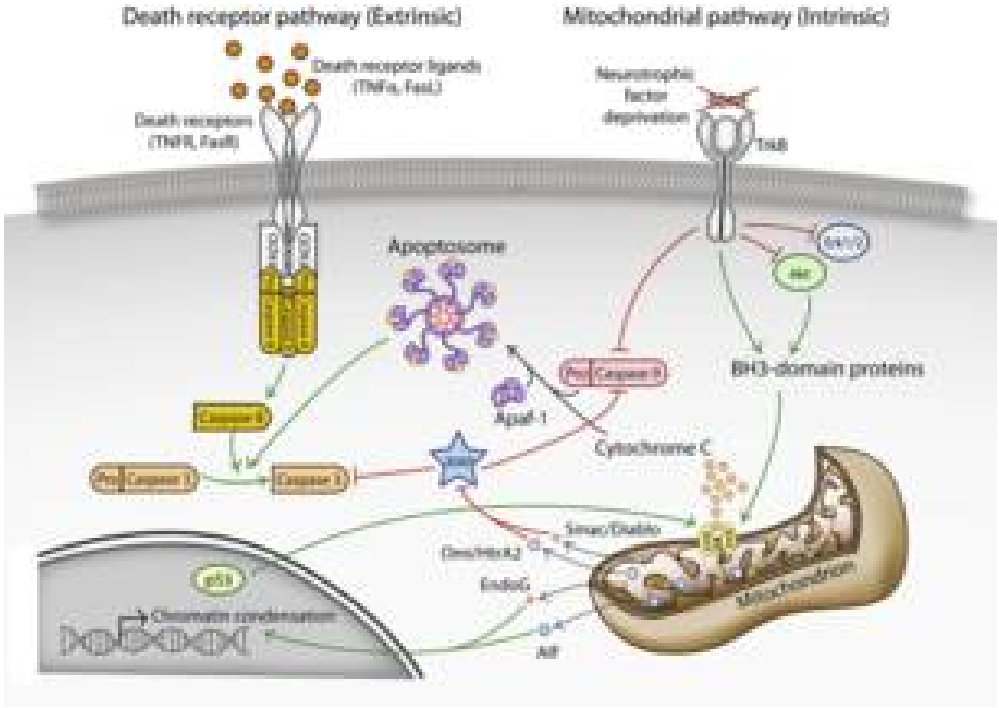


Figure 1.2: Main apoptotic pathways in cells.
The extrinsic pathway is mediated through the activation of death receptors like TNFR and FasR. This leads to the activation of caspase-8 which in turn activates caspase-3, ultimately resulting in cell death. In the intrinsic pathway mitochondrial damage leads to cytochrome c release and the formation of apoptosomes with APAF1 and finally an activation of caspase-3. Mitochondria can also release other cell death mediators into the cytosol that activate transcription factors in the nucleus (from Almasieh et al. 2012).

1.2.2. Neurotrophic factor deprivation

Another pathomechanisms implicated in the progressive apoptotic degeneration of RGCs is their deprivation from target-derived neurotrophic factors. Neurotrophic factors or neurotrophins are diffusible molecules that wield a survival effect on degenerating CNS neurons (Almasieh et al. 2012). It is commonly accepted that neurotrophic factors promote neuronal survival by inhibiting apoptotic pathways (Raff et al. 1993). During development neurons require trophic factors for survival, differentiation and establishment of synaptic connections or otherwise succumb to apoptosis (Lewin et al. 1998). In glaucoma, neurotrophic factor deprivation may occur due to impaired retrograde axonal transport through the optic nerve to the RGC bodies (Anderson and Hendrickson 1974; Quigley et al. 2000) or because of reduced neurotrophic factor expression levels within the retina as a result of ocular hypertension (Rudzinski et al. 2004). For whatever reasons the photoreceptors or RGCs die, apoptotic pathways are finally induced, ultimately resulting initially in night blindness in RP and in visual field defects in glaucoma. Because of the often slowly progressing neurodegenerative disorders, such as RP or glaucoma, they are often diagnosed at advanced stages of the disease when a considerable proportion of cells is already lost (Almasieh et al. 2012; You et al. 2013; Abed et al. 2015).

1.3. Therapeutic approaches

Currently there are no effective therapies for the treatment of neurodegenerative retinal disorders affecting the RPE, the photoreceptors and/or the RGCs. Animal models of these conditions not only help to understand the disease mechanisms but eventually also to develop treatments for these disorders. In fact, a large number of naturally occurring animal models and a constantly increasing number of genetically engineered animal models of degenerative retinal disorders are currently being used to assess the efficacy of therapeutic interventions to delay or prevent retinal degeneration and vision loss. The major therapeutic approaches include pharmacological treatments, electronic retinal implants, cell replacement strategies, corrective gene therapy, optogenetic therapy, enzyme replacement therapy (ERT) and neuroprotection (Sahni et al. 2011; Almasieh et al. 2012).

1.3.1. Electronic retinal implants

For advanced stages of retinal diseases, restoration of visual function is required. A retinal electronic device could provide means to translate visual information into electrical signals that are then transmitted to the RGCs and subsequently to the brain (Chader et al. 2009). However, glaucoma requires an intracortical microstimulation with electronic devices that bridge the interrupted connections between the retina and visual cortex (Schmidt et al. 1996). Thus far technologies have advanced to restore light perception (Chader et al. 2009; Zrenner et al. 2011), but there are issues with retinal devices concerning real-time vision, material stability, biocompatibility and a permanent power supply.

1.3.2. Cell replacement

Cell replacement strategies have been investigated intensively. The idea is to functionally replace irreversibly lost cells, such as RPE cells or photoreceptor cells, to restore vision. For photoreceptor replacement, numerous studies have been performed during the last years. Most of these studies were of limited success because the donor cells either failed to integrate into the host retinas or did not differentiate into mature retinal cell types. Subsequently, MacLaren and colleagues (2006) found that committed postmitotic photoreceptor progenitor cells have to be transplanted in order to successfully replace photoreceptor cells in the mature mammalian retina. A subsequent study extended this work, and defined rod progenitors isolated from the retina of 4 or 5 days old mice as the cell population that showed the highest capacity of integration and differentiation into mature rods (Bartsch et al. 2008). Recent transplantation of rod precursors into *Gnat1*$^{-/-}$ mice (a model of congenital stationary night blindness, which

lacks rod function) showed that newly integrated photoreceptors formed synaptic connections with bipolar and horizontal cells and were light sensitive in a similar manner as wild-type photoreceptors. Furthermore, the transplanted and integrated cells mediated optokinetic head tracking under scotopic conditions (Pearson et al. 2012; Pearson 2014), demonstrating their functionality. Beside the isolation of photoreceptors from neonatal tissue several studies have demonstrated differentiation of photoreceptors from embryonic stem (ES) cells (Osakada et al. 2008; Yue et al. 2010; Eiraku et al. 2011). Although the eye is an immune tolerant compartment transplantation of ES cell derived photoreceptors requires immunosuppression. To circumvent additional treatments and ethical concerns regarding the use of ES cells, induced pluripotent stem (iPS) cells are currently being analyzed as an alternative source for photoreceptor cells. iPS cells display the properties of ES cells, and are generated by reprogramming differentiated somatic cells, such as skin fibroblasts (Singh and MacLaren 2011; Tucker et al. 2013).

In many retinal dystrophies, such as AMD, photoreceptor degeneration is the result of a dysfunction or loss of RPE cells. As for photoreceptor replacement, many studies have evaluated the possibility to replace RPE cells by cell transplantation. RPE cells derived from ES cells or iPS cells resemble major characteristics of mature RPE cells, such as cell polarity, the ability to phagocytose shed photoreceptor outer segments, or the expression of specific RPE proteins (Singh and MacLaren 2011; Krohne et al. 2012; Ramsden et al. 2013). In a recent study, human ES cell-derived RPE cells have been transplanted into the subretinal space of patients with AMD. The RPE cells showed good integration and survival, and were tolerated in the recipient eyes. Of note, the treated patients experienced an improvement in visual acuity (Schwartz et al. 2012).

In glaucoma, cell replacement strategies are unlikely to be successful given that RGCs not only have to correctly integrate into the host retinas and to differentiate into mature and functional ganglion cells, but additionally have to extend axons over long distances to innervate in a topographical correct manner into the visual centres of the brain (Harvey et al. 2006; Almasieh et al. 2012; Wilson and Di Polo 2012).

1.3.3. Enzyme replacement therapy

The first correcting effect on the metabolic defect of cultured fibroblasts isolated from Hurler or Hunter patients (two types of MPS) was seen after mixing these cultures with each other or with normal fibroblasts, or after cultivation of the affected fibroblasts with conditioned medium from normal fibroblasts. This effect was attributed to secretion of corrective factors from healthy cells or cells having another defect (Fratantoni et al. 1968). Later studies revealed the uptake of enzymes into the lysosomes by receptor-mediated endocytosis via the mannose-6-phosphate (M6P) receptor. Cross-correction is

based on replacing a defective soluble enzyme by the extracellular administration of a healthy variant of this enzyme, a treatment strategy designated as ERT. Particularly LSDs with defects in soluble lysosomal enzyme are considered to benefit from ERT. Intriguingly, ERT in different LSDs required a restoration of the dysfunctional enzyme activity to only 1-5% of the activity in healthy subjects to decrease accumulation of storage material and correct the metabolic defect (Beck 2007). Currently ERT is available for MPS I, II and VI. Of note, a systemic administration of a functional enzyme has been shown to exert positive effects on many tissues. However, this holds not true for the affected CNS, because systemically administered enzymes are incapable to cross the blood-brain-barrier (Harmatz et al. 2008; Wraith et al. 2008; Clarke et al. 2009; Beck 2010). Furthermore, some safety and tolerability concerns remain with this therapeutic strategy. For instance patients receiving regular intravenous injections of proteins may develop antibodies which may inactivate the administered enzyme and lead to allergic reactions. In addition, this approach requires repetitive injections due to short half-life time of the enzymes (Beck 2010). A sustained delivery system would circumvent the need of multiple injections and additionally ensure stable concentrations of the supplied enzyme.

1.3.4. Gene-based therapy

Corrective gene therapy targets the primary genetic defect of an inherited disease, and was studied intensively for retinal disorders (Farrar et al. 2012). Genomics are more and more easily accessible, for instance through next-generation sequencing, and facilitate a rapid identification of disease-causing mutations in patients with retinal degeneration (Bowne et al. 2011). Most corrective gene therapy studies in animal models of retinal degeneration have used adenoviral, adeno-associated viral (AAV), lentiviral or herpes simplex-I vectors to transfer a functional gene variant into affected retinal cell types (Harvey et al. 2006). Adenoviruses are non-enveloped, linear, double-stranded DNA viruses, which can bear genes up to 8 kb. In the past adenoviral vectors have been reported to induce local inflammatory reactions and cell-mediated immune-response (Hoffman et al. 1997; Cayouette et al. 1998; Isenmann et al. 2001; Isenmann et al. 2004) but a recent clinical phase I trial using adenoviruses for administration of pigment epithelium-derived factor (PEDF) in patients with AMD reported no serious side-effects over several months (Campochiaro et al. 2006). AAV is the viral vector of choice for gene-based therapy because of several reasons. AAV are non-enveloped single- or double-stranded DNA viruses that are replication defective, non-pathogenic and non-toxic. There are several frequently used serotypes for gene transfer strategies into retinal cell types, with AAV2/5 (AAV5) and AAV2/8 (AAV8) exhibiting a particularly high tropism

for photoreceptor cells and AAV2/4 preferentially transducing RPE cells (Weber et al. 2003; Surace and Auricchio 2008). A recent clinical trial for Leber's congenital amaurosis (LCA) which is caused by mutations in a gene encoding RPE-specific protein 65 kDa (RPE65) used subretinal AAV2/2 injections to express a functional RPE65 protein in the host RPE. Of note, these studies observed beneficial functional effects in the treated eye, including an increased retinal sensitivity to light (Cideciyan et al. 2008; Maguire et al. 2008; Simonelli et al. 2010). Despite this tremendous visual improvement later examinations showed a progressive thinning of the ONL to a similar extend as in untreated eyes. Remarkably however, experiments in a canine animal model of LCA (with a dysregulation of RPE65 and a photoreceptor cell degeneration starting later in life) revealed progressive retinal degeneration when the RPE65 gene transfer into RPE cells was performed after the onset of photoreceptor degeneration, whereas no photoreceptor loss was observed when injection of the viral vector was performed prior to the onset of retinal degeneration. Because therapeutic intervention in retinal degenerations are usually performed after the onset of the disease, a combination of gene therapeutic interventions with neuroprotective, anti-apoptotic factors or antioxidants might be necessary to preserve both, retinal function and retinal structure (Cideciyan et al. 2013).

1.3.5. Neuroprotection

Many retinal dystrophies, such as AMD or glaucoma, are not amenable to corrective gene therapy, and for others genetic mutations have not yet been identified (Hartong et al. 2006). For these conditions, a treatment independent of the etiology of the degeneration is required. Neuroprotective strategies do not target the specific cause of a disease but rather its consequences, and attempt to limit pro-apoptotic pathways to prevent the death of affected cells across a wide range of diseases characterized by retinal degeneration (Buch et al. 2007). Neurotrophic factors are basically soluble proteins secreted by target cells of neuronal growth. Upon binding to their specific receptors, neurotrophic factors prevent apoptosis in neurons (Purves et al. 2001) by upregulating anti-apoptotic signalling pathways (Buch et al. 2007).

The first neurotrophic factor described was nerve growth factor (NGF) which promoted neuronal survival in the CNS (Hamburger et al. 1981). Subsequently, a number of other neurotrophic factors was identified, including brain-derived neurotrophic factor (BDNF) (Hofer and Barde 1988), ciliary neurotrophic factor (CNTF) (Sendtner et al. 1990), basic fibroblast growth factor (bFGF) (Faktorovich et al. 1990), glial cell line-derived neurotrophic factor (GDNF) (Henderson et al. 1994), pigment epithelium-derived factor (PEDF) (Tombran-Tink et al. 1991; Cayouette et al. 1999), rod derived cone viability

factor (RdCVF) (Leveillard et al. 2004) and Osteopontin (Del Rio et al. 2011). The still increasing number of newly identified neurotrophic factors improves the insight into molecular mechanisms mediating neuronal survival and degeneration. There are three main categories of neurotrophic factor receptors involved in neuroprotection: receptor tyrosine kinase (RTK) family (including tropomyosin receptor kinase proteins A-C) (Buch et al. 2007), p75 neurotrophin receptor (Dechan and Neumann 2005), and GDNF family receptors (GFRs), which form heterodimers with the receptor tyrosine kinase RET (Airaksinen and Saarma 2002).

CNTF is one of the most extensively studied growth factor in retinal degeneration. It was purified from chicken embryo and protected ciliary ganglion neurons from death (Helfand et al. 1976; Adler et al. 1979; Barbin et al. 1984). CNTF is a cytosolic protein and contains 200 amino acids with a molecular weight of 22.7 kDa. Although this trophic factor lacks a signal peptide it is secreted through an unknown pathway (Stockli et al. 1989). Based on its structural similarities CNTF belongs to the interleukin-6 family of cytokines (Taga and Kishimoto 1997; Bauer et al. 2007). The CNTF receptor (CNTFα) initiates downstream signalling upon CNTF binding and formation of heterodimers with the co-receptor molecules gp130 and leukaemia inhibitor factor receptor-β (LIFR-β) (Sleeman et al. 2000). This formation leads to the activation of Jak/Tyk kinases which in turn phosphorylate the signal transducer and activator of transcription 3 (STAT3). STAT3 forms hetero- and homodimers with phosphorylated STAT1 and subsequently modifies gene transcription and mediates cell protection (Fig. 1.3) (Thanos and Emerich 2005; Wen et al. 2012).

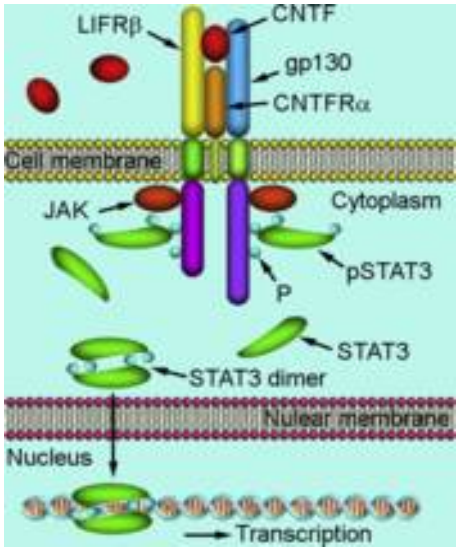


Figure 1.3: Scheme of signalling pathway of CNTF.
Binding of CNTF to the receptor CNTFRα leads to binding of the co-receptor LIFRβ and gp130. This activates the intracellular Jak/Tyk kinases which phosphorylates STAT3. Finally, the STAT3 dimers modify gene transcription in the nucleus (from Wen et al. 2012).

Several studies have shown strong protective effects of CNTF on the morphology of RGCs and photoreceptor cells. Intravitreal administration of recombinant CNTF in an elevated ocular hypertension rat model protected RGCs up to 4 weeks after injection (Ji et al. 2004). Injections of AAVs expressing a secretable form of CNTF have been shown to also confer significant protective effects on RGCs in different investigations (MacLaren et al. 2006; Hellstrom and Harvey 2011). In addition to RGC survival, CNTF has been demonstrated to promote regrowth of injured RGC axons after an optic nerve lesion (Leaver et al. 2006; Muller et al. 2009; Hellstrom and Harvey 2011; Pernet et al. 2013). Protective effects of CNTF on photoreceptors have been first shown in a photoreceptor degeneration mouse model induced by white light exposure. Intravitreal injections of CNTF significantly protected photoreceptors from degenerating up to 2 weeks (LaVail et al. 1998). Subsequent studies confirmed a significant neuroprotective effect of intraocularly administered CNTF in other animal models of photoreceptor loss such as the rd2 mouse (Cayouette et al. 1998; Liang et al. 2001; Schlichtenbrede et al. 2003), the rhodopsin S334ter and P23H transgenic rats (Liang et al. 2001), and the RCS rat (Huang et al. 2004).

Although CNTF exerts strong morphological protection on photoreceptors, functional analyses with ERGs showed decreasing light sensitivity of rods in response to sustained CNTF administration due to a negative impact of CNTF on the phototransduction cascade (Liang et al. 2001; Bok et al. 2002; Schlichtenbrede et al. 2003; Wen et al. 2006; McGill et al. 2007; Rhee et al. 2007). However, another study reported that a low dose application of CNTF protected photoreceptors without negatively affected ERG functions (Bush et al. 2004). Additionally, CNTF treatment in a rat model showed stable ERG data of cones (Li et al. 2010). In clinical trials protective effects of CNTF on photoreceptors are currently tested in patients with RP or AMD and have given promising results concerning the morphological and functional preservation of cone photoreceptors (Sieving et al. 2006; Talcott et al. 2011; Zhang et al. 2011). In fact, a recent study with long-term CNTF application in a mouse model with retinal degeneration has shown that surviving cone photoreceptors retain function (Lipinski et al. 2015). Since cones are responsible for visual acuity, protection of cones with CNTF would represent a promising therapeutic approach and has to be investigated further.

Another growth factor family that has been intensively studied is the vascular endothelial growth factor (VEGF) family. It consists of five secreted growth factors in mammals: VEGF (or VEGF-A), VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PIGF) (Rosenstein and Krum 2004; Holmes and Zachary 2005; Shibuya 2006). All VEGF growth factors are known for their functions in vascular development in physiological and pathological conditions (Carmeliet et al. 1996; Ferrara et al. 1996; Carmeliet and Jain

2000; Carmeliet et al. 2001; Luttun et al. 2002; Alitalo et al. 2005). VEGF-B is the only member of this family which has been reported to exert no or only limited angiogenic effects *in vivo* (Li et al. 2008; Bry et al. 2014). The VEGF growth factors bind to three transmembrane tyrosine kinase receptors (VEGFR-1 or fms-like tyrosine kinase 1 (Flt1), VEGFR-2 or mouse fetal liver kinase 1 (Flk1) and VEGFR-3 or fms-like tyrosine kinase 4 (Flt4)) and two semaphorin receptors (neuropilin-1 (NRP-1) and NRP-2) with differing specificities (Fig. 1.4) (Bry et al. 2014).

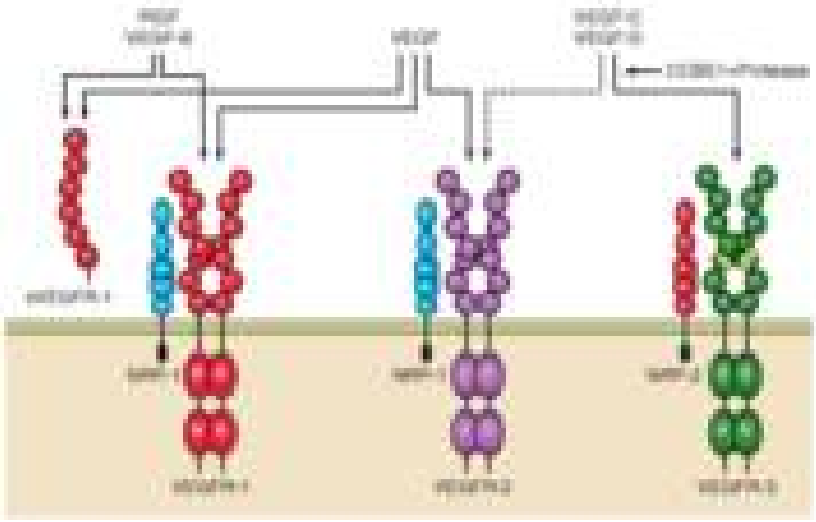


Figure 1.4: Transmembrane tyrosine kinase receptors of the VEGF family.
The VEGF-B family members bind with differing specificities to the three receptors VEGFR-1, VEGFR-2 and VEGFR-3 and their co-receptors NRP-1 and NRP-2. Angiogenic effects of VEGF result from VEGF binding to the VEGFR-2, whereas VEGF-B effects are mainly mediated through VEGFR-1 and NRP-1 (from Bry et al. 2014).

VEGF-B, also known as VEGF-related factor (VGF), was first discovered as a structural homologue of VEGF (Grimmond et al. 1996; Olofsson et al. 1996). In contrast to VEGF, VEGF-B does not have a crucial role in embryonic vasculature development and VEGF-B deficient mice are viable (Aase et al. 2001). VEGF-B rather acts as a survival factor for endothelial cells (Zhang et al. 2009b). Involvement of VEGF-B in fatty acid transport in endothelial cells has also been reported (Hagberg et al. 2010). Interestingly, anti-apoptotic and neuroprotective effects of VEGF-B *in vitro* and *in vivo* have been reported recently. VEGF-B was first described to stimulate proliferation in neuronal cell cultures (Sun et al. 2004) and has subsequently been implicated in the regulation of neurogenesis in adults (Sun et al. 2006). Later studies delineated neuroprotective effects of exogenously administrated VEGF-B on motor neurons, brain and retinal cell types (Li et al. 2008; Poesen et al. 2008). Further investigations on Parkinson disease models showed protective effects of VEGF-B on dopaminergic and sensory neurons *in vitro* and *in vivo* (Falk et al. 2009; Dhondt et al. 2011; Falk et al. 2011).

In initial experiments, neurotrophic factor administration to the retina was achieved by injections of recombinant proteins into the vitreous cavity. To estimate potential neuroprotective effects of intravitreal injections of recombinant growth factors, a variety of different factors was tested in a number of different animal models of inherited retinal degeneration, including bFGF in the Royal College of Surgeons (RCS) rat (Faktorovich et al. 1990), PEDF in *Pde6b*^{rd1} and rd2 mouse model (Cayouette et al. 1999) and CNTF in the *Pde6b*^{rd1} and Q334ter mouse with photoreceptor degeneration (LaVail et al. 1998). Intravitreal injections or different recombinant growth factors were also performed in animal models of glaucoma (Chen and Weber 2001; van Adel et al. 2003; Zhang et al. 2005; Lingor et al. 2008; Parrilla-Reverter et al. 2009). In all these experiments, some protection of photoreceptor cells or ganglion cells was observed. However, the neuroprotective effects were weak and only detectable for a short period of time after growth factor administration. As mentioned above for the administration of enzymes in ERT, the short half-life time of proteins requires multiple injections to achieve significant long-term therapeutic effects. Furthermore, a systemic administration of neurotrophic factors for ocular treatment is ineligible because of the blood-retina barrier. Consequently, a long-term therapy with neuroprotective factors requires a sustained intraocular administration of these proteins. Application systems that are currently being evaluated for a prolonged intraocular neurotrophic factor delivery include implantable miniature pumps (Saati et al. 2009), biodegradable factor-loaded slow release devices (Aburahma and Mahmoud 2011) or therapeutic strategies that either use viral vectors encoding neurotrophic factors or cell-based neuroprotective strategies.

Preclinical studies using adeno-associated or lentiviral vectors to express neurotrophic factors in endogenous ocular cell types gave promising results in protecting RGCs in glaucoma (in optic nerve crush (ONC) models (Leaver et al. 2006; MacLaren et al. 2006) or in laser-induced ocular hypertension models (Pease et al. 2009) and in protecting photoreceptors in RP models (Liang et al. 2001; Dalkara et al. 2011; Ali 2012). However, gene-based strategies for neurotrophic factor delivery face several problems. For instance, there is only limited control of the genetic modification of the host cells and the effective concentrations of neurotrophic factors derived to the retina. To circumvent genetic modifications of host cells and to deliver defined quantities of neurotrophic factors to the retina, cell-based neuroprotective strategies are currently being investigated intensively.

Cell transplantation strategies with a variety of different cell types have been evaluated as another possibility to prevent or delay retinal cell degeneration. Mesenchymal stem cells (MSCs) are the most frequent applied cell type in the context, because MSCs display some characteristics that make them a promising candidate cell type for cell-

based neuroprotective strategies. For instance, they can be isolated from a variety of tissues from affected patients, including the adult bone marrow, adipose tissue, muscle and umbilical cord (Caplan 1991; Erices et al. 2000; Qu-Petersen et al. 2002; Zuk et al. 2002), and can thus be used in autologous transplantation settings. Several studies have demonstrated that transplantation of MSCs into the eye results in attenuation of photoreceptor or ganglion cell loss in various animal models (Yu et al. 2006; Inoue et al. 2007; Li et al. 2009; Zwart et al. 2009; Joe and Gregory-Evans 2010; Johnson et al. 2010; Wang et al. 2010). For example subretinal transplantation of bone marrow MSCs delayed photoreceptor degeneration in the RCS rat model (Inoue et al. 2007) and intravitreal injections of MSC into a laser induced hypertension rat model of glaucoma resulted in significant RGC axon survival (Johnson et al. 2010). The protective effects of MSCs are most likely mediated through secretion of endogenously expressed neurotrophic factors and/or the induction of neurotrophic factor expression in endogenous ocular cells by the transplanted cells (Li et al. 2009; Johnson et al. 2010). Transplantation experiments of MSCs into the vitreous cavity of an ischemic retina rat model have shown that MSCs secrete CNTF, bFGF and BDNF after maturation over a time period of at least 4 weeks (Li et al. 2009). A number of studies have suggested that MSCs are capable of “transdifferentiate” into neurons (Sanchez-Ramos et al. 2000; Woodbury et al. 2000; Deng et al. 2001; Suzuki et al. 2004) but recent investigations have provided conflicting results concerning the ability of MSCs to generate authentic nerve cells (Phinney and Prockop 2007; Joe and Gregory-Evans 2010). Therefore, neural stem (NS) cells likely represent a better cell type for therapeutic applications in neural tissue such as the retina.

NS cells are multipotent cells that display the ability of self-renewal through symmetric or asymmetric divisions and the capability to differentiate into the three major neural cell types, neurons, astrocytes and oligodendrocytes (McKay 1997; Breunig et al. 2011). Ongoing neurogenesis has been demonstrated in most regions of the postnatal brain (Gage 2000), and NS cells have been identified in adult brains (Reynolds and Weiss 1996; Eriksson et al. 1998). Of interest, in the context of potential therapeutic applications, NS cells can be isolated from the embryonic or adult brain, or derived from ES and iPS cells (Temple 1989; Reynolds and Weiss 1992; Okabe et al. 1996; Thomson et al. 1998; Wernig et al. 2008). To evaluate whether NS cells comprise a suitable cell type for retinal cell protection, NS cells have been grafted intravitreally or subretinally into different animal models. After intraocular transplantation into the RCS rat, NS cells differentiated preferentially into astrocytes and neurons, and in contrast to MSCs neither integrated into the host retinas nor exerted adverse effects on the host eyes. NS cells, like MSCs, secrete different neurotrophic factors which protect retinal structure and

function (McGill et al. 2007). Long-term survival of intraocularly grafted neural stem or progenitor cells has also been reported in other studies (Ader et al. 2000; Pressmar et al. 2001; Conti et al. 2005; Glaser et al. 2007; Wang et al. 2008; Francis et al. 2009; Jung et al. 2013).

To enhance the neuroprotective potential of grafted stem cells, genetic modifications prior to transplantations have been performed to increase neurotrophic factor expression in these cells. In the context of glaucoma, cell-based delivery systems have mainly focused on the growth factors CNTF and BDNF. A study with mesenchymal stem cells lentivirally modified to express BDNF has demonstrated protective effects on RGCs *in vitro* in models of glutamate- and hydrogen peroxidase-mediated cell death (Harper et al. 2009)and *in vivo* in a rat model of laser-induced chronic ocular hypertension (Harper et al. 2011). Subretinal transplantations of MSCs adenovirally modified to express PEDF into the RCS rat resulted in significant protection of photoreceptor cells. Additionally, the grafted MSCs resembled RPE cells and integrated into the host RPE (Arnhold et al. 2007). Other investigative approaches for a sustained delivery of neurotrophic factors used encapsulated cell technology (ECT) devices filled with genetically engineered cells secreting recombinant proteins. The encapsulated cell implants contained immortalized human RPE cells that were modified to express CNTF. To evaluate the therapeutic potential of these implants, they were transplanted intravitreally into the rapid retinal degeneration S334ter-S rat model and the *rcd1* canine model of RP. Rats were injected at postnatal day 9 and CNTF-treated eyes showed a significant protection of photoreceptors after 2 weeks of treatment compared to the contralateral eye which received nonmodified cells. Dogs were grafted at 7 weeks of age and after 7 weeks eyes treated with CNTF showed significantly higher photoreceptor numbers compared to untreated contralateral eyes. After explantation, the implants still showed low level expression of CNTF, and the encapsulated RPE cells were still viable. In addition, protection of CNTF was dose dependent with a minimum protection at CNTF doses of 0.2 to 1.0 ng/day (Tao et al. 2002). Another study in New Zealand White albino rabbits showed a correlation between the morphological and functional changes and the amount of CNTF secreted from the implants. CNTF doses below 5 ng/day neither induced alterations in retinal morphology nor in ERG recordings (Bush et al. 2004).

Because the encapsulated cell implants are too big for the use in small-sized animals, such as mice, other investigations used NS cells as a vehicle to deliver for neurotrophic factors to the dystrophic retina. Recently, intravitreal transplantations of NS cells lentivirally modified to express CNTF gave promising results in hereditary and acutely induced retinal degeneration mouse models and are discussed below (Jung et al. 2013; Flachsbarth et al. 2014; Jankowiak et al. 2015).

Of note, the therapeutic potential of cell-based neuroprotective strategies is currently also being investigated in clinical trials in patients with RP or geographic atrophy using the encapsulated cell technology (Wen et al. 2012). This technology is based on the spontaneously immortalized human RPE cell line ARPE19 that has been modified to overexpress CNTF. The modified cells are encapsulated in a small device with semipermeable membranes, and implanted into the vitreous cavity of the patients. Patients of first clinical trials reported an improvement in visual acuity suggesting that therapeutically effective CNTF dose is below the threshold for suppression of retinal function (see above) but high enough to protect retinal structure. Furthermore, after two years of treatment anti-CNTF antibodies were not detected in the serum of patients (Sieving et al. 2006; Talcott et al. 2011; Zhang et al. 2011; Kauper et al. 2012; Wen et al. 2012; Birch et al. 2013).

The preclinical and clinical studies discussed above indicate that a sustained cell-based intraocular administration of neurotrophic factors represents a promising therapeutic strategy to delay retinal degeneration. Importantly, this therapeutic approach might be efficient in different degenerative retinal disorders irrespective of the specific cause of the diseases, making it particularly interesting for potential clinical applications. The search for new neurotrophic factors and the evaluation of their efficacy to attenuate retinal degeneration in appropriate animal models is therefore among the important future tasks of studies aimed at further improving the therapeutic potential of neuroprotective treatment strategies for degenerative retinal disorders.

2. General discussion

The causes of progressive dysfunction and degeneration of retinal cells are diverse. Glaucomatous optic neuropathies are among the leading causes of blindness in patients aged over 40, and are characterized by a progressive degeneration of retinal ganglion cells (RGCs) and their axons in the optic nerve (Quigley and Broman 2006; Qu et al. 2010; Almasieh et al. 2012). While the exact pathomechanisms leading to the apoptotic death of RGCs are not fully understood, an elevated intraocular pressure (IOP) is considered as a major risk factor to develop this multifactorial and age-related disease (Agarwal et al. 2009; Pascale et al. 2012). Retinitis pigmentosa (RP) is the most common cause of inherited photoreceptor degeneration and mutations in more than 60 genes have been identified to cause RP (Sahni et al. 2011). A frequent ocular involvement is also present in lysosomal storage disorders (LSDs), a heterogeneous group of rare inherited diseases. LSDs are subdivided according to the accumulated substances resulting from dysfunctional lysosomal transporters or enzymes (Klein and Futerman 2013). Effective therapies for all these degenerative retinal disorders are currently not available.

2.1. Identification of new gene mutation to cause retinal dysfunction

In numerous retinopathies, photoreceptor cells comprise the retinal nerve cell type that is primarily affected. In genetically heterogeneous groups, such as RP or age related macular degeneration (AMD), mutations in many different genes can cause a dysfunction and subsequent loss of photoreceptor cells, or of other retinal cell types which in turn might cause a secondary degeneration of photoreceptors (Wright et al. 2010). Recently the impact of neurodegenerative lysosomal storage disorders on retinal structure and function has gained more attention. Advances in molecular biology and animal reproductive physiology have increased the generation of transgenic animal models, in particular rodent models that closely resemble human diseases, both genetically and phenotypically. Although much progress has been made in defining the molecular basis of inherited diseases, the phenotypic expression abnormalities of the underlying genetic defects are not fully understood (Haskins et al. 2006).

In the present thesis we describe the retinal phenotype of a novel LSD mouse model of mucopolysaccharidosis (MPS) type IIIE. MPSs are characterized by pathogenic mutations in lysosomal enzymes that are involved in the degradation of sulfated glycosaminoglycans (GAGs) (Muenzer 1986; Neufeld 2001; Muenzer 2011; Coutinho et al. 2012). One MPSs subtype is the Sanfilippo syndrome, also known as MPS III. MPS III

is caused by mutations in genes encoding enzymes involved in the degradation of heparan sulfate (HS), including N-sulfoglucosamine sulfohydrolase (*SGSH*; MPS IIIA), N-α-acetylglucosaminidase (*NAGLU*; MPS IIIB), heparan-α-glucosaminide N-acetyltransferase (*HGSNAT*; MPS IIIC), and N-acetylglucosamine-6-sulfatase (*GNS*; MPS IIIE) (Valstar et al. 2008; Coutinho et al. 2012). It has recently been shown that arylsulfatase G (ARSG, also termed N-sulfoglucosamine-3-O-sulfatase) is another enzyme that is involved in the degradation of HS (Fig. 2.1) (Kowalewski et al. 2012; Kowalewski et al. 2014).

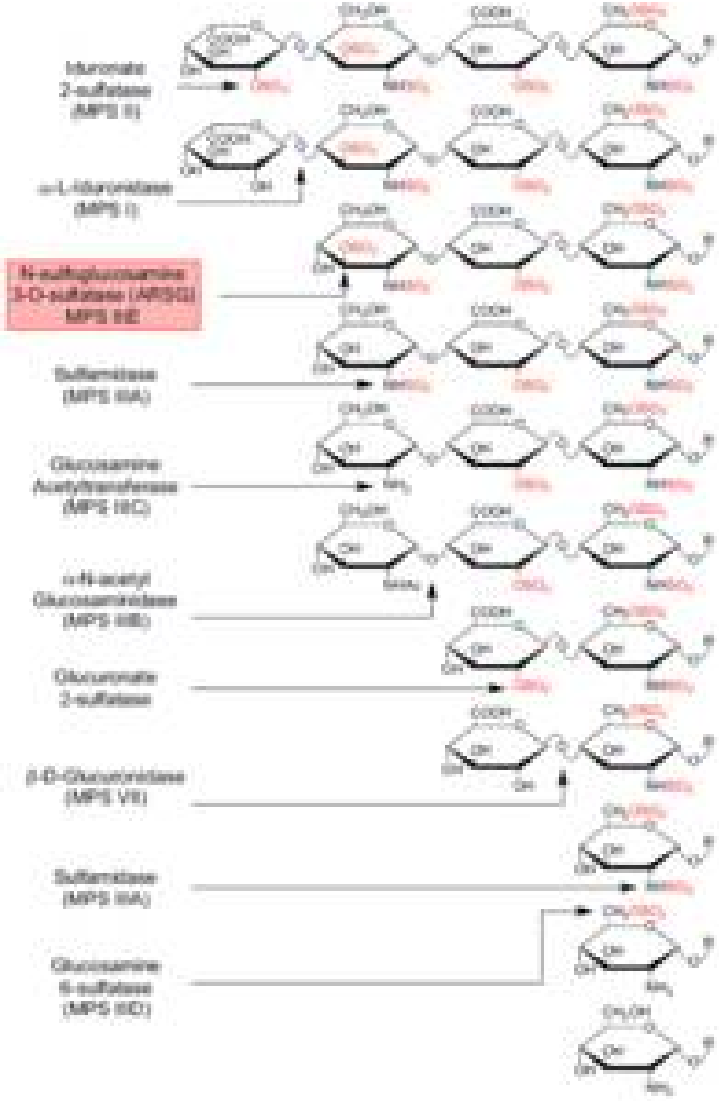


Figure 2.1: Schematic presentation of all nine enzymes involved in sequential catabolism of nonreducing ends (NRE) saccharides containing N-sulfoglucosamine-3-O-sulfate.
Defective enzymes involved in the degradation of different NRE saccharides and the resulting MPS types are indicated. Mutations in the *Arsg* gene and thus loss of ARSG activity leads to an accumulation of 3-O-sulfated substrate (from Kowalewski et al. 2012).

A recent analysis of *Arsg* knockout (KO) mice has demonstrated accumulation of HS in many different organs (Kowalewski et al. 2012). In comparison to mouse models of other

MPS III subtypes (Bhaumik et al. 1999; Li et al. 1999; Martins et al. 2015) *Arsg* KO mice display a milder phenotype with Purkinje cell degeneration in the cerebellum as the prominent neurological defect (Kowalewski et al. 2014). In the first part of this thesis we extended this initial phenotypic characterization of *Arsg* KO mice. This more detailed analysis revealed that ARSG deficiency in mice results in a degeneration of photoreceptor cells, starting between 1 month and 6 months of age (Kruszewski et al., submitted for publication), when neuronal loss in the brain is not yet detectable (Kowalewski et al. 2014). The slowly progressing degeneration of photoreceptor cells was accompanied by a reactive astrogliosis and a dysregulation of several lysosomal proteins. A more detailed analysis of the outer nuclear layer of 24 months old *Arsg* KO mice revealed that rod photoreceptor cells comprise the photoreceptor cell type that is mainly affected in the mutant mouse, whereas the number of cone photoreceptors remained normal. These results are in line with findings in a mouse model of MPS IIIB, where diminished electroretinographic (ERG) dark-adapted retinal responses indicated a loss of rod function, whereas normal retinal function after light adaptation suggested normal cone function (Heldermon et al. 2007). Of note, in most genetic conditions primary rod photoreceptor degeneration is followed by secondary cone degeneration. Normal numbers of cones in aged *Arsg* KO mice are likely related to the fact that rod photoreceptor degeneration progresses slowly, and cones are thus not yet affected in 2 year old mutant mice (Madreperla et al. 1990; Hamel 2007; Punzo et al. 2009). The thinning of the outer nuclear layer was accompanied by an accumulation of microglia cells / macrophages in the outer retina suggesting that retinal nerve cell types other than photoreceptor cells were not affected in the *Arsg* KO mutant. In addition, a recent study identified a novel mutation in the *HGSNAT* gene in patients displaying symptoms characteristic for RP (Haer-Wigman et al. 2015).

In an attempt to identify the actual cause of the photoreceptor cell degeneration in *Arsg* KO mice, we found that expression of ARSG in healthy adult murine retinas was restricted to the retinal pigment epithelium (RPE). Of interest in this context, RPE cells are vital for normal photoreceptor function and for photoreceptor survival, and a dysfunctional RPE causes photoreceptor cell degeneration (Strauss 2005). Furthermore, previous studies showed that RPE cells significantly contributed to the synthesis and degradation of all major mucopolysaccharides and impaired GAG degradation was suggested to contribute to RP (Del Monte et al. 1991). However, ultrastructural analyses of the RPE of aged *Arsg* KO retinas did not reveal obvious pathological alterations, such as storage vacuoles which are typically found in different tissues of animal models or patients of other MPS III subtypes (Del Monte et al. 1983; Lavery et al. 1983; Bhaumik et al. 1999; Heldermon et al. 2007; Jolly et al. 2007). While it is possible that RPE cells in

Arsg KO mice are functionally impaired but morphologically intact, we cannot exclude the possibility of a low level expression of ARSG in photoreceptor cells that was below the detection level of the immunohistochemical technique used in our study.

In summary, our work extends the phenotypic characterization of *Arsg* KO mice and demonstrates that in addition to Purkinje cell degeneration in the cerebellum. ARSG deficiency additionally causes a slowly progressing degeneration of rod photoreceptor cells. Loss of rod photoreceptor cells was accompanied by reactive astrogliosis, reactive microgliosis and a dysregulation of several lysosomal proteins. We suggest that the retinal phenotype caused by ARSG deficiency might help to identify human patients suffering from MPS caused by mutations in *ARSG*.

2.2. Therapeutic approaches for an ocular treatment independent of the etiology

Currently, there are no effective therapies for the treatment of degenerative retinal disorders affecting different retinal cell types, such as RPE cells, photoreceptor cells or RGCs. Animal models of degenerative retinal disorders provide the possibility to develop novel therapies for these conditions, and to assess the efficacy of these therapeutic interventions in delaying retinal degeneration and preserving visual function. The major strategies to establish effective therapies for retinal dystrophies include pharmacological treatments, the implantation of electronic retinal implants, cell replacement strategies, corrective gene therapy, optogenetic therapy, and neuroprotection (Sahni et al. 2011; Almasieh et al. 2012).

The aim of neuroprotective strategies is not to target the specific cause of the disease but rather its consequences, i.e. to delay the progressive degeneration of retinal cells such as photoreceptors or RGCs. In numerous preclinical studies several proteins have been identified that exert neuroprotective effects on different retinal cell types. One of the first identified neurotrophic factor is nerve growth factor (NGF), which has been shown to protect various nerve cell types from degeneration, including photoreceptor cells (Hamburger et al. 1981). Since then a number of other neuroprotective factors has been identified (Buch et al. 2007). Among those, ciliary neurotrophic factor (CNTF) is by far the most extensively studied neuroprotective factor in the context of degenerative retinal disorders (Wenzel et al. 2005; Wen et al. 2012). For instance, injections of recombinant CNTF into the vitreous cavity of an elevated ocular hypertension rat model of glaucoma significantly protected RGCs from degeneration for up to 4 weeks after the treatment (Ji et al. 2004). In a photoreceptor degeneration mouse model where retinal degeneration was induced by white light exposure, CNTF significantly protected photoreceptors up to 2 weeks after an intravitreal injection of the recombinant protein (LaVail et al. 1998). A number of subsequent studies confirmed the neuroprotective effects of intravitreally

injected recombinant CNTF on photoreceptor cells and RGCs (Maier et al. 2004; Zhang et al. 2005; Wen et al. 2006; Lingor et al. 2008; Parrilla-Reverter et al. 2009). However, attenuation of photoreceptor loss or RGC loss in all these studies was only detectable over a short period of time, most likely due to the short half-life of the cytokine. These results indicate that a continuous intraocular administration of neuroprotective factors is required in order to achieve significant and long-lasting therapeutic effects on diseased photoreceptors or RGCs.

2.2.1. Genetically modified neural stem cells for a sustained intraocular administration of neurotrophic factors

The main aim of this thesis was to establish a cell-based delivery system that allows a sustained and controlled administration of neurotrophic factors to the dystrophic mouse retina, and that is suitable for the application in a wide range of preclinical animal models. Of interest in the cell-based neuroprotective strategies is the fact that various unmodified cell types have been demonstrated to exert neuroprotective effects on photoreceptor cells or RGCs after intraocular transplantations into various animal models of retinal dystrophies. The most frequently cell type used in these intraocular transplantation experiments are mesenchymal stem cells (MSCs). For instance, intravitreal injections of mouse bone marrow cells in two mouse models of RP, the *Pde6b*^{rd1} and the *Pde6b*^{rd10} mouse mutant, attenuated photoreceptor degeneration and preserved ERG recordings (Otani et al. 2004). MSC transplantations also preserved the photoreceptor layer of rhodopsin knockout mice, another animal model of RP (Arnhold et al. 2007). In an ischemia/reperfusion rat model, grafted bone marrow-derived MSCs delayed the loss of RGCs by approximately 25% for up to 4 weeks (Li et al. 2009). Furthermore, intravitreal transplantations of MSCs have been shown to promote RGC survival in an ocular hypertension rat model (Johnson et al. 2010). It has been suggested that MSCs confer their neuroprotective activity on retinal nerve cell types through the secretion of endogenously expressed neurotrophic factors, or through an induction of neurotrophic factor expression in the host cells. In fact, intravitreal transplantations of genetically modified MSCs with an elevated expression of neurotrophic factors, such as neurotrophin-4, resulted in significantly higher protection of damaged retinal cells in an acute retinal injury mouse model (Machalinska et al. 2013). In a chronic ocular hypertension rat model, MSCs modified to secrete brain-derived neurotrophic factor (BDNF) preserved retina and optic nerve function more efficiently than MSCs modified to express green fluorescent protein only (GFP). Preservation of RGCs numbers was also greater in BDNF-MSC treated eyes compared to GFP-MSC treated eyes (Harper et al. 2011).

In addition to MSCs, other unmodified and modified cell types have also been tested for their ability to attenuate retinal degeneration, also with promising results regarding retinal cell protection. Intraocular transplantations of unmodified NS cells exerted neuroprotective effects on photoreceptor cells in dystrophic Royal College of Surgeons (RCS) rats, an animal model for RP (Gamm et al. 2007), and on RGCs in the optic nerve crush (ONC) rat model, an animal model for glaucoma (Satarian et al. 2013). We also tested several cell types as cellular vectors to administer neurotrophic factors to the murine retina, including MSCs, the immortalized neural progenitor cell line C17.2, neurosphere cells or primary retinal progenitor cells. However, all these cell types either did not survive for a prolonged period of time in the vitreal cavity after intraocular transplantation, or they integrated and damaged the host retina. Adherently cultivated neural stem (NS) cells isolated from the cerebral cortex of embryonic mice, in contrast, survived for extended periods of time after intravitreal transplantations and exerted no adverse effects on the host retina (Jung et al. 2013; Flachsbarth et al. 2014). NS cell were therefore selected to establish a cell-based intraocular delivery system for neurotrophic factors. The cultivation of NS cells under adherent conditions in the presence of endothelial growth factor (EGF) and fibroblasts growth factor-2 (FGF-2) gives rise to pure populations of symmetrically dividing clonogenic neural stem cells (Conti et al. 2005). Furthermore, NS cells remain tripotent after prolonged expansions of over 100 passages and give rise to neurons, astrocytes and oligodendrocytes when induced to differentiate *in vitro* and after transplantation into the adult brain or spinal cord *in vivo* (Conti et al. 2005; Glaser et al. 2007). Based on all these properties, we selected NS cells as the cell type to establish a cell-based intraocular delivery system for neurotrophic factors, and evaluated the neuroprotective potential of unmodified NS cells on dysfunctional photoreceptor cells and axotomized RGCs. These experiments revealed no evidence for neuroprotective effects of intravitreally grafted NS cells on photoreceptor cells in *Pde6b*^{rd1} and *Pde6b*^{rd10} mutant mice, nor on RGCs in the mouse ONC model. We therefore decided to establish genetically modified clonal NS cell lines with an ectopic expression of neurotrophic factors that are known to exert neuroprotective effects on retinal cell types.

2.2.2. Lentiviral modification of NS cells to achieve a stable expression of neurotrophic factors

After selecting NS cells as a vehicle for intraocular neurotrophic factor delivery, we next evaluated how to modify the NS cells to achieve a stable neurotrophic factor expression in undifferentiated NS cells and their differentiated progeny. To this aim, we tested different techniques, including electroporation, nucleofection or lipofection and finally

ended up with lentiviral vectors that are based on the lentiviral “gene ontology” (LeGO) vectors (Fig. 2.2) (Weber et al. 2008; Weber et al. 2010).

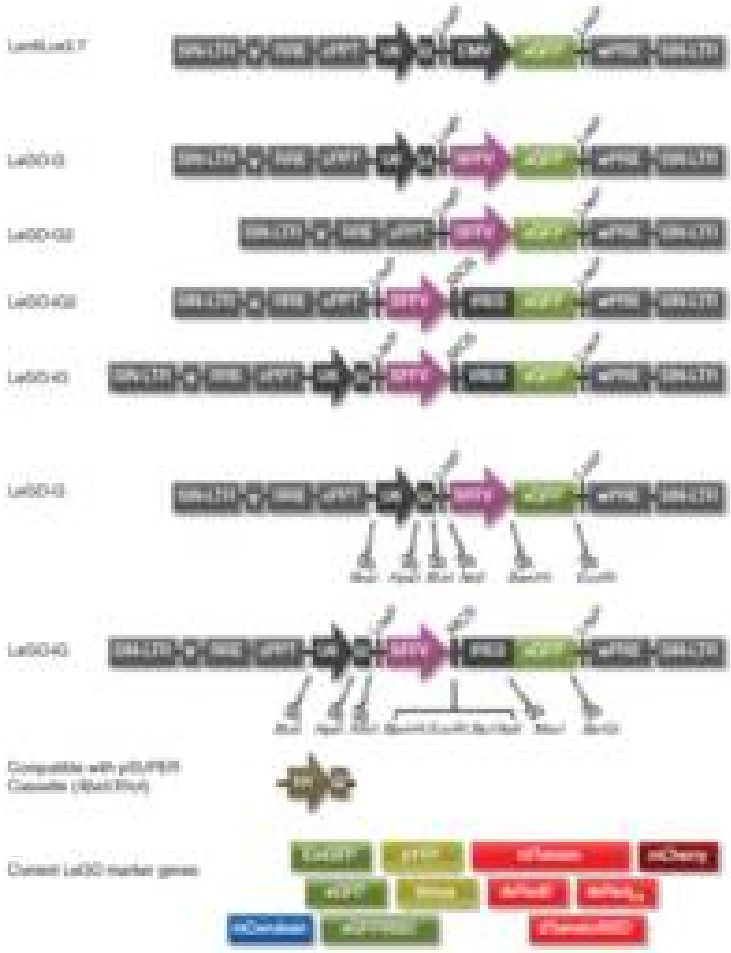


Figure 2.2: Structure of lentiviral “gene ontology” (LeGO) vectors.
Shown are different LeGO vectors that are derived from pLentiLox3.7. Unique restriction sites facilitate the exchange of the gene of interest or the reporter gene. The basic construct is available with different fluorescent markers (from Weber et al. 2008).

First, we evaluated which promoter ensures strong and long-lasting expression of transgenes in undifferentiated and neutrally differentiated NS cells. To this aim, we tested a number of different promoters, including cytomegalovirus (CMV), spleen focus-forming virus (SFFV), phosphoglycerate kinase 1 (PGK), elongation factor 1 alpha (EF1 alpha) and cytomegalovirus enhancer/chicken β-actin (CAG) promoter (data not shown). We decided to use the CAG promoter because we observed stable expression of different genes of interest and different fluorescent reporter proteins in undifferentiated NS cells for more than 20 passages *in vitro*. Furthermore, the CAG promoter also ensured stable transgene expression in neurons and astrocytes derived from lentivirally modified NS cells. The internal ribosome entry site (IRES) was used to separate the genes of interest from the reporter genes to ensure expression of a fully functional neurotrophic protein without fusion to the fluorescent reporter protein.

2.2.3. Sustained neural stem cell-based intraocular administration of CNTF attenuates photoreceptor cell loss in different mouse models

2.2.3.1. CNTF preserves photoreceptor cells in two mouse models of RP

The aim of the first therapeutic approach was to establish a sustained cell-based neurotrophic factor delivery to the dystrophic mouse retina. To this aim, we cloned a secretable variant of mouse ciliary neurotrophic factor (CNTF), a Venus reporter gene and a zeocin resistance gene into the LeGO vector under regulatory control of the CAG promoter. The CNTF and the reporter gene were separated from each other by an IRES sequence, while the reporter and the resistance gene were separated from each other by a P2A sequence of porcine teschovirus-1, giving rise to the polycistronic lentiviral vector pCAG-CNTF-IRES-Venus-2A-Zeo. The same vector but lacking the CNTF cDNA (pCAG-IRES-Venus-2A-Zeo) was generated for control experiments. CNTF was selected as a neurotrophic factor in these initial experiments, because this cytokine has been shown to exert strong neuroprotective effects on photoreceptor cells and RGCs in different animal models of retinal dystrophies (Wenzel et al. 2005; Harvey et al. 2006; Almasieh et al. 2012; Wen et al. 2012; Wilson and Di Polo 2012).

Of particular interest in this context, encapsulated human RPE cells modified to express CNTF are currently used in clinical trials to evaluate the therapeutic potential of cell-based neuroprotective approaches in patient with RP or AMD (Sieving et al. 2006; Talcott et al. 2011; Zhang et al. 2011; Kauper et al. 2012). The CNTF-secreting implant is composed of a semi-permeable membrane which allows oxygen and nutrients to diffuse in and CNTF to diffuse out of the device, thus keeping the cells alive and protecting them from immune responses after intravitreal implantations into the patients' eyes. In comparison to a virus-mediated gene transfer of neurotrophic factors, this encapsulated cell technology offers the advantage to continuously administer neurotrophic factors to dystrophic retinas without the need to genetically modify endogenous retinal cells. Other advantages of this cell-based delivery approach include (i) the possibility to determine the amount of the neurotrophic factor secreted from the encapsulated cell devices prior to implantation and (ii) the possibility to simply retrieve the implant from the eye in case of possible adverse effects of the treatment. In a phase I clinical trial, encapsulated cell devices secreting either low or high amounts of CNTF were implanted into the vitreous cavity of the patients. This safety study revealed a good survival of the encapsulated cells over extended periods of time, and intraocular tolerability of the devices (Sieving et al. 2006). A phase II trial then demonstrated that therapeutically relevant amounts of CNTF were delivered to the diseased retinas, as indicated by an attenuation of cone photoreceptor cells loss over a time period of 24 months when compared with sham-treated eyes (Talcott et al. 2011). The increase in

retinal thickness and the stabilization of visual acuity in CNTF-treated eyes was dose-dependent, with a significantly better preservation of visual acuity after implantation of encapsulated cell devices secreting high amounts of the cytokine (20 ng/day). These findings suggest that a cell-based sustained intraocular administration of CNTF via implantations of encapsulated and genetically modified cells can slow the progression of photoreceptor degeneration in patients with geographic atrophy (Zhang et al. 2011). A further study showed that the intraocular delivery of CNTF through encapsulated cells over a time period of up to 2 years did not result in the production of antibodies against CNTF or the encapsulated cell. Thus the encapsulated cell technology seems to represent a safe and suitable neuroprotective approach for the treatment of slowly progressing retinal dystrophies (Kauper et al. 2012). While the encapsulated cell technology is applicable in human patients and large animal models of retinal dystrophies, the large size of the cell implants precludes their use for experiments on the numerous genetic and acutely induced mouse models of degenerative disorders, making a systemic evaluation and optimization of this approach difficult. We therefore aimed at establishing a cell-based intraocular delivery system for neurotrophic factors with non-encapsulated cells that closely mimics properties of the encapsulated cell technology and that can be used to continuously deliver neurotrophic factors and other secretable proteins to the dystrophic mouse retina.

To examine the efficacy of a NS cell-based intraocular delivery of CNTF to attenuate retinal degeneration, we used two animal models of RP, the *Pde6b*^{rd1} and *Pde6b*^{rd10}mutant mouse. Both animal models represent naturally occurring mouse mutants carrying mutations in the β-subunit of the *PDE6B* gene. Mutations in the β-subunit of the *PDE6B* gene lead to an impaired activity of cyclic guanosine monophosphate (cGMP)-phosphodiesterase type 6 (cGMP-PDE) of rod photoreceptors. Thus, retinal degeneration results from accumulation of cGMP in rods. cGMP is a messenger molecule in rod photoreceptors which is involved in transduction of light photons into electrical signals. In *Pde6b*^{rd1} mice, rod photoreceptor degeneration starts around postnatal day 8. At postnatal day 15 all rod photoreceptors are degenerated while cone photoreceptors are completely lost by 4 weeks of age. *Pde6b*^{rd10} mice bear another mutation in the *Pde6b* gene which leads to a later onset of rod photoreceptor degeneration, starting at 2 weeks of age. In contrast to *Pde6b*^{rd1} mice, photoreceptor degeneration of *Pde6b*^{rd10} mice is light dependent and no ERG recordings are detectable at 2 months of age. Of note, mutations in the *PDE6B* gene have also been identified in patients with autosomal recessive RP. Thus, both the *Pde6b*^{rd1} and the *Pde6b*^{rd10} mouse represent suitable animal models of RP (Chang et al. 2002; Chang et al. 2007).

Intravitreal transplantations of modified NS cells were performed at postnatal day 7 in *Pde6b*^{rd1} mice and at postnatal day 14 in *Pde6b*^{rd10} mice, prior to the onset of photoreceptor degeneration. The grafted NS cells survived for at least two weeks in the host eyes, mainly differentiated into astrocytes and exerted no adverse effects on the morphology of the host retinas. Importantly, the CAG promoter ensured a high and stable expression of CNTF in undifferentiated and differentiated NS cells *in vitro* and in grafted cells *in vivo*. Determination of photoreceptor numbers in CNTF treated eyes of *Pde6b*^{rd1} mice 8 days post-transplantation and of *Pde6b*^{rd10} mice 16 days post-transplantation revealed significant protection of photoreceptors throughout the entire retina in both mouse models when compared with control retinas. These observations indicate that the modified NS cells had secreted functionally relevant amounts of CNTF into the aqueous humour from where the cytokine entered the dystrophic retinas to attenuate retinal degeneration. Clonal selection and subsequent expansion of modified NS cells with high expression levels of the fluorescent reporter protein gave rise to homogeneous clonal NS cell lines with defined and increased expression levels of the cytokine, when compared to the original bulk cultures composed of cells with different expression levels of CNTF. Furthermore, we demonstrated that CNTF protected photoreceptors in both mouse models in a dose-dependent manner, with more surviving photoreceptors in eyes that had received grafts of clonal NS cells with high expression levels of CNTF than in eyes that had received grafts of bulk NS cell cultures with lower expression levels of the cytokine (Jung et al. 2013).

Administration of CNTF to the retina has previously been shown to preserve photoreceptor morphology in a variety of animal models (Wen et al. 2012) but functional analyses of rod photoreceptors revealed a negative impact of CNTF on the light sensitivity of rods and their phototransduction cascade (Wen et al. 2006; Wen et al. 2008). However, some studies showed that low doses of CNTF did not affect ERG functions (Bush et al. 2004; Li et al. 2010). Additionally, clinical trials provided evidence that a long-term administration of CNTF preserved the morphology and function of cone photoreceptors (Sieving et al. 2006; Talcott et al. 2011; Zhang et al. 2011). Of note, a recent study confirmed that CNTF protected cone photoreceptors and preserved their function (Lipinski et al. 2015).

In summary, we have demonstrated that a sustained NS cell-based intraocular delivery of CNTF resulted in the administration of functionally relevant amounts of the cytokine to the dystrophic mouse retina, as indicated by the significant attenuation of photoreceptor cell loss in two mouse models of RP that normally display a rapid degeneration of photoreceptor cells. Moreover, we have also demonstrated that the neuroprotective effect of CNTF in both mouse models was widespread and evident over the entire retina.

Importantly, adverse effects of the grafted NS cells on the morphology of the host retinas were not observed.

2.2.3.2. CNTF preserves photoreceptors in a mouse model of NCL

To further evaluate the therapeutic potential of a sustained NS cell-based neurotrophic factor delivery in mouse models of retinal dystrophies, we intravitreally transplanted CNTF-NS cells into the *nclf* mouse, an animal model of variant late infantile neuronal ceroid lipofuscinosis (vLINCL). NCL is a genetically heterogeneous group of LSDs with an onset mainly in childhood and youth. Although mutations in various genes (encoding either soluble lysosomal enzymes or intracellular transmembrane proteins) have been identified to cause NCL, the clinical manifestation of these mutations results in similar symptoms, including progressive mental and motor deterioration, brain atrophy, seizures, and premature death. Retinal degeneration and vision loss represent another typical symptom of some NCL variants (Jalanko and Braulke 2009; Anderson et al. 2013; Schulz et al. 2013).

vLINCL is caused by mutations in the *CLN6* gene, which encodes a transmembrane protein of the endoplasmic reticulum with yet unknown function (Gao et al. 2002; Wheeler et al. 2002; Heine et al. 2004; Mole et al. 2004). Dependent on the pathogenic mutation, age of onset and severity of the disease can differ greatly (Kousi et al. 2012). The *nclf* mouse is a naturally occurring mouse model of vLINCL (Bronson et al. 1998). The retinal phenotype of the *nclf* mouse is characterized by an early-onset retinal degeneration, beginning in the first postnatal month. In one year old mutants, photoreceptor cells are almost completely lost. The progressive degeneration of photoreceptors is accompanied by an accumulation of storage material in various retinal cell types, and a dysregulation of several lysosomal proteins. Furthermore, amplitudes of ERG recordings decrease with increasing age of the mutants, and *nclf* mice perform poorly in optokinetic tracking experiments and visual cliff tests (Bartsch et al. 2013; Mirza et al. 2013; Morgan et al. 2013).

Several therapeutic approaches are currently being tested as potential treatment options of NCL, including enzyme replacement therapy (ERT), gene therapy, stem cell therapy and pharmaceutical treatment (Wong et al. 2010; Kohan et al. 2011; Shacka 2012; Bond et al. 2013). In the context of retinal degenerations associated with NCL, neural progenitor cell injections into the vitreal cavity of CLN8 mice and adeno-associated virus (AAV) mediated gene transfer of palmitoyl protein thioesterase-1 into the retina of CLN1 mutant mice significantly delayed photoreceptor degeneration (Griffey et al. 2005; Meyer et al. 2006). Another study reported reduced reactive microgliosis in the degenerating retina of *nclf* mice in response to dietary supplementation enriched in immuno-regulatory compounds (Mirza et al. 2013).

In the second neuroprotection study, we used the lentivirally modified NS cells to continuously administer CNTF to the dystrophic *nclf* mouse retina. We first established a clonal CNTF-NS cell line that expressed higher levels of the cytokine than the cell line used for the experiments on the *Pde6b*^{rd1} and *Pde6b*^{rd10} mutant mice. To this aim, we transduced the original clonally derived CNTF-NS cell line again, selected single cells with the highest expression level of the fluorescent reporter protein by fluorescent activated cell sorting (FACS), and clonally expanded these cells. This procedure was repeated several times until a clonally derived CNTF-NS cell culture with high expression of the cytokine was established for all further experiments. Intravitreal transplantations were performed in 2 week old mice, prior to the onset of photoreceptor degeneration. The intravitreally injected NS cells survived over a time period of at least 6 weeks in the host eyes where they predominantly differentiated into glial fibrillary acidic protein (GFAP)-positive astrocytes. Six weeks after transplantation, expression of CNTF was detectable in astrocytes derived from grafted CNTF-NS cells, but not in astrocytes derived from grafted control-NS cells. Analyses of CNTF treated eyes 2, 4, and 6 weeks post-transplantation revealed significantly higher photoreceptor numbers when compared to eyes that had received injections of control-NS cells. The protective effects of CNTF on photoreceptors were evident in all retinal regions, similar to observations in *Pde6b*^{rd1} and *Pde6b*^{rd10} mice, indicating a sustained delivery of CNTF to the entire *nclf* retina over a period of at least 6 weeks. Similar to the results obtained for the *Pde6b*^{rd1} and *Pde6b*^{rd10} retinas, we observed no adverse effects of the transplanted NS cells on the host retina of *nclf* mice. However, we occasionally found small-sized retinal folds in restricted retinal regions of *nclf* mice treated with CNTF-NS cells, which were absent from retinas of mice that had received injections of control-NS cells. Similar retinal folds were found in healthy or dystrophic feline retinas after repeated intravitreal injections of the CNTF analogue axokine (Chong et al. 1999). Therefore, we concluded that the retinal folds represent a complication of the prolonged administration of CNTF rather than a complication of the NS cell transplantation itself (Jankowiak et al. 2015). Thus, retinal fold are among the complications of long-term administration of high-doses of CNTF, in addition to the dose-dependent dysregulation of some components of the phototransduction cascade (Liang et al. 2001; Schlichtenbrede et al. 2003; Bush et al. 2004; Wen et al. 2006; McGill et al. 2007).

Taken together, this study provides evidence that a sustained intraocular NS cell-based delivery of CNTF attenuates retinal degeneration in another mouse model of photoreceptor cell loss. Although the pathomechanisms leading to the photoreceptor degeneration in *nclf* mice and both *Pde6b* mouse mutants are different, application of CNTF significantly attenuated photoreceptor cell loss in all three mouse models.

Accordingly, we conclude that a sustained cell-based CNTF delivery results in significant protection of photoreceptors over a prolonged period of time, irrespective of the specific etiology of the photoreceptor degeneration.

2.2.3.3. CNTF attenuates RGC loss and promotes axonal regeneration in a mouse ONC model

It has been proposed that an elevated IOP can result in an impaired axonal transport of neurotrophic factors, ultimately leading to the apoptotic death of RGCs (Fig. 2.3) (Anderson and Hendrickson 1974; Quigley et al. 2000; Pease et al. 2009; Almasieh et al. 2012). Several investigations have identified a number of neurotrophic factors that are capable to delay RGC degeneration in various animal models of glaucomatous optic neuropathies (Harvey et al. 2006; Lebrun-Julien and Di Polo 2008; Johnson et al. 2011; Wilson and Di Polo 2012). For instance, protective effects of BDNF have been intensively investigated in preclinical animal models of optic nerve injury and ocular hypertension (Mansour-Robaey et al. 1994; Di Polo et al. 1998; Isenmann et al. 1998; Ko et al. 2000; Martin et al. 2003). CNTF is another neurotrophic factor that has been shown to rescue RGCs from degeneration in various studies (Mey and Thanos 1993; Weise et al. 2000; van Adel et al. 2003; Ji et al. 2004; Maier et al. 2004; MacLaren et al. 2006; Parrilla-Reverter et al. 2009; Pease et al. 2009; Hellstrom and Harvey 2011). Additionally, CNTF not only promoted survival of degenerating RGCs but additionally induced long-distance regrowth of injured RGC axons into the distal optic nerve stump in animal models of a lesion-induced RGC loss (Cui et al. 2003; Leaver et al. 2006; Lingor et al. 2008; Muller et al. 2009; Hellstrom and Harvey 2011; Pernet et al. 2013).

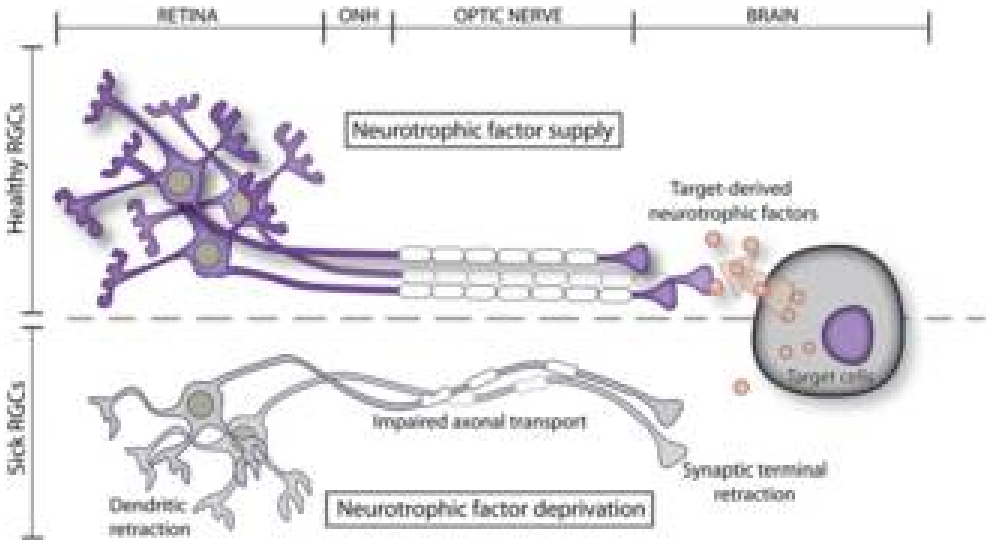


Figure 2.3: Apoptotic RGC degeneration in glaucoma as a result of neurotrophic factor deprivation.
Elevated IOP leads to an impaired axonal transport of target-derived neurotrophic factors which may represent one pathomechanism in glaucoma (from Almasieh et al. 2012).

To further investigate whether a NS cell-based intraocular delivery of CNTF is capable to attenuate the degeneration of retinal nerve cell types other than photoreceptor cells, and to evaluate whether neuroprotective effects of this approach can be detected over a time period longer than six weeks, CNTF expressing NS cells were transplanted into an ONC mouse model. In this study, we established another clonally derived NS cell line with an ectopic expression of high levels of CNTF. Immunocytochemical and immunoblot analyses confirmed a stable expression of CNTF for more than 30 passages also in this cell line. To induce degeneration of RGCs in adult mice, animals received an intraorbital crush of the optic nerve. The modified CNTF secreting clonal NS cell line was intravitreally grafted one day after the lesion. Determination of the number of surviving RGCs in flat-mounted retinas 1, 2, 3, and 4 months post-lesion revealed a significant protection of the axotomized RGCs in CNTF treated retinas when compared to control retinas at all post-lesion intervals analyzed. Examination of the transplanted NS cells confirmed that the donor cells had survived in the vitreous cavity over a time period of at least four months, the longest post-transplantation interval investigated. The transplanted NS cells were preferentially attached to the posterior pole of the lenses and were differentiated into astrocytes, similar to our previous observations in the *Pde6b*^{rd1}, *Pde6b*^{rd10}, and *nclf* mouse models of inherited photoreceptor degeneration. As mentioned before for CNTF treated *nclf* retinas, adverse effects of the transplanted NS cells on the host retinas were not observed, with the only exception of regionally restricted retinal folds that formed as a consequence of the sustained administration of high doses of the cytokine (Chong et al. 1999). Because CNTF is also known to promote axonal regeneration, we additionally analyzed whether sustained intraocular CNTF delivery had induced regrowth of the lesioned RGC axons in the ONC mouse model. To this aim, we performed anterograde axonal tracing experiments in NS cell treated animals one month after the optic nerve crush. While the grafted CNTF expressing NS cells promoted long distance regeneration of the axotomized RGC axons with some axons extended for more than 2 mm across the lesion site into the distal nerve stump, grafted control-NS cells induced only aberrant axonal sprouting over a distance of approximately 0.5 mm across the lesion site (Flachsbarth et al. 2014).

In summary, this study demonstrates that intravitreally grafted CNTF secreting NS cells not only efficiently attenuate the degeneration of photoreceptor cells, but also the lesion-induced loss of RGCs. This observation confirms and extends the view that therapeutic neuroprotective approaches can be potentially applied to diverse neurodegenerative diseases, irrespective of the specific etiology of the disease or the specific cell type affected. Another important result of the present study is the finding that intravitreally grafted NS cells survived for at least four months in the host eyes without exerting

adverse effects on the host retinas. The NS cell-based delivery system can therefore be used in long-term studies that are aimed to evaluate the therapeutic potential of secreted gene products in mouse models of retinal disorders.

2.2.3.4. VEGF-B, a novel neurotrophic factor for axotomized RGCs

After having demonstrated the therapeutic potential of a sustained NS cell-based delivery of CNTF in four different mouse models of retinal dystrophies, we next wanted to use this approach to investigate the neuroprotective effects of new neurotrophic factors on retinal cell types *in vivo*. Specifically, we decided to test the therapeutic potential of vascular endothelial growth factor B (VEGF-B) in a mouse model of lesion-induced RGC loss.

VEGF-B is a member of the VEGF family which consists of 5 structurally related proteins, VEGF (or VEGF-A), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). Because VEGFs and their receptors are known to play a pivotal role in both vasculogenesis and angiogenesis, VEGF-B has long been implicated in angiogenesis as well (Brockington et al. 2004; Holmes and Zachary 2005; Bry et al. 2014). However, studies aimed at evaluating the angiogenic activity of VEGF-B have provided only inconsistent results (Bellomo et al. 2000; Aase et al. 2001; Li et al. 2008; Lahteenvuo et al. 2009; Zentilin et al. 2010). Furthermore, recent investigations surprisingly demonstrated potent neurotrophic activities of classical angiogenic factors, such as VEGF-A. However, the use of VEGF for neuroprotective interventions is limited due to its potent angiogenic and permeability promoting activity (Sondell et al. 2000; Oosthuyse et al. 2001; Jin et al. 2002; Rosenstein et al. 2003; Khaibullina et al. 2004; Storkebaum et al. 2004; Kingham et al. 2014; Pelletier et al. 2015). The novel findings of neuroprotective functions of classical angiogenic factors stimulated work aimed at identifying possible neuroprotective activities of VEGF-B. Interestingly, various studies indeed showed that VEGF-B exerts neuroprotective effects on cerebral cortical neurons, dorsal root ganglion neurons and motor neurons *in vitro* (Sun et al. 2004; Poesen et al. 2008; Dhondt et al. 2011), and on sensory neurons and dopaminergic neurons *in vivo* (Poesen et al. 2008; Dhondt et al. 2011).

In our study, we cloned the mouse VEGF-B cDNA of the isoform 167 into a lentiviral vector additionally encoding the fluorescent reporter gene tdTomato in fusion with the resistance gene blasticidin under regulatory control of the CAG promoter, giving rise to the bicistronic vector pCAG-VEGF-B-IRES-tdTomato/BSD. For control experiments, the same vector but lacking the VEGF-B cDNA was used (pCAG-IRES-tdTomato/-BSD). After having established clonally derived NS cell lines with high expression levels of VEGF-B, cells were first characterized *in vitro*. Immunocytochemical analyses with anti-VEGF-B antibodies confirmed a stable expression of VEGF-B and reporter protein in undifferentiated NS cells, and in differentiated neurons and astrocytes derived from the

modified stem cells. To evaluate the neuroprotective potential of the VEGF-B-NS cell line *in vivo*, intravitreal injections of the modified NS cells were performed one day after an optic nerve lesion in adult wild-type mice. Quantitative analyses of flat-mounted retinas revealed the presence of significantly more surviving RGCs in VEGF-B treated retinas than in control retinas 14, 28, and 56 days after the nerve lesion. The results are in line with a recent report demonstrating significant protection of axotomized RGCs two weeks after an intravitreal injection of recombinant VEGF-B_{167} into adult mice (Li et al. 2008). The sustained NS cell-based treatment used in the present study significantly prolonged the protective effects of VEGF-B on lesioned RGCs for up to 1.5 month after the optic nerve injury when compared to the injections of recombinant VEGF-B. To further confirm that the delay of RGC loss was a specific effect of VEGF-B, several studies injected VEGFR-1 inhibitors which abolished VEGF-B mediated neuroprotection (Li et al. 2008). Unlike VEGF-B_{187}, VEGF-B_{167} is partly sequestered in the extracellular matrix, which possibly reduces the neuroprotective effects of the latter VEGF-B isoform. It will thus be interesting to compare the neuroprotective activities of both VEGF-B isoforms in future studies (Grimmond et al. 1996; Olofsson et al. 1996; Bry et al. 2014). In addition to analyzing RGC survival, we also performed anterograde axonal tracing experiments one month after the optic nerve crush to evaluate whether VEGF-B promotes axonal regeneration. In contrast to the long-distance regrowth of axotomized axons observed in CNTF-NS cell treated eyes, we did not observe significant regrowth of injured axons in VEGF-B cell treated eyes. A study in superoxide dismutase 1 mutant mice, an animal model of amyotrophic lateral sclerosis, also failed to observe neurite outgrowth promoting effects of VEGF-B on motor neurons (Poesen et al. 2008). Another investigation also confirmed that administration of VEGF-B does not induce axonal regeneration of the central nervous system neurons. However, VEGF-B restored sensation in the peripheral nervous system suggesting that the protein stimulates peripheral neurons to regrow their axons (Guaiquil et al. 2014). Finally, we analyzed the retinal vasculature of VEGF-B treated and control retinas, but found no differences with regard to total vessel length and number of vessel branching points between those two treatments, confirming that VEGF-B does not induce retinal neovascularization, even after prolonged intraocular administration of the protein. Of interest, a genome survey of the nematode *Caenorhabditis elegans* identified four receptors which are structurally related to VEGF receptors. Since nematodes lack a vascular system and the receptors are expressed on cells of neural origin it is intriguing to conclude that VEGFs and their receptors developed from one protein which may have had a neuroprotective rather than an angiogenic function (Popovici et al. 2002).

In summary, we confirmed that a sustained intraocular NS cell-based administration of VEGF-B significantly protected axotomized RGCs from degeneration over a time period of at least 2 months post-lesion. Furthermore, results of the present study also demonstrate that a continuous delivery of VEGF-B to the dystrophic retina does not induce retinal neovascularization. In contrast to CNTF which rescued axotomized RGCs from death and promoted long distance regeneration of injured RGC axons, VEGF-B protected lesioned RGCs from degeneration but exerted no effects on axonal regrowth.

3. Project 1:

Degeneration of photoreceptor cells in Arylsulfatase G-deficient mice.

Abstract

Purpose. Retinal degeneration is a common feature of several lysosomal storage disorders including the mucopolysaccharidoses, a group of metabolic disorders that is characterized by widespread accumulation of glycosaminoglycans due to lysosomal enzyme dysfunction. Here, we used a new mouse model of mucopolysaccharidosis IIIE to study the effect of Arylsulfatase G (ARSG) deficiency on retina integrity.

Methods. The retina of *Arsg* knockout mice aged between 1 and 24 months was analyzed by immunohistochemistry. Electron microscopic analyses were performed on retinas from 15 and 22 months old animals. Photoreceptor and microglia cell numbers and retina thickness was determined to quantitatively characterize retinal degeneration in ARSG-deficient mice.

Results. *Arsg* knockout mice showed a progressive degeneration of photoreceptor cells starting between 1 and 6 months of age, resulting in the loss of more than 50% of photoreceptor cells in 24 months old mice. Photoreceptor loss was accompanied by reactive astrogliosis, reactive microgliosis that was evident in the outer but not the inner retina, and elevated expression levels of some lysosomal proteins. Ultrastructural analyses of retinas revealed no evidence for the presence of storage vacuoles. Of note, expression of ARSG protein in wild-type mice was only detectable in the retinal pigment epithelium which, however, appeared unaffected in knockout mice at the ultrastructural level.

Conclusions. This is the first study demonstrating that ARSG deficiency results in progressive photoreceptor degeneration. The ARSG-related retinal phenotype might help to identify possible patients suffering from mucopolysaccharidosis caused by mutations in *ARSG*.

3.1. Introduction

The lysosomal degradative pathway of sulfated glycosaminoglycans (GAG) comprises a sophisticated hydrolytic network of highly specific glycosidases and sulfatases for complete degradation of these complex polysaccharides to sulfate and monosaccharides (Muenzer 1986; Neufeld 2001; Clarke 2008; Muenzer 2011; Coutinho et al. 2012). For each sulfate residue in different positions of the sugar moiety, distinct sulfatases are indispensable for their desulfation, which in turn is a prerequisite for glycosidic hydrolysis. Pathogenic mutations in genes coding for these hydrolytic enzymes lead to impaired degradation of GAGs and as a consequence to an accumulation of the corresponding substrates in lysosomes, a clinical situation described as lysosomal storage disorder (LSD). Disorders resulting from impaired lysosomal degradation of sulfated GAGs (heparan sulfate, dermatan sulfate, chondroitin sulfate and keratan sulfate) are summarized as mucopolysaccharidoses (MPSs) (Muenzer 1986; Neufeld 2001; Clarke 2008; Muenzer 2011; Coutinho et al. 2012; Kowalewski et al. 2012). One sub-group of the MPSs is Sanfilippo syndrome (MPS type III), which exclusively affects the degradation of heparan sulfate (HS). Mutations in genes coding for four different enzymes, needed for the removal of sulfated glucosamine residues of HS, are known to cause MPS III subtypes in humans, including N-sulfoglucosamine sulfohydrolase (encoded by *SGSH*; MPS IIIA), N-α-acetylglucosaminidase (encoded by *NAGLU*; MPS IIIB), heparan-α-glucosaminide N-acetyltransferase (encoded by *HGSNAT*; MPS IIIC) and N-acetylglucosamine-6-sulfatase (encoded by *GNS*; MPS IIID) (Valstar et al. 2008; Coutinho et al. 2012). Taking all pathogenic mutations in these four genes together, MPS III is the most frequently occurring type of MPS with a reported prevalence in different populations of 0.28 to 4.1 per 100,000 births (Valstar et al. 2008). We have recently shown that a fifth enzyme is critical for complete degradation of HS glucosamine residues when sulfated in the C3 position of glucosamine: Arylsulfatase G (ARSG), also termed N-sulfoglucosamine-3-O-sulfatase (Kowalewski et al. 2012; Kowalewski et al. 2014). We have generated *Arsg* knockout (KO) mice, and have demonstrated accumulation of HS in different organ systems including liver, kidney and brain (Kowalewski et al. 2012). Due to its assigned role in the degradation of HS and the resulting Sanfilippo syndrome-like pathological alterations, we tentatively assigned this MPS type as MPS IIIE. Compared to mouse models of the other MPS III subtypes (Bhaumik et al. 1999; Li et al. 1999; Martins et al. 2015), *Arsg* KO mice presented with a milder phenotype and a later onset of the disease, with Purkinje cell degeneration in the cerebellum as the major neurological phenotype (Kowalewski et al. 2015). Severe ataxia and Purkinje cell degeneration was also observed in an American Staffordshire Terrier dog pedigree that lacks functional ARSG due to a point mutation in the *ARSG* gene

(Abitbol et al. 2010). This canine model was assigned as a model of neuronal ceroid lipofuscinosis (NCL) because of the large amounts of accumulated lipofuscin in neurons. Human patients carrying pathogenic mutations in *ARSG* have not been identified until now.

MPSs are multisystemic disorders affecting most cell types of the body. However, the impaired cellular clearance of GAGs and the resulting lysosomal dysfunction is of particular detrimental significance for postmitotic cells such as neurons, as reflected by the profound neurological symptoms of most MPS patients. In MPS III patients, neurodegeneration is the key clinical feature, ultimately leading to premature death (Valstar et al. 2008; Muenzer 2011; Coutinho et al. 2012). The four human MPS III subtypes are clinically similar, with typical symptoms including developmental delay, mild coarse facial features, progressive loss of mental and motor functions, and epileptic seizures. Cortical atrophy and ventricular enlargement are common findings. Patients usually die at the end of the second or beginning of the third decade of life, often due to respiratory insufficiencies (Muenzer 1986; Zafeiriou et al. 2001; Valstar et al. 2008; Muenzer 2011; Coutinho et al. 2012; Wijburg et al. 2013). In addition to the brain, the retina is affected to a significant extent in two prominent groups of LSDs: retinal degeneration is a characteristic feature of several neuronal ceroid lipofuscinoses (NCL) (Koike et al. 2003; Weleber et al. 2004; Bozorg et al. 2009; Jalanko and Braulke 2009; Anderson et al. 2013; Bartsch et al. 2013; Schulz et al. 2013) and is also frequently seen in MPSs. Ocular involvement was reported in both patients and animal models of the majority of MPS subtypes, including MPSIII (Caruso et al. 1986; Lazarus et al. 1993; Bhaumik et al. 1999; Ashworth et al. 2006b; Ashworth et al. 2006a; Heldermon et al. 2007; Ferrari et al. 2011; Haer-Wigman et al. 2015). MPS III patients typically present with progressive photoreceptor loss closely resembling that occurring in retinitis pigmentosa, whereas the ganglion cells and optic nerve are usually unaffected (Del Monte et al. 1983; Lavery et al. 1983; Caruso et al. 1986; Ashworth et al. 2006b; Ashworth et al. 2006a; Ferrari et al. 2011; Ganesh et al. 2013; Haer-Wigman et al. 2015). In this study we describe degenerative changes in the retina of ARSG-deficient mice, a new mouse model of mucopolysaccharidosis type III. Progressive photoreceptor loss in the mouse starts between 1 and 6 months of age and is accompanied by reactive astrogliosis and microgliosis, and a dysregulation of several lysosomal proteins. We define retinal degeneration as an important and early onset pathological feature of ARSG-deficiency which might serve as a new predictive criterion to identify possible human MPS IIIE patients.

3.2. Materials and Methods

3.2.1. Animals

Arylsulfatase G knockout (*Arsg* KO) mice were generated as described elsewhere (Kowalewski et al. 2012). Mice were maintained on a mixed C57BL/6 129/Ola genetic background and housed according to the institutional guidelines of the University Bielefeld, with ad libitum access to food and water. Genotyping of mice was performed as described (Kowalewski et al. 2012). In all experiments, age-matched C57BL/6 129/Ola wild-type mice served as a control.

3.2.2. Electron microscopy

For electron microscopy, 15 and 22 months old *Arsg* KO and age-matched wild-type mice were deeply anesthetized and transcardially perfused with phosphate-buffered saline followed by perfusion with 6% glutaraldehyde (Merck, Darmstadt, Germany) in phosphate buffer. Eyes were enucleated, the lenses were removed, and the bulbs were post-fixed with 2% osmium tetroxide, dehydrated and embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM900 electron microscope (Zeiss, Jena, Germany) equipped with a Megaview III digital camera (Albert Tröndle, Moorenweis, Germany).

3.2.3. Immunohistochemistry of retina sections

Immunohistochemical analyses were performed on retinas from 1, 6, 12 and 24 months old *Arsg* KO and age-matched wild-type mice. Animals were sacrificed and eyes were quickly removed and fixed overnight in phosphate buffered saline (PBS; pH 7.4) containing 4% paraformaldehyde (PA). After dehydration in an ascending series of sucrose, eyes were frozen in Tissue-Tek (Sakura Finetek, Zouterwoude, Netherlands) and serially sectioned with a cryostat at a thickness of 25 µm. Central (i.e. in the plane of the optic disc) retina sections were first blocked in PBS containing 0.1% bovine serum albumin (BSA) and 0.3% Triton X-100 (both from Sigma-Aldrich, St. Louis, CA) for 1 hour and then incubated with primary antibodies (see table 1) overnight at room temperature. After washing with PBS, sections were incubated with Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 4 hours, stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and mounted onto slides. For detection of mannose 6-phosphate- (M6P) containing proteins, retina sections were incubated with the myc-tagged single-chain antibody fragment scFv M6P-1 (Muller-Loennies et al. 2010) followed by polyclonal rabbit anti-myc antibodies (Sigma-Aldrich) and Cy3-conjugated donkey anti-rabbit antibodies. To visualize cones, sections were stained with biotinylated peanut agglutinin (BPA; Vector Laboratories, Burlingame, CA)

overnight at room temperature, followed by Cy3-conjugated Streptavidin (Jackson ImmunoResearch) and DAPI. In all experiments sections from *Arsg* KO and age-matched wild-type mice were processed in parallel and under identical conditions. At least 6 animals were analyzed for each antigen, developmental age and genotype.

For immunohistochemical analyses of ARSG expression in retinal pigment epithelial (RPE) cells, the melanin pigment was bleached (Mishima et al. 1999). Sections were incubated in 0.05% potassium permanganate (Merck, Darmstadt, Germany) for 25 minutes, washed with PBS, incubated for 5 minutes in 0.5% oxalic acid (Carl Roth GmbH, Karlsruhe, Germany) and washed again with PBS before they were incubated with anti-ARSG antibodies.

Table 3.1: Primary antibodies used for immunohistochemistry

antigen	dilution	Company/reference
Arylsulfatase G (ARSG)	1:100	R&D Systems, Minneapolis, MN
Brain-specific homeobox/POU domain protein 3A (Brn-3a)	1:200	Santa Cruz Biotechnology Inc., Santa Cruz, CA
Cathepsin D	1:4.000	(Claussen et al. 1997)
Cluster of Differentiation 68 (CD68)	1:1.000	AbD Serotec, Kidlington, UK
Glial fibrillary acidic protein (GFAP)	1:500	Dako Cytomation GmbH, Hamburg, Germany
Ionized calcium-binding adapter molecule 1 (Iba1)	1:200	Wako Chemicals GmbH, Neuss, Germany
Lysosomal-associated membrane protein 1 (Lamp1)	1:200	Developmental Studies Hybridoma Bank, Iowa City, IA
Mannose 6-phosphate (M6P)	1:2.000	(Muller-Loennies et al. 2010)
Protein kinase C alpha (PKCα)	1:500	Santa Cruz Biotechnology Inc., Santa Cruz, CA
Recoverin (Rec)	1:3.000	Merck, Darmstadt, Germany
Retinal pigment epithelium-specific 65 kDa protein (RPE65)	1:2.000	Neuromics Antibodies, Edina, MN
Rhodopsin (Rho)	1:5.000	Merck, Darmstadt, Germany
Saposin D	1:2.000	(Klein et al. 1994)

For each antigen, sections from *Arsg* KO and age-matched wild-type mice were analyzed in parallel and with the same microscope settings using an Olympus FV 1000 confocal microscope (Olympus, Hamburg, Germany).

3.2.4. Photoreceptor counts and retina and outer nuclear layer thickness

To quantify the loss of photoreceptor cells in *Arsg* KO mice, central retina sections from mutant and age-matched wild-type mice were stained with anti-recoverin antibodies and DAPI. A merged confocal image of the entire retina section was prepared using Photoshop CS6 software (Adobe Systems Inc., San Jose, CA), and photoreceptor nuclei were counted at three defined positions corresponding to 25%, 50% and 75% of the distance between the optic disc and the peripheral margin of the nasal and temporal retina, respectively. Each area defined for photoreceptor counts covered the outer nuclear layer over a length of 220 µm (Jung et al. 2013). Statistical analyses of data were performed with the Student's *t*-test using GraphPad software (GraphPad Software, La Jolla, CA).

The thickness of the retina and the outer nuclear layer (i.e. photoreceptor cell bodies and inner and outer photoreceptor segments) was measured in central retina sections at nine equidistant positions between the optic disc and the periphery of the nasal and the temporal retinal half, respectively. Numbers of Iba1- and CD68-positive cells with a clearly visible DAPI-positive nucleus were determined in the inner retina (i.e. nerve fiber layer, ganglion cell layer, inner plexiform layer and inner nuclear layer) and outer retina (i.e. outer plexiform layer, photoreceptor cell bodies and inner and outer photoreceptor segments) of *Arsg* KO and wild-type mice aged between 1 and 24 months. The area of the inner and outer retina was measured using Photoshop CS6 software, and the density of positive cells was calculated. Statistical analyses of data were performed with the two-way ANOVA test followed by a Bonferroni Posthoc test using GraphPad software.

To determine the density of retinal ganglion cells (RGCs), eyes of 18 months old *Arsg* KO and age-matched wild-type mice (n=5 for each genotype) were fixed in 4% PA and retinas were flat-mounted on nitrocellulose membranes (Sartorius AG, Göttingen, Germany) as described (Flachsbarth et al. 2014). After blocking in PBS containing 0.1% BSA and 1% Triton X-100, retinas were incubated with polyclonal goat anti-Brn-3a antibodies overnight at room temperature. Primary antibodies were detected with Cy3-conjugated secondary antibodies, and flat-mounted retinas were stained with DAPI and mounted onto slides. Five images were taken from the center to the periphery of the superior, inferior, nasal and temporal retinal quadrant, covering a total area of approximately 1.9 mm². All Brn-3a-positive RGCs visible on these images were counted using Adobe Photoshop CS6 software, and the density of RGCs per mm² retinal area was calculated. Statistical analysis of data was performed using the Student's *t*-test.

3.3. Results

3.3.1. Retinal degeneration in ARSG-deficient mice

To characterize the retinal phenotype of mice deficient in Arylsulfatase G (ARSG) (Kowalewski et al. 2012; Kowalewski et al. 2015), we stained central retina sections from *Arsg* KO and age-matched wild-type mice with antibodies to glial fibrillary acidic protein (GFAP). In 1 month old mutants (Fig. 3.1b) and wild-type mice (Fig. 3.1a), expression of GFAP was restricted to retinal astrocytes located at the vitreal margin of the retinas. A similar pattern of GFAP expression was observed in the retina of wild-type mice aged between 6 and 24 months (not shown). In 6 (Fig. 3.1c), 12 (Fig. 3.1d) and 24 (Fig. 3.1e) months old *Arsg* KO mice, in comparison, expression of GFAP was elevated in retinal astrocytes and became additionally detectable in Müller cells. Reactive astrogliosis in *Arsg* KO was accompanied by a progressive thinning of the outer nuclear layer. Analyses of retina sections from 1 month old *Arsg* KO and age-matched wild-type mice stained with anti-recoverin antibodies to label photoreceptor cells revealed a similar thickness of the outer nuclear layer of both genotypes (compare Fig. 3.1f and g). In 6 (Fig. 3.1h), 12 (Fig. 3.1i) and 24 months (Fig. 3.1j) old *Arsg* KO mice, the thickness of the photoreceptor layer decreased with increasing age of the animals.

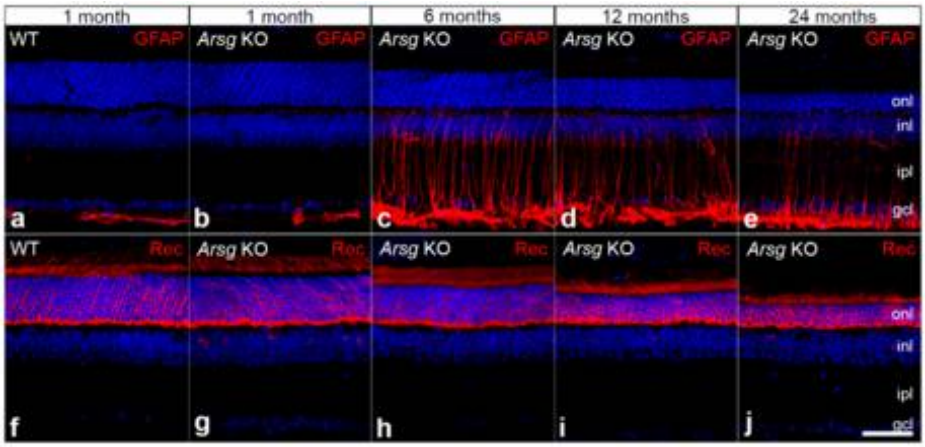


Figure 3.1: Expression of GFAP and recoverin in retinas of *Arsg* KO and wild-type mice at different developmental ages.
In 1 month old wild-type (a) and *Arsg* KO mice (b), expression of GFAP was restricted to astrocytes located at the vitreal margin of the retina. In 6 (c), 12 (d) and 24 (e) months old mutants, in comparison, expression of GFAP was strongly elevated in retinal astrocytes and was additionally detectable in Müller cells. Immunostainings with anti-recoverin antibodies revealed a similar thickness of the outer nuclear layer (onl) in 1 month old wild-type (f) and age-matched *Arsg* KO mice (g). In 6 (h), 12 (i) and 24 (j) months old mutants, the thickness of the outer nuclear layer decreased significantly with increasing age of the animals. All sections were stained with DAPI to label cell nuclei. gcl: ganglion cell layer; GFAP: glial fibrillary acidic protein; inl: inner nuclear layer; ipl: inner plexiform layer, Rec: recoverin. Scale bar in j (for a-j): 50 µm.

Measurements of the retina thickness at 9 equidistant positions between the optic disc and the periphery of the nasal and temporal retina, respectively (Fig. 3.2A), revealed similar values for 1 month old wild-type and age-matched *Arsg* KO mice at all retinal

positions analyzed (Fig. 3.2Ba). In 6 months old mutants, in comparison, retina thickness was significantly decreased at all retinal positions when compared to 1 month old *Arsg* KO mice (Fig. 3.2Ba). Retina thickness was further decreased in 24 months old mutants when compared to 6 months old *Arsg* KO mice (Fig. 3.2Ba), in line with the immunohistochemical data. Similarly, we observed no significant differences in the thickness of the outer nuclear layer between one month old *Arsg* KO and wild-type mice, but a progressive thinning of this layer in older *Arsg* KO mice (Fig. 3.2Bb). The thickness of the inner retina, in contrast, was similar in mutant and wild-type mice at all ages analyzed (data not shown), indicating that the retinal dystrophy in *Arsg* KO mice is mainly or exclusively due to a progressive loss of photoreceptor cells. In line with these results, we found similar densities of PKCα-positive bipolar cells in 24 months old animals of both genotypes (data not shown). Furthermore, the density of retinal ganglion cells (RGCs) in 18 months old animals did not differ significantly between both genotypes, with 3,688 ±165 RGCs/mm2 retina area (mean ±SEM) in wild-type mice and 3,932 ±180 RGCs/mm2 retina area in *Arsg* KO mutants (n=5 for each genotype; Supplementary Fig. 3.S1).

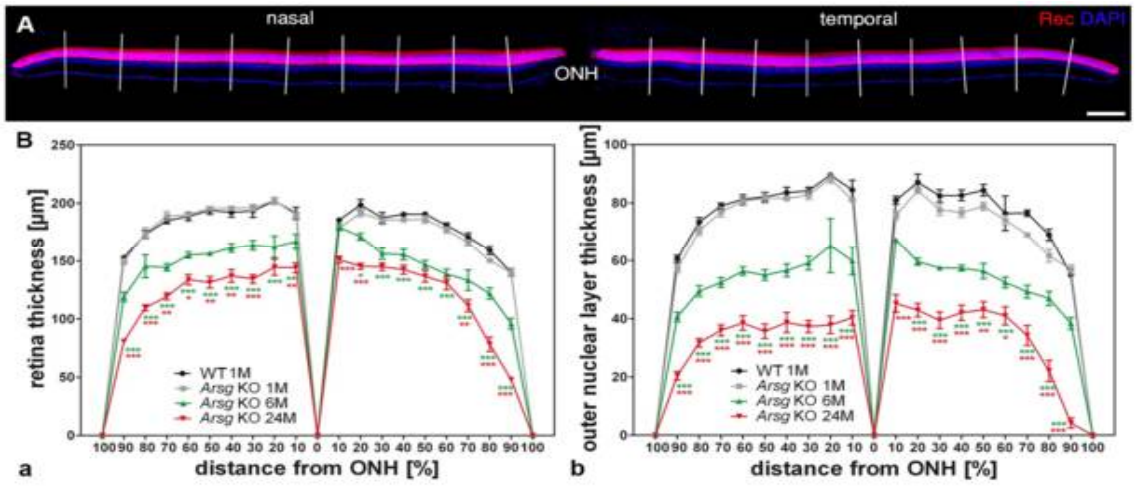


Figure 3.2: Thickness of the retina and the outer nuclear layer of *Arsg* KO and wild-type mice.
The thickness of the retina and the outer nuclear layer (i.e. photoreceptor cell bodies and inner and outer photoreceptor segments) was measured in central retina sections stained with anti-recoverin antibodies and DAPI (A) at nine equidistant positions (indicated with white lines in A) between the optic nerve head (ONH) and the periphery of the nasal and temporal retinal half, respectively. Analyses revealed a similar thickness of the retina and the outer nuclear layer in 1 month old *Arsg* KO (grey squares in Ba and Bb, respectively) and age-matched wild-type mice (black circles in Ba and Bb, respectively). In 6 months old mutants, the thickness of the retina and outer nuclear layer (green triangles 475 in Ba and Bb, respectively) was significantly decreased at all positions when compared with 1 month old mutants. Retina and outer nuclear layer thickness was further decreased in 24 months old mutants (red triangles in Ba and Bb, respectively) when compared with 6 months old *Arsg* KO mice. Each symbol represents the mean value (±SEM) of 6 animals. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ according to the two-way ANOVA followed by a Bonferroni posthoc test. M: month; Rec: recoverin. Scale bar in A: 200 µm.

To further quantify the loss of photoreceptor cells in the mutant, photoreceptor cell nuclei were counted in three areas located at defined positions of the nasal and temporal retina, respectively, each covering the outer nuclear layer over a length of 220 µm. In 1 month old animals, we found similar numbers of photoreceptor cells in wild-type mice (427.1 ±3.5 photoreceptor cells/area; mean ±SEM; n=6) and *Arsg* KO mutants (434.3 ±9.5; n=6; Fig. 3.3). In older mutants, the number of photoreceptor cells decreased significantly with increasing age of the animals. In 6, 12 and 24 months old mutants, we found 302.1 ±7.6, 258.0 ±8.3, and 179.5 ±6.6 photoreceptor cells/area, respectively (Fig. 3.3). Retinas from wild-type mice analyzed for comparison contained 424.8 ±5.3, 404.4 ±4.7, and 404.2 ±5.6 photoreceptor cells/area at the age of 6, 12 and 24 months, respectively (Fig. 3.3). To analyze whether the retinal dystrophy is the result of a progressive loss of rods or cones or both photoreceptor cell types, retina sections were labelled with anti-rhodopsin antibodies or peanut agglutinin. These experiments revealed the presence of apparently normal numbers of rods and cones with normal inner and outer segments in one month old mutants (Supplementary Fig. 3.S2). In older ARSG-deficient mice, the length of the outer segments of rods and cones was shortened when compared to wild-type mice. Furthermore, we found a similar density of cones in *Arsg* KO mice and wild-type mice at all developmental ages analyzed (for 24 months old mice, see Supplementary Fig. 3.S2).

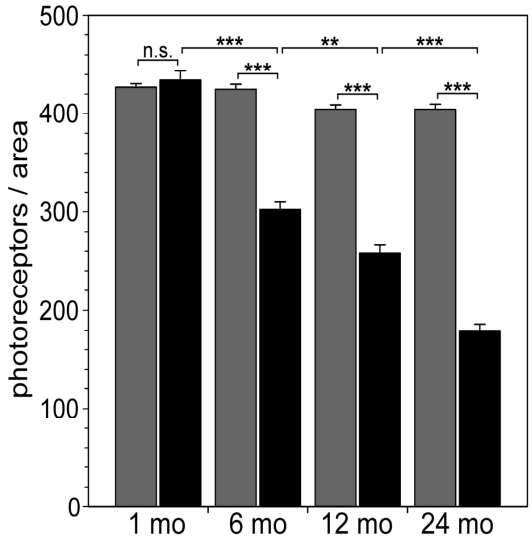


Figure 3.3: Photoreceptor numbers in *Arsg* KO and wild-type mice at different developmental ages.
Photoreceptor counts in 1 month old *Arsg* KO (black bar) and wild-type mice (grey bar) revealed similar cell numbers in both genotypes. In 6, 12 and 24 months old animals, photoreceptor numbers were significantly lower in *Arsg* KO mice when compared with age-matched wild-type mice, and decreased significantly with increasing age of the mutant. Each bar represents the mean value (±SEM) from 6 animals. n.s.: not significant; **: $p < 0.01$; ***: $p < 0.001$ according to the Student's *t*-test; mo: month.

3.3.2. Microglial cells in ARSG-deficient and wild-type retinas

Retina sections from mutant and wild-type mice of different developmental ages were stained with antibodies to Iba1 and CD68 to study reactive microgliosis in *Arsg* KO mice. In 1 month old animals, ramified Iba1-positive cells were found in the ganglion cell layer, inner plexiform layer, inner nuclear layer and outer plexiform layer, with no obvious differences in cell density or cell morphology between both genotypes (compare Fig. 3.4Aa and Ab). In older *Arsg* KO mice, Iba1-positive cells with a rounded amoeboid-like morphology became additionally detectable in the subretinal space of *Arsg* KO retinas (Fig. 3.4Ad). CD68-positive activated microglia/macrophages were essentially absent from wild-type retinas (for a 24 months old wild-type retina, see Fig. 3.4e) and from 1 month old *Arsg* KO retinas. In comparison, CD68-positive cells with an amoeboid morphology were frequently found in 6, 12 and 24 months old mutant retinas where they were mainly localized in the subretinal space (Fig. 3.4Af). Quantitative analyses revealed a similar density of Iba1-positive cells in the inner retina (defined as ganglion cell layer, inner plexiform layer and inner nuclear layer) of *Arsg* KO mutants and wild-type mice aged between 1 and 24 months, and in the outer retina (defined as outer plexiform layer, photoreceptor cell bodies and inner and outer photoreceptor segments) of 1 month old wild-type and *Arsg* KO mice (Fig. 3.4Ba). In the outer retina of older *Arsg* KO mice, however, the number of Iba1-positive cells increased significantly with increasing age of the mutants (Fig. 3.4Ba). CD68-positive cells were essentially absent from wild-type retinas, and were only rarely observed in the inner retina of the ARSG-deficient mice (Fig. 3.4Bb). In the outer retina of the mutant, in contrast, CD68-positive cells were frequently found in 6 months old animals, and their number was significantly increased in 24 months old *Arsg* KO mice (Fig. 3.4Bb).

3.3.3. Arylsufatase G expression in the adult mouse retina

Immunohistochemical analyses of the expression pattern of ARSG were performed on sections of adult wild-type retinas. The melanin pigment in RPE cells was bleached prior to the immunostainings to exclude quenching of the immunofluorescence. Double immunostainings revealed expression of ARSG in RPE65-positive retinal pigment epithelial cells of wild-type retinas (Fig. 3.5a-f). In retina sections from *Arsg* KO mice that were processed in parallel as a negative control, RPE cells were ARSG-negative as expected (Fig. 3.5g-l). The weak fluorescence associated with photoreceptor outer segments, the outer and inner plexiform layer and the ganglion cell layer of wild-type retinas was also observed in *Arsg* KO retinas (Fig. 3.5), and thus likely represents unspecific background labelling. Given that RPE cells were the only retinal cell type with detectable expression levels of ARSG, the retinal pigment epithelium of aged *Arsg* KO

mice was analyzed at the ultrastructural level. These experiments revealed no obvious morphological alterations of RPE cells in 15 or 22 months old ARSG-deficient mice when compared with age-matched wild-type mice (Supplementary Fig. 3.S3). Macrophages were frequently observed in the subretinal space of mutant retinas, but not in the subretinal space of wild-type retinas (Supplementary Fig. 3.S3).

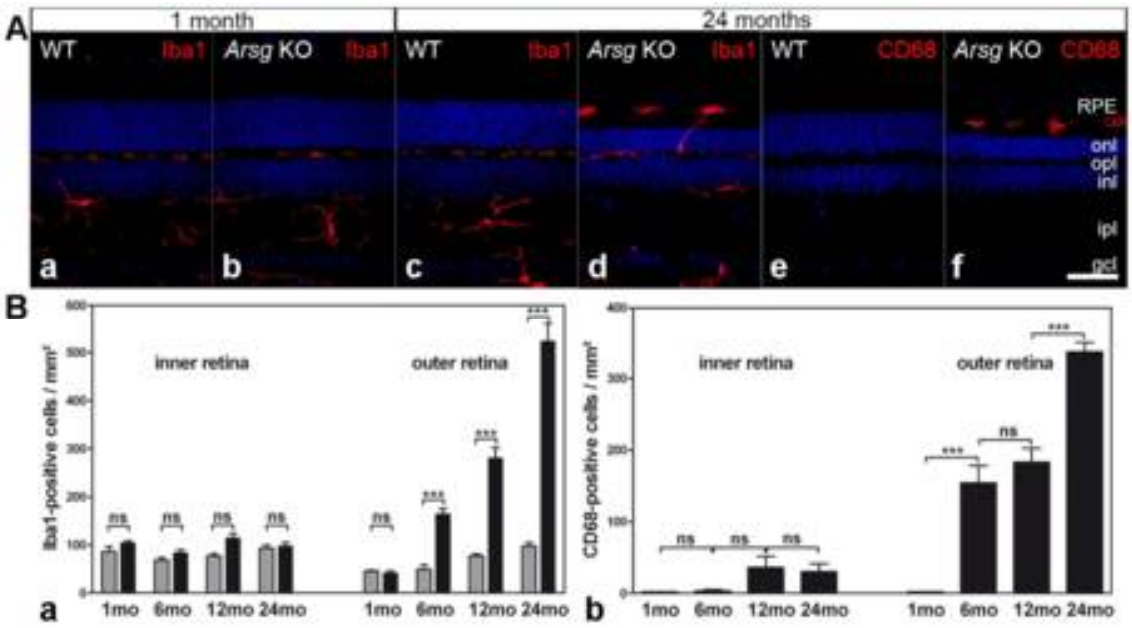


Figure 3.4: Distribution and density of Iba1-positive and CD68-positive cells in the retina of *Arsg* KO and wild-type mice.
Analyses of 1 month old animals revealed a similar distribution and density of Iba1-positive cells in wild-type (Aa) and *Arsg* KO mice (Ab). In 24 months old *Arsg* KO retinas (Ad), the number of Iba1-positive cells was significantly increased when compared to age-matched wild-type retinas (Ac), and positive cells were now additionally detectable between photoreceptor outer segments and the retinal pigment epithelium (RPE). CD68-positive cells were absent from retinas of 24 months old wild-type mice (Ae) but numerous in retinas 501 of age-matched *Arsg* KO mice where they were mainly located in the subretinal space (Af). Quantitative analyses revealed similar numbers of Iba1-positive cells in the inner retina (i.e. nerve fiber layer, ganglion cell layer, inner plexiform layer and inner nuclear layer) of *Arsg* KO (black bars in Ba) and wild-type mice (grey bars in Ba) aged between 1 and 24 months, and in the outer retina (i.e. outer plexiform layer, photoreceptor cell bodies and inner and outer photoreceptor segments) of 1 month old *Arsg* KO and wild-type mice (Ba). In the outer retina of 6, 12 and 24 months old mutants, the number of Iba1-positive cells increased significantly with increasing age of the animals, and was significantly higher than in age-matched wild-type mice (Ba). CD68-positive cells were essentially absent from wild-type retinas at all developmental ages analyzed, and were only occasionally observed in the inner retina of *Arsg* KO mice aged between 1 and 24 months and in the outer retina of 1 month old mutants (Bb). In the outer retina of older *Arsg* KO mice, the number of CD68-positive cells increased with increasing age of the animals (Bb). Each bar represents the mean value (±SEM) of 6 animals. gcl: ganglion cell layer; inl: inner nuclear layer; ipl: inner plexiform layer; mo: month; ns: not significant; onl: outer nuclear layer; opl: outer plexiform layer; ***: p<0.001 according to a two-way ANOVA followed by a Bonferroni posthoc test. Scale bar in f (for a-f): 50 µm.

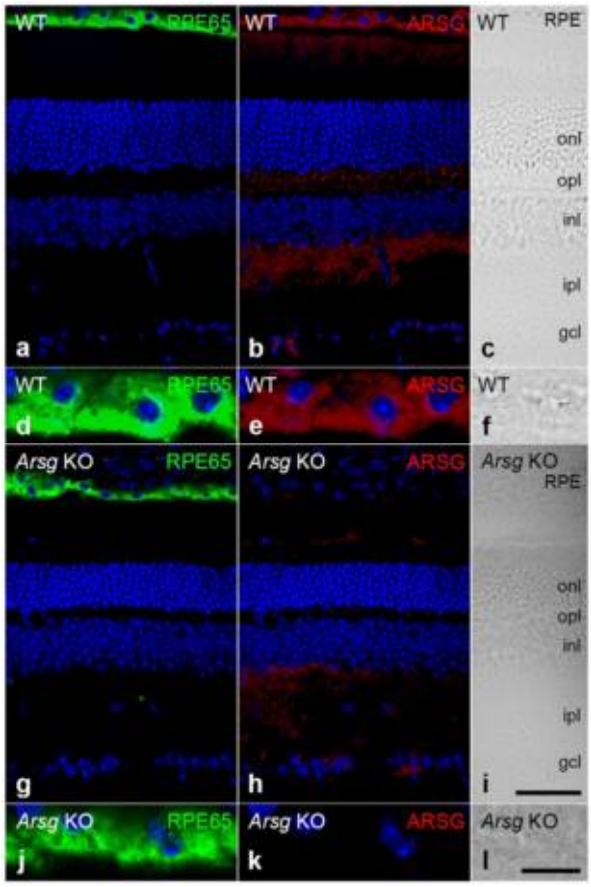


Figure 3.5: Expression of ARSG in the mouse retina.
In adult wild-type mice, ARSG-immunoreactivity (b, e) colocalized with RPE65 (a, d) in retinal pigment epithelial (RPE) cells. RPE cells in *Arsg* KO retinas, in comparison, were ARSG-negative as expected (h, k). The weak fluorescence of photoreceptor outer segments, outer plexiform layer, inner plexiform layer and ganglion cell layer in wild-type retinas stained with anti-ARSG antibodies (b) was also evident in *Arsg* KO retinas (h), and thus likely represents unspecific background labelling. (c), (f), (i) and (l) are phase contrast photomicrographs of (a, b), (d, e), (g, h) and (j, k), respectively to demonstrate complete bleaching of the melanin pigment in RPE cells. (d), (e), (j) and (k) are higher magnifications of the RPE shown in (a), (b), (g) and (h), respectively. All sections were stained with DAPI to label cell nuclei. gcl: ganglion cell layer; inl: inner nuclear layer; ipl: inner plexiform layer; onl: outer nuclear layer; opl: outer plexiform layer; RPE65: retinal pigment epithelium-specific 65 kDa protein. Scale bar in i (for a-c and g-i): 50 µm; in l (for d-f and j-l): 20 µm

3.3.4. Expression of lysosomal proteins in ARSG-deficient and wild-type retinas

To study the impact of ARSG-deficiency on the expression of lysosomal proteins, we analyzed the expression pattern of lysosomal enzymes containing the mannose 6-phosphate (M6P) recognition marker and the expression pattern of the lysosomal marker lysosomal-associated membrane protein-1 (Lamp1), the lysosomal protease cathepsin D (Ctsd) and Saposin D in retinas of 1 and 24 months old *Arsg* KO mice and age-matched wild-type mice (Fig. 3.6).

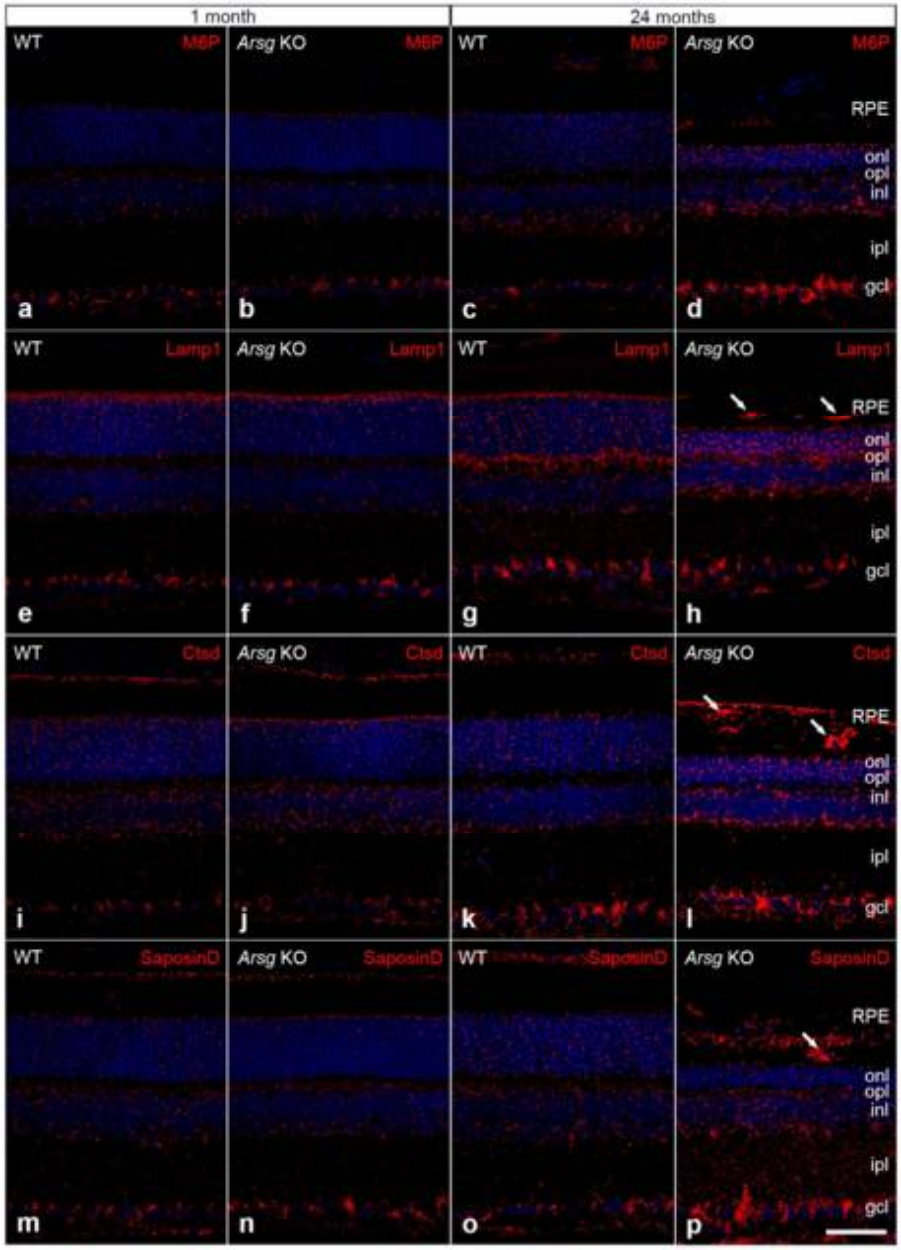


Figure 3.6: Expression of lysosomal proteins in the retina of *Arsg* KO and age-matched wild-type mice.
The distribution and expression levels of M6P, Lamp1, Ctsd and Saposin D were similar in 1 month old *Arsg* KO and wild-type retinas (compare a and b, e and f, i and j, and m and n, respectively). In 24 months old animals, expression of M6P, Ctsd and Saposin D were significantly increased in *Arsg* KO retinas when compared to age-matched wild-type retinas (compare c and d, k and l, and o and p, respectively). Expression levels of Lamp1, in comparison, were not detectably different between both genotypes at this age (compare g and h). Note the accumulation of lysosomal proteins in macrophages (labelled with white arrows in h, l and p) located between the outer nuclear layer (onl) and the retinal pigment epithelium (RPE) of 24 months old mutant mice. All sections were stained with DAPI to label cell nuclei. Ctsd: cathepsin D; gcl: ganglion cell layer; inl: inner nuclear layer; ipl: inner plexiform layer; Lamp1: lysosomal-associated membrane protein 1; M6P: mannose 6-phosphate; opl: outer plexiform layer. Scale bar in p (for a-p): 50 µm.

No detectable differences in the expression pattern and expression level of M6P and the different lysosomal proteins were observed between 1 month old *Arsg* KO retinas and age-matched wild-type retinas (Fig. 3.6; compare a and b for M6P, e and f for Lamp1, i and j for Ctsd, and m and n for Saposin D). In 24 months old animals, in comparison, expression levels of M6P were significantly increased in *Arsg* KO retinas when compared to wild-type retinas, particularly in the ganglion cell layer (compare Fig. 3.6c and d). Expression of Lamp1 was only slightly elevated in 24 months old mutants (compare Fig. 3.6g and h), while immunoreactivity for Ctsd (compare Fig. 3.6k and l) and Saposin D (compare Fig. 3.6o and p) was strongly increased in *Arsg* KO retinas when compared to age-matched wild-type retinas. Upregulation of Ctsd was particularly evident in the ganglion cell layer, inner nuclear layer and outer nuclear layer (Fig. 3.6l), while elevated expression of Saposin D was mainly detected in the ganglion cell layer (Fig. 3.6p). Furthermore, Lamp1, Ctsd and Saposin D were strongly accumulated in phagocytotic microglial cells located in the subretinal space of 24 months old *Arsg* KO retinas (Fig. 3.6h, l, p).

3.4. Discussion

A recent analysis of the *Arsg* KO mouse provided further insight into the degradative pathway of heparan sulfate (HS) and the endogenous substrate of ARSG, 3-O sulfated glucosamine. Moreover, we depicted the *in vivo* relevance of the substrate and its degradation by ARSG through manifestation of lysosomal storage in several tissues including the liver, the kidney and the brain in the absence of ARSG, and identified Purkinje cell degeneration and ataxia as prominent neurological symptoms in *Arsg* KO mice (Kowalewski et al. 2012; Kowalewski et al. 2015). In the present study, we extended the phenotypic characterization of the *Arsg* KO mouse, tentatively assigned as a mouse model of MPS IIIE (Kowalewski et al. 2012), to an early onset degeneration of photoreceptor cells starting between 1 and 6 months of age, when neuronal loss in the brain is not yet detectable. Retinal degeneration was accompanied by reactive astrogliosis, the appearance of phagocytic microglia/macrophages in the outer retina, and elevated expression of several lysosomal proteins. Loss of photoreceptor cells is thus among the earliest phenotypic manifestations of ARSG deficiency in the central nervous system.

An intriguing question raised by our observations is the actual cause of the progressive photoreceptor degeneration in the *Arsg* KO mouse. Of interest in this context, we found that expression of ARSG in the adult murine retina is confined to the RPE. RPE cells perform multiple functions that are vital for normal photoreceptor cell function and photoreceptor cell survival, and loss or dysfunction of RPE cells results in photoreceptor degeneration (Strauss 2005). However, ultrastructural analyses or ARSG-deficient retinas did not reveal obvious pathological alterations of the RPE, such as RPE atrophy or typical storage vacuoles as they have been observed in the kidney of *Arsg* KO mice and the RPE of animal models or patients of other MPS variants and MPS III subtypes (Del Monte et al. 1983; Lavery et al. 1983; Bhaumik et al. 1999; Ashworth et al. 2006a; Heldermon et al. 2007; Jolly et al. 2007; Kowalewski et al. 2012). Although the specific cause of the progressive photoreceptor cell loss in *Arsg* KO mouse has thus to be elucidated, it is tempting to speculate that heparan sulfate fragments and oligosaccharides released into the extracellular matrix might interfere with the proper function of the RPE (Valstar et al. 2008) or that a subtle lysosomal dysfunction causes functional alterations of RPE cells, ultimately resulting in photoreceptor cell death. Of note, the RPE is known to significantly contribute to the synthesis and degradation of all major mucopolysaccharides in the interphotoreceptor matrix (Del Monte et al. 1991).

Significant thinning of the outer nuclear layer and preferential accumulation of activated microglia cells/macrophages in the outer retina suggests that neurodegeneration in the retina of *Arsg* KO mice is mainly or exclusively confined to the photoreceptor cell layer.

Analysis of the outer nuclear layer revealed essentially normal numbers of cone photoreceptor cells in *Arsg* KO mice as old as 24 months, demonstrating that rod photoreceptor cells comprise the retinal cell type that is mainly affected in the absence of ARSG. Evidence for a significant loss of other retinal cell types than rod photoreceptors was not observed, as indicated by the normal thickness of the inner retina, normal numbers of bipolar cells and normal numbers of retinal ganglion cells in *Arsg* KO mice.

While immunohistochemical analyses revealed detectable levels of ARSG expression in RPE cells only, elevated levels of mannose 6-phosphate, Saposin D and Cathepsin D were mainly observed in the inner retina, particularly in retinal ganglion cells. The expression of most lysosomal proteins is coordinated and regulated by the transcription factor EB (TFEB) (Sardiello et al. 2009). Retinal ganglion cells and other retinal cell types might therefore respond to extrinsic pathological stimuli by upregulating TFEB-mediated lysosomal biogenesis or react to intrinsic alterations in the endolysosomal/lysosomal system such as a subtle accumulation of 3-O sulfated heparan sulfate. The latter hypothesis implies weak expression of ARSG below the detection level of our immunohistochemical analyses in retinal cell types other than RPE cells.

A characteristic feature of degenerating Purkinje cells in the cerebellum of *Arsg* KO mice was the presence of large intracellular aggregates that were immunoreactive for ubiquitin and p62, an autophagy adapter protein (Kowalewski et al. 2015). We hypothesized that these aggregates were the result of an impaired clearance of damaged lysosomes by autophagy. In the retina, however, similar p62-positive aggregates were not observed (data not shown), indicating that neuronal cell death in the cerebellum and the retina follows different mechanisms.

MPS IIIC is caused by mutations in *HGSNAT*, the gene encoding heparan-α-glucosaminide N-acetyltransferase, and retinal degeneration is among the typical symptoms of MPS IIIC patients (Ashworth et al. 2006a; Ganesh et al. 2013). Interestingly, a recent study identified novel mutations in *HGSNAT* in six patients that presented with retinitis pigmentosa but without any other clinical symptoms normally associated with MPS IIIC, such as neurological deterioration or visceral manifestations (Haer-Wigman et al. 2015). The mutations led to significantly reduced HGSNAT activities in these patients, ranging slightly above the level of typical MPS IIIC patients but considerably below the level of healthy subjects. As none of the patients manifested additional extraocular symptoms, the authors concluded that tissues usually affected by mutations in *HGSNAT* (especially the brain) express sufficient levels of residual enzymatic activity, but that the retina requires higher levels of HGSNAT activity to maintain normal structure and function (Haer-Wigman et al. 2015). Basically similar findings have recently been reported for another lysosomal storage disorder, CLN7

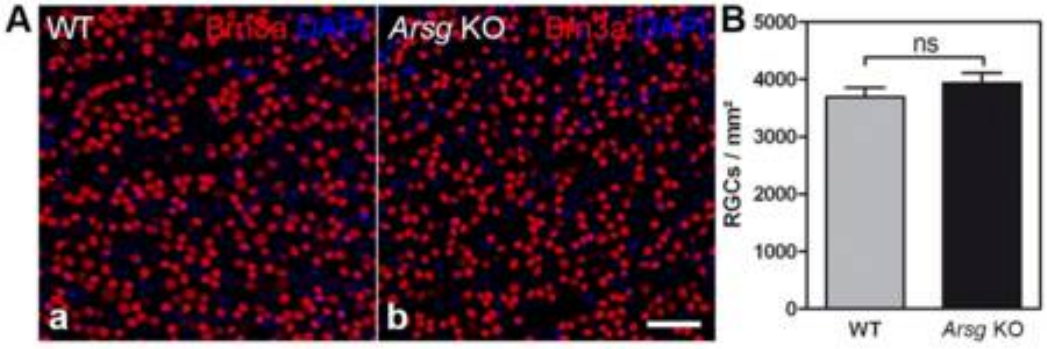
disease (Haer-Wigman et al. 2015). Similar to MPS IIIC, progressive photoreceptor loss is a typical feature of CLN7 disease both in patients (Kousi et al. 2009) and in a mouse model of this condition (Damme et al. 2014). Using genome-wide linkage analysis and exome sequencing, the study identified compound heterozygous variants in *MFSD8*, the gene affected in CLN7 disease, in two families presenting with macular dystrophy with central cone involvement (Roosing et al. 2015). Characteristic neurological symptoms normally associated with CLN7 disease, including mental regression, motor impairment or seizures were, however, not observed in these patients. Because both families carried a severe heterozygous mutation in combination with a missense mutation predicted to have a mild effect on the protein, it was proposed that there was sufficient residual activity of MFSD8 in all tissues of the patients, except in the retina (Roosing et al. 2015). Together, these studies point to a high susceptibility of the retina already to subtle changes in the lysosomal system, and might thus provide an explanation for the frequent involvement of the retina in various LSDs and for the progressive photoreceptor loss in *Arsg* KO mice despite the absence of detectable lysosomal storage.

American Staffordshire Terrier dogs suffering from ataxia have been shown to carry a point mutation in *Arsg* in a triplet coding for an amino acid in the vicinity of the catalytic domain of the protein (Abitbol et al. 2010). Evidence was presented that this missense mutation resulted in a significant reduction of ARSG activity (Abitbol et al. 2010). In close analogy to *Arsg* KO mice (Kowalewski et al. 2012; Kowalewski et al. 2015), affected dogs showed marked Purkinje cell degeneration in the cerebellum (Abitbol et al. 2010). However, different from our observations in the mouse model, retinal degeneration was not observed in this canine model of MPS IIIE (Abitbol et al. 2010). Similar to photoreceptor cells, Purkinje cells are affected in various LSDs (Sleat et al. 2004; Ko et al. 2005; Macauley et al. 2008; Macauley et al. 2009; Damme et al. 2011; Kollmann et al. 2012; Hassiotis et al. 2014), indicating that this neuronal cell type is also highly susceptible to lysosomal dysfunction. It is thus tempting to speculate that the species-specific differences in the phenotypic expression of ARSG dysfunction are related to the residual enzyme activity in dogs which is sufficient to maintain photoreceptor cells but not Purkinje cells as opposed to the complete absence of ARSG in the *Arsg* KO mouse where both nerve cell types are affected. Alternatively, these findings may reflect species-specific differences in the functional relevance of ARSG for photoreceptor cell integrity.

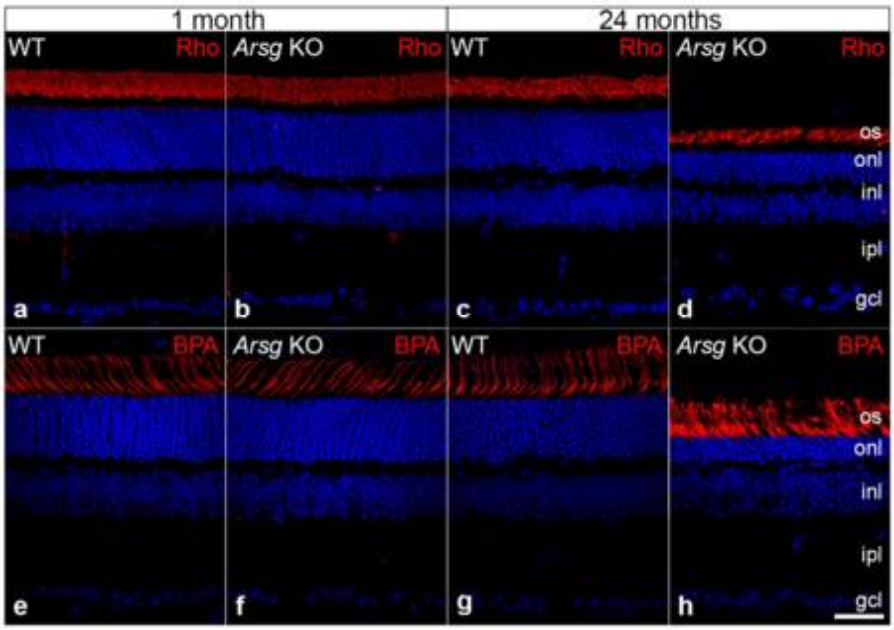
In conclusion, the present study demonstrates an early onset retinal degeneration in *Arsg* KO mice that in many aspects resembles the retinal dystrophy observed in other MPS III types and other LSDs. We suggest that the retinal phenotype caused by ARSG

deficiency might be helpful in the identification of possible human subjects suffering from MPS IIIE caused by mutations in *ARSG*.

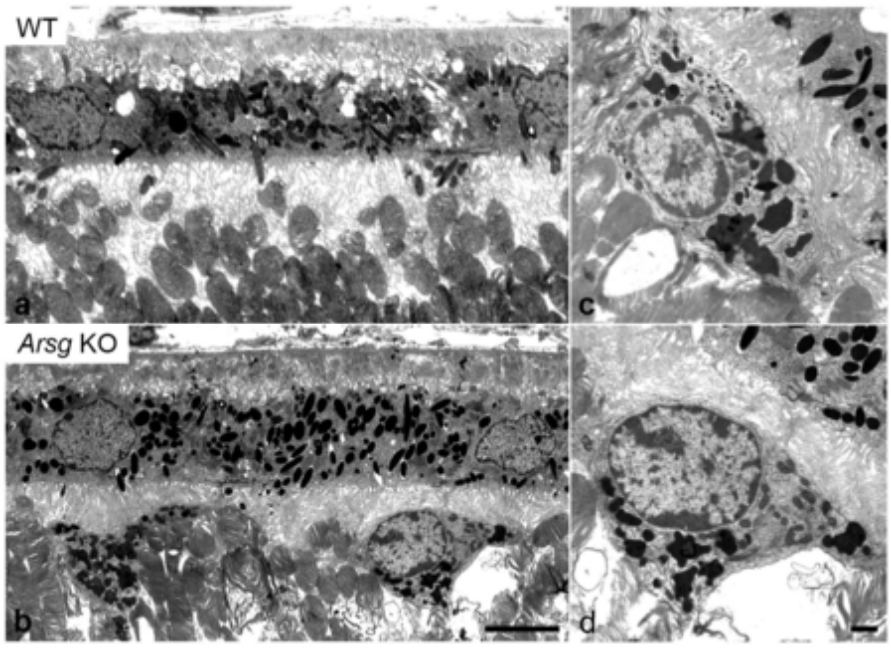
3.5. Supplementary figures



Supplementary Fig. 3.S1: Retinal ganglion cell density in 18 months old Arsg KO mutants and age-matched wild-type mice.
Representative images of retina flat mounts from wild-type mice (Aa) and Arsg KO mutants (Ab) stained with anti-Brn-3a antibodies. Quantitative analyses revealed similar densities of retinal ganglion cells (RGCs) in mutant (black bars in B) and wild-type mice (grey bars in B). Bars indicate mean values ±SEM from 5 animals for each genotype. Brn3a: brain-specific homeobox/POU domain protein 3A. ns: not significant. Statistical analysis of data was performed with the Student´s t-test. Scale bar in b (for a and b): 50 µm.



Supplementary Fig. 3.S2: Analyses of rod and cone photoreceptor cells in Arsg KO and wild-type mice.
In 1 month old animals, the morphology and density of rod (a, b) and cone (e, f) photoreceptor cells was similar in both genotypes. In 24 months old Arsg KO mice, outer segments (os) of rod (d) and cone (h) photoreceptor cells were reduced in length when compared to age-matched wild-type mice (c for rods, g for cones). Note the similar density of cones in mutant (h) and wild-type (g) retinas at this age. All sections were stained with DAPI. BPA: biotinylated peanut agglutinin; gcl: ganglion cell layer; inl: inner nuclear layer; ipl: inner plexiform layer; onl: outer nuclear layer; Rho: rhodopsin. Scale bar in h (for a-h): 50 µm.



Supplementary Fig. 3.S3: Electron microscopic analysis of the retinal pigment epithelium in 22 months old Arsg KO and wild-type mice.
The ultrastructure of retinal pigment epithelial (RPE) cells in aged Arsg KO mice (b) showed no obvious pathological alterations when compared to RPE cells of age-matched wild-type mice (a). Activated microglia cells with an amoeboid-like morphology were observed in the subretinal space of ARSG-deficient mice (asterisks in b, c and d), but not of wild-type mice (a). (d) is a higher magnification of the microglia cell shown in (b). Scale bar in b (for a and b): 5 µm; scale bar in d (for c and d): 1 µm.

4. Project 2:

Intravitreal transplantations of VEGF-B expressing neural stem cells delay the degeneration of axotomized retinal ganglion cells in adult mice.

Abstract

A sustained intraocular delivery of neurotrophic factors is among the strategies to develop treatments for yet untreatable retinal dystrophies. In the present study we analyzed the neuroprotective effects of a sustained cell-based intraocular delivery of vascular endothelial growth factor (VEGF-B) on axotomized retinal ganglion cells (RGCs) in adult mice. To this aim, we generated clonally derived neural stem (NS) cell lines lentivirally modified to stably express VEGF-B and the fluorescent protein tdTomato (VEGF-B-NS cells). NS cell lines for control experiments expressed tdTomato but not VEGF-B (control-NS cells). The modified NS cells were intravitreally grafted into adult mice one day after an intraorbital nerve crush. VEGF-B-NS and control-NS cells survived for at least 2 months *in vivo* and preferentially differentiated into astrocytes that were attached to the posterior pole of the lenses and stably expressed the transgenes. Analyses of flat mounted retinas 14, 28 and 56 days after the lesion revealed a significant neuroprotective effect of VEGF-B on the axotomized RGCs at all post-lesion time-points. The neuroprotective effect of VEGF-B-NS cells was strongest 1 month after the optic nerve crush with 1.9-fold more surviving RGCs than in control eyes, and decreased to 1.5-fold more surviving RGCs than in controls 56 days after the lesion. Anterograde tracing experiments performed 1 month after the optic nerve crush revealed no growth promoting effect of VEGF-B on the injured RGC axons. Although members of the VEGF family have strong angiogenic activities, VEGF-B did not induce angiogenesis. Moreover, adverse effects of the transplanted NS cells on the host retinas were not observed. Taken together, the present study demonstrates that a sustained cell-based intravitreal delivery of VEGF-B attenuates a lesion-induced loss of RGCs for up to two months in a mouse model of glaucoma.

4.1. Introduction

Glaucomatous optic neuropathies are among the leading causes of blindness worldwide, with an estimated 80 million glaucoma patients in 2020 (Quigley and Broman 2006). These retinal disorders are characterized by a progressive degeneration of retinal ganglion cells (RGCs) and their axons in the optic nerve (Qu et al. 2010; Almasieh et al. 2012). The pathomechanisms leading to glaucomatous optic neuropathies are not fully understood. Risk factors implicated in glaucoma include age, genetic background, reduced thickness of the cornea, vascular dysregulation, inflammation, exocitoxicity, and oxidative stress (Qu et al. 2010; Almasieh et al. 2012; You et al. 2013). Furthermore, an elevated intraocular pressure (IOP) is considered as a major risk factor for glaucomatous optic neuropathies (Newman 1996; O'Neill et al. 2009; You et al. 2013). Lowering the IOP is currently the only clinically proven treatment of glaucoma. However, degeneration of RGCs progresses in a significant portion of patients despite successful reduction of the IOP (Caprioli 1997; Leske et al. 2003).

Deprivation of target-derived neurotrophic factors due to an impaired axonal transport as a result of an elevated IOP has also been proposed to result in RGC degeneration (Pease et al. 2000; Quigley et al. 2000). The administration of neurotrophic factors to glaucomatous retinas has therefore been extensively studied as a strategy to protect RGCs and their axons from degeneration. These preclinical studies have identified a number of neurotrophic factors that are capable to attenuate the loss of RGCs in a variety of animal models of glaucoma, including glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) or ciliary neurotrophic factor (CNTF) (Klocker et al. 1997; Isenmann et al. 1998; Koeberle and Ball 2002; Ji et al. 2004; MacLaren et al. 2006).

The vascular endothelial growth factor (VEGF) family consists of five secreted growth factors in mammals: VEGF (or VEGF-A), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) (Brockington et al. 2004; Holmes and Zachary 2005; Bry et al. 2014). The VEGF growth factors bind with differing specificities to three transmembrane tyrosine kinase receptors (VEGFR-1 or fms-like tyrosine kinase 1 (Flt1), VEGFR-2 or mouse fetal liver kinase 1 (Flk1) and VEGFR-3 or fms-like tyrosine kinase 4 (Flt4)) and two semaphorin receptors (neuropilin-1 (NRP-1) and NRP-2). Members of the VEGF families and their receptors are known to play a pivotal role in both vasculogenesis and angiogenesis in embryos and adults (Brockington et al. 2004; Holmes and Zachary 2005; Bry et al. 2014).

VEGF is the best studied member of this family due to its prominent role in vascular development under physiological and pathological conditions (Carmeliet et al. 1996; Ferrara et al. 1996; Carmeliet and Jain 2000). VEGF was first identified as a

permeability-inducing factor secreted by tumor cells (Senger et al. 1983). Subsequent studies revealed a role of VEGF as a growth factor for vascular endothelial cells (Ferrara and Henzel 1989; Leung et al. 1989). PIGF has been implicated in angiogenesis and arteriogenesis in different diseases (Carmeliet et al. 2001; Luttun et al. 2002), while VEGF-C and VEGF-D have been demonstrated to be mainly involved in lymphangiogenesis (Alitalo et al. 2005). VEGF-B is the only member of this family with only minor angiogenic activity *in vivo* (Li et al. 2008; Bry et al. 2014).

Recent investigations demonstrated neuroprotective effects of VEGF on various nerve cell types, including motor neurons, cortical neurons, superior cervical ganglion neurons, and dorsal root ganglion neurons *in vitro* (Sondell et al. 2000; Jin et al. 2002; Rosenstein et al. 2003; Khaibullina et al. 2004; Storkebaum et al. 2004).

Furthermore, deletion of the hypoxia-response element of the VEGF promoter led to motor neuron fibre degeneration resulting in amyotrophic later sclerosis (Oosthuyse et al. 2001) and intraventricular administration of VEGF has been shown to stimulate neurogenesis *in vivo* (Jin et al. 2002). In addition, an investigation demonstrated a VEGF-mediated pro-angiogenic capacity during cronic nerve compression in rats which switched to a neurotrophic role after nerve decompression to promote recovery and nerve regeneration (Pelletier et al. 2015). However, the potent angiogenic activity of VEGF limits its use for neuroprotective therapeutic interventions. Thus, these data stimulated studies aimed at evaluating the other members of the VEGF family for potential neurotrophic functions. VEGF-B, also known as VEGF-related factor (VRF), was first discovered as a structural homologue of VEGF (Grimmond et al. 1996; Olofsson et al. 1996). VEGF-B is expressed in two isoforms that are generated by alternative splicing of exon 6, the short heparin-binding isoform VEGF-B_{167} and the long and more diffusible isoform VEGF-B_{186} (Grimmond et al. 1996; Olofsson et al. 1996). Unlike VEGF, VEGF-B is not essential for vasculature development, and mice deficient for VEGF-B are viable with atrial conduction abnormalities as the major phenotype (Aase et al. 2001). VEGF-B has been shown to act as a survival factor for endothelial cells (Zhang et al. 2009b). Furthermore, recent studies have reported anti-apoptotic and protective effects of VEGF-B on a variety of nerve cell types *in vitro* and *in vivo*. In a cerebral ischemia model, for instance, brain infarct volumes were increased by approximately 40% and neurologic impairment was more pronounced in VEGF-B-deficient mice when compared to wild-type control animals (Sun et al. 2004). Subsequent studies revealed proliferative effects of VEGF-B on neurons *in vitro* and *in vivo,* and an impaired neurogenesis in VEGF-B deficient mice (Sun et al. 2006). Later studies delineated neuroprotective effects of exogenously administrated VEGF-B on primary motor neurons (Poesen et al. 2008), on cerebral neurons in a middle cerebral artery occlusion stroke mouse model (Li et al.

2008) on dopaminergic neurons *in vitro* (Falk et al. 2009), in a rat model of Parkinson disease *in vivo* (Falk et al. 2011), and on dorsal root ganglion neurons *in vitro* and in a mouse model of distal neuropathy (Dhondt et al. 2011). Of note in the present context, exogenously administered recombinant VEGF-B has also been shown to attenuate the loss of axotomized RGCs in the optic nerve crush (ONC) mouse model (Li et al. 2008).

Given the potent neuroprotective effects of VEGF-B on a variety of neuronal cell types, the present study was performed to evaluate the neuroprotective effects of a sustained cell-based administration of VEGF-B on RGCs in a mouse model of glaucoma. To this aim, we generated clonally derived neural stem cell cultures overexpressing VEGF-B and grafted the modified clonal stem cell into the vitreous cavity of adult wild-type mice one day after an intraorbital optic nerve lesion.

4.2. Material and Methods

4.2.1. Animals

Neural stem (NS) cells were isolated from the cerebral cortex of 14 days old C57BL/6J mouse embryos. Intraorbital lesions of the optic nerve and intravitreal injections of cells were performed on adult (i.e. at least two months old) C57BL/6J mice. All animal experiments were approved by the University and State of Hamburg Animal Care Committees and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

4.2.2. Lentiviral vectors, generation of modified clonal NS cell lines and Western blot analysis

The mouse VEGF-B_{167} cDNA was cloned into the bicistronic lentiviral vector pCAG-IRES-tdTomato/BSD, giving rise to pCAG-VEGF-B-IRES-tdTomato/BSD. This vector is based on the lentiviral "gene ontology" (LeGO) vectors (Weber et al. 2010) and encodes the internal ribosome entry site (IRES) from the encephalomyocarditis virus and a tdTomato reporter gene in fusion with a blasticidin resistance gene under regulatory control of the cytomegalovirus enhancer/chicken β-actin (CAG) promoter. Lentiviral particles were pseudotyped with the envelope G protein of the vesicular stomatitis virus (VSV-G), produced by transient transfection of HEK 293T cells as described elsewhere (http://www.LentiGO-Vectors.de).

Generation of clonal NS cell lines with high expression levels of transgenes was performed as described elsewhere (Jung et al. 2013; Flachsbarth et al. 2014). In brief, adherently cultivated cells were seeded into plates coated with 0.1% Matrigel (BD Bioscience, Heidelberg, Germany) and spinoculated with pCAG-VEGF-B-IRES-tdTomato/BSD (VEGF-B-NS cells) or pCAG-IRES-tdTomato/BSD (control-NS cells) in the presence of 8 µg/ml hexadimethrine bromide (Polybrene; Sigma-Aldrich, St. Louis, CA). To select for positive cells, the transduced NS cells were further expanded in the presence of 4 µg/ml blasticidin (Life Technologies, Darmstadt, Germany) in NS cell medium (DMEM/F12 (Life Technologies) supplemented with 2 mM glutamine, 5 mM Hepes, 3 mM sodium bicarbonate, 0.3% glucose (all from Sigma-Aldrich), 10 ng/ml epidermal growth factor (EGF) and 10 ng/ml fibroblast growth factor-2 (FGF-2; both from TEBU, Offenbach, Germany), and 1% N2 and 1% B27 (both from Life Technologies). To establish clonal NS-cell lines with high expression levels of VEGF-B, single cells with the highest expression level of the fluorescent reporter protein were selected using fluorescence activated cell sorting (FACS; FACSAriaIIIu, BD Bioscience, San Diego, CA), and clonally expanded. To determine secretion levels of VEGF-B_{167}, 5 x 10^5 VEGF-B-NS cells or control-NS cells were cultivated in 0.5 ml NS cell medium for 24 h in the

presence of 100 µg/ml heparin (Sigma-Aldrich) to release VEGF-B_{167} from the extracellular matrix (ECM) (Olofsson et al. 1996). Culture supernatants of the clonal cell lines were analyzed by Western blot using polyclonal goat anti-VEGF-$B_{167/186}$ antibodies (R&D Systems, Minneapolis, MN) and horseradish peroxidase-conjugated anti-goat secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA). Serial dilutions of recombinant mouse VEGF-B_{167} (R&D Systems) were loaded as a reference. SuperSignal West Dura Extended Duration Substrate (ThermoFisher Scientific, Rockford, IL) was used to visualize immunoreactive bands, and the amount of secreted VEGF-B in the culture supernatants was estimated by densiometric analyses of immunoreactive bands using ImageJ software (http://imagej.nih.gov/ij/).

4.2.3. Intraorbital optic nerve lesions and intravitreal NS cell transplantations

Animals were deeply anesthetized by an intraperitoneal injection of Ketanest S (Parke Davis GmbH, Berlin, Germany) and Rompun (Bayer Vital GmbH, Leverkusen, Germany), and the optic nerve crush was intraorbitally crushed for 15 sec with a watchmaker's forceps as described elsewhere (Bartsch et al. 1992; Bartsch et al. 1995; Flachsbarth et al. 2014). Criteria for a successful nerve crush included loss of the pupillary light reflex, presence of well-preserved blood vessels and lack of retinal bleeding. One day after the crush 2 µl of PBS containing either $7.6x10^5$ VEGF-B-NS cells or control-NS cells were slowly injected into the vitreous cavity as described elsewhere (Jung et al. 2013; Flachsbarth et al. 2014; Jankowiak et al. 2015). Particular care was taken to not damage the lens during the injection procedure.

4.2.4. Immunocytochemical analyses of NS cells *in vitro* and *in vivo*

To analyze transgene expression in differentiated neural cell types, NS cells were induced to differentiate into astrocytes by cultivation for 5 days in NS cell medium supplemented with 1% fetal calf serum (Life Technologies) and 2% B27. Differentiation of NS cells into neurons was induced by maintaining the cells for 3 days in NS cell medium containing 5 ng/ml FGF-2, 1% N2 and 2% B27, followed by a cultivation period of 3 days in a 1:1 mixture of NS cell medium and Neurobasal medium (Life Technologies) supplemented with 0.25% N2 and 2% B27.

For immunocytochemical analyses of VEGF-B expression, cells were fixed with 4% paraformaldehyde (PA; Carl Roth GmbH, Karlsruhe, Germany) in phosphate buffered saline (PBS; pH 7.4), and blocked in PBS containing 0.1% bovine serum albumin (BSA) and 0.3% Triton X-100 (both from Sigma-Aldrich). Undifferentiated NS cell cultures were incubated with polyclonal goat anti-VEGF-B antibodies (R&D Systems) only. NS cell cultures that had been differentiated into astrocytes or neurons were simultaneously

incubated with anti-VEGF-B antibodies and polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) antibodies (Dako Cytomation GmbH, Hamburg, Germany) or monoclonal mouse anti-microtubule associated protein 2 (MAP2) antibodies (Sigma-Aldrich), respectively. Primary antibodies were detected with Cy2- and Cy5-conjugated secondary antibodies (Jackson Immunoresearch Laboratories), and cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

For the characterization of intravitreally grafted NS cells, animals were sacrificed 2 months after the ONC, and eyes were fixed for 1 h in 4% PA. To evaluate the expression of VEGF-B and to analyse the differentiation of the grafted NS cells, lenses with attached donor cells were removed from the eyes and incubated with goat anti-VEGF-B antibodies (R&D Systems) and rabbit anti-GFAP antibodies (DAKO Cytomation GmbH) or mouse anti-MAP2 antibodies (Sigma-Aldrich). Primary antibodies were detected with anti-goat Cy2- and anti-rabbit Cy5-conjugated secondary antibodies (Jackson Immunoresearch Laboratories). Lenses with attached donor cells were stained with DAPI, and confocal z-stacks were prepared with an Olympus FV 1000 confocal microscope (Olympus, Hamburg, Germany) and further processed using FV10-ASW software (Olympus) and Adobe Photoshop CS6 software (Adobe Systems, Inc., San Jose, CA, USA).

4.2.5. Anterograde axonal tracing

For anterograde labelling of RGC axons, a saturated solution of biotin-N-hydroxysuccinimidester (Sigma-Aldrich) in dimethylformamide (Carl Roth GmbH) was diluted 1:1 with ethanol. Intravitreal injection of anterograde tracer was performed 1 month after the optic nerve lesion. 24 hours later, animals were sacrificed and eyes with attached optic nerves were immersion-fixed in 4% PA, cryoprotected and frozen. Longitudinal sections of optic nerves were prepared at a thickness of 25 µm in thickness, and incubated with Cy3-conjugated streptavidin (Jackson Immunoresearch Laboratories). The distance between the distal margin of the lesion site and the tip of the longest regrown axon was determined in mice with grafted VEGF-B-NS or control-NS cells (n=6 for each experimental group). Analysis of optic nerve sections was done with an Olympus IX51 fluorescence microscope (Olympus). Statistical analysis of data was performed using the Student's *t*-test.

4.2.6. Analyses of retinal ganglion cell survival

To analyze survival of axotomized RGCs, animals were sacrificed 14 days post lesion (dpl), 28 dpl and 56 dpl. Eyes were fixed and retinas were flat-mounted on nitrocellulose membranes as described above. After blocking in PBS containing 0.1% BSA and 1% Triton X-100, retinas were incubated with polyclonal goat anti-Brn-3a antibodies (Santa

Cruz Biotechnology Inc.) overnight at room temperature. Subsequently, retinas were incubated with Cy3-conjugated secondary antibodies, stained with DAPI and mounted onto slides. Retinas from eyes with grafted VEGF-B-NS or control-NS cells were number-coded and 5 images were taken from the center to the periphery of the superior, inferior, nasal and temporal retinal quadrant, covering a total area of approximately 1.9 mm^2 (Flachsbarth et al. 2014). Brn-3a-positive RGCs were counted using Adobe Photoshop CS6 software (Adobe Systems Incorporated, San Jose, CA) and the density of RGCs per mm^2 was calculated. Retinas from eight animals with grafted VEGF-B-NS cells or control-NS cells were analyzed for each post-lesion interval. Statistical analyses of data were performed using the Student's *t*-test.

4.2.7. Analysis of retinal vascularization

To analyse retinal vascularization in mice with grafted VEGF-B-NS cells or control-NS cells, animals were sacrificed two months after the optic nerve crush and eyes were fixed for 15 min in 4% PA. Retinas were flat-mounted on nitrocellulose membranes (Sartorius AG, Göttingen, Germany), fixed again for 1 h, blocked in PBS containing 0.1% BSA and 1% Triton X-100 and incubated with biotinylated isolectin B_4 (IB_4; Life Technologies) in the presence of 1 mM $CaCl_2$ overnight at room temperature. Subsequently, retinas were incubated with Cy2-conjugated Streptavidin (Jackson Immunoresearch Laboratories), stained with DAPI and mounted onto slides. For analyses of vascularization in VEGF-B-NS and control-NS cell treated eyes (n=6 for each experimental group), confocal images of the superficial, intermediate and deep vascular layer were taken from each retinal quadrant, and the total length of all vessels and the total number of branching points were quantified using ImageJ software. Statistical analyses of data were performed using the Student's *t*-test.

4.3. Results

4.3.1. Derivation and characterization of genetically modified clonal NS cell lines

To establish VEGF-B expressing clonal NS cell lines, NS cells were isolated from the cerebral cortex of embryonic C57BL/6J mice and adherently cultivated. Modification of NS cells with lentiviral vectors encoding the mouse vascular endothelial growth factor B 167 isoform (VEGF-B) together with the reporter gene tdTomato and a blasticidin resistance gene under regulatory control of the CAG promoter gave rise to VEGF-B secreting cells (in the following termed VEGF-B-NS cells). NS cells for control experiments were modified with the same vector but lacking the VEGF-B cDNA (in the following termed control-NS cells). To generate clonal VEGF-B-NS or control-NS cell lines with high expression levels of the transgenes, modified NS cells were cultivated in the presence of blasticidin giving rise to pure tdTomato-positive NS cell cultures. Single cells with the highest reporter gene expression in these bulk cultures were selected by FACS and clonally expanded.

Analyses of undifferentiated clonal VEGF-B-NS and control-NS cell lines revealed expression of tdTomato in all cells (Fig. 4.1Aa, Ac). Expression of VEGF-B, in comparison, was only detectable in the VEGF-B-NS cell line (Fig. 1Ab) but not in the control-NS cell line (Fig. 4.1Ad). Western Blot analyses revealed the presence of VEGF-B in culture supernatants from the VEGF-B-NS cell clone, but not in culture supernatants from the control-NS cell clone (Fig. 4.1B). Quantitative analyses of culture supernatants from a clonal VEGF-B-NS cell line that was selected for all transplantation experiments revealed secretion of 270.4 ± 12.9 ng (mean ± SEM; n=3) VEGF-B per 10^6 cells in 24 hours at passage 25. Analysis of this cell line at passage 42 revealed similar VEGF-B expression levels, indicating stable transgene expression over at least 17 passages.

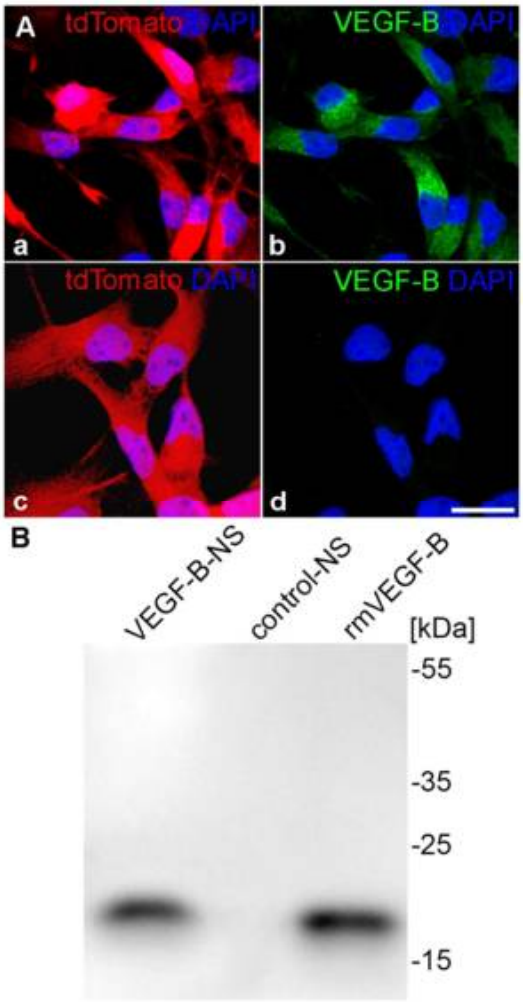


Figure 4.1: Expression of VEGF-B and tdTomato in lentivirally modified clonal NS cell lines. All cells in the clonal VEGF-B-NS cell line co-expressed the tdTomato reporter gene (Aa) and VEGF-B (Ab). Modified control-NS cells, in comparison, expressed the fluorescent reporter tdTomato (Ac) but no detectable levels of VEGF-B (Ad). Immunoblot analyses revealed VEGF-B in culture supernatants of VEGF-B-NS cells, but not in culture supernatants of control-NS cells (B). DAPI, 4',6-diamidino-2-phenylindole; rmVEGF-B, recombinant mouse vascular endothelial growth factor. Scale bar in Ad (for Aa-Ad): 25µm.

4.3.2. Characterization of NS cells *in vitro* and after transplantation *in vivo*

NS cells differentiate into neural cell types after intravitreal transplantations. Transplantation of undifferentiated NS cells results in differentiation of grafted cells *in vivo*. We therefore differentiated the VEGF-B-NS and control-NS cell line into astrocytes (Fig. 4.2a-f) and neurons (Fig. 4.2g-l) to analyze transgene expression in differentiated neural cell types. Immunocytochemical analyses of these cultures revealed that all GFAP-positive astrocytes (Fig. 4.2a, d) and MAP2-positive neurons (Fig. 4.2g, j) expressed the fluorescent protein tdTomato. VEGF-B immunoreactivity was only detectable in astrocytes (Fig. 4.2c) and neurons (Fig. 4.2i) derived from VEGF-B-NS cells, whereas differentiated astrocytes (Fig. 4.2f) and nerve cells (Fig. 4.2l) derived from control-NS cells lacked detectable levels of VEGF-B.

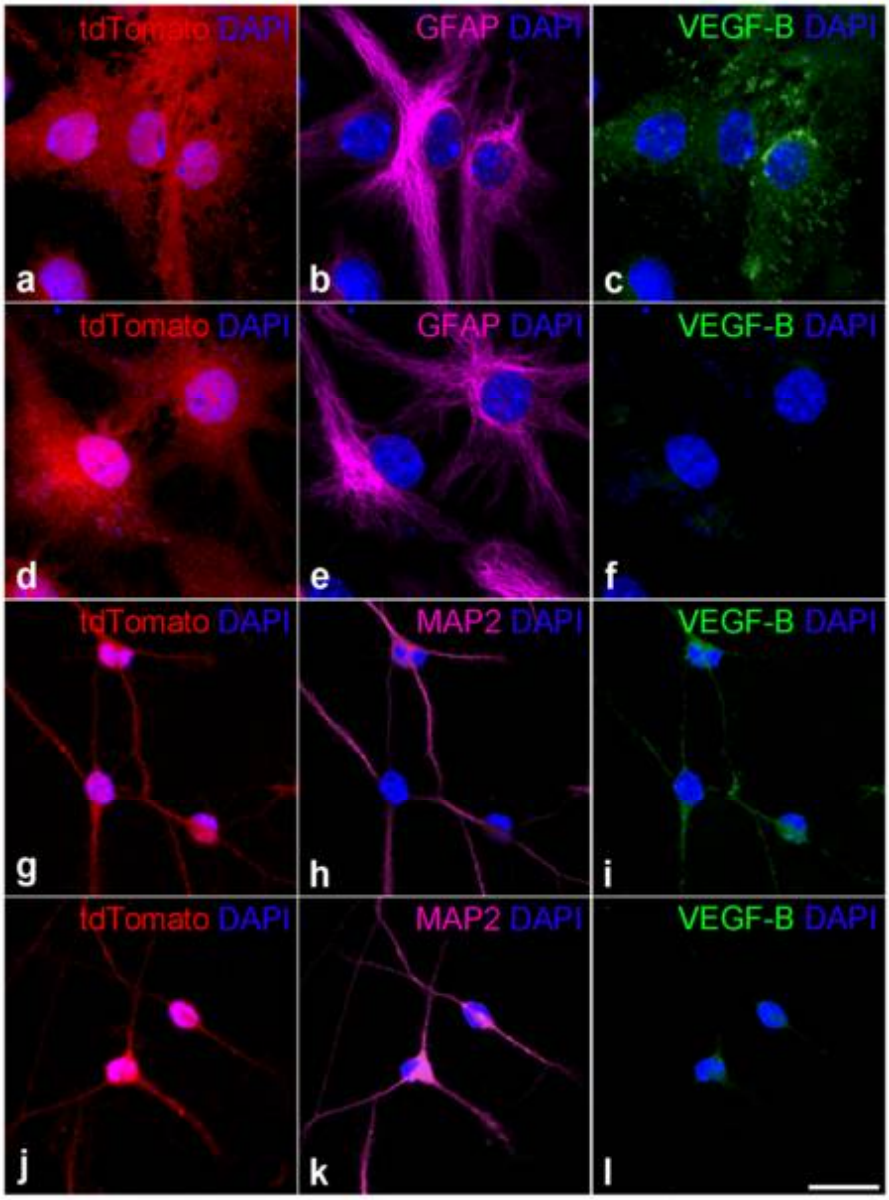


Figure 4.2: Analysis of VEGF-B and reporter gene expression in differentiated NS cells *in vitro*.
Clonally derived VEGF-B-NS cells (a-c, g-i) and control-NS cells (d-f, j-l) were differentiated into astrocytes (a-f) and neurons (g-l). All GFAP-positive astrocytes and MAP2-positive neurons derived from VEGF-B-NS cells co-expressed tdTomato (a, g) and VEGF-B immunoreactivity (c, i), whereas astrocytes and neurons derived from control-NS cells expressed tdTomato (d, j) but no detectable levels of VEGF-B (f, l). DAPI, 4',6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; VEGF-B, vascular endothelial growth factor. Scale bar in l (for a-l): 25µm.

Analyses of intravitreally grafted VEGF-B-NS and control-NS cells 2 months after intravitreal transplantation revealed a layer of tdTomato-positive cells that was attached to the posterior poles of the lenses (Fig. 4.3b, e). Immunostainings of the grafted cells with antibodies to cell type-specific antigens revealed that virtually all grafted VEGF-B-NS and control-NS cells were differentiated into GFAP-positive astrocytes (Fig. 4.3a, d). Differentiation of grafted VEGF-B-NS or control-NS cells into MAP2-positive nerve cells or myelin-basic protein-positive oligodendrocytes was not observed (data not shown). A

robust VEGF-B expression was only detectable in astrocytes derived from VEGF-B-NS cells (Fig. 4.3c) for at least two months, the longest post-transplantation period analyzed. Astrocytes derived from grafted control-NS cells expressed no detectable levels of VEGF-B (Fig. 4.3f). Of note, tumor formation or integration of grafted VEGF-B-NS or control-NS cells into host retinas was not observed.

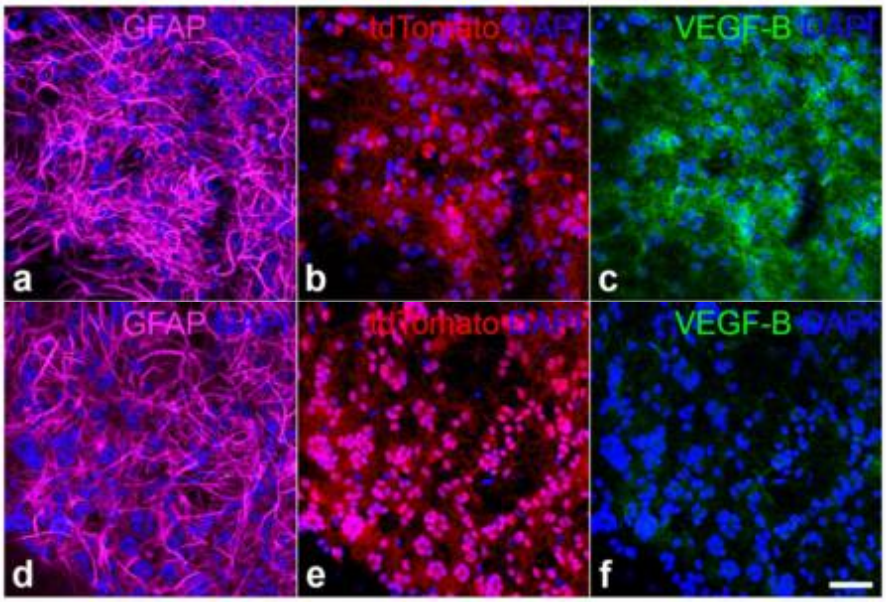


Figure 4.3: Survival and differentiation of intravitreally grafted NS cells.
Analyses of recipient eyes 2 months after intravitreal transplantation of VEGF-B-NS cells (a-c) and control-NS cells (d-f) revealed tdTomato-positive donor cells (b, e) that were attached to the posterior pole of the lenses. VEGF-B-NS cells and control-NS cells were differentiated into GFAP-positive astrocytes (a, d). Expression of VEGF-B was detectable in astrocytes derived from VEGF-B-NS cells (c), but not in astrocytes derived from control-NS cells (f). DAPI, 4',6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; VEGF-B, vascular endothelial growth factor. Scale bar in f (for a-f): 50µm.

4.3.3. Intravitreally grafted VEGF-B-NS cells attenuate the loss of axotomized RGCs

To analyze the neuroprotective effects of exogenously administered VEGF-B on axotomized RGCs, VEGF-B treated and control retinas were flat-mounted 14, 28 and 56 days after the optic nerve crush and stained with anti-Brn3a antibodies, a reliable marker of RGCs (Nadal-Nicolas et al., 2009). Qualitative inspections of these retinas consistently revealed the presence of more surviving RGCs in eyes with grafted VEGF-B-NS cells than in eyes with grafted control-NS cells (Fig. 4.4). Determination of RGC numbers in VEGF-B treated retinas revealed 484.4 ± 19.6 (mean ± SEM) RGCs/mm² 14dpl, 314.0 ± 12.7 RGCs/mm² 28dpl, and 144.6 ± 5.6 RGCs/mm² 56dpl (n=8 for each post-lesion time point analyzed; Fig. 4.5). In comparison, eyes with grafted control-NS cells contained 316.0 ± 9.8 RGCs/mm², 162.6 ± 12.2 RGCs/mm², and 95.0 ± 4.2 RGCs/mm² 14, 28, and 56dpl, respectively (n=8 for each post-lesion time point analyzed; Fig. 4.5).

Thus, eyes with grafted VEGF-B-NS cells contained 53.3%, 93.1%, and 52.2% more RGCs than eyes with grafted control-NS cells 14, 28, and 56 dpl, respectively. Statistical analyses revealed a significant rescue of axotomized RGCs in VEGF-B treated retinas when compared to control retinas at all post-lesion time points analyzed ($P<0.001$ according to the Student's *t*-test).

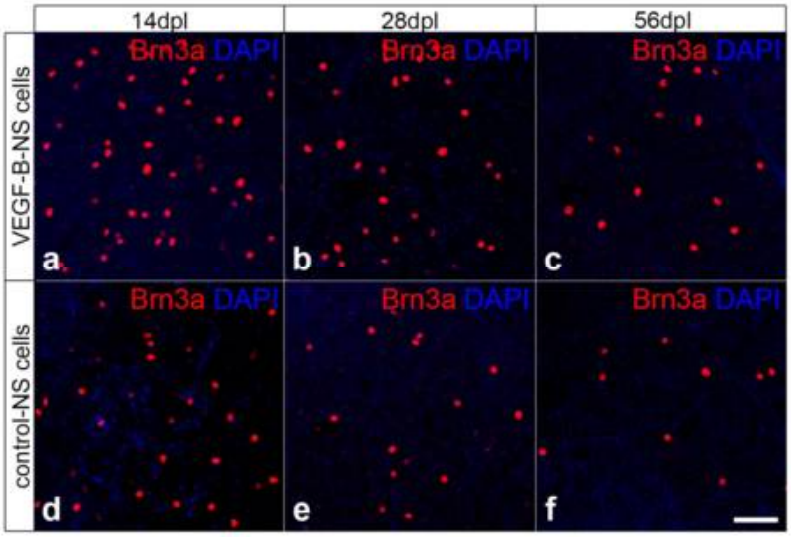


Figure 4.4: Intravitreally transplanted VEGF-B-NS cells attenuate degeneration of axotomized retinal ganglion cells in adult mice.
VEGF-B-NS cells (a-c) and control-NS cells (d-f) were intravitreally injected into adult mice one day after an intraorbital optic nerve crush. Flat-mounted retinas stained with anti-Brn3a antibodies 14 days post lesion (dpl; a, d), 28 dpl (b, e) and 56 dpl (c, f) revealed the presence of significantly more Brn3a-positive ganglion cells in animals with grafted VEGF-B-NS cells (a-c) compared to animals with grafted control-NS cells (d-f) at all post-lesion intervals. Brn3a, brain-specific homeobox/POU domain 3A; DAPI, 4',6-diamidino-2-phenylindole; VEGF-B, vascular endothelial growth factor B. Scale bar in f (for a-f): 50µm.

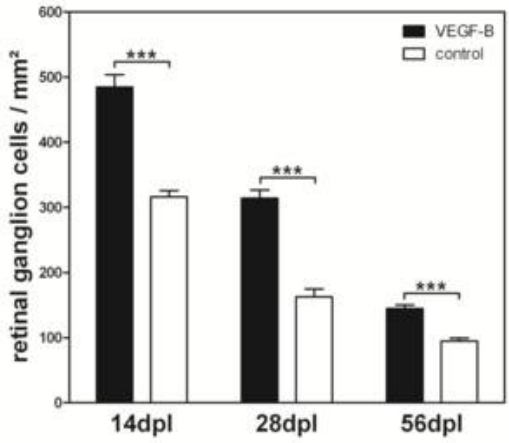


Figure 4.5: Quantitative analysis of the survival of axotomized retinal ganglion cells in VEGF-B treated and control retinas.
The numbers of Brn3a-positive ganglion cells were determined in flat-mounted retinas from animals with grafted VEGF-B-NS cells (filled bars) and control-NS cells (open bars) 14, 28 and 56 days post lesion (dpl). VEGF-B-treated retinas contained significantly more ganglion cells than control retinas at all post-lesion intervals. Each bar represents the mean number (±SEM) of RGCs per mm² from eight retinas. ****P* < 0.001 according to the Student's *t*-test. VEGF-B, vascular endothelial growth factor B.

4.3.4. Analysis of axonal regeneration and vascularization in VEGF-B treated retinas

To analyze whether a sustained intraocular administration of VEGF-B stimulates regeneration of axotomized RGC axons, we performed anterograde axonal tracings of the optic nerve one month after the nerve crush. Regrowth of lesioned RGC axons was assessed by measuring the distance between the distal margin of the lesion site and the tip of the longest regrown axon in longitudinal sections of the traced nerves (Fig. 4.6). In both VEGF-B treated (Fig. 4.6a) and control animals (Fig. 4.6b), injured RGC axons regrew for only a short distance across the lesion site into the distal optic nerve stump. In eyes with grafted VEGF-B-NS cells, the length of the longest regrown axons was 361.1 ± 20.4 µm (mean ± SEM), compared to 369.5 ± 28.7 µm in eyes with grafted control-NS cells (n=6 for each experimental group; Fig. 4.6c).

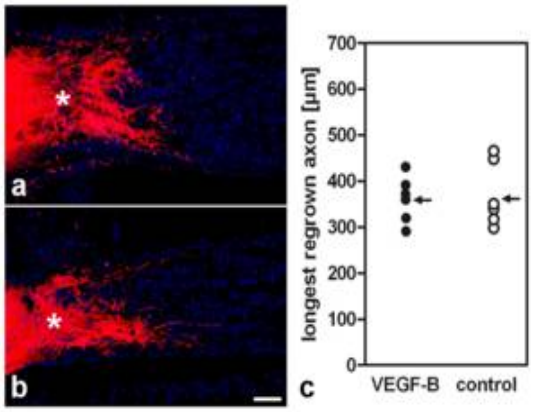


Figure 4.6: Regrowth of axotomized RGC axons in animals with grafted VEGF-B-NS and control-NS cells.
RGC axons were anterogradely labeled one month after an intraorbital optic nerve crush and intravitreal transplantation of VEGF-B-NS cells (a) or control-NS cells (b). In both VEGF-B-treated (a) and control retinas (b), axons extended for only a short distance across the lesion site (asterisk in a and b) into the distal optic nerve stump. The length of the longest regrown axon was not significantly different between VEGF-B-treated eyes (filled circles in c) and control eyes (open circles in c; n=6 for each experimental group, c) according to the Student's *t*-test. Arrows in c indicate mean values. VEGF-B, vascular endothelial growth factor B. Scale bar in a (for a and b): 50µm.

Because members of the VEGF family are known for their potent angiogenic activity, we additionally analyzed the retinal vascular network of the superficial, intermediate and deep retinal layers in eyes with transplanted VEGF-B-NS cells or control-NS cells 2 months after an optic nerve crush (Fig. 4.7). The arterial and venous vascular network in VEGF-B treated (Fig. 4.7Aa-Ac) and control retinas (Fig. 4.7Ad-Af) were visualized using isolectin B_4, and the complexity of the vascular network was evaluated by analyzing vessel branching and vessel length. This analysis revealed no significant differences in the number of vessel branching points between VEGF-B treated retinas (59.6 ± 3.8 branching points per mm^2 (mean ± SEM)) and control retinas (57.3 ± 3.8 branching

points per mm^2; n=6 for each experimental group; *P*>0.05 according to the Student's *t*-test; Fig. 4.7Ba). Similarly, intravitreally grafted VEGF-B-NS cells had no significant effect on vessel density (21.4 ± 12.0 mm vessel length/mm²) when compared to control-NS cells (21.2 ± 6.6 mm vessel length/mm2; n=6 for each experimental group; P>0.05 according to the Student's *t*-test; Fig. 4.7Bb).

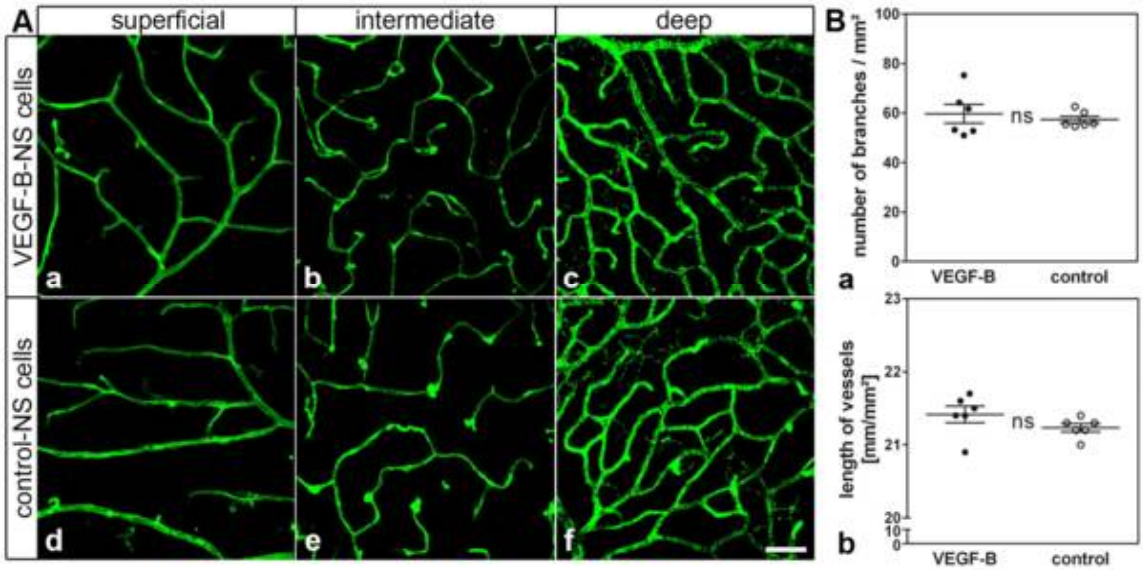


Figure 4.7: Comparison of the retinal vasculature in eyes with grafted VEGF-B-NS or control-NS cells.
The retinal vasculature of animals with grafted VEGF-B-NS (Aa-Ac) and control-NS cells (Ad-Af) was labelled with isolectin IB_4 56 days post lesion. Analyses of the superficial (Aa, Ad), intermediate (Ab, Ae) and deep (Ac, Af) retinal layers revealed no significant differences in the number of branches (Ba) and the total length of vessels (Bb) per retinal area between VEGF-B-treated (filled circles in Ba and Bb) and control retinas (open circles in Ba and Bb; n=6 for each experimental group). ns, not significant according to Student's *t*-test. VEGF-B, vascular endothelial growth factor B. Scale bar in Af (for Aa-Af): 50µm.

4.4. Discussion

Glaucomatous optic neuropathies, a leading cause of visual impairment in developed countries, are characterized by a progressive degeneration of retinal ganglion cells and their axons in the optic nerve (Quigley and Broman 2006; Qu et al. 2010; Almasieh et al. 2012). Efficient therapies for these degenerative disorders of the inner retina are currently not available. Risk factors implicated in glaucomatous optic neuropathies include an age, genetic disposition, vascular dysfunction, or an elevated intraocular pressure (Agarwal et al. 2009; Qu et al. 2010; Almasieh et al. 2012). Neurotrophic factor deprivation of RGCs as a result of an impaired axonal transport has been suggested as another cause of glaucomatous optic neuropathies (Pease et al. 2000; Quigley et al. 2000; Almasieh et al. 2012; Pascale et al. 2012). The administration of exogenous neurotrophic factors to glaucomatous retinas is therefore being evaluated in various preclinical studies as a potential strategy to treat glaucoma (Caprioli 1997; Lebrun-Julien and Di Polo 2008; Johnson et al. 2011).

Because neurotrophic factors usually have a short half-life time, do not cross the blood-retina barrier and may exert side effects when administered systematically, a local and sustained intraocular delivery of these proteins is required to achieve long-term neuroprotective effects in the retina. A continuous intraocular administration of neurotrophic factors and significant attenuation of RGC loss has been achieved in various animal models of glaucoma by genetically modifying endogenous retinal cells with viral and nonviral expression vectors or by intraocular transplantation of slow-release devices (Harvey et al. 2006; Johnson et al. 2011; Almasieh et al. 2012; Wilson and Di Polo 2012). Intraocular transplantation of genetically modified cells represents another strategy to continuously deliver neurotrophic factors to the glaucomatous retina. For instance, transplantation of lentivirally modified BDNF-secreting mesenchymal stem cells have been shown to attenuate RGC loss in a rat model of ocular hypertension (Harper et al. 2011). The simultaneous intravitreal transplantation of fibroblasts ectopically expressing BDNF, FGF-2 or neurotrophin-3 synergistically promoted survival of axotomized RGCs and synergistically stimulated axonal regeneration in the ONC rat model (Logan et al. 2006). Furthermore, we have recently shown that intravitreally grafted clonal neural stem (NS) cell lines lentivirally modified to secrete ciliary neurotrophic factor (CNTF) attenuate the loss of RGCs and stimulate long distance axonal regrowth in a mouse ONC model (Flachsbarth et al. 2014). In the present study, we used this NS cell-based delivery approach to investigate the effects of a continuous intraocular administration of VEGF-B on survival and axonal regeneration of intraorbitally lesioned RGCs in the adult mouse.

Initially, VEGF-B has been mainly implicated in angiogenesis because of its high sequence homology to VEGF and other members of the VEGF family. Functional analyses of VEGF-B, however, revealed only a minor role of the protein in angiogenesis (Bellomo et al. 2000; Aase et al. 2001; Li et al. 2008; Lahteenvuo et al. 2009; Zentilin et al. 2010). Of note in the present context, other studies revealed evidence for neuroprotective activities of several angiogenic factors, including VEGF (Sondell et al. 2000; Oosthuyse et al. 2001; Jin et al. 2002; Rosenstein et al. 2003; Khaibullina et al. 2004; Storkebaum et al. 2004; Kingham et al. 2014; Pelletier et al. 2015). However, the therapeutic potential of VEGF as a neuroprotective factor is limited due to its potent angiogenic and permeability promoting activity. These neuroprotective effects drew attention on VEGF-B as a possible new neurotrophic factor in regard of its low angiogenic activity. Indeed, several studies have demonstrated neuroprotective effects of VEGF-B on different nerve cell types *in vitro*, such as cerebral cortical neurons, dorsal root ganglion neurons or motor neurons (Sun et al. 2004; Poesen et al. 2008; Dhondt et al. 2011). Of note, VEGF-B has also been shown to exert neuroprotective effects on sensory neurons, in a mouse model of paclitaxel-induced sensory nerve degeneration and on dopaminergic neurons in a mouse model of Parkinson disease *in vivo* (Poesen et al. 2008; Dhondt et al. 2011).

In the present study, we used genetically modified NS cells as a vehicle to continuously administer VEGF-B to the dystrophic retina. To this aim, we transduced NS cells with a polycistronic lentiviral vector encoding VEGF-B together with the reporter gene tdTomato and a resistance gene blasticidin. Single cells with high expression levels of tdTomato were selected using FACS, and clonally expanded to derive cells lines with high expression levels of VEGF-B. Clonal NS cell lines for control experiments were modified with a lentiviral vector encoding the reporter and resistance gene but lacking the VEGF-B cDNA. Analyses of the modified cell lines revealed stable expression of the transgene in undifferentiated NS cells over several passages, and in neutrally differentiated NS cells *in vitro*. Following intravitreal transplantations, VEGF-B-NS cells preferentially differentiated into astrocytes which expressed VEGF-B and tdTomato for at least two months, the longest post-transplantation period analyzed. Grafted control-NS cells also differentiated into astrocytes which expressed the reporter gene but no detectable levels of VEGF-B. Adverse effects of the grafted cells on the morphology of the host eyes or integration of donor cells into the host retinas was not observed. All these observations are in line with our previous work where we grafted CNTF secreting NS cell lines into the *Pde6b*^{rd1} and *Pde6b*^{rd10} mouse models of retinitis pigmentosa (Jung et al. 2013), the nclf mouse model of neuronal ceroid lipofuscinosis (Jankowiak et al. 2015) and a mouse optic nerve crush model (Flachsbarth et al. 2014). The combined data suggest that

intravitreal transplantations of lentivirally modified clonal NS cell lines represent a useful methodology to continuously deliver secreted gene products to the retina of mouse models of degenerative retinal disorders.

Of note, quantitative analyses of RGC survival 14, 28, and 56 days after the optic nerve crush revealed the presence of significantly more surviving RGCs in eyes with grafted VEGF-B-NS cells than in eyes with grafted control-NS cells at all post-lesion intervals analyzed. This neuroprotective effect of a sustained cell-based administration of VEGF-B on axotomized RGCs is in line with a previous study which demonstrated neuroprotective effects of intravitreally injected recombinant VEGF-B_{167} on intraorbitally lesioned RGCs in a mouse ONC model two weeks after the nerve injury (Li et al. 2008). The latter work and several other studies of various nerve cell types additionally provided evidence that the neuroprotective effects of VEGF-B are mediated through VEGF receptor-1 (Storkebaum et al. 2004; Poesen et al. 2008; Falk et al. 2009; Dhondt et al. 2011; Falk et al. 2011). In future studies, it will be interesting to evaluate the neuroprotective effects of VEGF-B_{186}, because VEGF-B_{167} is partly sequestered in the ECM which might limit its therapeutic effects (Grimmond et al. 1996; Olofsson et al. 1996; Bry et al. 2014).

Given that VEGF-B protects axotomized nerve cells from degeneration and based on the recent observation that VEGF-B stimulates axonal regeneration in the peripheral nervous system (Guaiquil et al. 2014), we additionally analyzed whether the sustained administration of VEGF-B induced regeneration of the injured RGC axons. To this aim, we performed anterograde axonal tracing experiments one month after the optic nerve injury. These experiments revealed no significant difference between the length of the longest regrown axon in animals that received grafts of VEGF-B-NS cells and animals that received grafts of control-NS cells. These results are consistent with the observation that administration of VEGF-B to a superoxide dismutase 1 mutant mouse, an animal model of amyotrophic lateral sclerosis, failed to induce axonal regrowth (Poesen et al. 2008).

Finally, we also evaluated whether the sustained intraocular delivery of VEGF-B had induced neovascularization in the glaucomatous mouse retina. Morphometric analyses of the superficial, intermediate and deep retinal vasculature two months after transplantation of VEGF-B-NS cell revealed no effect of VEGF-B on total vessel length and the number of vessel branching points when compared to control retinas thus confirming the view that VEGF-B exerts only limited angiogenic activity (Li et al. 2008; Bry et al. 2014).

In summary, we have used a lentivirally modified NS cells as cellular vectors to continuously administer VEGF-B to the retina of a mouse model of glaucoma. Results demonstrate that VEGF-B promotes survival, but not axonal regeneration, of intraorbitally

lesioned RGCs. Evidence for an angiogenic activity of VEGF-B was not observed. We thus conclude that VEGF-B is among the candidate factors to develop neuroprotective strategies for degenerative retinal disorders. We also conclude that intravitreal transplantations of lentivirally modified clonal neural stem cell lines represent a useful methodology for preclinical studies aimed at evaluating the therapeutic potential of a cell-based administration of secreted gene products in mouse models of retinal disorders.

5. References

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I. Abbreviations

µg	microgram
µm	micrometer
AAV	adeno-associated virus
ACG	angle closure glaucoma
AIF	apoptosis inducing factor
AMD	age-related macula degeneration
ANOVA	analysis of variance
APAF	apoptosis protease-activating factor
ARSG	arylsulfatase G
ARVO	statement for the use of animal in ophthalmic and vision research
ASK	apoptosis regulating kinase
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BPA	biotinylated peanut agglutinin
Brn-3a	brain-specific homeobox/POU domain protein 3a
BSA	bovine serum albumin
BSD	blasticidin
$CaCl_2$	calcium chloride
CAG	CMV enhancer/chicken β-actin
CD68	cluster of differentiation 68
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
CMV	cytomegalovirus
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CNTFα	CNTF receptor alpha
Ctsd	cathepsin D
Cy	cyanine
DNA	desoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
dpl	days post lesion
DR	diabetic retinopathy
ECM	extracellular matrix
ECT	encapsulated cell technology
EF	elongation factor
EGF	endothelial growth factor
EndoG	endonuclease G
ERG	electroretinogram
ERT	enzyme replacement therapy
ES	embryonic stem
FACS	fluorescent activated cell sorting
FADD	Fas-associated death domain
FasL	Fas ligand
FASR	Fas receptor
FGF	fibroblast growth factor

Flk	fetal liver kinase
Flt	fms-like tyrosine kinase
GAG	glycosaminoglycans
GCL	ganglion cell layer
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GNS	N-acetylglucosamine-6-sulfatase
HEK	human embryonic kidney
HGSNAT	heparan-α-glucosaminide N-acetyltransferase
HS	heparan sulfate
HTRA2/OMI	high temperature requirement serine protease 2
i.e.	*id est*
Iba1	ionized calcium-binding adapter molecule 1
IRES	internal ribosome entry site
ILM	inner limiting membrane
INL	inner nuclear layer
IOP	intraocular pressure
IPL	inner plexiform layer
iPS	induced pluripotent stem
JAK	janus kinase
JNK	c-jun n-terminal kinase
kb	kilobase
kDa	kilodalton
KO	knockout
Lamp1	Lysosomal-associated membrane protein 1
LCA	Leber's congenital amaurosis
LeGO	lentiviral gene ontology
LIFR-β	leukaemia inhibitor factor receptor-β
LSD	lysosomal storage disorder
M/mo	month
M6P	mannose-6-phosphate
MAP2	microtubule associated protein 2
MAPK	mitogen-activated protein kinase
ml	milliliter
mm	millimeter
mm	millimolar
MPS	mucopolysaccharidosis
MSC	mesenchymal stem cell
MYOC	myocilin
n	statistical sample
NAGLU	N-α-acetylglucosaminidase
NCL or CLN	neuronal ceroid lipofuscinosis
ng	nanogram
NGF	nerve growth factor
NRE	nonreducing ends
NRP	neuropilin

NS	neural stem
OAG	open angle glaucoma
OFR	oxygen free radicals
OLM	outer limiting membrane
ONC	optic nerve crush
ONH	optic nerve head
ONL	outer nuclear layer
OPL	outer plexiform layer
P	phosphate
PA	paraformaldehyde
PBS	phosphate buffered saline
Pde6b	phosphodiesterase 6b
PEDF	pigment epithelium-derived factor
PGK	phosphoglycerate kinase
PKCα	protein kinase C alpha
PlGF	placental growth factor
Polybrene	hexadimethrine bromide
RCS	Royal College of Surgeons
rd	retinal degeneration
RdCVF	rod derived cone viability factor
Rec	recoverin
RGC	retinal ganglion cell
RHO/Rho	rhodopsin
RIP	receptor interacting protein
RP	retinitis pigmentosa
RPE	retinal pigment epithelium
RPE65	RPE-specific protein 65 kDa
RPGR	retinitis pigmentosa GFPase regulator
SEM	standard error of the mean
SFFV	spleen focus-forming virus
SGSH	N-sulfoglucosamine sulfohydrolase
SMAC/DIABLO	second mitochondrial-derived activator of caspases and Drosophila melanogaster homologue
STAT	signal transducer and activator of transcription
TNFR	tumor necrosis factor receptor
TNFα	tumor necrosis factor-alpha
TRAIL	TNF-related apoptosis-inducing ligands
TYK	tyrosine kinase
USH2A	usherin
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
VGF	VEGF-related factor
vLINCL	variant late infantile neuronal ceroid lipofuscinosis
VSV-G	envelope G protein of the vesicular stomatitis virus
zeo	zeocin

II. Publications, grants and participations

Publications:

Jung, G., Sun, J., Petrowitz, B., Riecken, K., Kruszewski, K., Jankowiak, W., Kunst, F., Skevas, C., Richard, G., Fehse, B. & Bartsch, U. 2013. Genetically modified neural stem cells for a local and sustained delivery of neuroprotective factors to the dystrophic mouse retina. *Stem Cells Transl Med*, 2, 1001-10.

- Experimental procedure (neural stem cell culture (CNTF-NS and control-NS cell culture), histological analyses)
- Preparation, review, and approval of the manuscript

Flachsbarth, K., Kruszewski, K., Jung, G., Jankowiak, W., Riecken, K., Wagenfeld, L., Richard, G., Fehse, B. & Bartsch, U. 2014. Neural stem cell-based intraocular administration of ciliary neurotrophic factor attenuates the loss of axotomized ganglion cells in adult mice. *Invest Ophthalmol Vis Sci*, 55, 7029-39

- Experimental procedure (neural stem cell culture (CNTF-NS and control-NS cells), histological analyses, Western Blot analyses of CNTF expression levels, preparation of lentiviral particles, cell transductions, generation of clonal cell lines by FACS)
- Preparation, review, and approval of the manuscript

Jankowiak, W., Kruszewski, K., Flachsbarth, K., Skevas, C., Richard, G., Ruther, K., Braulke, T. & Bartsch, U. 2015. Sustained neural stem cell-based intraocular delivery of CNTF attenuates photoreceptor loss in the nclf mouse model of neuronal ceroid lipofuscinosis. *PLoS One*, 10, e0127204

- Experimental procedure (neural stem cell culture (CNTF-NS and control-NS cells), Western Blot analyses of CNTF expression levels, preparation of lentiviral particles, cell transductions, generation of clonal cell lines by FACS)
- Preparation, review, and approval of the manuscript

Kruszewski, K., Lüllmann-Rauch, R., Dierks, T., Bartsch, U. & Damme, M. Degeneration of photoreceptor cells in Arylsulfatase G-deficient mice. *Invest Ophthalmol Vis Sci*, in revision (part of the present thesis)

- All experimental work (except electron microscopy)
- Preparation, review, and approval of the manuscript
- First author of the manuscript

Kruszewski, K., Flachsbarth, K. & Bartsch, U. Intravitreal transplantations of VEGF-B expressing neural stem cells delay the degeneration of axotomized retinal ganglion cells in adult mice. Manuscript in preparation (part of the present thesis)

- All experimental work (except cloning of lentiviral vector, optic nerve lesions and cell transplantations)
- Preparation, review, and approval of the manuscript
- First author of the manuscript

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- Experimental procedure (neural stem cell culture of ADAM10 cell lines, cell transductions, differentiations and immunocytochemistry)
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STEM CELLS TRANSLATIONAL MEDICINE

TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Genetically Modified Neural Stem Cells for a Local and Sustained Delivery of Neuroprotective Factors to the Dystrophic Mouse Retina

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Key Words. Neural stem cell • Stem cell transplantation • Retinal photoreceptors • Lentiviral vector • Ciliary neurotrophic factor

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ABSTRACT

A continuous intraocular delivery of neurotrophic factors (NFs) is being explored as a strategy to rescue photoreceptor cells and visual functions in degenerative retinal disorders that are currently untreatable. To establish a cell-based intraocular delivery system for a sustained administration of NFs to the dystrophic mouse retina, we used a polycistronic lentiviral vector to genetically modify adherently cultivated murine neural stem (NS) cells. The vector concurrently encoded a gene of interest, a reporter gene, and a resistance gene and thus facilitated the selection, cloning, and in vivo tracking of the modified cells. To evaluate whether modified NS cells permit delivery of functionally relevant quantities of NFs to the dystrophic mouse retina, we expressed a secretable variant of ciliary neurotrophic factor (CNTF) in NS cells and grafted the cells into the vitreous space of *Pde6b*^{rd1} and *Pde6b*^{rd10} mice, two animal models of retinitis pigmentosa. In both mouse lines, grafted cells attached to the retina and lens, where they differentiated into astrocytes and some neurons. Adverse effects of the transplanted cells on the morphology of host retinas were not observed. Importantly, the CNTF-secreting NS cells significantly attenuated photoreceptor degeneration in both mutant mouse lines. The neuroprotective effect was significantly more pronounced when clonally derived NS cell lines selected for high expression levels of CNTF were grafted into *Pde6b*^{rd1} mice. Intravitreal transplantations of modified NS cells may thus represent a useful method for preclinical studies aimed at evaluating the therapeutic potential of a cell-based intraocular delivery of NFs in mouse models of photoreceptor degeneration. STEM CELLS TRANSLATIONAL MEDICINE *2013; 2:1001–1010*

INTRODUCTION

Progressive dysfunction and degeneration of photoreceptors, as it occurs in retinitis pigmentosa or age-related macular degeneration, results in currently incurable visual impairment or blindness [1]. Corrective gene therapy, optogenetic therapy, implantations of electronic retinal prostheses, cell replacement strategies and neuroprotective strategies are among the approaches aimed at establishing treatments for such conditions [2–6]. All these therapeutic strategies have achieved remarkable results in animal models, and some have entered clinical trials [4, 7–9].

Neuroprotective strategies do not target the specific cause of a disease but instead attempt to limit the consequences (i.e., degeneration of photoreceptors and deterioration of visual function) and may thus be applicable across a broad range of degenerative retinal disorders. During the last two decades, a number of neurotrophic factors (NFs) have been shown to attenuate photoreceptor degeneration and to partly preserve retinal function in a variety of animal models of hereditary retinal degeneration and other diseases involving photoreceptor loss [3, 10]. Because NFs normally have short half-life times, do not ordinarily cross the blood-retina barrier, and bear the risk of unacceptable side effects when administered systemically, strategies are being developed that permit a local and sustained delivery of these factors to the retina. These include intravitreal implantations of biodegradable factor-loaded delivery devices, forced expression of NFs in endogenous retinal cells using viral or nonviral vectors, and intraocular transplantations of cells genetically modified to secrete such factors [11–16].

Intraocular implantations of encapsulated and genetically modified cells provide a straightforward strategy to use cells as vectors to deliver NFs to dystrophic retinas, as the encapsulation

protects not only the grafted cells from the immune system of the host but also the host retina from potential adverse effects of the grafted cells. Studies with encapsulated human retinal pigment epithelium (RPE) cells modified to secrete ciliary neurotrophic factor (CNTF) have indeed demonstrated the feasibility of this approach in large animal models [17, 18] and human patients with degenerative retinal disorders [19–22]. However, the size of the encapsulated cell implants precludes their use in preclinical studies aimed at evaluating and optimizing this therapeutic approach in small species, such as the mouse with its numerous genetic or acutely induced models of degenerative retinal disorders.

Because stem cells are highly expandable and amenable to genetic modifications, they not only hold promise for cell replacement strategies but also may serve as cellular vectors to deliver therapeutic gene products to diseased retinas. Neurally committed stem and progenitor cells are of particular interest in this context, as they have been shown to survive for extended periods of time in host eyes after transplantation [23–27] and to confer some neuroprotective activity to photoreceptors even when grafted without prior genetic modifications [13, 23, 28, 29]. Neural stem/progenitor cells are usually expanded as free-floating cellular aggregates, called neurospheres [30], which contain only a few stem cells but instead are mainly composed of committed neural progenitor cells [31]. When neurosphere cells are cultured under adherent conditions, however, they give rise to pure populations of continuously self-renewing clonogenic neural stem cells, which can be extensively expanded in culture [32]. These cells, which in analogy to continuously self-renewing embryonic stem (ES) cells have been termed neural stem (NS) cells, differentiate into neurons, astrocytes, and oligodendrocytes in vitro and after transplantation into the brain and spinal cord [32, 33] and are thus among the candidate cell types to deliver therapeutic gene products to the diseased nervous system.

CNTF is a member of the interleucin-6 cytokine family and has neuroprotective effects on various nerve cell types of the central and peripheral nervous system [34]. In the retina, CNTF protects photoreceptors and ganglion cells from degeneration, and it is probably the most extensively studied NF in the context of degenerative retinal disorders [3, 7]. Although CNTF potently attenuates photoreceptor loss in various animal models of inherited and acquired retinal disorders, it negatively affects retinal function in a dose-dependent and reversible manner [35–38]. Of note, CNTF is currently being evaluated in clinical trials for the treatment of inherited retinal degenerations and geographic atrophy, and some positive effects have been reported [19–22].

To establish a cell-based intraocular delivery system for a sustained administration of NFs to the dystrophic mouse retina, we took advantage of the strong protective effect of CNTF on retinal structure and expressed this cytokine in NS cells. The neuroprotective effect of the CNTF-secreting NS cells on photoreceptor cells was evaluated in *Pde6b*^{rd1} and *Pde6b*^{rd10} mutant mice, two animal models of autosomal recessive retinitis pigmentosa [39, 40].

Materials and Methods

Animals

Neural stem cells were isolated from the cerebral cortex of 14-day-old C57BL/6J wild-type mouse embryos. *Pde6b*^{rd1} and *Pde6b*^{rd10} mutant mice were maintained on a C57BL/6J background and genotyped by polymerase chain reaction (PCR) [40, 41]. All animal experiments were approved by the local ethics committee and were in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Isolation, Cultivation, and Differentiation of NS Cells

To establish NS cell cultures [32] from the cerebral cortex of mouse embryos, we first generated neurosphere cultures according to standard protocols [24, 42]. After two or three passages, neurospheres were enzymatically dissociated, and cells were further cultivated under adherent conditions in tissue culture flasks coated with 0.1% Matrigel (BD Biosciences, Heidelberg, Germany, http://www.bd.com) in NS-A medium (Euroclone, Pero, Italy, http://www.euroclonegroup.it) supplemented with 10 ng/ml fibroblast growth factor-2 (FGF-2) and 10 ng/ml epidermal growth factor (EGF; both from TEBU, Offenbach, Germany, http://www.tebu-bio.com), 1% modified N2 [32], and 1% B27 (Life Technologies, Darmstadt, Germany, http://www.lifetech.com). Astrocytic differentiation of NS cells was induced by maintaining cultures for 5 days in NS-A medium containing 1% fetal calf serum (Life Technologies) and 2% B27. Neuronal differentiation was induced by cultivating NS cells for 5 days in NS-A medium supplemented with 5 ng/ml FGF-2, 1% N2, and 2% B27, followed by a further cultivation period of 5 days in a 1:1 mixture of NS-A and Neurobasal medium (Life Technologies) containing 0.25% N2 and 2% B27.

Lentiviral Vectors and NS Cell Transduction

The open reading frame of mouse CNTF was PCR amplified from mouse brain cDNA and ligated in-frame with the Ig κ-chain leader sequence of pSecTag2 B (Life Technologies). The secretable variant of CNTF was then cloned into pCAG-IRES-Venus-2A-ZEO, giving rise to pCAG-CNTF-IRES-Venus-2A-ZEO. The vector is based on the lentiviral "gene ontology" (LeGO) vectors [43, 44] and contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus and a Venus reporter gene separated from a zeocin (ZEO) resistance gene by a P2A sequence of porcine teschovirus-1 under regulatory control of the cytomegalovirus enhancer/chicken β-actin (CAG) promoter (Fig. 1A). Lentiviral particles, pseudotyped with the envelope G protein of the vesicular stomatitis virus, were produced as described (http://www.lentigo-vectors.de).

NS cells were spinoculated with pCAG-CNTF-IRES-Venus-2A-ZEO to derive CNTF-secreting NS cells (CNTF-NS cells) or with pCAG-IRES-Venus-2A-ZEO to derive NS cells for control experiments (control-NS cells), and further expanded in the presence of 200 μg/ml zeocin (InvivoGen, San Diego, CA, http://www.invivogen.com/) to select for positive cells. To establish clonally derived control-NS and CNTF-NS cell lines, single cells with the highest expression levels of the reporter gene were plated into 96-well plates by fluorescence activated cell sorting (FACS; FACSAria; BD Biosciences, San Diego, CA, http://www.bdbiosciences.com).

Immunocytochemistry, Immunoblot Analysis, and Enzyme-Linked Immunosorbent Assay

Cells were fixed in 4% paraformaldehyde (PA), blocked, and stained with polyclonal rabbit anti-CNTF antibodies (kind gift of Dr. M. Sendtner). Astrocytes and neurons in differentiated NS cell cultures were identified with monoclonal mouse anti-glial fibrillary acidic protein (GFAP) and anti-microtubule associated protein 2 antibodies (both from Sigma-Aldrich, St. Louis, MO,

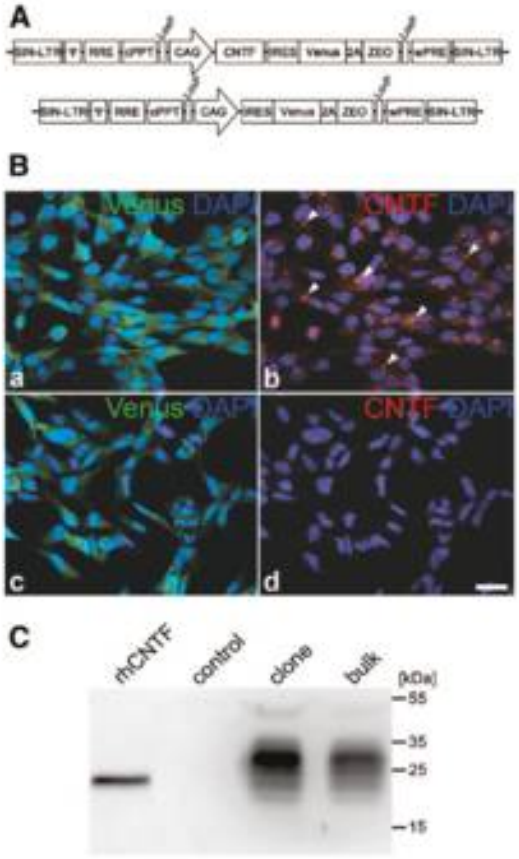

Figure 1. Generation of CNTF-secreting neural stem (NS) cell cultures. **(A):** The lentiviral vector used in this study encoded a secretable variant of mouse CNTF under regulatory control of the human CAG promoter. The vector additionally encoded a Venus reporter gene and a zeocin resistance gene, both being located downstream of an internal ribosome entry site of the encephalomyocarditis virus and separated from each other by a P2A sequence (top). The same construct, but lacking the CNTF cDNA, served as a control vector (bottom). **(B):** NS cells were transduced with pCAG-CNTF-IRES-Venus-2A-ZEO. Cells with high expression levels of the reporter gene were clonally expanded and immunostained with anti-CNTF antibodies **(Ba, Bb)**. Note that all cells in the CNTF-NS clone were positive for Venus **(Ba)** and showed strong CNTF immunoreactivity in a perinuclear location **(Bb)**. A clonal NS cell line derived from cultures transduced with the control vector pCAG-IRES-Venus-2A-ZEO, in comparison, expressed Venus **(Bc)** but no detectable levels of CNTF **(Bd)**. Scale bar = 20 μm. **(C):** CNTF was detected in the culture supernatants from CNTF-NS cell bulk cultures (bulk) and clonally derived CNTF-NS cell lines (clone), but not in supernatants from control-NS cell cultures (control). Abbreviations: Ψ, packaging signal; CAG, cytomegalovirus enhancer/chicken β-actin; CNTF, ciliary neurotrophic factor; cPPT, central polypurine tract; DAPI, 4′,6-diamidino-2-phenylindole; IRES, internal ribosome entry site; LoxP, recognition site of Cre recombinase; rhCNTF, recombinant human ciliary neurotrophic factor; RRE, rev-responsive element; SIN-LTR, self-inactivating long-terminal repeat; wPRE, woodchuck hepatitis virus posttranscriptional regulatory element; ZEO, zeocin.

http://www.sigmaaldrich.com), respectively. Primary antibodies were detected with Cy3- or Cy5-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com).

To evaluate secretion of CNTF, culture supernatants were concentrated with Amicon Ultra Centrifugal Filter devices (Millipore, Bedford, MA, http://www.millipore.com) and analyzed in immunoblots using polyclonal rabbit anti-CNTF antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) and horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Laboratories). Immunoreactive bands were visualized using SuperSignal West Dura Extended Duration Substrate (ThermoFisher Scientific, Rockford, IL, http://www.thermofisher.com/global/en/home.asp). Secretion of CNTF was quantified with an enzyme-linked immunosorbent assay (ELISA) kit for mouse CNTF (USCN Life Science Inc., Wuhan, China, http://www.uscnk.com). ELISA plates were analyzed with a Sunrise microplate reader and Magellan software (Tecan, Männedorf, Switzerland, http://www.tecan.com).

Intravitreal Cell Transplantations and Immunohistochemistry

NS cells were intravitreally grafted into 7-day-old *Pde6b*^{rd1} and 14-day-old *Pde6b*^{rd10} mice. Animals were deeply anesthetized, a glass micropipette was inserted into the vitreous space at the junction between sclera and cornea, and 1 and 2 μl of vitreous fluid were removed from the eyes of *Pde6b*^{rd1} and *Pde6b*^{rd10} mice, respectively. *Pde6b*^{rd1} mice received injections of 1 μl of phosphate-buffered saline (PBS) containing 3.8×10^5 CNTF-NS cells or no cells into one eye, and 3.8×10^5 control-NS cells in 1 μl of PBS into the contralateral eye. *Pde6b*^{rd10} mice received injections of 2 μl of PBS containing 7.6×10^5 CNTF-NS cells or no cells into one eye, and 7.6×10^5 control-NS cells in 1 μl of PBS into the contralateral eye. Care was taken not to damage the lens during the removal of vitreous fluid or the injection of cells or vehicle solution. *Pde6b*^{rd1} mutants were analyzed at postnatal day 15 (P15) and P25. *Pde6b*^{rd10} mice were kept in a 12-hour-light/12-hour-dark cycle with illumination levels of ~200 lux and were analyzed at P30.

Eyes from *Pde6b*^{rd1}, *Pde6b*^{rd10}, or age-matched untreated wild-type mice were fixed in 4% PA, cryoprotected, frozen, serially sectioned at a thickness of 25 μm, and stained with polyclonal rabbit anti-recoverin antibodies (Millipore). Lenses with attached donor cells were stained with polyclonal rabbit anti-CNTF and anti-GFAP (Dako, Glostrup, Denmark, http://www.dako.com) or anti-β-tubulin III (Sigma-Aldrich) antibodies. Lenses from *Pde6b*^{rd10} mice with grafted CNTF-NS or control-NS cell bulk cultures, or from *Pde6b*^{rd1} mice with grafted CNTF-NS or control-NS cell clones ($n = 3$ for each genotype and cell population) were stained with polyclonal rabbit anti-Ki67 antibodies (Abcam, Cambridge, U.K., http://www.abcam.com) to identify proliferating donor cells. Between 150 and 400 donor cells were analyzed per lens to determine the percentage of Ki-67-positive donor cells. Sections and lenses were analyzed with an Olympus FV 1000 confocal microscope (Olympus, Hamburg, Germany, http://www.olympus-global.com).

Photoreceptor Counts

Merged images of the entire nasal halves of central (i.e., in the plane of the optic disc) retinal sections were prepared using Photoshop CS3 software (Adobe Systems Inc., San Jose, CA, http://www.adobe.com). Photoreceptors were counted in three areas located at defined distances from the optic disc, corresponding to 25%, 50%, and 75% of the length of the nasal retina. Each area covered the outer nuclear layer over a length of 220 μm. Photoreceptor numbers were determined in *Pde6b*^{rd1} and *Pde6b*^{rd10} mice with intravitreally injected NS cells or PBS, and in 15- and

30-day-old untreated wild-type mice. Six animals from three independent experiments were analyzed for each experimental group. Statistical analysis of data was performed with the two-way analysis of variance test.

Results

Lentiviral Expression of CNTF in NS Cells

To establish NS cell cultures from the cerebral cortex of embryonic mice, we converted neurosphere cultures into adherently growing cultures and further expanded the cells in the presence of EGF, FGF-2, and N2. Under these conditions, cultures consisted of a pure population of symmetrically dividing clonogenic NS cells with molecular features reminiscent of neurogenic radial glia [32, 33]. To genetically modify these cells, we generated a polycistronic lentiviral vector encoding a secretable variant of mouse CNTF, a Venus reporter gene, and a zeocin resistance gene under control of the CAG promoter (pCAG-CNTF-IRES-Venus-2A-ZEO; Fig. 1A). The same construct but lacking the CNTF cDNA served as a control vector (pCAG-IRES-Venus-2A-ZEO; Fig. 1A). NS cells were spinoculated with pCAG-CNTF-IRES-Venus-2A-ZEO and pCAG-IRES-Venus-2A-ZEO to derive CNTF-NS and control-NS cells, respectively. Further cultivation of cells in the presence of zeocin gave rise to CNTF-NS and control-NS cell cultures exclusively composed of Venus-positive cells. Immunocytochemical analysis of these bulk cultures revealed expression of CNTF in virtually every CNTF-NS cell, whereas control-NS cells lacked detectable levels of CNTF immunoreactivity (not shown, but clonal cell lines are given in Fig. 1B). Immunoblot analysis of culture supernatants confirmed secretion of CNTF from CNTF-NS cell bulk cultures, whereas supernatants from control-NS cell bulk cultures lacked detectable levels of the cytokine (Fig. 1C). Because NS cells differentiated into astrocytes and neurons after intravitreal transplantations, we additionally analyzed expression of CNTF in differentiated NS cell cultures. CNTF immunoreactivity was detectable in virtually all astrocytes and neurons derived from CNTF-NS cells, but not in astrocytes and neurons derived from control-NS cells (Fig. 2).

CNTF-Secreting NS Cell Clones

To establish NS cell cultures with elevated expression levels of CNTF, we next generated clonally derived CNTF-NS cell lines. Assuming that expression levels of CNTF and the reporter gene from the polycistronic lentiviral vector are proportional to each other, we isolated individual NS cells with the highest expression levels of Venus using FACS to establish clonal cell lines with high levels of CNTF expression. Immunocytochemistry (Fig. 1B) and immunoblot analysis of culture supernatants (Fig. 1C) demonstrated expression of CNTF in all CNTF-NS cell clones established, whereas control-NS cell clones lacked expression of the cytokine. ELISA analysis of culture supernatants from a CNTF-NS cell clone that showed high expression levels of CNTF in immunoblots revealed secretion of 5.4 ng of CNTF per 1×10^6 cells in 24 hours. The original CNTF-NS cell bulk culture, in comparison, produced 4.0 ng of CNTF per 1×10^6 cells in 24 hours. Expression of CNTF in the clonal cell line was thus increased by ~35% when compared with the original bulk culture, and the clone was therefore selected for transplantation experiments. Expression of CNTF in the CNTF-NS cell clone remained detectable by immunocytochemistry and immunoblot analysis for at least 40 passages, and ELISA analysis of culture supernatants revealed secretion of similar quantities of CNTF at passages 33 and 46, demonstrating stable expression of the cytokine.

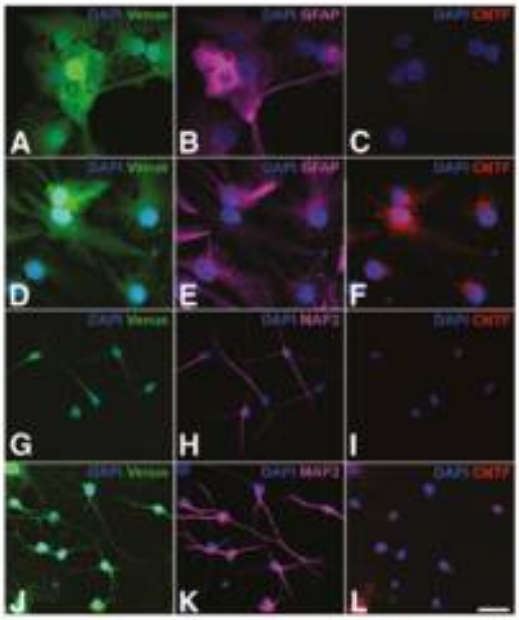

Figure 2. Analysis of CNTF expression in differentiated neural stem (NS) cell cultures. NS cells were transduced with pCAG-CNTF-IRES-Venus-2A-ZEO **(D–F, J–L)** or the control vector pCAG-IRES-Venus-2A-ZEO **(A–C, G–I)**, and positive cells were selected with zeocin and differentiated into astrocytes **(A–F)** or neurons **(G–L)**. Note the co-expression of Venus and CNTF in virtually all GFAP-positive astrocytes **(D–F)** and MAP2-positive neurons **(J–L)** derived from CNTF-NS cells. Astrocytes **(A–C)** and neurons **(G–I)** derived from control-NS cells, in comparison, expressed Venus but no detectable levels of CNTF. All cultures were counterstained with DAPI to visualize cell nuclei. Scale bar = 20 μm. Abbreviations: CNTF, ciliary neurotrophic factor; DAPI, 4′,6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2.

Characterization of Intravitreally Grafted NS Cells In Vivo

Analysis of eyes from *Pde6b*^{rd10} and *Pde6b*^{rd1} mice with grafted CNTF-NS or control-NS cells revealed the presence of numerous Venus-positive donor cells that were attached to the posterior pole of the lens (Fig. 3; supplemental online Fig. 1) and the vitreal side of the retina (Fig. 4). Integration of donor cells into the host retinas was not observed (Fig. 4). Immunohistochemical analysis of lenses with attached donor cells revealed that grafted CNTF-NS and control-NS cell bulk cultures (Fig. 3) or clonal cell lines (supplemental online Fig. 1) were differentiated into GFAP-positive astrocytes, and some β-tubulin III-positive nerve cells. In addition, we found expression of Ki-67 in 2.6% and 1.8% of the grafted CNTF-NS cells and in 3.7% and 1% of the grafted control-NS cells in *Pde6b*^{rd1} and *Pde6b*^{rd10} mice, respectively. Furthermore, CNTF immunoreactivity was detectable only in donor cells derived from grafted CNTF-NS cell bulk cultures or clones, but not in donor cells derived from grafted control-NS cell bulk cultures or clones (Fig. 3; supplemental online Fig. 1). Finally, we observed no adverse effects of the grafted NS cells on the morphology of either *Pde6b*^{rd10} (Fig. 5) or *Pde6b*^{rd1} retinas (Fig. 4; supplemental online Figs. 2, 3).

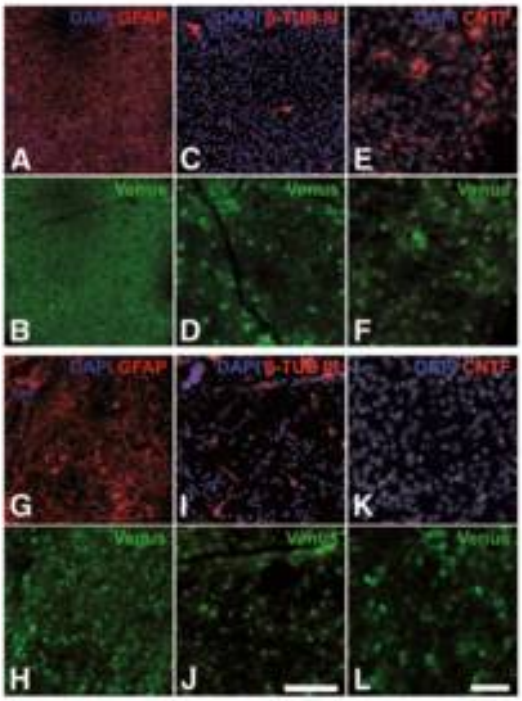

Figure 3. Characterization of intravitreally grafted CNTF-neural stem (NS) and control-NS cell bulk cultures in *Pde6b*^{rd10} mice. Analysis of eyes from *Pde6b*^{rd10} mutant mice with intravitreally grafted CNTF-NS **(A–F)** and control-NS cell bulk cultures **(G–L)** revealed the presence of dense layers of Venus-positive cells **(B, D, F, H, J, L)** that were attached to the posterior poles of the lenses. The majority of Venus-positive donor cells were differentiated into GFAP-positive astrocytes (compare **[A]** with **[B]**, and **[G]** with **[H]**), and some into β-tubulin III-positive neurons (compare **[C]** with **[D]**, and **[I]** with **[J]**). Expression of CNTF was detectable in donor cells derived from CNTF-NS cells **(E)** but not in donor cells derived from control-NS cells **(K)**. Scale bars = 100 μm (bar in **[J]** applies to **[A–D, G–J]**) and 50 μm (bar in **[L]** applies to **[E, F, K, L]**). Abbreviations: β-TUB III, β-tubulin III; CNTF, ciliary neurotrophic factor; DAPI, 4′,6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein.

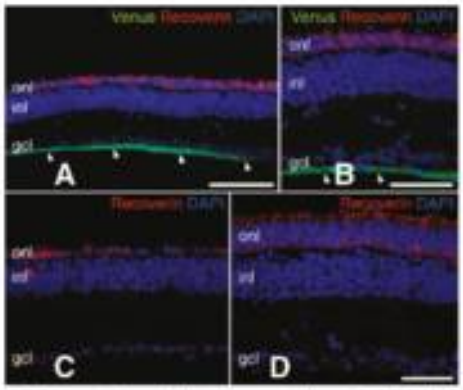

Figure 4. Attenuation of photoreceptor loss in *Pde6b*^{rd1} mice at advanced stages of retinal degeneration. The clonal ciliary neurotrophic factor (CNTF)-neural stem (NS) and control-NS cell lines were intravitreally grafted into 7-day-old *Pde6b*^{rd1} mice, and animals were analyzed at postnatal day 25. Note that the transplanted cells had formed a layer of Venus-positive cells that was attached to the vitreal side of the retina (arrowheads in **[A, B]**). Integration of donor cells into the host retinas was not detectable **(A, B)**. The outer nuclear layer of eyes with the grafted control-NS cell line consisted of only one row of photoreceptor nuclei at this developmental age **(C)**. The outer nuclear layer of the CNTF-treated contralateral retina, in comparison, still consisted of several rows of photoreceptor nuclei **(D)**, demonstrating significant attenuation of photoreceptor loss by the grafted CNTF-NS cell clone. Scale bars = 100 μm **(A)**, 50 μm **(B–D)**. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer.

CNTF-NS Cells Protect Photoreceptors in *Pde6b*^{rd10} and *Pde6b*^{rd1} Mice

The neuroprotective effect of CNTF-NS cell bulk cultures on photoreceptors was analyzed in 30-day-old Pde6b^{rd10} mice (Fig. 5). A comparison of eyes with grafted CNTF-NS cell bulk cultures and contralateral eyes with grafted control-NS cell bulk cultures consistently revealed a significantly thicker outer nuclear layer in the CNTF-treated eyes (Fig. 5). Importantly, the photoreceptor rescue in CNTF-treated eyes was evident over the entire length of all retinal sections analyzed. To quantify the neuroprotective effect of the CNTF-NS cells, photoreceptor numbers were determined in the nasal halves of retinal sections at three positions, corresponding to 25%, 50%, and 75% of the distance between optic disc and periphery of the retina. In CNTF-treated retinas, 274.8 ± 31.6 (mean ± SEM), 311.5 ± 26.9, and 339.8 ± 23.5 photoreceptors were present at the 25%, 50%, and 75% positions, respectively. In the contralateral control retinas, in comparison, we counted 101.7 ± 18.9, 151.0 ± 25.3, and 181.7 ± 27.3 photoreceptors at the 25%, 50%, and 75% positions, respectively (Fig. 6A). CNTF-treated retinas of *Pde6b*^{rd10} mice thus contained 1.9- to 2.7-fold more photoreceptors than the contralateral control retinas ($p < .01$), depending on the retinal region analyzed. We additionally evaluated whether control-NS cells also protected photoreceptors in *Pde6b*^{rd10} mice but found no significant differences between photoreceptor numbers in eyes with grafted control-NS cell bulk cultures (132.7 ± 8.8, 134.3 ± 10.5, and 154.0 ± 15.3 cells at the 25%, 50%, and 75% positions, respectively) and contralateral eyes that had received injections of PBS only (129.0 ± 26.8, 128.7 ± 30.0, and 139.0 ± 29.9 cells at the 25%, 50%, and 75% positions, respectively; supplemental online Fig. 4). Retinas from untreated 30-day-old wild-type mice (supplemental online Fig. 4) analyzed for comparison contained 526.2 ± 11.8 (25% position), 531.2 ± 19.8 (50% position), and 503.9 ± 8.6 (75% position) photoreceptors.

CNTF-NS cell bulk cultures also potently protected photoreceptors in the *Pde6b*^{rd1} mutant mouse. In 15-day-old *Pde6b*^{rd1} mice, the outer nuclear layer was significantly thicker in eyes with grafted CNTF-NS cells than in the contralateral eyes with grafted control-NS cells (supplemental online Fig. 2). As in *Pde6b*^{rd10} mice, the rescue effect was widespread and evident in all retinal regions analyzed (supplemental online Fig. 2). Quantitative analysis confirmed a significant protective effect of the CNTF-NS cell bulk culture also in this mutant, with 253.7 ± 11.4, 260.5 ± 17.7, and 268.3 ± 13.7 photoreceptors at the 25%, 50%, and 75% positions, respectively, as compared with 109.2 ± 8.6, 110.5 ± 5.6, and 130.7 ± 8.9 photoreceptors at the 25%, 50%, and 75% positions in control retinas, respectively (Fig. 6B). CNTF-treated retinas thus contained 2.1- to 2.4-fold more photoreceptors than the contralateral control eyes ($p < .001$). Retinas from untreated 15-day-old wild-type mice (supplemental online Fig. 4) analyzed for comparison contained 527.8 ± 9.8, 513.0 ± 12.6, and 487.3 ± 16.3 photoreceptors at the 25%, 50%, and 75% positions, respectively.

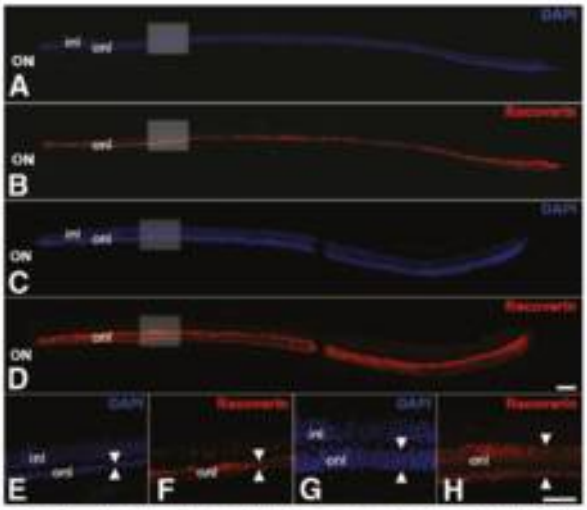

Figure 5. Intravitreally grafted ciliary neurotrophic factor (CNTF)-neural stem (NS) bulk cultures attenuate photoreceptor degeneration in *Pde6b*^{rd10} mice. Control-NS cell bulk cultures were grafted into one eye **(A, B, E, F)** and CNTF-NS cell bulk cultures into the contralateral eye **(C, D, G, H)** of 14-day-old *Pde6b*^{rd10} mice, and animals were analyzed 16 days later. Analysis of central retinal sections stained with DAPI and anti-recoverin antibodies revealed a significantly thicker outer nuclear layer in eyes that had received CNTF-NS cell grafts (**[C, D]**, arrowheads in **[G, H]**) than in the contralateral eyes that had received control-NS cell grafts (**[A, B]**, arrowheads in **[E, F]**). Note that the rescue effect of the grafted cells was evident along the entire length of the retinal section **(C, D)**. Adverse effects of the grafted cells on the morphology of the host retinas were not detectable **(A–H)**. **(E, F)** and **(G, H)** are higher magnifications of the boxed areas in **(A, B)** and **(C, D)**, respectively. Scale bars = 100 μm (bar in **[D]** applies to **[A–D]**) and 50 μm (bar in **[H]** applies to **[E–H]**). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; inl, inner nuclear layer; ON, optic nerve; onl, outer nuclear layer.

Neuroprotective Effect of a CNTF-NS Cell Clone in *Pde6b*^{rd1} Mice

Intravitreal transplantations of a clonal cell line with a ~35% increase in CNTF expression into *Pde6b*^{rd1} (compare supplemental online Figs. 2 and 3) or *Pde6b*^{rd10} mice (not shown) resulted in a photoreceptor rescue that appeared more pronounced than that observed with the original CNTF-NS cell bulk culture. Quantitative analysis of *Pde6b*^{rd1} retinas confirmed a profound protection of photoreceptors by the grafted CNTF-NS cell clone, with 283.5 ± 13.3 (25% position), 288.7 ± 11.4 (50% position), and 314.5 ± 11.5 (75% position) photoreceptors in CNTF-treated retinas, as compared with 87.8 ± 3.0, 92.7 ± 8.9, and 110.0 ± 6.7 photoreceptors at the 25%, 50%, and 75% positions in control retinas, respectively (Fig. 6C). CNTF-treated *Pde6b*^{rd1} eyes thus contained 2.9- to 3.2-fold more photoreceptors than the contralateral eyes with the grafted control-NS cell clone ($p < .001$). Transplantations of three other CNTF-NS cell clones also resulted in significant protection of photoreceptors in this mutant (data not shown). A significant neuroprotective effect of the CNTF-NS cell clone was still detectable in 25-day-old *Pde6b*^{rd1} mice (Fig. 4). At this advanced stage of retinal degeneration, CNTF-treated retinas contained 134.0 ± 15.5, 154.5 ± 13.7, and 177.2 ± 18.3 photoreceptors, whereas the contralateral control retinas contained 41.0 ± 5.3, 40.2 ± 4.4, and 44.2 ± 6.3 photoreceptors at the 25%, 50%, and 75% positions, respectively. CNTF-treated retinas thus contained up to 4.0-fold more photoreceptor cells than the control retinas ($p < .001$). We also analyzed whether the control-NS cell clone attenuate photoreceptor loss in *Pde6b*^{rd1} mice but found similar photoreceptor numbers in eyes with grafted control-NS cells (80.5 ± 4.9, 88.8 ± 4.3, and 126.2 ± 10.5 photoreceptors at the 25%, 50%, and 75% positions, respectively) and contralateral eyes injected with PBS only (97.0 ± 8.3, 97.2 ± 12.3, and 117.2 ± 12.3 photoreceptors at the 25%, 50%, and 75% positions, respectively; supplemental online Fig. 4).

To compare the neuroprotective activity of the CNTF-NS cell bulk culture and the CNTF-NS cell clone in *Pde6b*^{rd1} mice, we calculated the quotients of photoreceptor numbers in retinas with grafted CNTF-NS cells and contralateral retinas with grafted control-NS cells for both experimental groups at the three retinal positions. Values for animals that had received injections of NS cell bulk cultures and animals that had received injections of clonal NS cell lines were 2.42 ± 0.26 (mean ± SEM) and 3.25 ± 0.2 (25% position), 2.38 ± 0.17 and 3.24 ± 0.31 (50% position), and 2.1 ± 0.17 and 2.92 ± 0.23 (75% position), respectively (Fig. 6D). The CNTF-NS cell clone thus protected photoreceptors more effectively than the original CNTF-NS cell bulk culture ($p < .01$; Fig. 6D), in line with the elevated expression level of CNTF in the clonal cell line.

Discussion

The intraocular implantation of genetically modified and encapsulated cells is among the strategies to achieve a local and sustained administration of NFs to the retina, and it has been successfully used in large animal species [17, 18] and human patients with advanced retinitis pigmentosa or geographic atrophy [19–22]. However, the implantation of encapsulated cells is not easily applicable to the numerous genetic or acutely induced mouse models of degenerative retinal disorders because of the small eyes of this species. To establish a cell-based intraocular delivery system for preclinical studies aimed at evaluating and optimizing this therapeutic approach in mouse models of photoreceptor degeneration, we therefore searched for a cell type that allows a sustained administration of NFs to the dystrophic mouse retina without the need of prior encapsulation.

Neural stem/progenitor cells, isolated from the developing or adult brain or derived from ES cells, survive for extended periods of time after intraocular transplantation [23–27]. Furthermore, these cells have been shown to attenuate photoreceptor degeneration in the Royal College of Surgeons (RCS) rat [13, 28, 29] and a mouse model of neuronal ceroid lipofuscinoses [23], even when grafted without prior genetic modifications. We therefore argued that neural stem cells isolated from the embryonic mouse brain and cultivated under adherent conditions in the presence of EGF and FGF-2 may serve as vehicles to deliver NFs to the dystrophic mouse retina. Cells maintained under such conditions give rise to pure populations of symmetrically dividing clonogenic neural stem cells and have therefore been termed NS cells, in analogy to continuously self-renewing ES cells [32]. NS cells can be extensively expanded in vitro and give rise to neurons, astrocytes, and oligodendrocytes in culture and after transplantation into the brain [32] or spinal cord [33]. NS cell cultures have also been established from the adult mouse brain, fetal human brain, ES cells, and induced pluripotent stem cells [32, 45–47].

To analyze whether a sustained delivery of functionally relevant quantities of NFs to the dystrophic mouse retina can be

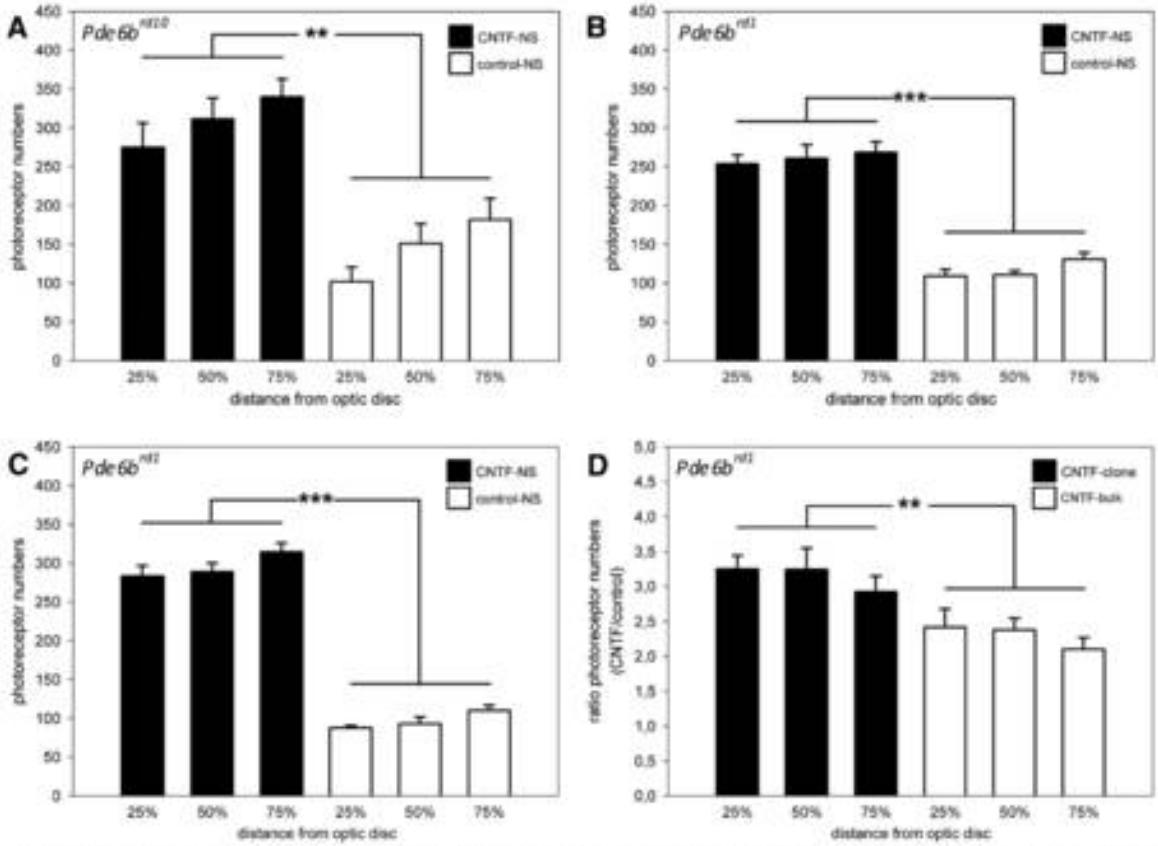

Figure 6. Photoreceptor numbers in *Pde6b*^{rd1} and *Pde6b*^{rd10} mice with intravitreally grafted CNTF-NS cells and control-NS cells. Eyes from *Pde6b*^{rd10} **(A)** and *Pde6b*^{rd1} **(B, C)** mice that received intravitreal injections of either CNTF-NS cell bulk cultures **(A, B)** or a clonally derived CNTF-NS cell line **(C)** contained significantly more photoreceptors than the contralateral eyes that received injections of the corresponding control cells. Note that the clonally derived CNTF-NS cell line selected for high levels of CNTF expression had a significantly more pronounced neuroprotective effect on photoreceptors in *Pde6b*^{rd1} mice than the original CNTF-NS cell bulk culture **(D)**. Each bar in **(A–C)** represents the photoreceptor number (mean ± SEM) from six retinas at the indicated retinal positions. Each bar in **(D)** represents the quotient (mean ± SEM) of photoreceptor numbers in eyes with the grafted CNTF-NS cell clone and the contralateral eyes with the grafted control-NS cell clone (n = 6; filled bars), or in eyes with the grafted CNTF-NS cell bulk culture and the contralateral eyes with the grafted control-NS cell bulk culture (n = 6; open bars) at the indicated retinal positions. **, $p < .01$; ***, $p < .001$, according to the two-way analysis of variance test. Abbreviations: CNTF, ciliary neurotrophic factor; NS, neural stem.

achieved by intraocular transplantations of modified NS cells, we expressed a secretable variant of this cytokine in NS cells. CNTF was selected because of its profound neuroprotective effect on photoreceptors, which we used as a measure for the efficacy of our approach. Initially demonstrated to attenuate light-induced degeneration of photoreceptors [48], CNTF has subsequently been shown to protect photoreceptors in a variety of animal models of inherited and acquired photoreceptor loss [3]. Of note, the therapeutic potential of CNTF for the treatment of inherited retinal degenerations and geographic atrophy is currently being evaluated in clinical studies using encapsulated human RPE cells modified to secrete this cytokine, and some positive results have been reported [19–22]. However, although CNTF potently preserves retinal structure, it negatively affects retinal function in a reversible and dose-dependent manner [35–38, 49]. These negative effects of CNTF are accompanied by a dysregulated expression of various genes, including some that encode components of the phototransduction cascade [50, 51].

To express CNTF in NS cells, we used a polycistronic lentiviral vector additionally encoding a resistance and a reporter gene. This construct facilitated the selection of modified NS cells, the characterization of the cells after transplantation, and the generation of clonal NS cell lines with elevated expression levels of CNTF. Analyses of modified NS cell cultures indeed revealed expression of the reporter gene in virtually every cell, and immunocytochemistry and immunoblot analysis of CNTF-NS cells demonstrated expression of the cytokine for at least 40 passages, corresponding to a culture period of more than 6 months. Robust expression of CNTF was also observed in astrocytes and neurons derived from CNTF-NS cells in vitro.

Expression of the Venus reporter gene allowed the identification and characterization of the modified NS cells after transplantation into *Pde6b*^{rd1} and *Pde6b*^{rd10} mice. In both mouse lines, grafted NS cells survived in the vitreous, where they formed dense layers of Venus-positive cells that were attached to the retina and lens. The vast majority of donor cells were identified as astrocytes, and some were identified as neurons. The detection of only a few proliferating donor cells in *Pde6b*^{rd1} and *Pde6b*^{rd10} mice is in line with the rapid neural differentiation of the grafted cells. Furthermore, we found sustained expression

of CNTF in donor cells derived from CNTF-NS cells, although donor cells derived from control-NS cell lacked detectable levels of the cytokine. Robust survival and sustained expression of transgenes in NS cell-derived donor cells were also observed in a mouse model of glaucoma, where astrocytes and neurons with an ectopic expression of NFs survived in the vitreous for at least 4 months after transplantations (Kai Flachsbarth and U.B., unpublished results). Despite the presence of numerous Venus-positive donor cells in the vitreous cavity, integration of grafted cells into the host retinas was not apparent. This observation differs from results of other studies that have shown extensive integration of intravitreally grafted neural stem/progenitor cells into developing or dystrophic retinas [26, 52]. Intravitreal transplantations of NS cells into *Pde6b*^{rd1} or *Pde6b*^{rd10} mice before the onset of retinal degeneration and/or intrinsic differences between the neural stem/progenitor cells used in the different studies might account for these discrepant results. In fact, although we have transplanted cell populations highly enriched in neural stem cells, the other studies have grafted murine neural progenitor cells expanded in neurosphere cultures [52] or neural progenitor cells from the hippocampus of adult rats [26, 52].

The neuroprotective potential of the CNTF-secreting NS cells was evaluated in *Pde6b*^{rd1} and *Pde6b*^{rd10} mice, two animal models of retinitis pigmentosa with an early onset and rapid degeneration of photoreceptor cells [39, 40]. Intravitreal injections of CNTF-NS cells from a bulk culture that produced 4.0 ng of CNTF per 1×10^6 cells in 24 hours resulted in significant protection of photoreceptors in *Pde6b*^{rd1} mice. Depending on the retinal region analyzed, CNTF-treated retinas contained up to 2.4-fold more photoreceptors than the contralateral retinas with grafted control-NS cells. Intravitreally grafted CNTF-secreting NS cells from the same bulk culture significantly attenuated photoreceptor degeneration also in *Pde6b*^{rd10} mice, with up to 2.7-fold more photoreceptors in CNTF-treated eyes than in control eyes. Of note, the neuroprotective effect was widespread in both mouse lines and evident in all retinal regions analyzed. Furthermore, the grafted NS cells had no adverse effects on the morphology of host retinas in either mouse strain, in contrast to other cell types that we tested in intravitreal transplantation experiments. Mesenchymal stem cells, for instance, attenuate photoreceptor degeneration without prior genetic modification [53] and thus represent another candidate cell type to deliver NFs to the dystrophic retina. In our hands, however, these cells caused local retinal detachments resulting in an aggravated loss of photoreceptors. Furthermore, the immortalized neural progenitor cell line C17.2 [54] continued to proliferate after intravitreal transplantations, and grafted retinal stem cells isolated from the developing neuroretina [55] survived only poorly in the vitreous.

Because nonmodified neural stem/progenitors have been shown to attenuate photoreceptor degeneration [13, 23, 28, 29], we additionally evaluated the neuroprotective potential of control-NS cells in *Pde6b*^{rd1} and *Pde6b*^{rd10} mutant mice. Photoreceptor numbers in eyes that had received injections of either control-NS cells or the vehicle only, however, were not significantly different from each other in either mouse line. These apparently discrepant observations might be related to particular properties of the cell populations used for transplantation, to the particular animal model analyzed, or both. For instance, nonmodified human neural progenitor cells attenuate photoreceptor degeneration in the RCS rat in which the inability of RPE cells to phagocytose shed photoreceptor outer segments leads to progressive retinal degeneration. This neuroprotective activity of the human neural progenitor cells has been attributed, at least in part, to the endogenous expression of the neurotrophic factors brain-derived neurotrophic factor, insulin-like growth factor 1, and FGF-2 in this cell population and/or to the capability of subretinally located donor cells to phagocytose shed photoreceptor outer segments [13, 28, 29].

Expression levels of transgenes in modified bulk cultures may differ between different cultures and between different passages of the same culture, impeding the delivery of defined quantities of transgenes and the analysis of dose-dependent effects of secreted gene products using a cell-based delivery system. We therefore took advantage of the fact that NS cell cultures consist of homogeneous populations of clonogenic stem cells, and we established CNTF-secreting clonal NS cell lines from NS cells with high expression levels of the reporter gene to derive NS cell clones with high expression levels of the cytokine. The clonal NS cell lines indeed stably expressed elevated levels of CNTF compared with the original bulk cultures, and they effectively protected photoreceptors in both mutant mouse lines. Furthermore, we found that intravitreal transplantations of a clonal NS cell line with an ~35% increase in CNTF expression resulted in a significantly more pronounced photoreceptor protection in *Pde6b*^{rd1} mice than intravitreal transplantations of the original NS cell bulk culture. Attenuation of photoreceptor degeneration by the clonal cell line was still apparent in *Pde6b*^{rd1} mice at developmental ages when the outer nuclear layer in this mutant is normally almost completely degenerated [56].

Conclusion

Intravitreal transplantations of adherently cultivated neural stem cells modified to secrete CNTF resulted in a sustained delivery of functionally relevant quantities of the cytokine to the dystrophic mouse retina, as indicated by the significant attenuation of photoreceptor loss in *Pde6b*^{rd1} and *Pde6b*^{rd10} mutant mice. Furthermore, a coexpressed reporter gene greatly facilitated the characterization of the modified stem cells after transplantation and the derivation of clonal NS cell lines with high expression levels of CNTF and profound neuroprotective effects on photoreceptors. Adverse effects of the grafted cells on the morphology of host retinas were not observed. The combined results demonstrate that intravitreal transplantations of genetically modified NS cells represent a useful method for preclinical studies aimed at evaluating the therapeutic potential of a cell-based administration of NFs in mouse models of photoreceptor degeneration.

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Author Contributions

G.J.: collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; J.S., B.P., K.R., K.K., and W.J.: collection and assembly of data, data analysis and interpretation, final approval of manuscript; F.K. and C.S.: collection and assembly of data; G.R.: financial support, final approval of manuscript; B.F.: provision of study material, data analysis and interpretation, final approval of manuscript; U.B.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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RESEARCH ARTICLE

Sustained Neural Stem Cell-Based Intraocular Delivery of CNTF Attenuates Photoreceptor Loss in the *nclf* Mouse Model of Neuronal Ceroid Lipofuscinosis

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Abstract

A sustained intraocular administration of neurotrophic factors is among the strategies aimed at establishing treatments for currently untreatable degenerative retinal disorders. In the present study we have analyzed the neuroprotective effects of a continuous neural stem (NS) cell-based intraocular delivery of ciliary neurotrophic factor (CNTF) on photoreceptor cells in the *nclf* mouse, an animal model of the neurodegenerative lysosomal storage disorder variant late infantile neuronal ceroid lipofuscinosis (vLINCL). To this aim, we genetically modified adherently cultivated NS cells with a polycistronic lentiviral vector encoding a secretable variant of CNTF together with a Venus reporter gene (CNTF-NS cells). NS cells for control experiments (control-NS cells) were modified with a vector encoding the reporter gene tdTomato. Clonal CNTF-NS and control-NS cell lines were established using fluorescent activated cell sorting and intravitreally grafted into 14 days old *nclf* mice at the onset of retinal degeneration. The grafted cells preferentially differentiated into astrocytes that were attached to the posterior side of the lenses and the vitreal side of the retinas and stably expressed the transgenes for at least six weeks, the latest post-transplantation time point analyzed. Integration of donor cells into host retinas, ongoing proliferation of grafted cells or adverse effects of the donor cells on the morphology of the host eyes were not observed. Quantitative analyses of host retinas two, four and six weeks after cell transplantation revealed the presence of significantly more photoreceptor cells in eyes with grafted CNTF-NS cells than in eyes with grafted control-NS cells. This is the first demonstration that a continuous intraocular administration of a neurotrophic factor attenuates retinal degeneration in an animal model of neuronal ceroid lipofuscinosis.

Introduction

Neuronal ceroid lipofuscinosis (NCL) comprises a heterogeneous group of neurodegenerative lysosomal storage diseases of mainly childhood and youth. At present, mutations in more than a dozen different genes have been identified that cause NCL. Most of these genes encode soluble lysosomal enzymes or transmembrane proteins localized in lysosomes or the endoplasmic reticulum (ER). Other locations described for some NCL proteins include the ER-Golgi intermediate complex, the cytosol, synaptic vesicles or the plasma membrane (http://www.ucl.ac.uk/ncl/mutation.shtml) [1–5]. Despite the heterogeneity of the disease-associated genes, several symptoms are common to most of these fatal storage disorders, including progressive mental deterioration, motor malfunctions, seizures, and premature death. Loss of vision due to retinal degeneration is another characteristic symptom of several NCL forms, and has been described in CLN1, CLN2, CLN3, CLN5, CLN6, CLN7 and CLN8 patients [4, 6, 7].

Mutations in the *CLN6* gene cause variant late infantile NCL (vLINCL), or in rare cases adult onset Kufs type A disease [8]. The function of CLN6, a polytopic membrane protein of the endoplasmic reticulum (ER) with 311 amino acids and 7 predicted transmembrane domains is unknown [9–12]. Until now, 71 pathogenic mutations have been identified in the *CLN6* gene (http://www.ucl.ac.uk/ncl/CLN6mutationtable.htm), which may differ significantly in their impact on the severity, time course and the age of onset of the disease [13]. While about 50% of the affected children present an early retinal phenotype [4], the retina has been reported to be unaffected in patients with an CLN6-linked adult onset of the disease [8].

The *nclf* mouse, a naturally occurring mouse model of CLN6 disease [14], carries a c.307insC mutation in the *CLN6* gene that is also present in CLN6 patients of Pakistani origin [9, 10]. The single base insertion leads to a frameshift, resulting in a truncated CLN6 protein with a reduced half-life [15, 16]. Similar to human patients carrying mutations in the *CLN6* gene, the *nclf* mouse is characterized by an early-onset retinal degeneration. Reactive gliosis and apoptotic degeneration of photoreceptor cells becomes detectable in the mutant as early as one month after birth. Other characteristic features of the retinal phenotype of *nclf* mice include accumulation of storage material in various retinal cell types, dysregulation of several lysosomal proteins, and activation of microglial cells. Progressive apoptotic degeneration of photoreceptors in *nclf* mice is nearly complete at the end of the first postnatal year, and paralleled by progressive visual deterioration as measured in electroretinogram (ERG) recordings, optokinetic tracking experiments, and visual cliff tests [17–19].

Approaches to develop treatments for the neurological symptoms associated with NCLs include enzyme replacement therapy, gene therapy, stem cell therapy, and immune therapy [20–23]. In the retina, a delay in photoreceptor degeneration and/or deterioration of visual function has been reported after intravitreal transplantations of neural progenitor cells in a mouse model of CLN8 disease [24], adeno-associated virus- (AAV) mediated ocular gene transfer of palmitoyl protein thioesterase-1 in a mouse model of CLN1 disease [25] and attenuation of reactive microgliosis in a mouse model of CLN6 disease [18]. Given that a number of growth factors and cytokines have been demonstrated to delay photoreceptor degeneration and visual impairment in various animal models of induced or inherited retinal degeneration [26–29], neuroprotective approaches may represent another strategy to ameliorate retinal degeneration in NCL. However, neurotrophic factors do not ordinarily cross the blood-retina barrier and have short half-life times. Robust neuroprotective effects therefore depend on a sustained intraocular administration of these factors which may be achieved by viral or non-viral gene transfer to endogenous retinal cell types, or by intraocular transplantations of cells that have been genetically modified to secrete these factors [26–29].

In the present study, we have evaluated the neuroprotective effects of a sustained neural stem cell-based intraocular administration of ciliary neurotrophic factor (CNTF), a member of the interleukin-6 family of cytokines [30] that has been shown to potently rescue retinal structure in various animal models of retinal degeneration [26, 28], on photoreceptor cells in the *nclf* mouse. To this aim, we used a polycistronic lentiviral vector to generate clonally derived neural stem (NS) cell lines with an ectopic expression of a secretable variant of the cytokine. The CNTF-secreting NS cells were grafted into the vitreous cavity of *nclf* mice at the onset of retinal degeneration, and the numbers of surviving photoreceptor cells were determined at different post-transplantation time points. This is the first report demonstrating that a sustained intraocular administration of a neurotrophic factor attenuates retinal degeneration in a mouse model of NCL.

Materials and Methods

Animals

Mutant mice harboring the c.307insC frameshift mutation in the *CLN6* gene (B6.Cg-Cln6^{nclf}/J; in the following termed *nclf* mice) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained on a C57BL/6J genetic background and genotyped by PCR analysis of DNA from tail biopsies and subsequent sequencing of the PCR product [9]. C57BL/6J wild-type mice were used for control experiments. All animal experiments were carried out in accordance with the German Animal Welfare Act on protection of animals and were approved by the local ethics committee (Freie und Hansestadt Hamburg—Amt für Gesundheit und Verbraucherschutz; permit number 59/12). All cell transplantations were performed under ketamine and xylazine anesthesia, and all efforts were made to minimize suffering of the animals.

Lentiviral vectors, NS cell transduction and derivation of a clonal NS cell line with elevated CNTF expression

To generate CNTF-secreting neural stem cells (CNTF-NS cells), the mouse CNTF cDNA was ligated in frame with the Ig κ-chain leader sequence of pSecTag2 B (Life Technologies, Darmstadt, Germany) and cloned into the polycistronic lentiviral vector pCAG-IRES-VENUS-2A-ZEO, encoding the internal ribosome entry site (IRES) of the encephalomyocarditis virus, a Venus reporter gene, the P2A sequence of porcine teschovirus-1 and a zeocin resistance gene under regulatory control of the cytomegalovirus enhancer/chicken ß-actin (CAG) promoter, giving rise to pCAG-CNTF-IRES-Venus-2A-ZEO. A vector containing the CAG promoter, the IRES sequence, and a tdTomato reporter gene fused to a blasticidin resistance gene (pCAG-IRES-tdTomato/BSD) was generated to establish NS cell lines for control experiments (control-NS cells). Lentiviral particles were produced by transient transfection of HEK 293T cells as described (http://www.LentiGo-Vectors.de).

To generate neural stem (NS) cell lines with high expression levels of CNTF, a previously established clonal CNTF-NS cell line [31] was again transduced with pCAG-CNTF-IRES-Venus-2A-ZEO. Positive cells were selected by cultivation in DMEM/F12 (Life Technologies) supplemented with 0.3% glucose, 2 mM glutamine, 3 mM sodium bicarbonate, 5 mM HEPES (all from Sigma-Aldrich, St. Louis, MO; in the following termed NS cell medium), 10 ng/ml epidermal growth factor (EGF) and 10 ng/ml fibroblast growth factor-2 (FGF-2; both from TEBU, Offenbach, Germany), 1% N2 and 1% B27 (both from Life Technologies) and 200 μg/ml zeocin (InvivoGen, San Diego, CA). Single cells with the highest expression level of the reporter gene in these cultures were selected using fluorescence activated cell sorting (FACS; FACSArialllu, BD Bioscience, San Diego, CA), plated into 96 well plates and again clonally expanded. For

control experiments, wild-type NS cells were transduced with pCAG-IRES-tdTomato/BSD (control-NS cells). Single cells with the highest expression levels of tdTomato were selected using FACS, and clonally expanded in the presence of 4 μg/ml blasticidin (Life Technologies) to establish clonal control-NS cell lines with high expression levels of the reporter gene.

To semi-quantitatively compare secretion levels of CNTF between different NS cell clones, 0.5 x 10^6 cells of each clonal cell line were cultivated for 24 hours in 0.5 ml medium, and equal volumes of culture supernatants were analyzed in Western blots using polyclonal rabbit anti-CNTF antibodies (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:15.000; Jackson Immunoresearch Laboratories, West Grove, PA). The clonal cell line with the highest expression level of CNTF was selected for further experiments, and the amount of CNTF secreted from this cell line at passage 11 and 19 was estimated in three independent Western blot analyses of culture supernatants using serial dilutions of recombinant mouse CNTF (Biomol, Hamburg, Germany) as a reference. Densitometric analysis of immunoreactive bands was performed using ImageJ software (NIH, Bethesda, MD).

In vitro differentiation of NS cells and immunocytochemistry

To analyze expression of CNTF in differentiated neural cell types *in vitro*, CNTF-NS and control-NS cells were differentiated into astrocytes by maintaining the cells for 7 days in NS cell medium supplemented with 1% fetal calf serum (Life Technologies) and 2% B27. To differentiate NS cells into neurons, cells were cultivated for three days in NS cell medium containing 5 ng/ml FGF-2, 1% N2 and 2% B27, followed by cultivation for additional four days in a 1:1 mixture of NS cell medium and Neurobasal medium (Life Technologies) supplemented with 0.25% N2 and 2% B27. Cells were fixed in 4% paraformaldehyde (PA) in phosphate buffered saline (PBS; pH 7.4), blocked in PBS containing 0.1% bovine serum albumin (BSA) and 0.3% Triton X-100 (both from Sigma-Aldrich), and simultaneously incubated with polyclonal rabbit anti-CNTF antibodies (1:100) and monoclonal mouse anti-glial fibrillary acidic protein (GFAP, 1:500; Sigma-Aldrich) or monoclonal mouse anti-microtubule associated protein 2 (MAP2, 1:200) antibodies (Sigma-Aldrich) overnight at room temperature. Cy2-, Cy3- or Cy5-conjugated secondary antibodies (all diluted 1:200; Jackson Immunoresearch Laboratories) were applied for 3 hours to detect primary antibodies, and cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

Intravitreal cell transplantations and immunohistochemistry

Nclf mice received intravitreal transplantations of NS cells at postnatal day 14. Animals were deeply anesthetized by an intraperitoneal injection of ketamine and xylazine, and 2 μl of vitreous fluid were slowly removed from the eyes using a glass micropipette that was inserted into the vitreous cavity at the junction between sclera and cornea. Subsequently, 7.6x10^5 CNTF-NS cells in 2 μl PBS were injected into one eye, and the same number of control-NS cells in the same volume of PBS into the contralateral eye. Care was taken to not damage the lens or the retina during the removal of the vitreous fluid or the injection of the cells. Eyes were analyzed two, four and six weeks after transplantation.

Eyes from untreated wild-type mice and *nclf* mice with grafted CNTF-NS or control-NS cells were immersion-fixed in 4% PA, cryoprotected in an ascending series of sucrose, frozen and serially sectioned with a cryostat at a thickness of 25 μm. For determination of photoreceptor numbers, central (i.e. in the plane of the optic disc) retina sections were incubated with polyclonal rabbit anti-recoverin antibodies (Millipore, Bedford, MA). To study expression of CNTF and differentiation of grafted cells, lenses with attached donor cells were simultaneously

incubated with polyclonal rabbit anti-CNTF (1:100) and either monoclonal mouse anti-GFAP (1:500), monoclonal mouse anti-ß-tubulin III (1:1000; Sigma-Aldrich), or monoclonal rat anti-myelin basic protein (MBP; 1:200; Millipore, Bedford, MA) antibodies. Proliferation of grafted NS cells was evaluated by incubating lenses with attached CNTF-NS or control-NS cells with polyclonal rabbit anti-Ki-67 antibodies (1:200; Abcam, Cambridge, MA) one week and six weeks after transplantation (n = 6 for each cell population and post-transplantation time point). At least 1,000 CNTF-NS or control-NS cells were analyzed for expression of Ki-67 at each post-transplantation interval, and the percentage of positive cells was calculated. Primary antibodies were detected with Cy2-, Cy3- or Cy5-conjugated secondary antibodies (all diluted 1:200; Jackson Immunoresearch Laboratories), and retinal sections and lenses with attached NS cells were stained with DAPI and analyzed with an Olympus FV 1000 confocal microscope (Olympus, Hamburg, Germany).

Photoreceptor counts and outer nuclear layer thickness

Entire central retinal sections stained with anti-recoverin antibodies and DAPI were photographed and the individual images were merged using Photoshop CS3 software (Adobe Systems Inc., San Jose, CA). Photoreceptors were counted in six defined areas (each covering the outer nuclear layer over a length of 220 µm) at positions corresponding to 25, 50 and 75% of the distance between the optic disc and the periphery of the temporal or nasal retina. Photoreceptor numbers were determined in *nclf* mice two, four and six weeks after transplantation (6 mice for each post-transplantation interval) and in one month old untreated wild-type mice (n = 6). Statistical analysis of data was performed with a mixed two-way ANOVA (having Time as between groups factor and Treatment as within groups factor) followed by Newman-Keuls post-hoc analyses.

The thickness of the outer nuclear layer (defined as DAPI-positive photoreceptor nuclei) was measured in central retinal sections at nine equidistant positions between the optic nerve head and the peripheral margin of the nasal and temporal retina, respectively. Statistical analysis of data was performed with the Student's t-test for paired samples.

Results

Generation of clonal CNTF-NS and control-NS cell lines

A previously established clonal NS cell line expressing CNTF together with a Venus reporter and a zeocin resistance gene (designated clone 1) [31] was again transduced with the polycistronic lentiviral vector pCAG-CNTF-IRES-Venus-2A-ZEO (Fig 1a) to generate a NS cell line with elevated expression levels of the cytokine. Cells with the highest expression levels of the Venus reporter gene were selected by FACS and clonally expanded. After five rounds of transductions and clonal expansions, semi-quantitative Western blot analyses of culture supernatants from the clonal cell lines revealed significantly increased levels of CNTF when compared to supernatants of the original CNTF-NS cell line, and one of these novel cell lines (designated clone 2) was selected for all further experiments (Fig 1c). Western blot analyses of culture supernatants from this cell line at passage 11 revealed secretion of about 83.7 ±6.8ng CNTF per 10^5 cells in 24 hours (mean ±SEM from three independent experiments). Similar amounts of CNTF were found in culture supernatants from passage 19, suggesting stable expression of the cytokine in this cell line. Clonal NS cell lines for control experiments were generated by transducing wild-type NS cells with the lentiviral vector pCAG-IRES-tdTomato/BSD (Fig 1b). Western blot analyses of these control-NS cell clones revealed no detectable amounts of CNTF in the culture supernatants (Fig 1c).

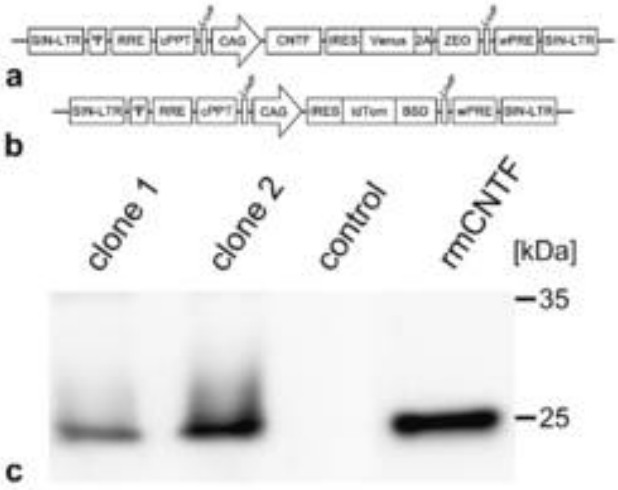

Fig 1. Lentiviral vectors and immunoblot analyses of culture supernatants from clonal CNTF-NS and control-NS cell lines. A lentiviral vector encoding a secretable variant of mouse ciliary neurotrophic factor (CNTF), an internal ribosome entry site (IRES) sequence of the encephalomyocarditis virus and a Venus reporter and a zeocin (ZEO) resistance gene separated by a P2A sequence of porcine teschovirus-1 (2A) under regulatory control of the cytomegalovirus enhancer/chicken β-actin (CAG) promoter (a) was used to generate CNTF-secreting NS cells. NS cells for control experiments were transduced with a vector containing the CAG promoter, an IRES sequence and a tdTomato (tdTom) reporter gene fused to a blasticidin (BSD) resistance gene (b). Immunoblot analysis (c) of culture supernatants from the newly established CNTF-NS cell clone (clone 2) revealed elevated secretion levels of CNTF when compared to the original clonal CNTF-NS cell line (clone 1). Supernatants from control-NS cell clones (control) lacked detectable levels of the cytokine (c). Recombinant mouse CNTF (rmCNTF) was loaded as a reference. Ψ, packaging signal; cPPT, central polypurine tract; LoxP, recognition site of Cre recombinase; RRE, rev-responsive element; SIN-LTR, self-inactivating long-terminal repeat; wPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

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Immunocytochemical analyses of undifferentiated NS cells revealed co-expression of CNTF (Fig 2b) and the fluorescent reporter protein Venus (Fig 2a) in all cells of the CNTF-NS cell line. Cells in the control-NS cell line, in comparison, expressed the reporter gene tdTomato (Fig 2c) but no detectable levels of the cytokine (Fig 2d). Moreover, CNTF-immunoreactivity was detectable at similar intensities in the CNTF-NS cell line for at least 23 passages (higher passages were not analyzed), in line with the data obtained by Western blot analyses.

To analyze transgene expression in neural cell types derived from CNTF-NS or control-NS cells *in vitro*, clonal cell lines were differentiated into nerve cells (Fig 3a–3f) or astrocytes (Fig 3g–3l) using directed differentiation protocols. Expression of CNTF in these cultures was analyzed seven days after induction of differentiation. While all MAP-2-positive neurons (Fig 3a–3c) and GFAP-positive astrocytes (Fig 3g–3i) derived from CNTF-NS cells co-expressed the reporter gene Venus and the cytokine, neurons (Fig 3d–3f) and astrocytes (Fig 3j–3l) derived from control-NS cells expressed the reporter gene tdTomato but no detectable levels of CNTF.

Analysis of intravitreally grafted CNTF-NS and control-NS cells *in vivo*

Six weeks after intravitreal transplantation into two weeks old *nclf* mice, grafted CNTF-NS and control-NS cells were identified in the recipient eyes by their expression of the fluorescent reporter proteins Venus (Fig 4a) and tdTomato (Fig 4d), respectively. Both CNTF-NS and control-NS cells had formed dense cell layers that were attached to the posterior poles of the lenses (Fig 4, S1 Fig) or to the vitreal surface of the host retinas (S1 Fig). Formation of tumors or

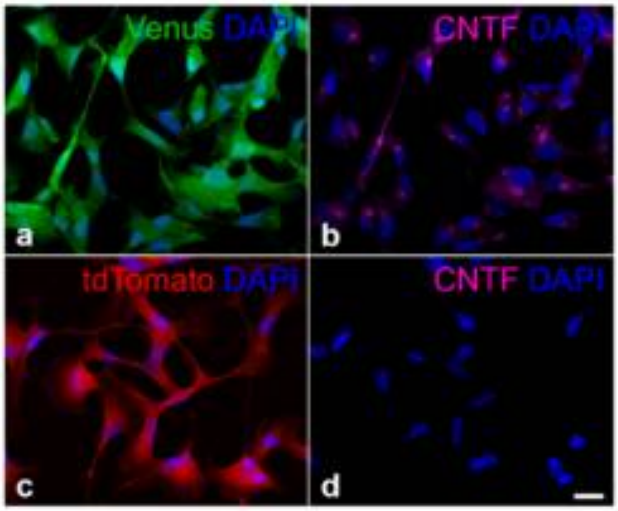

Fig 2. Expression of CNTF and the reporter genes in clonal CNTF-NS and control-NS cell lines. All cells in the clonal CNTF-NS cell line expressed the reporter gene Venus (a) and showed CNTF-immunoreactivity in the perinuclear region (b). Control-NS cells, in comparison, expressed the reporter gene tdTomato (c) but lacked detectable expression of the cytokine (d). CNTF, ciliary neurotrophic factor; DAPI, 4',6-diamidino-2-phenylindole. Bar in d (for a-d): 20 μm.

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integration of donor cells into the host retinas (S1 Fig) was not observed. Furthermore, we found that 2.1% ±0.36% (mean ± SEM; 6 eyes) of the grafted CNTF-NS cells and 1.8% ±0.38% (6 eyes) of the grafted control-NS cells expressed Ki-67 one week after the transplantation. In comparison, no Ki-67-positive donor cells were detectable six weeks after transplantation of CNTF-NS or control-NS cells (6 eyes for each cell population). The vast majority of the transplanted CNTF-NS and control-NS cells were identified as GFAP-positive astrocytes (Fig 4b and 4e). A few grafted NS cells were differentiated into ß-tubulin III-positive nerve cells, while differentiation of donor cells into myelin-basic protein-positive oligodendrocytes was not observed. Of note, immunocytochemical analyses revealed robust expression of CNTF in CNTF-NS cell-derived astrocytes for at least six weeks after transplantation (Fig 4c), the latest post-transplantation time point analyzed. Donor cells derived from control-NS cells, in comparison, lacked detectable expression of the cytokine (Fig 4f).

Intravitreally grafted CNTF-NS cells attenuate photoreceptor loss in the *nclf* mouse

CNTF-NS cells were intravitreally grafted into 14 days old *nclf* mice, and the neuroprotective effect of the modified cells on photoreceptors was analyzed two, four and six weeks after transplantation (Fig 5a, 5c and 5e). Intravitreal injections of control-NS cells into the contralateral eye of each animal served as a control (Fig 5b, 5d and 5f). Analyses of central (i.e. in the plane of the optic disc) retinal sections that were stained with anti-recoverin antibodies and DAPI consistently revealed a thicker outer nuclear layer of the CNTF-treated retinas when compared to the contralateral control retinas of the same animals at all post-transplantation time points (Fig 5; S2 and S3 Figs). Importantly, this neuroprotective effect on photoreceptor cells was not regionally restricted but evident in all regions of CNTF-treated retinas (S2 and S3 Figs). Adverse effects of the grafted CNTF-NS or control-NS cells on the general histology of the

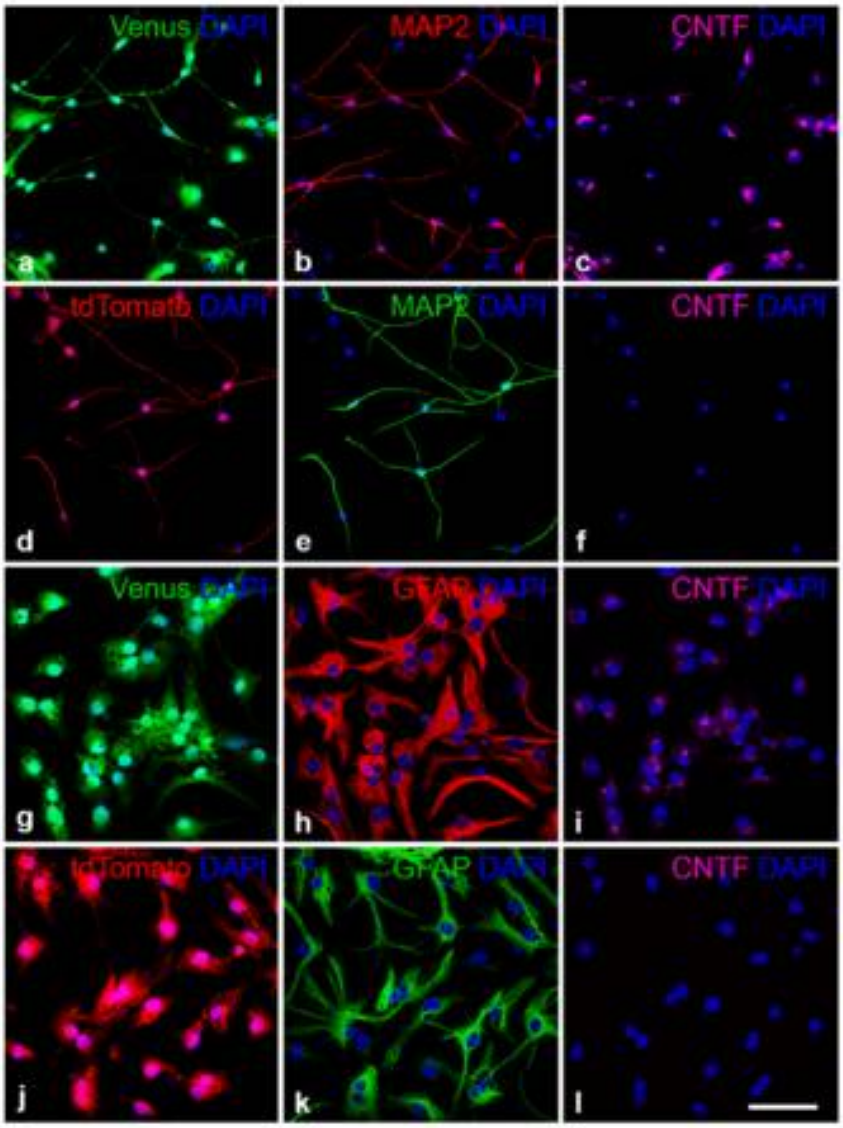

Fig 3. CNTF and reporter gene expression in neural cell types derived from CNTF-NS cells and control-NS cells *in vitro*. CNTF-NS (a-c, g-i) and control-NS cells (d-f, j-l) were differentiated into neurons (a-f) or astrocytes (g-l). Note that all MAP-2-positive neurons (b) and GFAP-positive astrocytes (h) derived from CNTF-NS cells co-expressed the reporter gene Venus (a, g) and CNTF (c, i). Neurons (e) and astrocytes (k) derived from control-NS cells, in comparison, expressed the reporter gene tdTomato (d, j) but no detectable levels of the cytokine (f, l). CNTF, ciliary neurotrophic factor; DAPI, 4',6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2. Bar in l (for a-l): 50 μm.

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recipient retinas were not observed (Fig 5; S1 and S2 Figs), with the exception of some small-sized retinal folds in a fraction of the CNTF-treated retinas.

Determination of photoreceptor numbers in six defined retinal areas located at positions that corresponded to 25%, 50% and 75% of the distance between the optic disc and the periphery of the nasal and temporal retina confirmed a significant protection of photoreceptor cells by the grafted CNTF-NS cells. Two weeks after transplantation, we counted 2,526.2 ±24.8

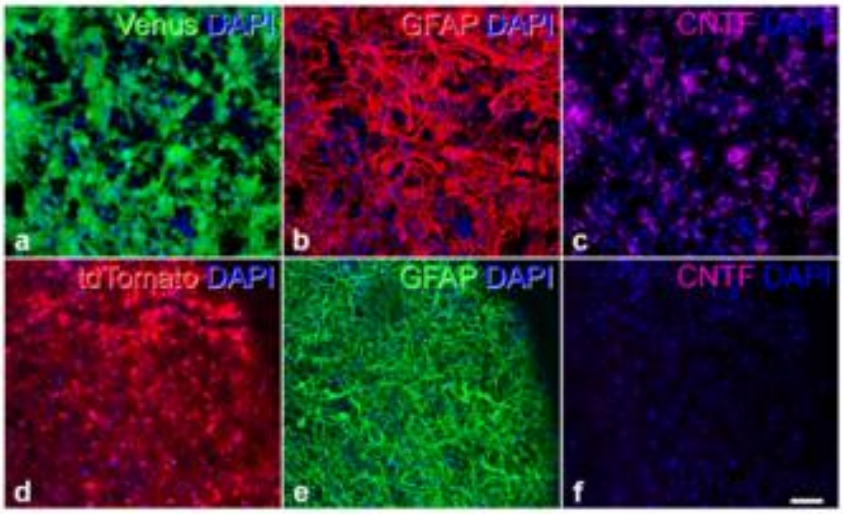

Fig 4. Characterization CNTF-NS and control-NS cells six weeks after intravitreal transplantation into *nclf* mice. Six weeks after intravitreal transplantation, CNTF-NS (a-c) and control-NS cells (d-f) were identified in the host eyes by their expression of the fluorescent reporter proteins Venus (a) and tdTomato (d), respectively. Both, Venus-positive CNTF-NS cells and tdTomato-positive control-NS cells were attached to the posterior poles of the lenses where they were mainly differentiated into GFAP-positive astrocytes (b, e). Expression of CNTF was detectable in astrocytes derived from CNTF-NS cells (c), but not in astrocytes derived from control-NS cells (f). CNTF, ciliary neurotrophic factor; DAPI, 4',6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein. Bar in f (for a-f): 50 μm.

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(mean ± SEM) photoreceptors in the eyes with grafted CNTF-NS cells, compared to 2,206.3 ±34.8 photoreceptors in the contralateral control eyes with grafted control-NS cells ($p<0.001$ according to the mixed two-way ANOVA test; Fig 6). At four and six weeks after transplantation, CNTF-treated retinas contained 2,150.5 ±45.9 and 1,921.0 ±29.8 photoreceptors, while the contralateral control retinas contained 1,740.2 ±49.5 and 1,570.0 ±29.8 photoreceptors, respectively ($p<0.001$ for both post-transplantation time points). CNTF-treated retinas thus contained 14.5%, 23.6% and 22.4% more photoreceptor cells than the control retinas two, four and six weeks after the transplantation, respectively (Fig 6). Untreated retinas from one month old wild-type mice (n = 6) were analyzed for comparison and contained 3,174.7 ±60.0 photoreceptor cells.

Discussion

The neuronal ceroid lipofuscinoses (NCLs) comprise a genetically heterogeneous group of neurodegenerative lysosomal storage disorders that are characterized by intracellular accumulation of autofluorescent material. Dementia, epilepsy and motor deterioration are among the characteristic symptoms of these fatal disorders. Visual impairment due to retinal degeneration is another typical symptom of most NCL forms [1, 3, 4, 6, 7]. For NCL forms caused by mutations in genes encoding soluble lysosomal enzymes, enzyme replacement therapy is among the strategies that are being explored as a potential treatment option. Therapeutic options for NCL forms caused by mutations in genes encoding transmembrane proteins, in comparison, are more limited [20–23, 32].

In the present study, we evaluated whether a sustained cell-based intraocular delivery of a neurotrophic factor ameliorates neurodegeneration in the retina of the *nclf* mouse, an animal model of CLN6 disease that harbors a frameshift mutation in a gene encoding a

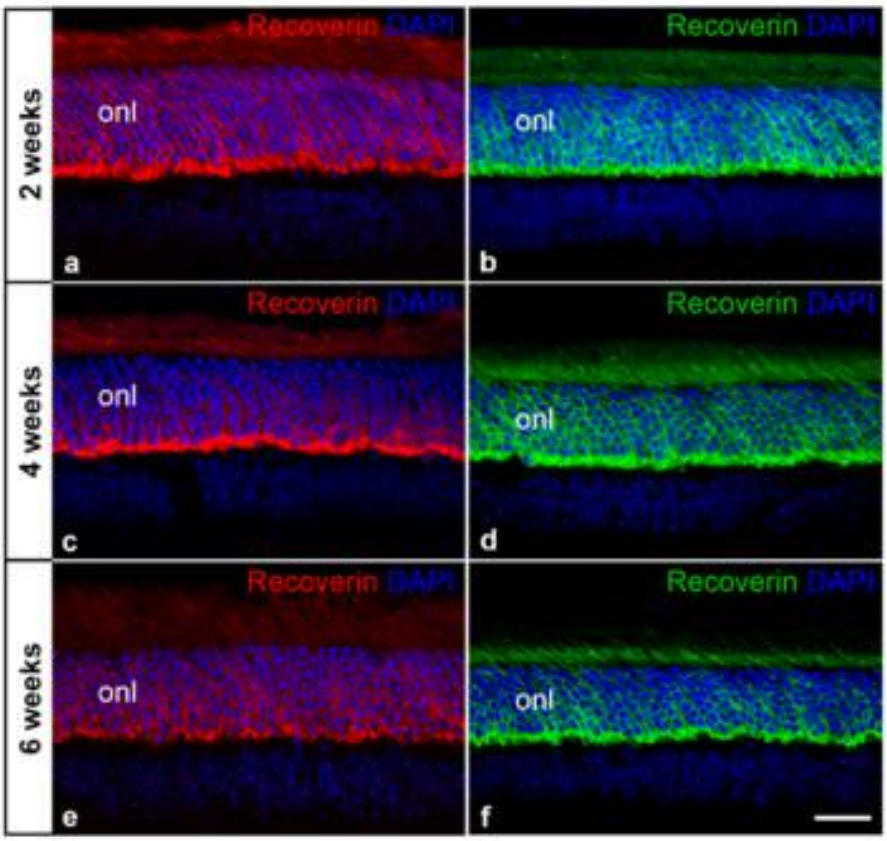

Fig 5. Intravitreally grafted CNTF-NS cells attenuate photoreceptor degeneration in *nclf* mice. A CNTF-NS cell clone was grafted into one (a, c, e) and a control-NS cell clone into the contralateral eye (b, d, f) of 14 days old *nclf* mice. Central retinal sections were stained with anti-recoverin antibodies and DAPI two (a, b), four (c, d) and six (e, f) weeks after transplantation. Note the thicker outer nuclear layer (onl) of CNTF-treated retinas when compared to control retinas at all post-transplantation time points. DAPI, 4',6-diamidino-2-phenylindole; onl, outer nuclear layer. Bar in f (for a-f): 50 μm.

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transmembrane protein [9, 10] of the endoplasmic reticulum (ER) [11]. To this aim, we generated a clonal NS cell line with high expression levels of a secretable variant of mouse CNTF and the fluorescent reporter protein Venus by repeated lentiviral transductions and subsequent clonal expansions. When these cells were grafted into the vitreous cavity of *nclf* mice, they rapidly stopped proliferating and preferentially differentiated into astrocytes that were attached to the posterior surface of the lenses and the vitreal surface of the retinas, similar to our observations in *Pde6b*^{rd1} or *Pde6b*^{rd10} mutant mice [31] and a mouse optic nerve crush model [33]. Integration of control-NS or CNTF-NS cells into the host retinas was not observed over the six weeks post-transplantation time period. The latter finding is in contrast to reports that have shown wide-spread integration of intravitreally grafted neural stem/progenitor cells into developing or dystrophic retinas [24, 34–37]. Intrinsic differences between the neural stem/progenitor cells used in these studies and adherently cultivated NS cells which represent a pure population of symmetrically dividing tripotent neural stem cells [38–40], or the time point of NS cell transplantation prior to the onset of retinal degeneration in the *nclf* mutant [17] may account for these apparently discrepant observations.

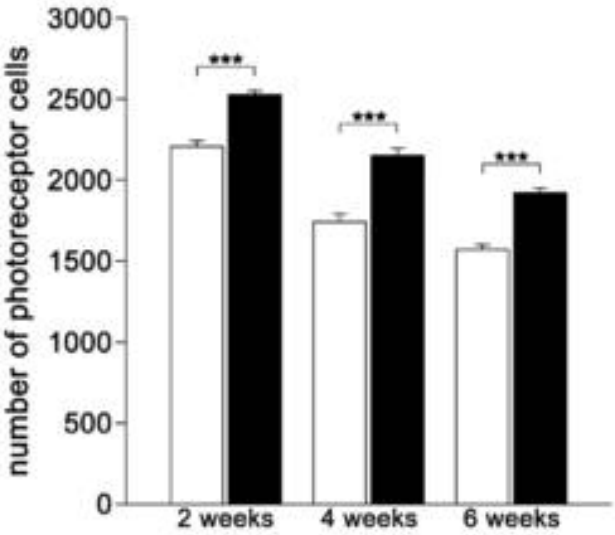

Fig 6. Photoreceptor numbers in eyes of *nclf* mice with grafted CNTF-NS or control-NS cells at different post-transplantation time points. A CNTF-NS and a control-NS cell line were intravitreally grafted into 14 days old *nclf* mice and photoreceptor numbers were determined in central retinal sections at six defined positions two, four and six weeks after transplantation. Note that CNTF-treated eyes contained significantly more photoreceptors (filled bars) than the contralateral eyes with grafted control-NS cells (open bars) at all post-transplantation time points. Each bar represents the mean value (±SEM) from six retinas. ***, $p<0.001$ (Newman-Keuls post hoc test after the mixed two-way ANOVA).

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Importantly, the NS cell-derived astrocytes survived in the vitreous cavity, stably expressed CNTF and the reporter gene, and significantly delayed the degeneration of photoreceptor cells over a time period of six weeks, the longest post-transplantation interval analyzed. These results are in line with our previous findings in a mouse optic nerve crush model where we observed survival of intravitreally grafted NS cells, stable expression of the cytokine in the donor cells and significant neuroprotective effects on axotomized retinal ganglion cells over a period of four months after cell transplantation [33]. Together, these data suggest that intravitreal transplantations of lentivirally modified NS cells may serve as a useful strategy for preclinical studies aimed at analyzing the long-term effects of cell-based neuroprotective approaches in mouse models of degenerative retinal disorders.

Of note, the protective effect of the CNTF-NS cells on photoreceptor cells in the *nclf* mouse was not regionally restricted but evident in all regions of the experimental retinas, as may be expected for a neuroprotective factor that is continuously released into the vitreous fluid from where it enters the dystrophic retina. Furthermore, we observed no adverse effects of the grafted control-NS cells on the general morphology of the host retinas. However, we found some small-sized retinal folds in restricted regions of a fraction of *nclf* retinas from eyes that had received CNTF-NS cell grafts. The formation of retinal folds has also been observed after repeated intravitreal injections of the CNTF analogue axokine in healthy or dystrophic retinas of cats [41] and in a mouse optic nerve crush model after intravitreal injections of CNTF-secreting neural stem cells [33]. Retinal folds thus appear to be among the complications associated with a sustained intraocular delivery of high doses of CNTF, such as a dysregulation of various genes including some encoding components of the phototransduction cascade and negative effects on visual function in a dose-dependent and reversible manner as measured by electroretinogram recordings [42–46].

Attenuation of photoreceptor degeneration after intraocular transplantations of cells genetically modified to secrete growth factors or cytokines that exert neuroprotective effects on photoreceptor cells, such as glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), neurotrophin-4 or CNTF, has also been observed in several other animal models of retinal degeneration, including the Royal College of Surgeon (RCS) rat [47, 48], the S334ter rat [49–51], a sodium iodate-induced mouse model of photoreceptor cell loss [52], the *rcd1* canine model [50] and the *Pde6b*^{rd1} and *Pde6b*^{rd10} mouse [31]. These reports together with the present study demonstrate that intraocular transplantations of genetically engineered cells represent a promising strategy to achieve a sustained administration of neuroprotective factors to the dystrophic retina. In fact, the therapeutic potential of a cell-based delivery of CNTF is currently being explored in human patients with retinitis pigmentosa or geographic atrophy [28] using intravitreal implants of a genetically modified and encapsulated human retinal pigment epithelial (RPE) cell line, indicating the potential relevance of cell-based neuroprotective approaches for clinical applications.

Dietary supplementation with the naturally occurring anti-inflammatory compounds docosahexaenoic acid (DHA) and curcumin has recently been evaluated as another strategy to slow down the progression of retinal degeneration in the *nclf* mouse [18]. DHA and curcumin have both been shown to reduce the production of nitric oxide and the expression of pro-inflammatory cytokines in microglial cells [53–56]. Analyses of retinas from DHA- and curcumin-treated animals revealed that the strong reactive microgliosis normally accompanying retinal degeneration in the *nclf* mutant was strongly diminished, as indicated by the significantly decreased numbers of amoeboid microglial cells. Interestingly, dietary supplementation with DHA and curcumin also delayed deterioration of visual function, while preservation of retinal structure was only observed in DHA-treated but not in curcumin-treated *nclf* mutants [18].

The motor neuron degeneration (*mnd*) mouse represents a naturally occurring animal model of CNL8 disease [57]. Similar to the *nclf* mouse, the *mnd* mouse harbors a mutation in a gene encoding a transmembrane protein of the ER [58, 59] and displays progressive apoptotic degeneration of photoreceptor cells [57, 60–62]. A recent study has analyzed the fate of intravitreally grafted neuralized mouse ES cells in this mutant, and observed wide-spread integration of the donor cells into the *mnd* retinas, where most of the cells acquired a neuronal phenotype. Furthermore and more interestingly, lysosomal storage bodies in *mnd* retinas were significantly reduced in size and fewer in number, and the loss of photoreceptor cells was significantly delayed in retinal regions with integrated donor cells when compared to regions of the same retinas that were devoid of donor cells or to sham-injected control retinas [24]. While the mechanisms by which the grafted cells exerted their neuroprotective effects in this mouse model of CNL8 disease remain to be elucidated, other studies have also observed amelioration of photoreceptor degeneration and partial preservation of visual function after intraocular transplantations of non-modified cell types, including such diverse cell types as neural progenitor cells [47, 63–65], bone marrow-derived stem cells [66–69] and Schwann cells [70]. In most studies, the neuroprotective effects have been attributed to the release of endogenously expressed growth factors and cytokines from the non-modified donor cells [47, 63, 68], or to the induction of neuroprotective factors in the recipient retinas by the grafted cells [65, 69]. Endogenous expression of neuroprotective factors known to rescue photoreceptor cells from degeneration, such as BDNF, FGF-2 or CNTF, in human neural progenitor cells and mesenchymal stem cells is in line with this view [47, 63, 68]. Although not specifically addressed in the present study, non-modified NS cells exerted no neuroprotective effects on photoreceptor cells in the *Pde6b*^{rd1} and *Pde6b*^{rd10} mouse [31], or on retinal ganglion cells in a mouse model of optic nerve injury [33]. To understand these apparently contradictory results, we analyzed non-modified NS cells for the expression of selected growth factors and cytokines by immunocytochemistry and Western blot

analyses of culture supernatants, and found no detectable expression levels of BDNF, GDNF or CNTF. Furthermore, the ability of a certain cell population to attenuate retinal degeneration may also depend on the specific pathomechanisms ultimately leading to the death of photoreceptor cells and may therefore differ in different animal models of retinal degeneration, as indicated by recent work on the RCS rat. Photoreceptor cells in this mutant die because phagocytosis of photoreceptor outer segments by RPE cells is impaired due to a mutation in the *Mertk* gene. Based on the observation that subretinally grafted human neural progenitor cells are capable to phagocytose shed outer segments in this rat mutant, it has been suggested that the non-modified neural progenitor cells confer their neuroprotective activity on photoreceptors in this animal model, at least in part, by functionally replacing the dysfunctional RPE [63, 71].

The therapeutic potential of a sustained cell-based administration of CNTF is currently being evaluated in patients with retinitis pigmentosa or geographic atrophy using intravitreal implants of an encapsulated human RPE cell line genetically modified to secrete this cytokine [72–76]. Analyses of the efficacy of this so-called encapsulated cell technology in mouse models of retinal degeneration are impeded by the large size of the encapsulated cell implants. The cell-based neuroprotective approach described in the present study mimics some aspects of the encapsulated cell technology, in that modified and intravitreally located cells provide a continuous supply of a neuroprotective factor to a dystrophic retina. We therefore suggest that this NS cell-based approach represents a useful methodology for preclinical studies aimed at analyzing the therapeutic potential of a cell-based intravitreal delivery of neurotrophic factors on retinal structure and function in mouse models of photoreceptor loss.

Supporting Information

S1 Fig. Localization of intravitreally grafted CNTF-NS cells in eyes of *nclf* mice. Analysis of eyes from *nclf* mice six weeks after intravitreal transplantation of CNTF-NS cells revealed the presence of Venus-positive donor cells (arrowheads in a) that were attached to the vitreal surface of the retinas. Integration of Venus-positive cells into the host retinas was not observed. Donor cells were also found on the posterior surfaces of the lenses (arrowheads in b). DAPI, 4',6-diamidino-2-phenylindole; gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer. Bar in f (for a-f): 50 μm.
(TIF)

S2 Fig. Morphology of host retinas after intravitreal transplantations of NS cells. CNTF-NS cells (a) and control-NS cells (b) were grafted into the vitreous cavity of 14 days old *nclf* mice, and retinas were analyzed six weeks after transplantation. Note the increased thickness of the outer nuclear layer (onl) in all regions of the CNTF-treated retina (a) when compared to the contralateral control retina (b). Adverse effects of the grafted cells on the general morphology of the host retinas were not detectable (a, b). Shown are overviews of the entire nasal half of a CNTF-treated and a contralateral control retina in central retinal sections. DAPI, 4',6-diamidino-2-phenylindole; ipl, inner plexiform layer; ON, optic nerve. Bar in b (for a and b): 200 μm.
(TIF)

S3 Fig. Thickness of the outer nuclear layer in CNTF-treated and control retinas. CNTF-NS cells were grafted into one and control NS-cells into the contralateral eye of 14 days old *nclf* mice, and the thickness of the outer nuclear layer was determined at 18 equally spaced positions between the peripheral margins of the nasal and temporal retina two (a), four (b) and six (c) weeks after transplantation. The outer nuclear layer was consistently thicker in CNTF-treated (red circles) when compared to control treated eyes (blue squares) at all post transplantation

time points. Each symbol represents the mean value (±SEM) from six retinas, *: $p<0.05$; **: $p<0.01$; ***$p<0.001$ according to the Student's t-test for paired samples. onh, optic nerve head. (TIF)

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Author Contributions

Conceived and designed the experiments: WJ UB. Performed the experiments: WJ KK KF CS. Analyzed the data: WJ UB. Contributed reagents/materials/analysis tools: KR TB. Wrote the paper: WJ GR KR TB UB.

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Investigative Ophthalmology & Visual Science

Glaucoma

Neural Stem Cell–Based Intraocular Administration of Ciliary Neurotrophic Factor Attenuates the Loss of Axotomized Ganglion Cells in Adult Mice

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Purpose. To analyze the neuroprotective effect of intravitreally grafted neural stem (NS) cells genetically modified to secrete ciliary neurotrophic factor (CNTF) on intraorbitally lesioned retinal ganglion cells (RGCs) in adult mice.

Methods. Adherently cultivated NS cells were genetically modified to express a secretable variant of mouse CNTF together with the fluorescent reporter protein Venus. Clonal CNTF-secreting NS cell lines were established using fluorescence activated cell sorting, and intravitreally grafted into adult mice 1 day after an intraorbital crush of the optic nerve. Ben-3a-positive RGCs were counted in flat-mounted retinas at different postlesion intervals to evaluate the neuroprotective effect of the CNTF-secreting NS cells on the axotomized RGCs. Anterograde axonal tracing experiments were performed to analyze the regrowth of the injured RGC axons in CNTF-treated retinas.

Results. Intravitreally grafted NS cells preferentially differentiated into astrocytes that survived in the host eyes, stably expressed CNTF, and significantly attenuated the loss of the axotomized RGCs over a period of at least 4 months, the latest postlesion time point analyzed. Depending on the postlesion interval analyzed, the number of RGCs in eyes with grafted CNTF-secreting NS cells was 2.8-fold to 6.4-fold higher than in eyes with grafted control NS cells. The CNTF-secreting NS cells additionally induced long-distance regrowth of the lesioned RGC axons.

Conclusions. Genetically modified clonal NS cell lines may serve as a useful tool for preclinical studies aimed at evaluating the therapeutic potential of a sustained cell-based intravitreal administration of neuroprotective factors in mouse models of glaucoma.

Keywords: axonal regeneration, ciliary neurotrophic factor, glaucoma, intraocular stem cell transplantation, lentiviral vector, neural stem cells, neuroprotection, optic nerve lesion, retinal ganglion cell

Glaucoma is among the leading causes of blindness, with an estimated 80 million people being affected worldwide in 2020.[1] It is a complex neurodegenerative disorder of the retina that is characterized by a progressive loss of retinal ganglion cells (RGCs) and their axons, resulting in visual field loss and eventually irreversible blindness.[2,3] The pathomechanisms leading to the apoptotic death of RGCs in this age-related and multifactorial disease are not fully understood. Clinically, an increased IOP is known to be a major risk factor for the development of glaucomatous optic neuropathy. Other factors that have been implicated in causing progressive degeneration of RGCs include neurotrophic factor deprivation, mitochondrial dysfunction, excitotoxic damage, vascular dysfunction, oxidative stress, inflammation, and glial cell activation.[2–5] Currently, lowering IOP is the only proven treatment for glaucoma. However, in a significant proportion of glaucoma patients, the disease progresses despite successful IOP reduction,[6,7] indicating the need for alternative treatments.

It has been proposed that impaired axonal transport as a result of elevated IOP leads to a scarcity of target-derived neurotrophic factors and subsequent apoptotic degeneration of RGCs.[8,9] Stimulation of prosurvival signaling pathways by the supplementation of neurotrophic factors has therefore been extensively explored as a strategy to protect RGCs from degeneration. These studies have identified a number of neurotrophic factors that are capable of delaying the degeneration of RGCs in various animal models of RGC loss.[2,10–13] For instance, intraocular administration of brain-derived neurotrophic factor (BDNF) or glial cell line–derived neurotrophic factor has been demonstrated to significantly delay the degeneration of RGCs in animal models of optic nerve injury[14–20] or ocular hypertension.[21–24]

Ciliary neurotrophic factor (CNTF), a member of the IL-6 family of cytokines,[25] is another neurotrophic factor that has been shown to potently rescue RGCs in various pathological conditions.[19,26–33] In addition to protecting RGCs from degeneration, the cytokine has been demonstrated to promote long-distance regrowth of injured RGC axons in the adult mammalian optic nerve.[20,34–38] However, single intravitreal injections of recombinant CNTF resulted in only limited neuroprotective and axon growth–promoting effects, due to the short half-life of the protein.[39] In comparison, more robust

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long-term effects were observed after sustained intraocular administration of the cytokine, which, in most studies, was achieved by virus-mediated gene transfer to the retina.[2,10,13]

Intraocular transplantations of genetically modified cells represent another strategy to continuously deliver neuroprotective factors to the retina.[40,41] Importantly, the use of ex vivo modified cells offers the possibility to adjust the amount of neurotrophic factors administered to the retina before the transplantation. Furthermore, in terms of potential clinical applications, the modified cells can be encapsulated into semipermeable polymer devices that allow diffusion of the neurotrophic factors from the implant to the retina, while protecting the transplanted cells from the immune system of the host and the host retina from potential adverse effects of the grafted cells. Moreover, the encapsulated cell implants can be retrieved from the vitreous in case of complications, adding another important safety aspect to this approach.[41–43] In fact, the therapeutic potential of a cell-based intraocular administration of a neurotrophic factor is currently being evaluated in patients with RP or geographic atrophy, using intravitreal implants of an encapsulated RPE cell line genetically modified to secrete CNTF.[44–48]

In the present study, we used a polycistronic lentiviral vector to generate clonal neural stem cell lines stably expressing a secretable variant of CNTF. The modified cells were grafted into the vitreous cavity of adult wild-type mice 1 day after an intraorbital optic nerve crush to evaluate the effects of a sustained cell-based intraocular administration of the cytokine on axonal regrowth and RGC survival in this animal model of injury-induced RGC loss.

Materials and Methods

Animals

Neural stem cells were isolated from the cerebral cortex of 14-day-old C57BL/6J mouse embryos. Intraorbital optic nerve lesions and intravitreal neural stem (NS) cell transplantations were performed on adult (i.e., at least 2 months old) C57BL/6J mice. Animals were obtained from the animal facility of the University Medical Center Hamburg-Eppendorf (Hamburg, Germany). All animal experiments were approved by the University and State of Hamburg Animal Care Committees and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Lentiviral Vectors, NS Cell Transductions, and Generation of Modified Clonal NS Cell Lines

The cDNA of mouse CNTF was ligated in frame with the Ig κ-chain leader sequence of pSecTag2 B (Life Technologies, Darmstadt, Germany) and cloned into the polycistronic lentiviral vector pCAG-IRES-Venus-2A-ZEO encoding the internal ribosome entry site from the encephalomyocarditis virus, a Venus reporter gene, a P2A sequence of porcine teschovirus-1, and a zeocin resistance gene under regulatory control of the cytomegalovirus enhancer/chicken β-actin promoter, giving rise to pCAG-CNTF-IRES-Venus-2A-ZEO.[49] Lentiviral particles were pseudotyped with the envelope G protein of the vesicular stomatitis virus and produced by transient transfection of HEK 293T cells as described elsewhere (http://www.LentiGo-Vectors.de).[50]

To generate clonal NS cell lines with high expression levels of transgenes, we again transduced previously established CNTF-secreting NS cell lines (CNTF-NS cells) and NS cell lines for control experiments (control-NS cells)[49] with pCAG-CNTF-IRES-Venus-2A-ZEO and pCAG-IRES-Venus-2A-ZEO, respectively. In brief, NS cells were seeded into 24-well plates coated with 0.1% Matrigel (BD Bioscience, Heidelberg, Germany) and cultivated in Dulbecco's modified Eagle's medium/F12 (Life Technologies) supplemented with 2 mM glutamine, 5 mM HEPES, 3 mM sodium bicarbonate, 0.3% glucose (all from Sigma-Aldrich Corp., St. Louis, MO, USA; in the following termed "NS cell medium"), 10 ng/mL epidermal growth factor (EGF) and 10 ng/mL FGF-2 (both from TEBU, Offenbach, Germany), and 1% N2 and 1% B27 (both from Life Technologies). Transduction of cells was performed by spinoculation in the presence of 8 μg/mL hexadimethrine bromide (Polybrene; Sigma-Aldrich). Positive cells were selected by further cultivating the cells under adherent conditions[49–51] in culture flasks coated with poly-L-ornithine (Sigma-Aldrich) and 0.1% Matrigel in the same medium but additionally containing 200 μg/mL zeocin (Invivogen, San Diego, CA, USA). Single CNTF-NS cells with the highest expression level of the reporter gene were then sorted into 96-well plates by fluorescence-activated cell sorting (FACS; FACSAriaIIIu, BD Bioscience, San Diego, CA, USA) to establish clonal CNTF-NS cell lines with elevated expression levels of the cytokine. CNTF levels in culture supernatants of the CNTF-NS cell clones were compared by Western blot analysis using polyclonal rabbit anti-CNTF antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and horseradish peroxidase–conjugated anti-rabbit secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA). The clonal cell lines with the highest expression levels of the cytokine were selected, and CNTF levels in the culture supernatants were estimated by Western blot analyses using recombinant mouse CNTF (Biomol, Hamburg, Germany) as a reference. Densitometric analysis of immunoreactive bands was performed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

In Vitro Differentiation of NS Cells and Immunocytochemistry

Differentiation of NS cells into astrocytes was induced by cultivating the cells in NS cell medium containing 1% fetal calf serum (Life Technologies) and 2% B27. Astrocytes were maintained for up to 2 months in culture. Neuronal differentiation of NS cells was induced by maintaining the cells for 3 days in NS cell medium supplemented with 5 ng/mL FGF-2, 1% N2, and 2% B27, followed by an additional cultivation period of 4 days in a 1:1 mixture of NS cell medium and Neurobasal medium (Life Technologies) supplemented with 0.25% N2 and 2% B27.

For immunocytochemical analyses of CNTF expression, CNTF-NS and control-NS cell cultures were fixed in 4% paraformaldehyde (PA; Carl Roth GmbH, Karlsruhe, Germany) in PBS (pH 7.4), blocked in PBS containing 0.1% BSA and 0.5% Triton X-100 (both from Sigma-Aldrich), and incubated with polyclonal rabbit anti-CNTF antibodies (Santa Cruz Biotechnology, Inc.). Cultures were simultaneously incubated with monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibodies (Sigma-Aldrich) or monoclonal mouse anti-microtubule associated protein 2 (MAP2) antibodies (Sigma-Aldrich) to identify astrocytes or neurons, respectively. Primary antibodies were detected with anti-rabbit Cy3- and anti-mouse Cy5-conjugated secondary antibodies (Jackson Immunoresearch Laboratories). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

Intraorbital Optic Nerve Lesions and Intravitreal NS Cell Transplantations

Animals were deeply anesthetized by an intraperitoneal injection of Ketanest S (Parke Davis GmbH, Berlin, Germany)

and Rompun (Bayer Vital GmbH, Leverkusen, Germany), and the optic nerve was intraorbitally crushed with watchmaker's forceps for 15 seconds at a distance of 0.5 to 1.0 mm from the eye.[52,53] Loss of the pupillary light reflex, the presence of well-preserved blood vessels, and lack of retinal bleeding were considered as criteria for a successful nerve crush. In a fraction of animals, RGC axons were anterogradely labeled (see below) to further control the nerve crush. One day after the crush, animals were again deeply anesthetized, and 2 µL vitreous fluid were removed from the eye with a fine glass micropipette that was inserted into the vitreous at the junction between sclera and cornea. Subsequently, 2 µL PBS containing either 7.6×10^5 CNTF-NS cells or control-NS cells were slowly injected into the vitreous cavity.[49,54] Some animals received intravitreal injections of 2 µL PBS without cells. Particular care was taken not to damage the lens during the removal of the vitreous fluid or the injection of the cells or the vehicle solution.

Characterization of Intravitreally Grafted NS Cells

Animals were killed 4 months after transplantation, and eyes were immersion-fixed for 1 hour in PBS containing 4% PA. Lenses with attached donor cells were removed and incubated with rabbit anti-CNTF antibodies, mouse anti-GFAP antibodies, rabbit anti-ß-tubulin III antibodies (Sigma-Aldrich), or rat anti-myelin basic protein (MBP) antibodies (Millipore, Bedford, MA, USA) to evaluate the expression of CNTF and to analyze the differentiation of the grafted NS cells. Primary antibodies were detected with anti-rabbit Cy3-, anti-rat Cy3-, or anti-mouse Cy5-conjugated secondary antibodies (Jackson Immunoresearch Laboratories). Lenses with attached donor cells were stained with DAPI, and confocal z-stacks of the posterior poles of the lenses with attached donor cells were prepared with an Olympus FV 1000 confocal microscope (Olympus, Hamburg, Germany) and processed using FV10-ASW software (Olympus). Confocal z-stacks of flat-mounted retinas that were prepared 4 months after cell transplantation and stained with anti-Brn-3a antibodies (see below) were taken with a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany), and processed using Imaris software (Bitplane, Zürich, Switzerland). Some eyes with grafted CNTF- or control-NS cells were immersion-fixed in 4% PA, cryoprotected in an ascending series of sucrose, frozen, serially sectioned at a thickness of 25 µm, and stained with DAPI.

Anterograde Axonal Tracing

To anterogradely label RGC axons, a saturated solution of biotin-N-hydroxysuccinimidester (Sigma-Aldrich) in dimethylformamide (Carl Roth GmbH) was diluted 1:1 with ethanol, and intravitreally injected into the eyes with crushed optic nerves.[53,55] After 24 hours, animals were killed and eyes with attached optic nerves were immersion-fixed in 4% PA, cryoprotected, and frozen. Longitudinal sections of optic nerves, 25 µm in thickness, were prepared with a cryostat and incubated with Cy3-conjugated streptavidin (Jackson Immunoresearch Laboratories). The distance between the distal margin of the lesion site and the tip of the longest regrown axon in mice with grafted control-NS or CNTF-NS cells ($n = 6$ for each experimental group) was determined 1 month after the crush. Analysis of sections was done with an Olympus IX51 fluorescence microscope (Olympus).

Analysis of RGC Survival

Animals were killed 1, 2, 3, or 4 months after the optic nerve crush, and eyes were fixed for 15 minutes in 4% PA. Retinas were flat-mounted on nitrocellulose membranes (Sartorius AG, Göttingen, Germany), fixed again in 4% PA for 1 hour, blocked in PBS containing 0.1% BSA and 1% Triton X-100, and incubated with polyclonal goat anti-Brn-3a antibodies (Santa Cruz Biotechnology, Inc.) overnight at room temperature. Subsequently, retinas were incubated with Cy3-conjugated secondary antibodies, stained with DAPI, and mounted onto slides. Retinas were number-coded, and five photomicrographs from the center to the periphery of the superior, inferior, nasal, and temporal retinal quadrant were taken, covering a total retinal area of approximately 1.9 mm^2. All Brn-3a–positive RGCs visible on these 20 photomicrographs were counted using Adobe Photoshop CS3 software (Adobe Systems, Inc., San Jose, CA, USA), and the number of RGCs per mm^2 was calculated. Six eyes with grafted CNTF-NS cells or control-NS cells were analyzed for each postlesion interval (i.e., 1, 2, 3, and 4 months after the optic nerve crush). Retinal ganglion cell densities were additionally determined in eyes with intravitreally injected PBS 1 month after the crush ($n = 6$), and in normal untreated eyes ($n = 6$). Statistical analysis of data was performed using the Student's *t*-test.

Results

Neural Stem Cells and Lentiviral Vectors

To express CNTF in adherently cultivated NS cells[51,56] from the embryonic mouse brain (in the following termed "CNTF-NS cells"), we generated a polycistronic lentiviral vector that is based on the lentiviral "gene ontology" (LeGO) vectors.[50,57] The vector encoded a secretable variant of mouse CNTF together with a Venus reporter gene and a zeocin resistance gene under regulatory control of the strong and ubiquitously active cytomegalovirus enhancer/chicken β-actin CAG promoter (pCAG-CNTF-IRES-Venus-2A-ZEO; Fig. 1Aa) to ensure robust transgene expression in undifferentiated NS cells and their differentiated progeny. Neural stem cells for control experiments (in the following termed "control-NS cells") were transduced with the same vector but lacking the CNTF cDNA (pCAG-IRES-Venus-2A-ZEO; Fig. 1Ab).[49]

Generation of Clonal NS Cell Lines With High Expression Levels of Transgenes

To further increase expression levels of transgenes in previously established clonal CNTF-NS and control-NS cell lines,[49] cells were again transduced with pCAG-CNTF-IRES-Venus-2A-ZEO and pCAG-IRES-Venus-2A-ZEO, respectively. Expansion of the transduced NS cells in the presence of zeocin gave rise to CNTF-NS and control-NS cell bulk cultures that were exclusively composed of Venus-positive cells. Single cells with the highest expression level of Venus in these bulk cultures were isolated using FACS and clonally expanded. After four rounds of transductions and clonal expansions, cell lines were analyzed by fluorescence microscopy, immunocytochemistry, and Western blot. Analyses confirmed elevated expression levels of CNTF and Venus or the reporter gene in the CNTF-NS or control-NS cell lines, respectively, when compared with the original clonal cell lines. The clones with the highest expression levels of transgenes were selected for further experiments. Immunocytochemical analyses revealed that all cells in the CNTF-NS cell line coexpressed Venus and CNTF (Figs. 1Ba, 1Bb). Control-NS cells were also Venus-positive, but lacked detectable levels of CNTF immunoreactivity (Figs. 1Bc, 1Bd). Immunoblot analyses demonstrated secretion of CNTF into the culture supernatant of the CNTF-NS cell lines, whereas supernatants of control-NS cell clones lacked detectable levels of the cytokine (Fig. 1C). Expression of CNTF in the clonal CNTF-NS cell line was

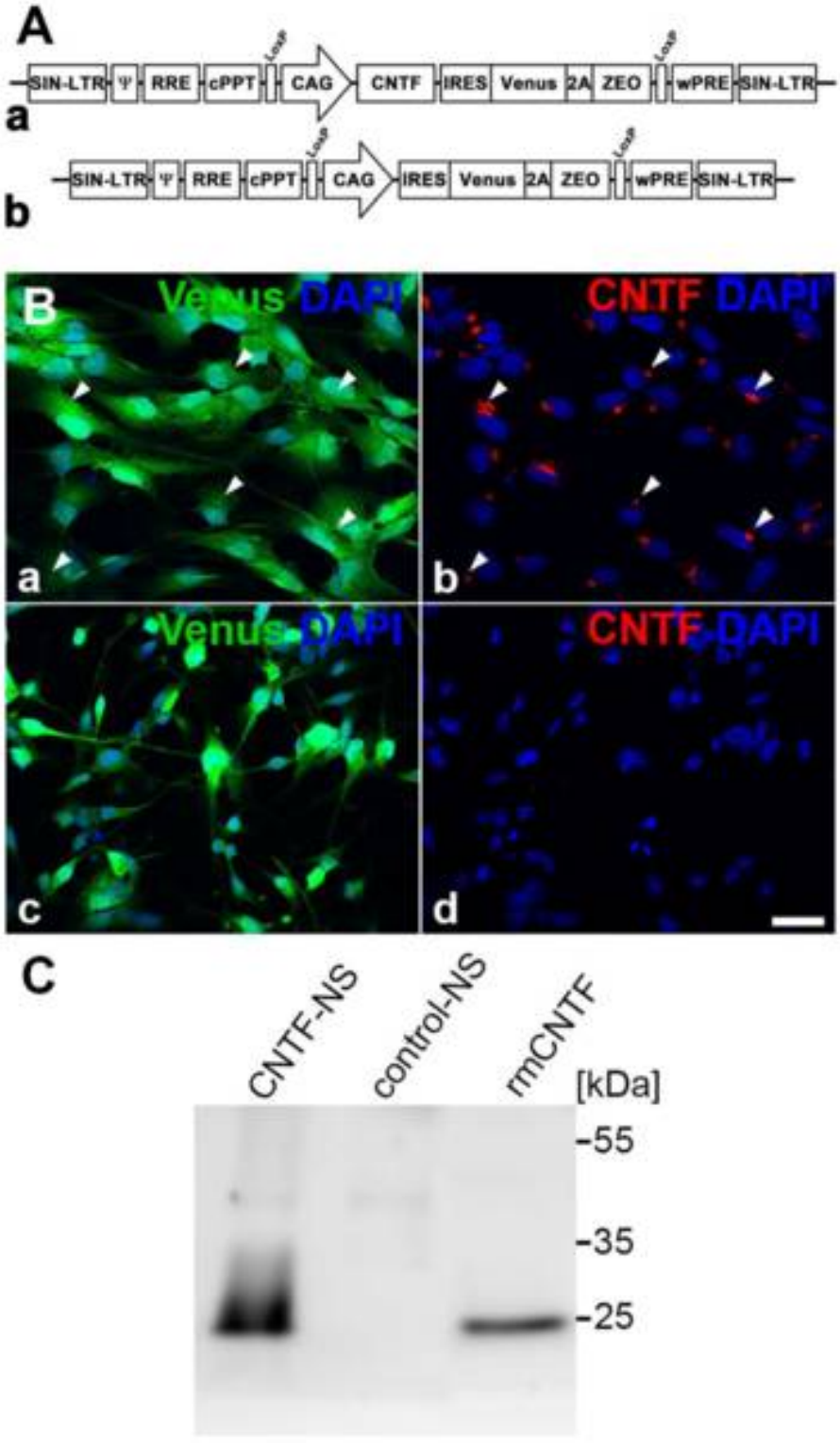

Figure 1. Lentiviral vectors and expression of CNTF and Venus in genetically modified clonal NS cell lines. (**A**) Neural stem cells were transduced with a polycistronic lentiviral vector encoding a secretable variant of mouse CNTF under regulatory control of the human CMV enhancer/chicken β-actin (CAG) promoter. The vector additionally encoded a Venus reporter gene and a zeocin (ZEO) resistance gene separated from each other by a P2A sequence (2A) of porcine teschovirus-1 (**Aa**). The same vector but lacking the CNTF cDNA was used to transduce NS cells for control experiments (**Ab**). (**B**) Clonally derived CNTF-NS (**Ba**, **Bb**) and control-NS cell lines (**Bc**, **Bd**) were generated using FACS and immunostained with

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anti-CNTF antibodies. Whereas all cells in the CNTF-NS (**Ba**) and control-NS cell lines (**Bc**) expressed Venus, expression of CNTF was detectable only in the CNTF-NS cell clone (**Bb**; some cells labeled with *arrowheads* in **Ba** and **Bb**), but not in the control-NS cell clone (**Bd**). (**C**) Western blot analyses revealed secretion of CNTF into the supernatant of CNTF-NS cell cultures (CNTF-NS), whereas culture supernatants from control-NS cells (control-NS) lacked detectable levels of the cytokine. Ψ, packaging signal; cPPT, central polypurine tract; IRES, internal ribosome entry site; LoxP, recognition site of Cre recombinase; rmCNTF, recombinant mouse ciliary neurotrophic factor; RRE, rev-responsive element; SIN-LTR, self-inactivating long-terminal repeat; wPRE, Woodchuck hepatitis virus posttranscriptional regulatory element. *Scale bar* in (**Bd**) for (**Ba**–**Bd**): 25 μm.

detectable by immunocytochemical and immunoblot analyses for more than 30 passages, corresponding to a culture period of approximately 6 months. Furthermore, immunoblot analyses ($n = 3$) of culture supernatants from this cell line revealed secretion of 87.2 ± 10.1 ng (mean ± SEM) CNTF per 10^5 cells in 24 hours at passage 15. Culture supernatants from passage 34 contained similar quantities of CNTF, indicating stable expression of the cytokine in this clonal cell line.

To analyze expression of CNTF in differentiated neural cell types in vitro, we next differentiated the CNTF-NS and control-NS cell lines into neurons or astrocytes (Fig. 2). Whereas all MAP2-positive neurons derived from CNTF-NS and control-NS cells were positive for Venus, expression of CNTF was detectable only in neurons derived from CNTF-NS cells, but not in neurons derived from control-NS cells (Figs. 2A–F). Similarly, GFAP-positive astrocytes from both CNTF-NS and control-NS cells were positive for Venus, whereas expression of CNTF was detectable only in astrocytes derived from CNTF-NS cells, but not in astrocytes derived from control-NS cells (Figs. 2G–L). Of note, expression of CNTF in cultured astrocytes derived from the CNTF-NS cell line remained detectable for at least 2 months (Fig. 2I), the longest cultivation period analyzed.

Intravitreal Transplantations of CNTF-NS and Control-NS Cells

Intravitreally grafted CNTF-NS (Figs. 3A–D; Supplementary Fig. S1) and control-NS cells (Figs. 3E–H) were identified in the host

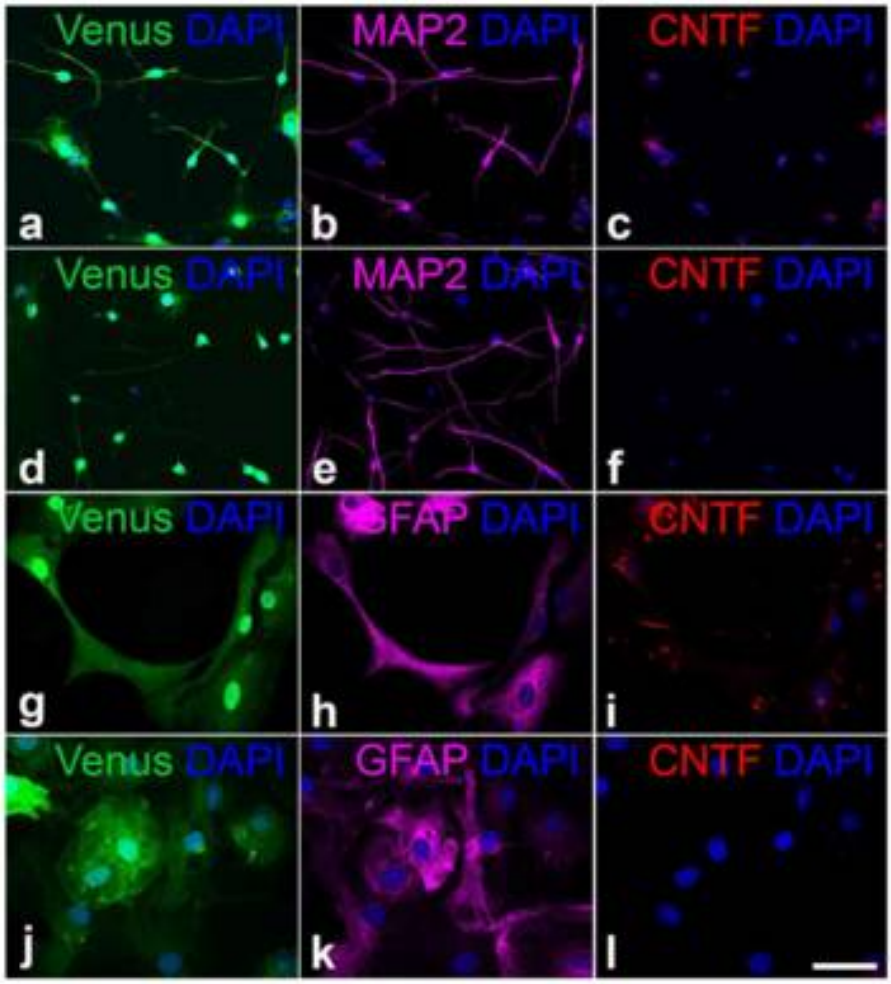

Figure 2. Expression of CNTF and Venus in differentiated clonal NS cell lines. CNTF-NS (**a**–**c**, **g**–**i**) and control-NS cell clones (**d**–**f**, **j**–**l**) were differentiated into MAP2-positive neurons (**a**–**f**) or GFAP-positive astrocytes (**g**–**l**). The reporter gene Venus was expressed in all neurons (**a**, **d**) and astrocytes (**g**, **j**) derived from the CNTF-NS (**a**, **g**) and control-NS cell lines (**d**, **j**). CNTF was detectable only in neurons (**c**) and astrocytes (**i**) derived from CNTF-NS cells, but not in neurons (**f**) and astrocytes (**l**) derived from control-NS cells. Neurons and astrocytes were analyzed for expression of Venus and CNTF 1 week and 2 months after induction of differentiation, respectively. *Scale bar* in (**l**) (for **a**–**l**): 50 μm.

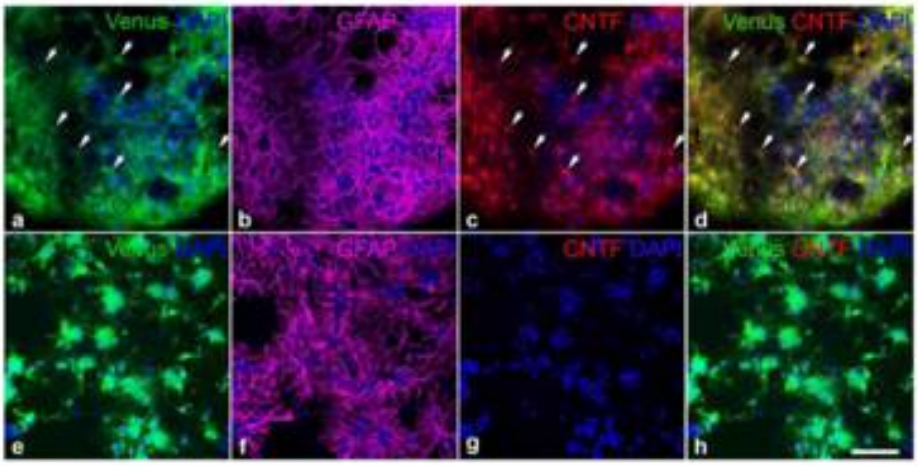

FIGURE 3. Neural differentiation and expression of CNTF in intravitreally grafted NS cell lines. Analyses of eyes 4 months after intravitreal transplantations of CNTF-NS (**a**–**d**) and control-NS cell lines (**e**–**h**) revealed the presence of Venus-positive donor cells (**a**, **e**) that were attached to the posterior poles of the lenses. Virtually all CNTF-NS cells (**b**) and control-NS cells (**f**) were differentiated into GFAP-positive astrocytes. Expression of CNTF was detectable in cells derived from CNTF-NS cells (**c**), but not in cells derived from control-NS cells (**g**). (**d**) and (**h**) are merged images of (**a**) and (**c**), and (**e**) and (**g**), respectively. Some donor cells coexpressing Venus and CNTF are marked with *solidus arrows* in (**a**), (**c**) and (**d**). *Scale bar* in (**h**) (for **a**–**h**): 100 μm.

eyes by their expression of the reporter gene Venus (Figs. 3A, 3E; Supplementary Figs. S1A–S1E). Of note, both cell populations survived in the vitreous cavity of the recipient eyes for at least 4 months, the longest posttransplantation interval investigated. Both, CNTF-NS and control-NS cells had formed layers of Venus-positive cells that were either attached to the posterior pole of the lenses (Fig. 3; Supplementary Fig. S1A) or to the vitreal surface of the retinas (Supplementary Figs. S1B–E). Evidence for the formation of tumors by the grafted cells, or for integration of Venus-positive donor cells into the host retinas (Supplementary Figs. S1B, S1D, S1E) was not observed. However, we found some small-sized retinal folds in locally restricted regions of the flat-mounted host retinas. These retinal folds were observed in a fraction of animals with grafted CNTF-NS cells, but not in animals with grafted control-NS cells.

Immunostainings of donor cells that were attached to the posterior poles of the lenses revealed that most of the CNTF-NS and control-NS cells were differentiated into GFAP-positive astrocytes (Figs. 3B, 3F). A few CNTF-NS and control-NS cells were differentiated into ß tubulin-III–positive nerve cells (data not shown). Differentiation of grafted cells into MBP-positive oligodendrocytes was not observed. Importantly, robust expression of CNTF was detectable in astrocytes derived from the grafted CNTF-NS cell clone for at least 4 months after transplantation, the latest posttransplantation time point investigated (Figs. 3C, 3D). Astrocytes derived from the control-NS cell clone, in contrast, lacked detectable expression of the cytokine (Figs. 3G, 3H).

Intravitreally Grafted CNTF-NS Cells Attenuate the Degeneration of Axotomized RGCs

The numbers of RGCs in untreated animals and animals that had received an intraorbital crush of the optic nerve were determined in flat-mounted retinas that were stained with antibodies to Brn-3a, a reliable marker for RGCs.[38] Quantitative analysis of retinas from untreated animals ($n = 6$) revealed the presence of 3993.8 ± 54.4 (mean ± SEM) RGCs/mm^2. In animals that received an intraorbital crush of the optic nerve and intravitreal transplantation of control-NS cells, RGC numbers decreased to 189.8 ± 13.5 RGCs/mm^2, 97.0 ± 12.8 RGCs/mm^2, 50.2 ± 4.2 RGCs/mm^2, and 62.7 ± 5.0 RGCs/mm^2 at 1, 2, 3, and 4 months after the lesion, respectively ($n = 6$ for each postlesion interval; Figs. 4E–H, Fig. 5). In animals with intravitreally grafted CNTF-NS cells, in comparison, we detected 529.0 ± 20.9 RGCs/mm^2 1 month after the lesion, 429.8 ± 30.7 RGCs/mm^2 2 months after the lesion, 320.5 ± 11.5 RGCs/mm^2 3 months after the lesion, and 302.7 ± 4.3 RGCs/mm^2 4 months after the lesion ($n = 6$ for each postlesion interval; Figs. 4A–D, Fig. 5). CNTF-treated retinas thus contained 2.8-, 4.4-, 6.4-, and 4.8-fold more surviving RGCs than control retinas at the 1-, 2-, 3-, and 4-month postlesion interval, respectively. This difference between RGC numbers in CNTF-treated and control retinas was statistically significant at all postlesion time points analyzed ($P < 0.001$ according to the Student's *t*-test; Fig. 5). Anterograde axonal tracing experiments performed in a fraction of animals from the different experimental groups at the different postlesion intervals confirmed complete transections of RGC axons in all lesioned nerves analyzed.

To evaluate whether control-NS cells also exerted neuroprotective effects on axotomized RGCs, we additionally compared the number of RGCs between eyes that had received intravitreal grafts of control-NS cells and eyes that had received intravitreal injections of the vehicle solution only. One month after an optic nerve crush, eyes with grafted control-NS cells contained 189.8 ± 13.5 RGCs/mm^2, a value not significantly different from that obtained for retinas that received injections of the vehicle (156.2 ± 23.0 RGCs/mm^2; $n = 6$ for each experimental group).

Regrowth of Axotomized RGC Axons in CNTF-Treated Retinas

Anterograde axonal tracing experiments were performed to analyze whether the sustained cell-based intraocular administration of CNTF had stimulated regrowth of the injured RGC axons. To estimate the extent of axonal regrowth, we determined the distance between the distal margin of the lesion site and the tip of the longest regrown axon in longitudinally sectioned optic nerves from animals with grafted

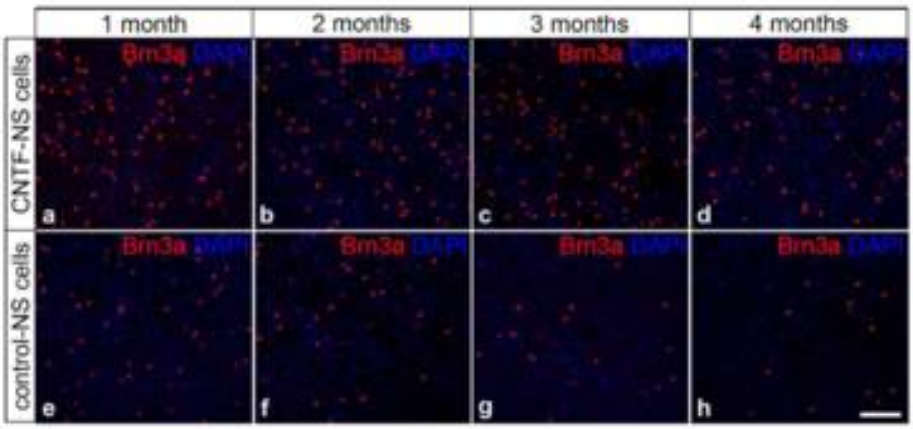

FIGURE 4. Intravitreally grafted CNTF-NS cells attenuate degeneration of axotomized retinal ganglion cells in adult mice. Adult mice received intravitreal injections of a CNTF-NS cell clone (**a**–**d**) or a control-NS cell clone (**e**–**h**) 1 day after an intraorbital optic nerve crush. Analysis of flat-mounted retinas 1 (**a**, **e**), 2 (**b**, **f**), 3 (**c**, **g**), and 4 months (**d**, **h**) after the lesion revealed the presence of significantly more Brn-3a–positive ganglion cells in eyes with grafted CNTF-NS cells (**a**–**d**) than in eyes with grafted control-NS cells (**e**–**h**) at all postlesion intervals. *Scale bar* in (**h**) (for **a**–**h**): 100 μm.

control-NS cells and grafted CNTF-NS cells ($n = 6$ for each experimental group) 1 month after an intraorbital nerve crush (Fig. 6). RGC axons in control animals extended for only short distances (459.3 ± 47.0 μm (mean ± SEM); Figs. 6A, 6B, 6E) across the lesion site into the distal optic nerve stump. In CNTF-treated animals, in comparison, axotomized RGC axons regrew for up to 2800 μm across the lesion site into the distal optic nerve stump (2302.7 ± 162.8 μm; Figs. 6C, 6D, 6E). Axons in the distal optic nerve stump of CNTF-treated animals followed an irregular course (Figs. 6C, 6D), indicating that they corresponded to regrown axons and not to axons that had escaped the nerve crush.

Discussion

Cell-replacement strategies are among the approaches that are currently being explored to develop therapies for yet untreatable degenerative retinal disorders. Recent studies have indeed demonstrated the feasibility to replace dysfunctional or degenerated photoreceptor cells or RPE cells by cell transplantation.[59–62] However, for retinal disorders characterized by the loss of RGCs, cell-replacement strategies are complicated by the fact that the transplanted cells not only have to integrate as functional RGCs into the host retinas, but additionally have to grow their axons over long distances to project in a topographically appropriate manner to the visual centers of the brain. Current therapeutic strategies for glaucomatous or nonglaucomatous optic neuropathies are therefore primarily aimed at delaying the loss of endogenous RGCs rather than at replacing degenerated RGCs.[2,3,5,10,13]

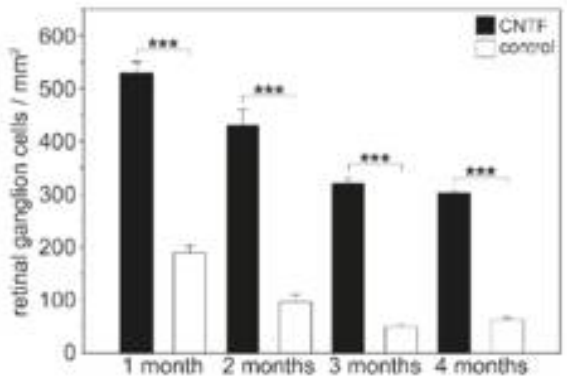

FIGURE 5. Quantitative analysis of the neuroprotective effect of intravitreally grafted CNTF-NS cells on axotomized RGCs. The number of Brn-3a–positive RGCs was determined in eyes with intravitreally grafted CNTF-NS cells (*filled bars*) or control-NS cells (*open bars*) 1, 2, 3, and 4 months after an intraorbital optic nerve crush. Note that the CNTF-treated retinas contained significantly more RGCs than the control retinas at all postlesion intervals. Each *bar* represents the mean number (±SEM) of RGCs per mm² from six retinas. ***$P < 0.001$ according to the Student's *t*-test.

Neurotrophic factor deprivation due to impaired axonal transport has been suggested to contribute to the loss of RGCs in glaucomatous optic neuropathies, and neurotrophic factor supplementation has therefore been extensively studied as a potential treatment option for glaucoma. Because neurotrophic factors usually have short half-life times and do not ordinarily cross the blood-retina barrier, robust and long-lasting neuroprotective effects likely depend on a sustained intraocular delivery of these factors. A sustained intraocular supply of neurotrophic factors has been achieved by intravitreal implantations of slow-release devices and viral or nonviral gene transfer to retina cells.[2,10,11,13] Transplantations of cells that have been genetically modified to overexpress neurotrophic factors represent another strategy to continuously deliver these factors to glaucomatous retinas that has been successfully used in preclinical studies. For instance, intravitreal implants of encapsulated cells engineered to secrete glucagon-like peptide-1 have recently been reported to delay degeneration of RGCs in a rat model of optic nerve crush.[63] Moreover, intravitreal injections of mesenchymal stem cells lentivirally modified to secrete BDNF resulted in significant attenuation of RGC loss in a rat model of ocular hypertension.[64] Furthermore, simultaneous intraocular administration of neurotrophin-3, FGF-2, and BDNF through intravitreal transplantations of transfected fibroblasts has been shown to synergistically promote survival and axonal regrowth of axotomized RGCs in adult rats.[65]

In the present study, we explored the use of neural stem cells from the embryonic mouse brain as cellular vectors to continuously deliver neurotrophic factors to adult mouse retinas with a lesion-induced degeneration of RGCs. When

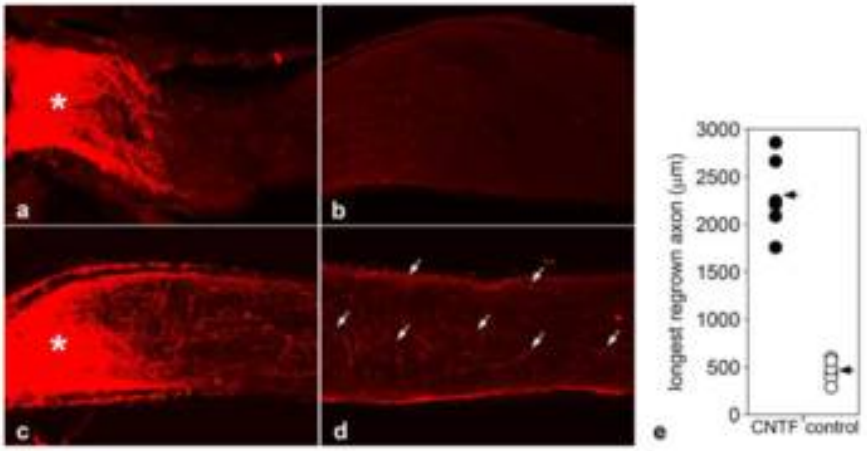

Figure 6. Long-distance regrowth of axotomized RGC axons in CNTF-treated retinas. Retinal ganglion cell axons in eyes with intravitreally grafted control-NS cells (**a**, **b**) or CNTF-NS cells (**c**, **d**) were anterogradely labeled 1 month after an intraorbital optic nerve crush. Retinal ganglion cell axons in control animals extended only a short distance across the lesion site (*asterisk* in [**a**]) into the distal optic nerve stump (**a**), and no regrown axons were present 1 mm distal to the lesion (**b**). In CNTF-treated retinas, in comparison, numerous axons were grown across the lesion site (*asterisk* in [**c**]) into the distal nerve stump, and some were detectable 1 mm distal to the lesion (*arrows* in [**d**]). Note the irregular trajectory of the labeled axons (**c**, **d**). The length of the longest regrown axon in animals with grafted control-NS cells was 459.3 ± 47.0 µm (mean ± SEM), compared with 2502.7 ± 162.8 µm in animals with grafted CNTF-NS cells ($n = 6$ for each experimental group; [**e**]). *Arrows* in (**e**) indicate mean values.

these cells are cultivated under adherent conditions in the presence of EGF and FGF-2, they give rise to cultures consisting of homogeneous populations of symmetrically dividing clonogenic stem cells which, in analogy to embryonic stem cells, have been termed NS cells.[51,56] To evaluate the efficacy of an NS cell–based neuroprotective approach in an animal model of optic nerve injury, we took advantage of the potent rescue effects of CNTF on RGCs in various pathological conditions,[19,20,33] and expressed the cytokine in NS cells using polycistronic lentiviral vectors. In a recent study, we analyzed the neuroprotective potential of CNTF-NS cells in *Pde6b*^{rd1} and *Pde6b*^{rd10} mutant mice, two animal models of retinitis pigmentosa characterized by an early onset and rapid degeneration of photoreceptor cells.[49] We found that intravitreal transplantations of CNTF-NS cells resulted in significant attenuation of photoreceptor degeneration in both mouse mutants, in line with reports that have demonstrated protective effects of the cytokine on photoreceptor cells in a variety of animal models of inherited or acquired retinal degeneration.[43,66] However, retinas of *Pde6b*^{rd1} and *Pde6b*^{rd10} mice were analyzed already 18 and 16 days after transplantation of the CNTF-NS cells, respectively.[49] One major interest of the present study was therefore to evaluate the survival, transgene expression, and neuroprotective effects of the CNTF-NS cells on axotomized RGCs in long-term experiments.

To this aim, we first increased the expression level of CNTF in a previously established clonal CNTF-NS line[49] by repeated transductions and subsequent clonal expansions of NS cells with the strongest expression of the reporter gene. After several rounds of transductions and clonal expansions, a clonal cell line with high expression levels of CNTF was selected for all further experiments. When this cell line was differentiated into astrocytes in vitro, the glial cells stably expressed the reporter gene and the cytokine for at least 2 months, the longest cultivation period evaluated. More importantly, grafted NS cells survived for at least 4 months in the vitreous cavity of the recipient eyes. Here, the Venus-positive donor cells formed dense cell layers that were attached to the posterior pole of the lenses or the vitreal surface of the retinas. Integration of donor cells into the host retinas was not observed. In addition, there was no evidence for ongoing proliferation of the grafted cells, in line with our previous findings that the proliferation marker Ki-67 was expressed in only less than 4% and 2% of the donor cells 8 and 16 days after intravitreal transplantations of NS cells into *Pde6b*^{rd1} and *Pde6b*^{rd10} mice, respectively.[49] Furthermore, we found some small-sized and locally restricted retinal folds in a fraction of eyes with grafted CNTF-NS cells. Retinal folds also have been described in dystrophic and normal retinas of cats that had received repeated intravitreal injections of the human CNTF analogue axokine.[67] Formation of retinal folds thus appears to be among the complications associated with the intraocular delivery of CNTF,[67–71] provided the cytokine is administered in high amounts and over an extended period of time.

The vast majority of grafted CNTF-NS cells were differentiated into astrocytes that still expressed CNTF 4 months after transplantation. Astrocytes derived from control-NS cells, in contrast, lacked detectable expression levels of the cytokine. Of note, sustained expression of the cytokine in eyes with CNTF-NS cell–derived astrocytes correlated with a significant attenuation of RGC loss, as demonstrated by the presence of 2.8-, 4.4-, 6.4-, and 4.8-fold more surviving RGCs in CNTF-treated retinas than in control retinas at the 1-, 2-, 3-, and 4-month postlesion intervals, respectively. Although protection of axotomized RGCs by the CNTF-NS cell grafts was transient and degeneration of RGCs progressed also in CNTF-treated eyes, we consider it remarkable that the NS cell–based intraocular delivery of CNTF attenuated RGC loss over an extended period. Given that intraorbital optic nerve lesions induce a rapid apoptotic degeneration of RGCs,[72] it will be interesting to evaluate the neuroprotective potential of the clonal CNTF-NS cell line in animal models of ocular hypertension that more closely mimic the slowly progressing RGC loss in human glaucoma patients.[73–75]

In addition to protecting RGCs from degeneration, CNTF has been shown to promote regrowth of injured RGC axons.[26,35,37,38] In line with these studies, we observed long-distance regeneration of lesioned RGC axons in eyes with grafted CNTF-NS cells. One month after the crush, some axons in CNTF-treated eyes were regrown for more than 2 mm into the distal nerve stump, whereas axons in eyes with grafted control-NS cells extended for only approximately 0.5 mm into

the distal nerve stump. These data further confirm sustained delivery of functionally relevant quantities of the cytokine from the intravitreally located CNTF-secreting donor cells to the adult murine retina.

Recent transplantation studies have identified a variety of cell types that exert neuroprotective effects on RGCs without prior genetic modification, including Schwann cells, olfactory ensheathing cells, oligodendrocyte precursor cells, and mesenchymal stem cells.[40,74] Although the precise mechanisms by which these cell types rescued RGCs from degeneration are largely unknown, the neuroprotective effects have usually been attributed to the secretion of endogenously expressed neurotrophic factors and/or to immune modulatory effects of the grafted cell types. To analyze whether nonmodified NS cells also exert neuroprotective effects on RGCs, we determined the number of RGCs in animals that had received intravitreal injections of control-NS cells or the vehicle only, and found similar numbers of surviving RGCs in both experimental groups 1 month after the nerve crush.

In summary, we have shown that an intravitreally grafted CNTF-secreting clonal NS cell line survived in the vitreous cavity of the host eyes and significantly attenuated a lesion-induced degeneration of RGCs over a period of 4 months, the longest posttransplantation time period analyzed. Furthermore, we also demonstrated that the grafted NS cells promoted long-distance regrowth of intraorbitally lesioned RGC axons in adult mice. The combined data suggest that genetically modified clonal NS cell lines may represent a useful tool for preclinical studies aimed at evaluating the therapeutic potential of a sustained cell-based intraocular administration of neuroprotective factors in mouse models of optic nerve injury or other retinal disorders characterized by a loss of RGCs.

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Declaration on oath

I hereby declare, on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aid.

Hamburg, 30.09.2015

City and date

Signature